Resistance to *Phytophthora cinnamomi* in American Chestnut (*Castanea dentata*) Backcross Populations that Descended from Two Chinese Chestnut (*Castanea mollissima*) Sources of Resistance

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Abstract

Restoration of American chestnut (*Castanea dentata*) depends on combining resistance to both the chestnut blight fungus (*Cryphonectria parasitica*) and *Phytophthora cinnamomi*, which causes Phytophthora root rot, in a diverse population of *C. dentata*. Over a 14-year period (2004 to 2017), survival and root health of American chestnut backcross seedlings after inoculation with *P. cinnamomi* were compared among 28 BC₃, 66 BC₄, and 389 BC₃F₃ families that descended from two BC₁ trees (Clapper and Graves) with different Chinese chestnut grandparents. The 5% most resistant Graves BC₃F₃ families survived *P. cinnamomi* infection at rates of 75 to 100% but had mean root health scores that were intermediate between resistant Chinese chestnut and susceptible American chestnut families. Within Graves BC₃F₃ families, seedling survival was greater than survival of Graves BC₃ and BC₄ families and was not genetically correlated with

chestnut blight canker severity. Only low to intermediate resistance to *P. cinnamomi* was detected among backcross descendants from the Clapper tree. Results suggest that major-effect resistance alleles were inherited by descendants from the Graves tree, that intercrossing backcross trees enhances progeny resistance to *P. cinnamomi*, and that alleles for resistance to *P. cinnamomi* and *C. parasitica* are not linked. To combine resistance to both *C. parasitica* and *P. cinnamomi*, a diverse Graves backcross population will be screened for resistance to *P. cinnamomi*, survivors bred with trees selected for resistance to *C. parasitica*, and progeny selected for resistance to both pathogens will be intercrossed.

Keywords: backcross breeding, chestnut blight, *Cryphonectria parasitica*, heritability, host resistance, ink disease, *Phytophthora* root rot.

Although the introduction of the fungus that causes chestnut blight (*Cryphonectria parasitica* (Murr.) Barr) in the early 20th century eventually caused the functional extinction of American chestnut trees (*Castanea dentata* (Marsh.) Borkh.) in the eastern United States, Phytophthora root rot, caused by the soilborne oomycete *Phytophthora cinnamomi* Rands (Crandall et al. 1945; Tucker 1933), is thought to have extirpated American chestnut trees from lower-elevation forests in the southeastern United States prior to the introduction of *Cryphonectria parasitica* (Anagnostakis 2012; Crandall et al. 1945; Freinkel 2007; Zentmyer 1980). A disease suspected to be Phytophthora root rot (also known as ink disease) was first reported in 1824 killing Allegheny chinquapin shrubs (*Castanea pumila* Mill.) in Georgia (Anagnostakis 2001, 2012), and American chestnut is also highly susceptible to *P. cinnamomi* (Crandall et al. 1945; Jeffers et al. 2009). Therefore, extensive mortality of American chestnut trees in the southeastern United States in the early to mid-1800s has been speculated to be from Phytophthora root rot (Crandall et al. 1945; Zentmyer 1980). Today, Phytophthora root rot has the potential to limit reintroduction of American chestnut trees with improved resistance to

Cryphonectria parasitica across large areas of the native range of *Castanea dentata* (Anagnostakis 2001; Jacobs 2007). Whereas American chestnut resprouts from the roots after stems are killed by chestnut blight, the whole plant is killed by Phytophthora root rot.

Until recently, Phytophthora root rot on American chestnut was reported to be caused only by *P. cinnamomi* (Crandall et al. 1945; Tucker 1933). The source, location, and timing of the earliest introduction of *P. cinnamomi* to the United States is not known but *P. cinnamomi* is speculated to have been imported into the southeastern United States on exotic plants from southeastern Asia, where *P. cinnamomi* is thought to have originated (Crandall and Gravatt 1967; Crandall et al. 1945; Zentmyer 1980, 1988). Currently, *P. cinnamomi* is the most common and virulent species of *Phytophthora* on American chestnut but *P. cambivora* (most likely *P. × cambivora*) (Jung et al. 2017), *P. cryptogea*, and *P. heveae* recently were isolated from American and backcross hybrid seedlings and have been found to cause lesions on roots of American chestnut seedlings after artificial inoculation (Sharpe 2017).

The chestnut blight fungus produces propagules that are both wind-borne and splash dispersed, and this pathogen is ubiquitous throughout the native range of American chestnut (Anagnostakis 2012; Freinkel 2007). By contrast, *P. cinnamomi* is a soilborne pathogen with a random distribution in time and space (Crandall et al. 1945; Meadows and Jeffers 2011). Soil moisture is essential for the production of sporangia and release of zoospores by *P. cinnamomi* (Zentmyer 1980). Phytophthora root rot on American chestnut seedlings was most damaging in moist soils in controlled experiments (Rhoades et al. 2003). *P. cinnamomi* spreads to host plants in water or soil containing infective propagules (i.e., motile zoospores or chlamydospores), by growth of plant roots into infested soil, or by root-to-root contact (Ristaino and Gumpertz 2000; Zentmyer 1980). The current geographic range of *P. cinnamomi* in the eastern United States overlaps with the range of American chestnut from Alabama to southern Pennsylvania. *P. cinnamomi* is not prevalent north of 40° N latitude at the present time, presumably due to the adverse effects of prolonged cold temperatures

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Funding: This material is based on research that was supported by The American Chestnut Foundation and by the United States Department of Agriculture National Institute of Food and Agriculture, under project numbers SC-1700309, SC-1700445, SC-1700481, and SC-1700534 at Clemson University. Technical Contribution No. 6715 of the Clemson University Experiment Station.

The author(s) declare no conflict of interest.

Accepted for publication 1 February 2019.

during winter (Balci et al. 2007; Benson 1982). However, as the climate warms, the range of *P. cinnamomi* is predicted to expand into the northeastern region of the United States by 2080 (Burgess et al. 2017).

Recent efforts to restore the American chestnut to its native range have focused on improving resistance to *Cryphonectria parasitica* in American chestnut populations (Anagnostakis 2012). Since 1983, The American Chestnut Foundation (TACF) has pursued backcross breeding to generate hybrids that combine resistance to *C. parasitica* from Chinese chestnut (*Castanea mollissima* Blume) with the timber-type form of American chestnut (Burnham et al. 1986). In a TACF breeding program, two sources of resistance to *C. parasitica*, which descended from two distinct *C. mollissima* trees, have been advanced to the third backcross generation (BC₃) with *C. dentata*. Two generations of intercrossing between backcross trees selected for resistance to *C. parasitica* have been performed to enhance this resistance in the BC₃F₂ and BC₃F₃ generations (Steiner et al. 2017).

Chinese chestnut also is resistant to *P. cinnamomi* (Crandall et al. 1945; Jeffers et al. 2009, 2012). Thus, American chestnut backcross descendants selected for resistance to *C. parasitica* potentially inherited alleles for *P. cinnamomi* resistance from *C. mollissima*. However, TACF has not selected for resistance to *P. cinnamomi* in backcross generations. Several coauthors of this study (i.e., S. N. Jeffers, J. B. James, and P. H. Sisco) began screening seedlings of American chestnut backcross families for resistance to *P. cinnamomi* in 2004 after *P. cinnamomi* had killed over 80% of BC₂ American chestnut seedlings growing in a planting in Oconee County, SC between 2001 and 2003 (James 2011a,b; Laurie 2014). In annual trials conducted from 2004 to 2011, these collaborators evaluated seedling resistance to *P. cinnamomi* among American chestnut BC₃ and BC₄ progeny of BC₂ and BC₃ trees selected for resistance to *C. parasitica* (Jeffers et al. 2009, 2012). In 2012, this group began screening seedlings in BC₃F₃ families that were derived from open pollination among BC₃F₂ trees. TACF collaborated with J. Frampton (another coauthor) at North Carolina State University (NCSU) to evaluate additional BC₃F₃ families from 2014 to 2016. Beginning in 2017, evaluation of seedlings in BC₃F₃ families was conducted by TACF in collaboration with personnel at the United States Department of Agriculture (USDA) Forest Service Resistance Screening Center in Asheville, NC.

