Identifying Host Resistance to *Phytophthora cinnamomi* in Hybrid Progeny of *Castanea dentata* and *Castanea mollissima*

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Additional index words. American chestnut, Chinese chestnut, ink disease, phytophthora root rot, germplasm conservation, disease resistance breeding, tree breeding

Abstract. *Phytophthora cinnamomi* Rands, the causal pathogen of phytophthora root rot (PRR) of chestnut, is one of the main obstacles to growth of American chestnut (*Castanea dentata* (Marsh.) Bork.) in the southern part of its distribution. To facilitate introgression of PRR resistance of Chinese chestnut (*C. mollissima* Blume) into a *C. dentata* genetic background, we assessed the disease resistance of 10 interspecific hybrid families derived from potentially resistant *C. mollissima* cultivars. Hybrid progeny were inoculated with *P. cinnamomi* in the nursery and assessed for root lesion severity after 1 year of growth. Asymptomatic plants were transplanted to a *P. cinnamomi*-positive orchard and evaluated for survival midway through the following growing season. During the nursery experiment, 8 of 10 hybrid families were not significantly different from susceptible *C. dentata* controls for average disease resistance scores. However, multiple asymptomatic individuals were identified in each of the eight families. Two of the 10 hybrid families were not significantly different from the resistant *C. mollissima* and *C. henyii* controls. In the *P. cinnamomi*-positive orchard, the prescreened hybrid families displayed a greater proportion of survivors than backcross families that had not been prescreened for *P. cinnamomi* resistance. Hybrid plants that have survived 2 years of growth in *P. cinnamomi*-infested potting media and soils represent an important step toward the production of genetically diverse chestnut populations in the southeastern United States that combine the PRR resistance of *C. mollissima* with the morphology and local adaptation of *C. dentata*.

PRR is a catastrophic disease of the three North American *Castanea* taxa: American chestnut (*C. dentata* (Marsh.) Bork.), Allegheny chinquapin (*C. pumila* (L.) Mill. var. *pumila*), and Ozark chinquapin (*C. pumila* (L.) Mill. var. *ozarkensis* (Ashe) Tucker) (Crandall et al., 1945; Jeffers et al., 2009, 2012; Sina et al., 2018). The principal causal pathogen, the oomycete *Phytophthora cinnamomi* Rands, is suspected to have been introduced to one of the coastal ports of the southeastern United States via ornamental plants from eastern Asia in the early 19th century (Anagnostakis, 2001; Crandall et al., 1945). Analyses of historical records have implicated *P. cinnamomi* in the widespread dieback of *C. dentata* and *C. pumila* var. *pumila* in the southeastern Coastal Plain and Piedmont during the 19th century (Anagnostakis, 2001; Crandall et al., 1945). *Phytophthora cinnamomi* has been isolated from dying chestnuts and chinquapins across the southeastern United States, as far north as Pennsylvania and as far west as Arkansas (Crandall et al., 1945; Fitzsimmons, 2016; Jeffers et al., 2009). Symptoms of PRR include necrosis of roots, leaf chlorosis, wilting of foliage, and branch dieback (Crandall et al., 1945; Jeffers et al., 2009). Unless symptoms are controlled with potassium phosphites or similar chemicals (James, 2011a), PRR will cause the death of susceptible plants 3 weeks to several years after the initial infection (Crandall et al., 1945; Jeffers et al., 2009). *Phytophthora cinnamomi* is one of the most serious obstacles to growth of *C. dentata* throughout its former range because of the prevalence of the pathogen in a variety of soils across the southeast and the fact that PRR is ultimately fatal to North American *Castanea* species.

In addition to PRR, the North American *Castanea* species are severely affected by chestnut blight, caused by the ascomycete *Cryphonectria parasitica* (Murr.) Barr (Alexander et al., 2005; Anagnostakis, 2001; Paillot, 1993, 2002). Since 1989, the American Chestnut Foundation (TACF) and collaborators have used the backcross method to introgress blight resistance from Chinese chestnut (*C. mollissima* Blume) into a *C. dentata* genetic background (Hebard, 2005; Westbrook, 2017). In a recent review of TACF’s backcross breeding program, Steiner et al. (2017) reported that progeny tests have validated experimentally expectations that a blight-resistant population of *C. dentata*-type trees can be constructed using introgression of extraspicific alleles for blight resistance. However, because *P. cinnamomi* was only recently recognized as an impediment to *C. dentata* restoration, TACF’s breeding program has not selected for PRR resistance. As a result, PRR has caused the failure of entire experimental orchards and forest progeny tests before advanced backcross trees could be assessed for chestnut blight resistance (Clark et al., 2014; Jeffers et al., 2009, 2012; Sisco, 2009).