The overall objective of this project was to evaluate first-year hybrid American chestnut seedlings for resistance to *P. cinnamomi* under standardized conditions. If resistance was identified, a secondary objective was to identify the source or sources of the resistance. We present results of evaluating seedlings of American chestnut, Chinese chestnut, and American chestnut backcross progeny for resistance to *P. cinnamomi* over 14 growing seasons (2004 to 2017). Genetic variation in resistance to *P. cinnamomi* was estimated among three generations of American chestnut backcross families (BC₃, BC₄, and BC₃F₃) that descended from two BC₁ trees. This dataset enabled us to test for loss of *P. cinnamomi* resistance with successive backcrosses to *C. dentata* and for increases in resistance through intercrossing backcross trees. Among BC₃F₃ families, *P. cinnamomi* resistance was compared for a subset of families screened at multiple locations using different isolates of *P. cinnamomi* and different procedures for disease phenotyping. A genetic correlation between resistance to *P. cinnamomi* and *C. parasitica* was estimated among American chestnut BC₃F₃ families that were screened for resistance to both pathogens. Implications for breeding to combine resistance to *P. cinnamomi* and *C. parasitica* are discussed.

Materials and Methods

Sources of seed and research trial locations. The American chestnut backcross seedlings evaluated for resistance to *P. cinnamomi* were descendants of the Clapper and Graves sources of resistance to *C. parasitica*. Clapper and Graves are two BC₁ trees ((*C. mollissima* × *C. dentata*) × *C. dentata*) with different *C. mollissima* grandparents (Anagnostakis 2007; Clapper 1952; Hebard 2006). A subset of BC₂ and BC₃ descendants of Clapper and Graves, which were selected for resistance to *C. parasitica* at

TACF Meadowview Research Farms, were crossed with susceptible *C. dentata* parents to generate BC₃ and BC₄ progeny that were evaluated for resistance to *P. cinnamomi*. In total, progeny of 10 of 41 Clapper BC₂ selections, 20 of 83 Clapper BC₃ selections, 18 of 41 Graves BC₂ selections, and 46 of 71 Graves BC₃ selections were evaluated for resistance to *P. cinnamomi*.

The BC₃F₃ seed were obtained from TACF Meadowview Research Farms through open pollination of 124 Clapper and 265 Graves BC₃F₂ mothers by neighboring BC₃F₂ trees. Similarly, the BC₃F₂ parents were generated through open pollination among BC₃ trees. To put the number of BC₃F₃ families that were evaluated for *P. cinnamomi* resistance into context, approximately 36,000 BC₃F₂ progeny of 83 Clapper BC₃ trees and 27,000 BC₃F₂ progeny of 71 Graves BC₃ trees have been planted in Meadowview seed orchards since 2002. These approximately 63,000 BC₃F₂ trees were artificially inoculated with *C. parasitica*, and the most susceptible trees were culled based on severity of blight canker development (Steiner et al. 2017). At the time of the writing of this article, approximately 5,000 Clapper BC₃F₂ trees and 4,250 Graves BC₃F₂ trees remained in Meadowview seed orchards. The final objective is to cull all but 1% of trees that are most resistant to *C. parasitica* or *P. cinnamomi* (i.e., retain approximately 600 trees). Although we have evaluated a small subset of the total number of potential BC₃F₃ families for *P. cinnamomi* resistance, we have likely screened BC₃F₃ grand-progeny from a majority of the BC₃ trees selected for blight resistance. The BC₃F₂ mothers whose progeny were screened for resistance to *P. cinnamomi* were themselves the progeny of 41 of 71 Graves BC₃ selections and 34 of 83 Clapper BC₃ selections. It is likely that grand-progeny of additional BC₃ selections have been represented in the screening through the unknown paternal contribution in the BC₃F₂ and BC₃F₃ generations. In this article, seedlings derived from open pollination of a given mother tree or from a specific controlled pollination are referred to as a family. Seed were collected in the fall and stratified in moist peat in plastic bags over the winter at 4 to 8°C.

Chestnut seedlings were evaluated for resistance to *P. cinnamomi* at three locations: Clemson University personnel conducted trials in collaboration with colleagues at Chestnut Return Farms (Oconee County, SC); NCSU personnel conducted trials on the main campus in Raleigh, NC; and TACF personnel conducted trials in collaboration with colleagues at the USDA Forest Service Resistance Screening Center (Asheville, NC). At each location, different inoculation and phenotyping methods were used (detailed below) to insure that results were not artifacts of a single location, inoculation procedure, or evaluation method. To compare family rankings for *P. cinnamomi* resistance between pairs of locations and methods, subsets of 53 BC₃F₃ families were evaluated by Clemson University and NCSU in 2014, 2015, and 2016. Another 17 BC₃F₃ families were evaluated by Clemson University and TACF with the USDA Forest Service in 2017. In each year and at each location, resistant Chinese chestnut and susceptible American chestnut seedlings were included as controls.

Trials conducted by Clemson University at Chestnut Return Farms: 2004 to 2017. The 14 annual trials conducted at Chestnut Return Farms have been summarized, including dates of planting, inoculation, and evaluation of Phytophthora root rot phenotypes; lengths of time between when seedlings were planted, inoculated, and evaluated; and numbers of seedlings and families evaluated (Table 1). Among 505 American chestnut backcross families evaluated during this time, 62 were descendants of Clapper and 166 were descendants of Graves. Detailed analyses of resistance to *P. cinnamomi* within hybrid and backcross families that descended from additional Chinese chestnut and Japanese chestnut trees, which were included in these trials, are beyond the scope of this article. Preliminary results of *P. cinnamomi* resistance in hybrid and backcross American chestnut seedlings, including those that descended from sources of resistance other than Clapper or Graves, have been reported (Jeffers et al. 2009, 2012).

Seed were sowed outdoors in early to mid-April (plus late March 2008) in 568-liter tubs (Structural Foam Stock Tank FG424500BLA:

147 cm long by 99 cm wide by 64 cm tall; Rubbermaid Commercial Products, High Point, NC) when radicles had emerged from approximately 50% of the seed. The tubs contained a soilless growing mix composed primarily of peat and bark (Fafard 3B Mix; currently produced by Sun Gro Horticulture, Agawam, MA). A subset of seed from each family was planted in 3 to 10 tubs. Between 1 and 25 seeds from each family (mean = 6) were planted in each tub, depending on the year. Seed from individual families were planted together in a row, with approximately 1 to 3 cm between seeds and at a depth of 2 to 4 cm. The position of families within tubs was randomized among tubs. Tubs were watered as needed throughout the growing season. Once seedlings had emerged, slow-release fertilizer (Osmocote; The Scotts Company, Marysville, OH) was added to each tub in late April or early May.

Seedlings were inoculated with a combination of two isolates of *P. cinnamomi* (JJ-1 and JJ-52, both mating type A2) that were isolated in 2003 from backcross chestnut seedlings with Phytophthora root rot growing at Chestnut Return Farms. These isolates are stored in a permanent collection that is maintained by S. N. Jeffers at Clemson University. The two isolates were grown independently on sterile rice grains (Holmes and Benson 1994; Meadows et al. 2011) in 2004 to 2007 and then on sterile vermiculite moistened with V8 juice broth (Roiger and Jeffers 1991) from 2007 to 2017. Both types of inoculum were used in 2007. Equal volumes of inoculum containing each isolate were thoroughly mixed before inoculation. Seedlings were inoculated 12 to 16 weeks after planting, usually in early to mid-July (Table 1). To inoculate, furrows (2 to 3 cm deep and 95 to 97 cm long) were made between every other row of seedlings, and inoculum (i.e., 10 ml of rice or 50 ml of V8-vermiculite) was sprinkled in the furrow, evenly distributed, and covered. After inoculation, seedlings in all tubs were watered to incorporate the inoculum and prevent it from desiccating. Tubs were flooded each year for a 6- to 8-h period one to three times in July or August to enhance disease development. At 20 to 29 weeks (mean = 24 weeks) after inoculation, dormant seedlings were evaluated for disease severity (Table 1). Each seedling was removed carefully from a tub to keep the roots intact and washed in tap water. On each seedling, the roots and the lower stem

were inspected for typical symptoms of Phytophthora root rot. Disease severity was scored on a 0-to-3 scale, where 0 = no lesions on roots, plant healthy; 1 = lesions on some lateral roots or plant stunted; 2 = lesions on the tap root or severe root rot on lateral roots; and 3 = 100% root rot, plant dead (Jeffers et al. 2009).

Each year, one to three diseased seedlings and approximately 1 liter of container mix from each tub were collected and taken to the lab to confirm the presence of *P. cinnamomi*. Root systems were washed again under running tap water and blotted dry. Tissue pieces from advancing lesions were embedded in PARPH-V8 selective medium, and isolation plates were placed at 20°C for 5 to 7 days (Ferguson and Jeffers 1999; Jeffers 2015). Subsamples of container mix from each tub were assayed with a baiting bioassay using camellia and rhododendron leaf pieces as baits (Ferguson and Jeffers 1999; Meadows et al. 2011).

Trials conducted by NCSU: 2014 to 2016. During April and May of each year, 20 stratified seeds from each American chestnut, Chinese chestnut, and American chestnut backcross family were sowed individually into D40 Deepots (650 ml, 25 × 6 cm; Stuewe & Sons, Tangent, OR) containing a 1:1 peat-perlite medium (Table 2). After initial growth at TACF Meadowview Research Farms in Virginia, seedlings were transferred to the Horticultural Field Lab at NCSU in Raleigh, NC on 24 June 2014, 22 May 2015, and 10 June 2016. Seedlings were grown outdoors under 40% shade cloth in a completely randomized design and were inoculated 7 to 10 weeks after planting by inserting three infested rice grains about 2.5 cm deep into separate holes in the medium of each Deepot and covering the grains. The rice grains had been colonized by a single isolate of *P. cinnamomi* (23ss04) obtained from a Fraser fir tree (*Abies fraseri* (Pursh) Poir.) in North Carolina with Phytophthora root rot (Frampton et al. 2013). Eight weeks after inoculation, surviving seedlings were re-inoculated with the same isolate of *P. cinnamomi* to ensure that they had been challenged sufficiently by the pathogen. To promote disease development, seedlings were irrigated from above three times daily for 20 min (approximately 1.5 cm/day). Seedlings were fertilized periodically with 15-16-17 (N-P-K) Peters Peat-lite Special containing micronutrients (The Scotts Company) in an aqueous solution

Table 1. Summary of 14 consecutive annual trials to evaluate seedlings of American, Chinese, and backcross chestnut families for resistance to *Phytophthora cinnamomi* at Chestnut Return Farm in Oconee County, SS based on the percentage of seedlings surviving at the end of each trial

Year	Dates on which seedlings were ^a			Durations (weeks) ^b			Number of ^c		Number of families ^d				One-way ANOVA: <i>P</i> > <i>F</i> values ^e	
	Planted	Inoculated	Evaluated	P-I	I-E	P-E	Tubs	Seeds	BC	Am	Ch	Total	Am vs. Ch ^f	BC families ^g
2004	14 Apr 04	08 Jul 04	12 Jan 05	12	27	39	2	508	6	1	2	9	NC	NC
2005	05 Apr 05	28 Jun 05	20 Dec 05	12	25	37	6	1,250	26	1	1	28	<0.001	<0.001
2006	05 Apr 06	14 Jul 06	13 Dec 06	14	22	36	6	1,031	23	1	3	27	NC	<0.001
2007	04 Apr 07	19 Jul 07	18 Dec 07	15	22	37	7	1,545	42	5	1	48	<0.001	<0.001
2008	27 Mar 08	14 Jul 08	17 Dec 08	16	22	38	7	1,279	23	4	1	28	<0.001	<0.001
2009	08 Apr 09	09 Jul 09	28 Jan 10	13	29	42	7	1,020	32	3	2	37	<0.001	<0.001
2010	09 Apr 10	22 Jul 10	07 Dec 10	15	20	35	9	1,502	54	1	1	56	<0.001	<0.001
2011	05 Apr 11	11 Jul 11	13 Dec 11	14	22	36	10	1,633	48	1	1	50	<0.001	<0.001
2012	02 Apr 12	12 Jul 12	09 Jan 13	14	26	40	13	1,914	76	3	1	80	<0.001	<0.001
2013	17 Apr 13	30 Jul 13	18 Dec 13	15	20	35	11	1,480	71	1	1	73	<0.001	<0.001
2014	02 Apr 14	10 Jul 14	09 Dec 14	14	22	36	13	1,869	32	1	1	34	<0.001	<0.001
2015	08 Apr 15	07 Jul 15	13 Jan 16	13	27	40	13	1,639	29	1	1	31	<0.001	<0.001
2016	06 Apr 16	06 Jul 16	12 Jan 17	13	27	40	11	1,210	23	1	1	25	<0.001	<0.001
2017	13 Apr 17	06 Jul 17	23 Jan 18	12	29	41	5	645	20	1	1	22	<0.001	<0.001
Total	...	...	...	...	...	...	...	18,525	505	25	18	548	...	...

^a Abbreviations: April (Apr), July (Jul), January (Jan), Jun (June), December (Dec), and March (Mar) in years 2004 (04) through 2018 (18).

^b Lengths of time in weeks between activities involving seedlings: P = planted, I = inoculated, and E = evaluated.

^c Number of 568-liter tubs planted with seedlings and numbers of seeds planted each year.

^d Numbers of different families planted each year. A family is a specific chestnut genotype: Am = open-pollinated American chestnut, Ch = open-pollinated Chinese chestnut, and BC = open-pollinated and specific crosses of (American chestnut × Chinese chestnut) × American chestnut backcross hybrids.

^e Results from statistical analyses by one-way analysis of variance (ANOVA): *P* > *F* is the probability of a greater *F* statistic occurring. Analyses were not conducted for some data (NC) because of insufficient replication.

^f Results showing the significant difference in survival between the American and Chinese chestnut families each year based on a single-degree-of-freedom linear contrast.

^g Results showing the significant difference in survival among only the hybrid chestnut families evaluated each year based on a one-way ANOVA of a subset of the data from this trial.

prepared to deliver N at 200 ppm. Symptom development was visually assessed 8 and 16 weeks after inoculation in 2014 and biweekly for 16 weeks in 2015 and 2016. Foliage health was assessed by estimating percent shoot (leaf and stem) necrosis on a 0-to-100 scale in increments of 10 but also including 1, 2, and 95% levels. Seedlings were considered dead if 100% of the shoot was necrotic and alive (i.e., surviving) if otherwise. Each year, a control group of seedlings from each Chinese chestnut, American chestnut, and BC₃F₃ family was not inoculated with *P. cinnamomi* and served as controls to estimate mortality rates in the absence of *Phytophthora* root rot.

Trial conducted by TACF and the USDA Forest Service in 2017. Forty seeds from each American chestnut, Chinese chestnut, and American chestnut backcross family were sowed in Fafard 3B Mix in D40 Deepots in a greenhouse at the USDA Forest Service Resistance Screening Center in February (Table 2). The pots were arranged in 20-cell trays in a randomized complete block design. The blocks consisted of 10 trays placed in flood tables that measured 1.2 by 1.8 by 0.3 m. Once seedlings emerged, they were fertilized with time-release Osmocote fertilizer and irrigated overhead three times per week for 13 weeks prior to inoculation. After inoculation, seedlings were subirrigated three times each week for the duration of the trial. Subirrigation completely saturated the container mix in each Deepot and provided conducive conditions for disease development. Irrigation water was pumped from the flood tables to holding tanks and treated with bleach (NaOCl) after use to prevent contaminating the site with *P. cinnamomi*. Seedlings were inoculated once with the same isolate of *P. cinnamomi* (23ss04) used in the NCSU trials, and the inoculum was prepared as colonized V8-vermiculite, as in the Clemson University trials. A 5-ml aliquot of V8-vermiculite inoculum was placed in each pot, the inoculum was covered immediately with sand, and the pots were watered to prevent desiccation of the inoculum. Survival was assessed by visual observation of foliage symptoms and removing seedlings with wilted crowns at 4, 8, 16, and 24 weeks after inoculation. Seedlings with wilted crowns were scored as dead, and roots on these seedlings were inspected for typical symptoms of *Phytophthora* root rot.