In response to PRR-induced mortality of potentially blight-resistant hybrid seedlings, a screening program was initiated in 2004 to detect PRR-resistant seedlings in TACF’s backcross lines (James, 2011a, 2011b; Jeffers et al., 2009, 2012). Results of this screening program have indicated that PRR-resistant seedlings are present at low frequency in some, but not all, TACF advanced backcross lines (BC2-F5, BC3 F5, and BC5 F generations) that have been selected for blight resistance—most notably in lines derived from the blight-resistant hybrid cultivars Clapper and Graves (Jeffers et al., 2009). More recent resistance screening efforts showed that PRR resistance was also present in descendants of TACF’s third major source of blight resistance, *C. mollissima* ‘Nanking’ (Zhebentyayeva et al., 2014).

Studies of the quantitative trait loci (QTL) associated with resistance to *P. cinnamomi* have aided breeding efforts by demonstrating that this trait is controlled by a limited number of genomic regions and may therefore be a practicable target for marker-assisted selection (Santos et al., 2017; Zhebentyayeva et al., 2014). Santos et al. (2017) identified QTLs for PRR resistance on linkage groups B and K of Chinese chestnut (*C. crenata* Sieb. & Zucc.), and QTLs for PRR resistance were found on the same two linkage groups of *C. mollissima* (T.N. Zhebentyayeva, The Pennsylvania State University, personal communication). These findings suggest common resistance mechanisms to *P. cinnamomi* infection across different species within the genus *Castanea* (Santos et al., 2017). However, candidate genes within these QTLs have not been tested for their effects, and there exists the possibility that the East Asian *Castanea* species may have multiple alleles that encode PRR resistance. Until the genetic control of PRR resistance is better understood, additional sources of resistance are desirable for chestnut breeding programs in the eastern United States, because different *C. mollissima* and *C. crenata* cultivars may have different alleles encoding resistance. Efforts to identify multiple sources of PRR resistance are also important because they will facilitate the
introgression of different nut characteristics and other agronomically important traits into populations being tested for the commercial chestnut market. Because of the high susceptibility of *C. dentata* to both PRR and chestnut blight, the growth of *C. dentata*-type trees across large portions of the southeastern United States requires planting trees that have host resistance to both pathogens. The objective of this study was to evaluate PRR resistance in 10 hybrid families derived from crosses between susceptible *C. dentata* trees and potentially resistant *C. mollissima* cultivars in a containment system, followed by outplanting the least symptomatic plants into a *P. cinnamomi*-positive orchard and assessing their survival midway through the second growing season.

**Materials and Methods**

**Plant materials.** We used three approaches to generate hybrid progeny for PRR resistance screening. With the first approach, we performed controlled pollinations between interspecific *F*1 hybrids (*C. dentata* × *C. mollissima*) selected previously for high levels of resistance to chestnut blight (J.H. Craddock, unpublished data) and naturally occurring *C. dentata* trees from either Tennessee or Alabama, yielding eight first-backcross (*BC*1) families (Fig. 1A; Table 1). With the second approach, an *F*2 hybrid (*C. dentata* × *C. mollissima*) selected previously for blight resistance (J.H. Craddock, unpublished data) was open-pollinated by neighboring advanced backcross trees (either *BC*2-*F*1 or *BC*1-*F*2 generations) selected previously for chestnut blight resistance and several *C. dentata* morphological traits, yielding “better backcross” (*BB*1) progeny that we refer to as a family in this study (Fig. 1B; Table 1). All *F*1 hybrids in this orchard were male-sterile; thus, only the selected *BC*1-*F*1 and *BC*2-*F*1 plants could contribute pollen. The third crossing strategy is justified because the *BB*1 progeny could inherit blight resistance from two sources: TACF’s ‘Clapper’ line parent (i.e., paternal parent) and/or the *C. mollissima* cultivar used in our recent *F*1 cross (i.e., maternal parent). The site conditions of the *F*1 trees used for our *BC*1, *F*2, and *BB*1 crosses were of particular importance, because nearly all the *F*1 hybrids have exhibited several years of healthy growth in *P. cinnamomi*-positive orchards. The following *C. mollissima* cultivars were used as potential sources of resistance: ‘Amy’, ‘Byron’, ‘Gideon’, ‘Lindstrom 99’, ‘Nanking’, ‘Payne’, and ‘Petersburg’ (Table 1).