Evaluation of resistance to *C. parasitica*. In total, 103 Graves BC₃F₃ families that were evaluated for resistance to *P. cinnamomi* were also evaluated for resistance to *C. parasitica* at TACF Meadowview Research Farms. These 103 families were a subset of 544 families that had been evaluated for resistance to *C. parasitica* from 2011 to 2016 in separate experiments (Steiner et al. 2017). Thirty BC₃F₃ seeds per family were planted in the field at Meadowview Research

Farms at 1-by-2.3-m spacing in a randomized complete block design (2011 to 2013) or resolvable incomplete design (2014 to 2016). Progeny from each family was inoculated in the third growing season with two isolates of *C. parasitica*: SG2-3 with low virulence and Ep155 with high virulence. Cankers lengths and subjective canker severity ratings (1 = no canker expansion, 2 = some expansion beyond initial lesion, and 3 = large, sunken canker with sporulation) were assessed 6 months after inoculation. Canker severity was estimated from the sum of the normalized canker length and canker severity rating for the two isolates of *C. parasitica* (Steiner et al. 2017).

Data analysis. The original root rot severity scores of 0 to 3 used in the Clemson University trials were reversed to root health scores of 3 to 0, so that families could be ranked for resistance to *P. cinnamomi*. Seedlings scored as 3, 2, and 1 were alive while those scored as 0 were dead. An analysis of variance to detect differences in survival among families in each year and at each location was performed with the R package ‘easynova’ (Arnhold 2013). Tubs were treated as complete blocks in analysis of survival in trials conducted by Clemson University and by TACF and the USDA Forest Service, whereas results from the trials conducted by NCSU were analyzed as a completely randomized design. Family means were judged significantly different if *P* values from *F* tests of family effects were less than 0.05.

Mixed-model analyses of *Phytophthora* root rot phenotypes were performed in ASReml-R (Butler et al. 2009). Phenotypes of backcross descendants of the Clapper and Graves trees were analyzed separately. Root health rating (3-to-0 scale) or survival (0,1) of seedlings in the Clemson University trials were analyzed using univariate models that included the population mean and the random effects of inoculation year, tub, family, and error. Crown health (0 to 100) or survival (0,1) of seedlings in the NCSU trials were analyzed employing univariate models that included the population mean and the random effects of inoculation year, family, and error. Seedling survival in the TACF-USDA Forest Service trial was analyzed in a model that included the population mean and the random effects of block, family, and error.

The heritability of *Phytophthora* root rot phenotypes was estimated for individual BC₃F₃ trees with the formula:

$$h^2 = 4\sigma_{\text{family}}^2 / \sigma_{\text{phenotype}}^2 \quad (1)$$

where $\sigma_{\text{phenotype}}^2$ is the sum of the variance components associated with random effects in the model (i.e., year, family, block, and error).

Table 2. Summary of four annual trials to evaluate seedlings of American, Chinese, and BC₃F₃ families for resistance to *Phytophthora cinnamomi* at North Carolina State University (NCSU) and the USDA Forest Service Resistance Screening Center (RSC) in Asheville, NC based on the percentage of seedlings surviving at the end of each trial

Trial ^e	Year	Dates on which seedlings were ^a			Duration (weeks) ^b			Number of families ^c				Number of seedlings				One-way ANOVA: <i>P</i> > <i>F</i> values ^d	
		Planted	Inoculated	Evaluated	P-I	I-E	P-E	BC	Am	Ch	Total	BC	Am	Ch	Total	Am vs. Ch ^f	BC ₃ F ₃ ^g
NCSU	2014	15 May 14	23 Jul 14	12 Nov 14	10	16	26	95	0	0	95	2,259	0	0	2,259	NC	<0.001
NCSU	2015	01 Apr 15	29 May 15	18 Sep 15	8	16	24	97	2	2	101	1,892	40	32	1,964	<0.001	<0.001
NCSU	2016	11 May 16	01 Jul 16	21 Oct 16	7	16	23	74	3	1	78	1,482	40	20	1,542	<0.001	<0.001
RSC	2017	15 Feb 17	23 May 17	07 Nov 17	14	24	38	37	1	1	39	1,036	27	39	1,102	<0.001	<0.001
Total	...	...	...	...	...	...	...	287	6	4	297 ^h	6,669	107	91	6,897	...	...

^a Abbreviations: July (Jul), November (Nov), April (Apr), September (Sep), October (Oct), and February (Feb) in years 2014 (14) through 2017 (17).

^b Lengths of time in weeks between activities involving seedlings: P = planted, I = inoculated, and E = evaluated.

^c Numbers of different families planted each year. A family is a specific chestnut genotype: Am = open-pollinated American chestnut, Ch = open-pollinated Chinese chestnut, and BC = specific BC₃F₃ generation of American chestnut backcross hybrids.

^d Results from statistical analyses by one-way analysis of variance (ANOVA): *P* > *F* is the probability of a greater *F* statistic occurring. Analyses were not conducted for some data (NC) because of insufficient replication.

^e NCSU = North Carolina State University and RSC = The American Chestnut Foundation–United States Department of Agriculture Forest Service Resistance Screening Center.

^f Results showing the significant difference in survival between the American and Chinese chestnut families each year based on a single-degree-of-freedom linear contrast.

^g Results showing the significant difference in survival among only the American chestnut BC₃F₃ families evaluated each year based on a one-way ANOVA of a subset of the data from this trial.

^h Note that 16 families were each evaluated in two separate years; therefore, the sum of the number of families evaluated in all years (313) is greater than the total number of families evaluated (297).

This heritability estimate assumes random mating among BC₃F₂ trees and that BC₃F₃ progeny were half-siblings (Lynch and Walsh 1998). The family variance component and best linear unbiased predictions (BLUPs) of *Phytophthora* root rot phenotypes for each family were estimated by incorporating the inverse of the pedigree-derived additive genetic relationship matrix into the model (Butler et al. 2009). The BLUPs were scaled from 0 (American chestnut mean) to 100 (Chinese chestnut mean) with the formula:

$$\text{Scaled BLUP} = 100[1 - (\text{BLUP} - \mu_{\text{Chin}}) / (\mu_{\text{Am}} - \mu_{\text{Chin}})] \quad (2)$$

where BLUP is in the original scale and μ_{Chin} and μ_{Am} are the average BLUPs for Chinese chestnut and American chestnut families, respectively.

Bivariate analyses were performed to estimate genetic correlations between different *Phytophthora* root rot phenotypes from the same trees, survival of the same families inoculated with *P. cinnamomi* at different screening locations, and between *Phytophthora* root rot survival and chestnut blight canker severity. Covariance was estimated among all random effects (i.e., family, tub, and error) for analyses involving different phenotypes from the same trees. For bivariate analyses of *Phytophthora* root rot survival at different sites or between *Phytophthora* root rot survival and chestnut blight canker severity, experimental design effects and residuals were assumed to be uncorrelated while genetic covariance was estimated among families. Genetic correlations were estimated with the formula:

$$\text{Cov}_{\text{trait1,trait2}} / [\sqrt{\text{Var}_{\text{trait1}}} + \sqrt{\text{Var}_{\text{trait2}}}] \quad (3)$$

where Cov and Var are family covariance and variance components, respectively. Standard errors for genetic correlations and heritabilities were estimated with the Δ method (Lynch and Walsh 1998). Genetic correlations were considered significantly different than zero if the *P* value from the likelihood ratio test of the correlation was less than 0.05 (Butler et al. 2009). Figures were prepared using the R package ‘ggplot2’ (Wickham 2009).

Results

Seedling survival within families after inoculation with *P. cinnamomi*. Significant differences in seedling survival (*F* test, *P* < 0.001) were observed between resistant Chinese chestnut and susceptible American chestnut controls and among backcross families in all years and at all screening locations (Tables 1 and 2). In all, 11% of American chestnut seedlings inoculated with *P. cinnamomi* survived to the end of assessment periods—which, on average, was 38 weeks at Chestnut Return Farms, 24 weeks at the Resistance Screening Center, and 16 weeks at NCSU. In contrast, 97% of the Chinese chestnut seedlings survived over the same time period. In 111 Graves BC₃F₃ families evaluated in the Clemson University trials, an average of 32% of the seedlings survived, whereas 75 to 100% of the seedlings survived in the 5% most resistant families. Similar rates of survival were observed for seedlings in 156 Graves BC₃F₃ families evaluated in NCSU trials, where seedling survival averaged

24% overall, and 75 to 95% of the seedlings survived in the 5% most resistant families. Average survival of seedlings in Clapper BC₃F₃ families was higher in NCSU trials (99 families, mean survival = 24%) as compared with Clemson University trials (30 families, mean survival = 7%) (*F* test, *P* < 0.001).

High rates of survival were observed for control plants in the NCSU trials that were not inoculated with *P. cinnamomi*. Survival was 91% for American chestnuts (20 of 22 seedlings), 100% for Chinese chestnuts (20 of 20 seedlings), and 90% for BC₃F₃ trees (258 of 287 seedlings). In Clemson University trials, *P. cinnamomi* was isolated consistently from the roots of diseased plants and container mix samples collected annually from individual tubs. These results confirm that the majority of the mortality of inoculated seedlings could be attributed to *Phytophthora* root rot and not to other causes.

Heritability of *Phytophthora* root rot phenotypes among BC₃F₃ families. Phenotypes associated with resistance to *P. cinnamomi* (i.e., survival, root health, and crown health) were highly heritable ($h^2 = 0.85 \pm 0.09$ to 1.0 ± 0.11) among seedlings in Graves BC₃F₃ families over 6 years of evaluation at the three locations (Tables 3 and 4). For Graves BC₃F₃ families included in the 2015 and 2016 NCSU trials, the heritability of survival of seedlings increased rapidly following inoculation and approached a maximum of 1 at 10 to 12 weeks after the first of two inoculations (Fig. 1). In the TACF-USDA Forest Service trial, where seedlings were inoculated once with *P. cinnamomi*, heritability of seedling survival in Graves BC₃F₃ families increased more gradually to 0.8 ± 0.17 at 24 weeks (Fig. 1). The *Phytophthora* root rot phenotypes were less heritable among seedlings in the Clapper BC₃F₃ families ($h^2 = 0.08 \pm 0.07$ to 0.32 ± 0.08). Survival of seedlings in Clapper BC₃F₃ families was more strongly heritable in trials at NCSU ($h^2 = 0.27 \pm 0.09$) than in Clemson University trials ($h^2 = 0.11 \pm 0.08$) (Tables 3 and 4).

Resistance to *P. cinnamomi* inherited across backcross generations in Graves families. In Clemson University trials, seedlings in families from three backcross generations (BC₃, BC₄, and BC₃F₃) were evaluated, which enabled an intergenerational comparison of *P. cinnamomi* resistance. Among backcross descendants of the Graves BC₁ tree, variation in survival of seedlings in BC₃F₃ and BC₃ families was positively skewed and spanned the range of seedling survival in resistant Chinese chestnut and susceptible American chestnut control seedlings (Fig. 2A). By contrast, when root health ratings were used as the phenotype to assess *P. cinnamomi* resistance, the most resistant Graves BC₃F₃ families had resistance intermediate between Chinese chestnut and American chestnut (Fig. 2B). Although resistance of seedlings estimated from root health was less than that of seedlings estimated from survival, root health and seedling survival were strongly genetically correlated among Graves BC₃F₃ families ($r_{\text{genetic}} = 0.98 \pm 0.01$, likelihood ratio test *P* < 0.001).

Average survival of seedlings in BC₃F₃ families was greater than average survival of seedlings in BC₃ families, BC₄ families, and American chestnut families (Tukey test, *P* < 0.05). Survival BLUPs for multiple BC₃F₃ families were regressed on those of single BC₄

Table 3. Narrow-sense heritability (h^2) of survival and root health ratings at 35 to 42 weeks after inoculation with *Phytophthora cinnamomi* among seedlings of American chestnut BC₃F₃ families that descended from the Clapper (C) or Graves (G) BC₁ trees in six Clemson University trials (2012 to 2017)

Year	Survival ($h^2 \pm \text{SE}$) ^a		Root health ($h^2 \pm \text{SE}$) ^b		Number of families		Median number seedlings per family (maximum, minimum)	
	C	G	C	G	C	G	C	G
2012	...	0.64 ± 0.19	...	0.75 ± 0.20	...	39	...	14 (5,17)
2013	0.02 ± 0.12	1.12 ± 0.26	0	1.18 ± 0.25	19	35	13 (7,17)	14 (5,22)
2014	...	0.91 ± 0.28	...	0.72 ± 0.25	...	19	...	26 (6,30)
2015	0.14 ± 0.10	0.87 ± 0.38	0.12 ± 0.09	0.68 ± 0.32	13	9	34 (23,54)	35 (26,37)
2016	...	0.76 ± 0.22	...	0.84 ± 0.23	...	21	...	41 (34,47)
2017	...	0.88 ± 0.26	...	0.62 ± 0.22	...	17	...	27 (30,12)
All years	0.11 ± 0.08	0.90 ± 0.11	0.08 ± 0.07	0.87 ± 0.11	32	122	14 (7,54)	16 (5,54)

^a Survival of seedlings 20 to 29 weeks after inoculation. SE = standard error.

^b Root health ratings 20 to 29 weeks after inoculation: 3 = no lesions on roots, 2 = lesions on some lateral roots; 1 = lesions on the tap root or severe root rot on lateral roots; and 0 = severe root rot, plant dead.

families that descended from a common BC₃ relative, where the BC₃ tree was a grandparent of the BC₃F₃ trees and a parent of the BC₄ trees (Fig. 3). The slope of the regression line was positive—indicating that inheritance of resistance to *P. cinnamomi* from the BC₃ relative was detectable after two generations of intercrossing by open pollination to generate the BC₃F₃ trees. The intercept of the regression line was greater than zero, indicating that BC₃F₃ families were, on average, more resistant than their BC₄ relatives. There was a large variation in seedling survival BLUPs among BC₃F₃ families that descended from a common BC₃ grandparent.

Minimal *P. cinnamomi* resistance among Clapper families in Clemson University trials. Survival BLUPs for seedlings in Clapper BC₃, BC₄, and BC₃F₃ families were, on average, not significantly different from those of susceptible American chestnut families (Fig. 4). Root health BLUPs for Clapper BC₃ families were, on average, marginally greater than those for American chestnut (Tukey test, $P = 0.027$).

Resistance to *P. cinnamomi* in NCSU and TACF-USDA Forest Service trials. Similar to results observed in Clemson University trials, genetic variation in survival of seedlings in Graves BC₃F₃ families at 16 weeks spanned the range of variation in survival of seedlings between Chinese chestnut and American chestnut controls in NCSU and TACF-USDA Forest Service trials (Fig. 5A). Genetic variation in crown health was reduced relative to variation in seedling survival (Fig. 5B) but family rankings of seedling survival and crown health were strongly correlated ($r_{\text{genetic}} = 0.84 \pm 0.03$). Seedlings in Graves BC₃F₃ families had significantly higher average BLUPs for survival and crown health than those in Clapper BC₃F₃ families and American chestnut controls (Tukey test, $P < 0.05$). Seedlings in Clapper BC₃F₃ families with the greatest resistance in NCSU trials had BLUPs for survival and crown health that were intermediate between the American chestnut and Chinese chestnut controls (Fig. 5). This result contrasts with those from Clemson University trials, where no resistance to *P. cinnamomi* was detected among seedlings in Clapper BC₃F₃ families.