To perform controlled pollinations, we emasculated bisexual catkins and covered female flowers with corn pollinating bags about 1 week before anthesis. We collected staminate catkins from male parents, placed catkins on glass panes overnight to promote anther dehiscence, collected pollen in vials, and stored pollen at 3°C until pollination was performed. We applied pollen to female flowers using a plastic vial lid or glass microscope slide and placed pollination bags over female flowers until nuts were harvested. To detect unintended outcrossing, for every 10 flowering shoots that were hand-pollinated, we treated one flowering shoot as a no-pollen control. Seeds were harvested in Sept. 2014 and Oct. 2014, and were stored in slightly moistened peatmoss at 3°C through the winter. Chlorine tablets were added to the containment pools to kill *P. cinnamomi* in the irrigation effluent.

Seedlings were assessed for PRR symptom severity using the rating system developed by Jeffers et al. (2009). In January, we removed plants from their containers and gently washed the root systems to remove potting medium. We assigned symptom severity ratings to each of the plants using the root rot phenotyping system developed by Jeffers et al. (2009): 0 = healthy, no lesions observed on roots; 1 = lesions observed on at least one lateral root; 2 = lesions observed on the tap root; and 3 = severe root rot, plant dead.

**Orchard planting and survival.** In Spring 2016, plants in the lowest symptom severity category (plants rated 0) were planted in TACF’s Georgia Chapter’s Lake Allatoona Orchard (Allatoona, GA; lat. 34.1871 N, long. –84.7065 W), which was documented previously as *P. cinnamomi* positive. In Aug. 2016, survival was assessed of plants that had been prescreened for PRR resistance.

Data analysis. To evaluate the strength of PRR resistance in hybrid families compared with previous studies, we calculated a survival quotient (SQ) for each family, using the formula of Jeffers et al. (2009):

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\text{SQ} = \frac{(1 \times n_0) + (0.5 \times n_1) + (0.25 \times n_2)}{\text{Total number of seedlings} \times 100},
\]

where *n*0, *n*1, and *n*2 are the number of seedlings rated 0, 1, and 2, respectively. The SQ ranges from 0% to 100%. High SQ values indicate resistance whereas low SQ values indicate susceptibility.

Using the raw symptom severity data, the effects of treatment (family) and block, and interaction between treatment and block were modeled as a random-effects model with a two-way analysis of variance, and differences among families were evaluated with Duncan’s new multiple range test using the “easyanova” package (Arnholt, 2013) in R version 3.5.0 statistical software (R Core Team, 2018).
Results and Discussion

In the nursery trial, PRR symptoms and seedling mortality were observed in every interspecific hybrid family and the susceptible Castanea dentata control group, whereas no plant mortality or PRR symptoms were observed in the resistant Castanea mollissima and Castanea henryi control groups, as expected (Fig. 2). Three of the 13 Castanea dentata control plants exhibited no PRR symptoms at the end of the nursery trial, which indicates that some seedlings “escaped” infection during the first year of screening, probably because of uneven distribution of the pathogen across portions of the planting containers.