Correlations in survival of seedlings between trials. A strong genetic correlation was observed between survival of seedlings in trials conducted by Clemson University and those in trials conducted by NCSU ($r_{\text{genetic}} = 0.94 \pm 0.03$, likelihood ratio test $P < 0.001$) for 53 BC₃F₃ families (9 Clapper and 44 Graves) screened by both groups. To test whether mean seedling survival among all families differed between Clemson University and NCSU trials, a regression was performed between the BLUPs for survival of seedling in BC₃F₃ families in the NCSU and Clemson University trials. The slope of the regression line was greater than one (t test, $P < 0.001$), indicating that BLUP values for seedling survival in NCSU trials were greater, on average, than those for seedlings in Clemson University trials (Fig. 6A). For seedlings in 17 Graves BC₃F₃ families that were inoculated with *P. cinnamomi* in trials conducted by Clemson University and TACF-USDA Forest Service in 2017, the genetic correlation in seedling survival between the two trial locations was 0.81 ± 0.13 (likelihood ratio test $P = 0.002$), and the slope of the regression line was not significantly different than 1 (t test, $P > 0.05$) (Fig. 6B).

Resistance to *C. parasitica* and *P. cinnamomi* was not correlated. The genetic correlation between chestnut blight canker severity and seedling survival after inoculation with *P. cinnamomi* was not significantly different than zero ($r_{\text{genetic}} = -0.01 \pm 0.13$, likelihood ratio test $P = 0.1$) among 103 Graves BC₃F₃ families (Fig. 7).

Discussion

Since 1983, TACF has pursued backcross breeding to introduce Chinese chestnut resistance to the chestnut blight fungus (*C. parasitica*) into hybrids that are expected to have inherited 94% of their genome from American chestnut parents (Burnham et al. 1986; Diskin et al. 2006; Hebard 2006; Steiner et al. 2017). This program originally did not include evaluating backcross families for resistance to *P. cinnamomi*, which causes Phytophthora root rot. Fortunately, Chinese chestnut is also resistant to *P. cinnamomi* (Crandall et al. 1945; Jeffers et al. 2009, 2012). Therefore, personnel from Clemson University, Chestnut Return Farms, and TACF began evaluating seedling progeny from a variety of BC₂ and BC₃ American chestnut backcross trees that had been selected for resistance to *C. parasitica* to determine whether any of these backcross trees had also inherited resistance to *P. cinnamomi*. Significant differences in seedling survival among backcross families inoculated with *P. cinnamomi* were observed each year in trials conducted from 2004 to 2011. Based on these results, it was determined that some BC₂ and BC₃ American chestnut backcross parents inherited alleles for resistance to *P. cinnamomi*. Consequently, open-pollinated BC₃F₃ progeny of BC₃F₂ trees that descended from the BC₂ and BC₃ trees evaluated in earlier trials were evaluated for resistance to *P. cinnamomi* from 2012 to 2017 in trials conducted by personnel at Clemson University, NCSU, and TACF (with the USDA Forest Service) to determine which BC₃F₂ parents had inherited resistance to *P. cinnamomi*.

Over the 14-year duration of this project, 18 annual trials were conducted at three locations in two states. Significant differences in seedling survival at 16 to 29 weeks after inoculation with *P. cinnamomi* occurred consistently among American chestnut backcross families in all 18 trials. In these trials, seedling survival was determined at the end of one growing season and was based on a combination of three root rot severity scores that ranged from no symptoms to severe root rot symptoms. We know from years of experience (J. B. James and S. N. Jeffers, unpublished data) that many of the seedlings with severe root rot symptoms do not survive a second growing season after transplanting into pots or in the field; however, survival after one growing season has proven to be the best parameter for identifying differences in resistance to *P. cinnamomi* among seedlings in backcross families under the experimental conditions used in these trials.

Differences in survival and average root health ratings among backcross families were highly heritable, suggesting that the root health and survival phenotypes of individual trees accurately reflects their underlying genetic resistance to *P. cinnamomi*. Correlated survival within backcross families that were inoculated with different isolates of *P. cinnamomi* at multiple screening locations in different

Table 4. Narrow-sense heritability (h^2) of survival and crown health ratings at 16 weeks after inoculation with *Phytophthora cinnamomi* among seedlings of American chestnut BC₃F₃ families that descended from the Clapper (C) or Graves (G) BC₁ trees in three North Carolina State University trials (2014 to 2016) and one trial conducted by The American Chestnut Foundation and the United States Department of Agriculture Forest Service (2017)

Year	Survival ($h^2 \pm \text{SE}$) ^a		Crown health ($h^2 \pm \text{SE}$) ^b		Number families		Median number seedlings per family (maximum, minimum)	
	C	G	C	G	C	G	C	G
2014	0.33 $\pm$ 0.08	0.66 $\pm$ 0.24	0.38 $\pm$ 0.08	0.72 $\pm$ 0.25	78	17	25 (10,31)	23 (18,28)
2015	0.20 $\pm$ 0.11	0.92 $\pm$ 0.15	0.28 $\pm$ 0.13	1.0 $\pm$ 0.16	28	69	20 (14,21)	20 (10,22)
2016	...	1.13 $\pm$ 0.16	...	1.13 $\pm$ 0.16	...	75	...	20 (18,22)
2017	...	0.74 $\pm$ 0.17	...	...	...	37	...	30 (7,40)
All years	0.27 $\pm$ 0.09	0.85 $\pm$ 0.09	0.32 $\pm$ 0.08	1.0 $\pm$ 0.11	99	189	24 (10,49)	20 (7,57)

^a Survival of stems 16 weeks after inoculation. SE = standard error.

^b Crown health is percentage of crown that had wilted 16 weeks after inoculation.

years further confirmed that our trials accurately measured the genetic component of *P. cinnamomi* resistance despite differences in experimental conditions.

A number of observations supported the hypothesis that backcross descendants of the Graves BC₁ tree inherited major-effect alleles for *P. cinnamomi* resistance from Mahogany, the *C. mollissima* grandparent of Graves. Chinese chestnut control seedlings survived at a high level (97%) whereas American chestnut controls survived at a low level (11%), which confirmed that *P. cinnamomi* resistance in backcross trees was conferred by alleles inherited from *C. mollissima*. The Graves BC₃ and BC₃F₃ families that were most resistant to *P. cinnamomi* had seedling survival percentages that approached those of resistant Chinese chestnut seedlings. Third-backcross trees are expected to only have inherited 1/16th of their genome from *C. mollissima* (Steiner et al. 2017); therefore, major-effect alleles for *P. cinnamomi* resistance were expected to be located on small segments of backcross genomes inherited from *C. mollissima*. It is remarkable that alleles for resistance to *P. cinnamomi* that were inherited from *C. mollissima* had not been bred out through backcrossing to *C. dentata* because TACF did not select for resistance to *P. cinnamomi*.

We showed in this study that chestnut blight canker severity and Phytophthora root rot survival were not genetically correlated among Graves BC₃F₃ families; therefore, major-effect alleles for *P. cinnamomi* resistance are expected to be unlinked with alleles for blight resistance. Quantitative trait locus (QTL) mapping supports the hypothesis that alleles that confer resistance to *C. parasitica* and *P. cinnamomi* are not linked. Among *C. mollissima* × *C. dentata* F₂ progeny of the Mahogany *C. mollissima* tree, QTLs for blight resistance were detected on linkage groups B, F, and G (Kubisiak et al. 1997, 2013). Among BC₁ descendants of another *C. mollissima* tree, a QTL for resistance to *P. cinnamomi* was first detected on linkage group E by Kubisiak (2010). By reanalyzing Kubisiak's data, Olukolu et al. (2012) found an additional locus on linkage group E plus a locus on linkage group A. Using much larger BC₁ families from two *C. mollissima* trees (Mahogany and the one used by Kubisiak), T. Zhebentyayeva (personal communication) confirmed

the two resistance loci on linkage group E and found an additional locus for resistance on linkage group K. QTLs for resistance to *P. cinnamomi* also were discovered on linkage groups E and K among *C. sativa* × *C. crenata* F₁ hybrids (Santos et al. 2017). These results suggest that Japanese chestnut (*C. crenata*) and Chinese chestnut share common loci that confer resistance to *P. cinnamomi*.

Our results suggest that alleles for *P. cinnamomi* resistance have dominant effects. Average and maximum survival and root health scores among seedlings in BC₃ families, which are at best heterozygous for alleles that confer resistance to *P. cinnamomi*, had marginally lower survival and root health scores as compared with seedlings in BC₃F₃ families. The BC₃F₃ progenies were generated from two generations of intercrossing among BC₃ and BC₃F₂ trees. Thus, BC₃F₃ trees potentially inherited *P. cinnamomi* resistance alleles from both parents in a homozygous state. The observation that survival of the most resistant heterozygous BC₃ families was similar to that of the most resistant and potentially homozygous BC₃F₃ families suggests that random intercrossing among BC₃ and BC₃F₂ trees selected for resistance to *C. parasitica* rarely generates progeny that are homozygous for *P. cinnamomi* resistance alleles at one or more loci. Regardless, the greater average resistance and wide segregation for *P. cinnamomi* resistance among seedlings in BC₃F₃ families as compared with BC₄ progeny that descended from common BC₃ trees suggests that intercrossing backcross trees selected for resistance to *P. cinnamomi* has the potential to enhance *P. cinnamomi* resistance in the subsequent generation.

The observation that seedlings in the most resistant Graves BC₃F₃ families had root health that was intermediate between Chinese and American chestnut suggests that Graves BC₃F₂ and BC₃F₃ progeny did not inherit the full complement of *P. cinnamomi* resistance alleles from the *C. mollissima* Mahogany tree in a homozygous state. Resistance to *P. cinnamomi* may be enhanced to levels similar to *C. mollissima* trees through controlled pollinations between the most resistant backcross trees and additional selection for *P. cinnamomi* resistance among their progenies.

Our results were inconclusive as to whether descendants of Clapper inherited little to no resistance to *P. cinnamomi*, as suggested by Clemson University trials, or intermediate levels of resistance, as suggested by NCSU trials. Backcross descendants of Clapper may have inherited fewer or smaller-effect resistance alleles as compared with descendants of Graves. Alternatively, BC₃F₂ progeny of Clapper BC₃ mothers may have inherited resistance to *P. cinnamomi* from neighboring Graves BC₃ trees, more distant *C. dentata* backcross

Fig. 1. Heritability ($h^2 \pm 1$ standard error) of survival of chestnut seedlings in Graves BC₃F₃ families over time after inoculation with *Phytophthora cinnamomi*. Seedlings were inoculated twice in the North Carolina State University trials in 2015 (circles) and 2016 (triangles) at 0 and 8 weeks, whereas seedlings were inoculated once in The American Chestnut Foundation–United States Department of Agriculture Forest Service trial in 2017 (squares).

Fig. 2. Genetic variation in **A**, survival and **B**, root health among backcross descendants of the Graves chestnut tree 35 to 42 weeks after inoculation with *Phytophthora cinnamomi* in Clemson University trials. Box-plots were created from variation in best linear unbiased predictions (BLUPs) of survival and root health among 18 BC₃ families, 46 BC₄ families, 122 BC₃F₃ families, 13 Chinese chestnut families, and 13 American chestnut families. The BLUPs were scaled from 0 (American chestnut mean) to 100 (Chinese chestnut mean).

descendants of additional *C. mollissima* trees, or pure *C. mollissima* trees that were planted on the same farm as the Clapper BC₃ trees.

Large numbers of BC₃F₂ progeny were needed to generate a subset of progeny that were homozygous for multiple blight resistance alleles; therefore, open pollination among BC₃ trees was used to generate BC₃F₂ trees (Steiner et al. 2017). Individual Clapper BC₃ selections were planted within 2.1 to 475 m (average distance = 154 m) of other Clapper BC₃ selections. Maximum pollination occurs among trees that are less than 30 m apart and the likelihood of pollination

decreases exponentially as distance between trees increases (Rutter 1990). Consequently, it is likely that most of the Clapper BC₃F₂ trees were the progeny of Clapper BC₃ × BC₃ crosses. However, with open pollination, it is possible that a subset of the trees referred to as Clapper BC₃F₂s were the progeny of Clapper BC₃ mothers pollinated by trees other than Clapper BC₃ fathers. The Clapper BC₃ selections were planted 15 to 548 m (average distance = 234 m) from Graves BC₃ selections and a minimum of 140 m from Chinese chestnuts on TACF Glenn C. Price Farm in Meadowview, VA. These pollen sources may have contributed alleles for resistance to *P. cinnamomi* to Clapper BC₃F₂ and BC₃F₃ seedlings. A larger number of Clapper BC₃F₃ families were screened in NCSU trials (99 families) as compared with Clemson trials (32 families). Therefore, it is more likely that NCSU trials detected partial resistance in Clapper or pollen contamination. Genotyping of Clapper and Graves BC₃ and BC₃F₂ trees as well as pure Chinese and American chestnut trees is ongoing (J. Westbrook and J. Holliday, unpublished). With these data, we will be able to determine whether Clapper and Graves BC₃ trees intercrossed to generate Clapper × Graves BC₃F₂ trees and whether any of the BC₃F₂ trees were the progeny of outcrosses to Chinese chestnuts.

Selection for resistance to *P. cinnamomi* from within a diverse backcross breeding population is the first step toward breeding to combine resistance to *C. parasitica* and *P. cinnamomi*. Additional evaluation for resistance to *P. cinnamomi* will focus primarily on descendants of the Graves tree due to the greater potential for gains in resistance as compared with descendants of the Clapper tree. Within TACF's national breeding program, in total, 181 backcross lines that descended from the Graves tree have been generated by breeding BC₂ and BC₃ selections from the Meadowview farm with wild-type American chestnut trees with locations ranging from Maine to Georgia (Westbrook 2018a). To date, only BC₃F₃ trees from the Meadowview farm breeding program, which are descendants of American chestnuts from southwest Virginia, have been screened for resistance to *P. cinnamomi*. Starting in 2018, TACF began to increase the genetic diversity of backcross trees selected for resistance to *P. cinnamomi* by screening Graves BC₃F₂ and BC₄F₂ descendants of American chestnuts from Maine, Vermont, New Hampshire, Massachusetts, Pennsylvania, northern Virginia, Kentucky, North Carolina, South Carolina, and Georgia. Forty progeny per BC₃ or BC₄ parent were inoculated with *P. cinnamomi*. Progeny that survive inoculation with *P. cinnamomi* will be planted at orchard sites where *P. cinnamomi* is already present so that seedlings will

Fig. 3. In trials conducted by Clemson University, survival of chestnut seedlings after inoculation with *Phytophthora cinnamomi* in multiple Graves BC₃F₃ families versus single Graves BC₄ families that shared a common BC₃ ancestor. The BC₃ ancestor is a grandparent of the BC₃F₃ trees and a parent of the BC₄ trees. Best linear unbiased predictions (BLUPs) of survival were scaled from 0 (American chestnut mean) to 100 (Chinese chestnut mean). The regression line is the least squares regression of BC₃F₃ versus BC₄ survival in BLUPs.

Fig. 4. Genetic variation in **A**, survival and **B**, root health among backcross descendants from the Clapper chestnut tree at 35 to 42 weeks after inoculation with *Phytophthora cinnamomi* in Clemson University trials. Box-plots were created from variation in best linear unbiased predictions (BLUPs) of survival and root health among 10 BC₃ families, 20 BC₄ families, 32 BC₃F₃ families, 13 Chinese chestnut families, and 13 American chestnut families. The BLUPs were scaled from 0 (American chestnut mean) to 100 (Chinese chestnut mean).

Fig. 5. Genetic variation in **A**, survival and **B**, crown health among chestnut seedlings in BC₃F₃ families that descended from the Clapper and Graves trees. Box-plots were created from variation in best linear unbiased predictions (BLUPs) of survival and crown health 16 weeks after inoculation with *P. cinnamomi*; 99 Clapper BC₃F₃ families, 189 Graves BC₃F₃ families, 4 American chestnut families, and 4 Chinese chestnut families included in three North Carolina State University trials and one The American Chestnut Foundation–United States Department of Agriculture Forest Service trial. The BLUPs were scaled from 0 (American chestnut mean) to 100 (Chinese chestnut mean).

also be exposed to the pathogen in natural settings. The final objective is to obtain at least one flowering BC₃F₂ or BC₄F₂ seedling from each BC₃ or BC₄ mother tree with improved resistance to *P. cinnamomi*.

Considering that resistance to *C. parasitica* and resistance to *P. cinnamomi* are not correlated, additional breeding and selection will be required to combine the resistances to these two pathogens. It is not feasible to select trees in the current BC₃F₂ generation that inherited high levels of resistance to both *C. parasitica* and *P. cinnamomi*. In screening for resistance to *C. parasitica* in the BC₃F₂ generation, TACF selects 1 of 150 trees (Steiner et al. 2017). Even with this high selection intensity, the selected trees are predicted to have *C. parasitica* resistance that is intermediate between Chinese chestnut and American chestnut. Additional selection for *P. cinnamomi* resistance would be required, leaving too few selections for further intercrossing.

One potential breeding strategy to combine resistance to both pathogens is to cross BC₃F₂ (or BC₄F₂) trees selected for resistance to *P. cinnamomi* with BC₃F₂ trees that have been selected for resistance to *C. parasitica*. The BC₃F₃ progeny would be evaluated for resistance to *P. cinnamomi* first; then, survivors would be evaluated for resistance to *C. parasitica*. Another generation of intercrossing and selection for resistance to both pathogens would be required to obtain selections that are homozygous for alleles that confer resistance to both pathogens. A challenge to this strategy is that it requires large numbers of progeny from each cross due to multigenic control of the resistances to both pathogens, and it is difficult to obtain large numbers of seed through controlled pollinations. We estimate that at least 200 BC₃F₃ progeny per cross would need to be evaluated to obtain one progeny with high levels of resistance to both pathogens.

A second breeding strategy is to outcross descendants of transgenic *C. parasitica*-resistant American chestnut trees with *P. cinnamomi*-resistant backcross trees. Collaborators at the State University of New York in Syracuse have developed a transgenic American chestnut tree that is resistant to *C. parasitica* by overexpressing the oxalate oxidase (OxO) gene from wheat (Zhang et al. 2013). When the transgenic trees are outcrossed with wild-type American chestnut trees, approximately 50% of the progeny that inherited the OxO gene have been demonstrated to have high levels of resistance after artificial inoculation with *C. parasitica* (Newhouse et al. 2014). By simplifying the genetics of *C. parasitica* resistance to a single transgene with a dominant effect, as few as 40 to 80 progeny per cross between a transgenic tree and a *P. cinnamomi*-resistant backcross tree may be sufficient to generate selections that inherited the OxO gene and major-effect *P. cinnamomi* resistance alleles. The inheritance of the

OxO gene can be detected without inoculation with *C. parasitica* by using an enzymatic assay to detect OxO activity in plant tissue (Zhang et al. 2013). Progeny that inherit the OxO gene will then be screened for resistance to *P. cinnamomi*. Surviving plants will be planted at orchard locations where *P. cinnamomi* already is present in the soil. Selections will be intercrossed to generate progeny that inherited both the OxO gene and *P. cinnamomi* resistance alleles in a homozygous state.

Currently, one transgenic insertion of the OxO gene into one American chestnut tree from New York is being considered for deregulated status by the U.S. federal government (W. Powell, personal communication). If regulatory approval is obtained, three generations of outcrossing the transgenic founder clone to wild-type American chestnuts and backcross trees will be required to expand the effective population size to >500 (Westbrook 2018b). Resistance to *C. parasitica* and *P. cinnamomi* will be combined in the final generation of outcrossing so that selection for OxO activity and *P. cinnamomi* resistance will be required only after the final outcross and first intercross generations (Westbrook 2018a). It is estimated that it will take 15 to 35 years from the time of federal regulatory approval to release transgenic trees to complete five additional generations of breeding (i.e., three outcross generations and two intercross generations) to combine resistance to these two pathogens within a genetically diverse population that is homozygous for resistance to both pathogens (Westbrook 2018b). The intermediate objective of combining resistance to these pathogens in a less genetically diverse population may be accomplished within 10 years of the release of transgenic trees. This objective will require one generation of breeding between transgenic trees resistant to *C. parasitica* and backcross trees resistant to *P. cinnamomi*, selection among the progeny, and intercrossing among the selections to generate large quantities of seed.

In conclusion, restoration of American chestnut trees depends on combining resistance to *C. parasitica* and *P. cinnamomi*, two pathogens that have effectively eliminated this tree species from forests in the eastern United States. Resistance must be combined within breeding populations that are sufficiently diverse so that the species may continue to evolve within its native range. Our results demonstrate that American chestnut backcross descendants of one of TACF's sources of resistance to *C. parasitica* also have inherited major-effect

Fig. 6. Genetic correlation of chestnut seedling survival within BC₃F₃ families inoculated with *Phytophthora cinnamomi* at different geographic locations: **A**, 44 Graves families (triangles) and 9 Clapper families (circles) were evaluated in trials conducted simultaneously by Clemson University and North Carolina State University (NCSU); **B**, 17 Graves families were evaluated in trials conducted simultaneously by Clemson University and The American Chestnut Foundation—United States Department of Agriculture Forest Service Resistance Screening Center (RSC). The least squares regression line $\pm$ 95% confidence interval (solid line) is compared with the 1:1 line (dashed line).

Fig. 7. Genetic correlation between survival after inoculation with *Phytophthora cinnamomi* and chestnut blight canker severity (best linear unbiased predictions [BLUP] values) among chestnut seedlings from 103 Graves BC₃F₃ families.

alleles for resistance to *P. cinnamomi*. Future selection for *P. cinnamomi* resistance in backcross populations and breeding these trees with *C. parasitica*-resistant backcross and transgenic trees will be used to produce trees with resistance to both pathogens.

Acknowledgments

We thank the members and volunteers of and the donors to The American Chestnut Foundation who have provided long-term financial support, moral support, and labor for the mission to restore the American chestnut to its native range; the many people who assisted with the Clemson University trials that were conducted in collaboration with Chestnut Return Farms over 14 years, including R. Baker, S. Barilovitz, S. Barilovitz, Jr., P. Bowers, R. Cain, E. R. Camacho, C. Colburn, C. Collier, D. Dlugos, D. Drechsler, L. Georgi, A. Gitto, B. Glenn, M. Greene, B. Harrelson, F. Hebard, T. Hodge, J. Hodges, J. Hwang, J. Jeffers, M. Jeffers, C. Kennedy, P. Laurie, L. Luszczyk, F. McLaughlin, I. Meadows, S. Oak, B. Paris, J. Payne, T. Perkins, E. Perry, C. Santos, S. Schreier, E. Schwartzman, C. Scott, J. Sevic, S. Sharpe, J. Vissage, Y. Wamishie, T. Watson, W. White, T. Zhebentyayeva, and D. Zwart; R. Blaedow, K. Frick, B. Jarrett, B. Minton, E. Schwartzman, and B. Williams at the USDA Forest Service Resistance Screening Center, who assisted with *Phytophthora* root rot evaluation; A. M. Braham at NCSU for supervising and conducting the inoculation procedure; and T. Allison, M. Escanfarla, W. Kohlway, Y. Kurt, J. Pearson, R. Porter, and C. Williams for assisting with inoculum preparation, inoculation, and phenotypic assessments

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