SQ values of hybrid families ranged from 56.5% to 89.6%. The susceptible control species, Castanea dentata, had the lowest SQ value (48.1%), whereas both resistant control species, Castanea mollissima and Castanea henryi, had the highest SQ values observed in the trial (100%) (Fig. 2). A comparison of our SQ values with those of Jeffers et al. (2009) revealed that all but one of our hybrid families had greater SQ values than backcross families screened in an earlier study (Jeffers et al., 2009). Only one BC1 family in our study, TN-TTU-A4 × ALCLEB04, had an SQ value (56.5%) similar to that of the most resistant cross reported by Jeffers et al. (2009): Hyko × JB575 (56.3%). One explanation for this difference is that all hybrid families studied by Jeffers et al. (2009) resulted from a greater number of backcrosses to the susceptible species, Castanea dentata, than any of the families evaluated in our study. Furthermore, parent trees in the crosses of Jeffers et al. (2009) had not been selected for PRR resistance. Specifically, Jeffers et al. (2009) screened BC2F1, BC3 F1, and BC4F2 progeny, whereas we screened eight BC1 families, one F1 family, and one BB1 family. Because PRR resistance in Castanea dentata is thought to be under the control of multiple genes (Santos et al., 2017), it would be expected that Asian American BC1 hybrids would retain more alleles for resistance than later generation backcrosses to Castanea dentata if the parent trees were not selected for PRR resistance at every generation. A second possible explanation is that the strength of selection in the study of Jeffers et al. (2009) was greater. For example, the environment in the planting containers of Jeffers et al. (2009) may have been more favorable for the proliferation of P. cinnamomi, or our inoculum may not have been entirely successful. The latter scenario would have resulted in an uneven distribution of P. cinnamomi in the tubs. The existence of three asymptomatic Castanea dentata does indicate that P. cinnamomi was not present uniformly within the tubs.

Analysis of variance of the nursery screening data indicated that the effect of treatment (family)
Analysis of variance also indicated a highly significant effect of block ($\alpha < 0.001$). Multiple range test indicated that 8 of 10 hybrid families had mean symptom severity that was not significantly different from the susceptible Castanea dentata controls (Fig. 3). Although the average symptom severity of these families was high, all eight families contained multiple seedlings that were asymptomatic at the end of the nursery experiment. These individuals were retained for outplanting in the P. cinnamomi-positive orchard.

Two of 10 hybrid families (TN-TTU-A4 × ALCLEB05 and TN-TTU-A4 × ALCLEB01) had average symptom severity that was not significantly different from the PRR-resistant species Castanea mollissima and Castanea henryi (Fig. 3). Both of these BC$_1$ families are descended from the resistant cultivar grandparents of Castanea mollissima, ‘Gideon’ (Table 1). Interestingly, a closely related family that shares the ‘Gideon’ source of PRR-resistance, TN-TTU-A4 × ALCLEB04, was not significantly different from the Castanea dentata susceptible control. There are a few possible explanations for these seemingly incongruous results. The first is that some variation in PRR resistance may exist among the pollen parents of these backcross families (i.e., the naturally occurring Castanea dentata trees ALCLEB01, ALCLEB04, and ALCLEB05). This seems highly unlikely, because previous studies did not find evidence of PRR resistance in Castanea dentata (Crandall et al., 1945; Jeffers et al., 2009). A more likely explanation can be attributed to the small number of trees in TN-TTU-A4 × ALCLEB01 and TN-TTU-A4 × ALCLEB05 (with 27 and 12 seedlings, respectively), and that some plants escaped infection, depressing the average symptom severity for these two families.

The trial in a P. cinnamomi-infected orchard was designed to test plants that had missed pathogen exposure in the nursery trial, as well as provide results informative of field resistance.

In the assessment of orchard survival, 101 of 144 prescreened BC$_1$ and BB$_1$ plants were alive after 8 months of growth in the P. cinnamomi-positive orchard in Aug. 2016. These 101 plants represent 70% of the 144 seedlings identified as least symptomatic in the 2015 nursery trials that were transplanted to the P. cinnamomi-positive orchard. In previous plantings at the Allatoona orchard, hybrid seedlings that had not been prescreened for PRR resistance experienced very low survival compared with PRR-resistant Castanea mollissima controls. As of Aug. 2016, for example, only 6 of 46 (13%) Castanea dentata seedlings, 5 of 40 (13%) nonscreened BC$_1$:F$_2$ seedlings, 71 of 338 (21%) nonscreened BC$_1$:F$_2$ seedlings, 10 of 71 (14%) nonscreened BC$_1$ seedlings, and 36 of 47 (77%) Castanea mollissima seedlings were alive after at least 1 year of growth in the P. cinnamomi-positive orchard. Because hybrid families that had not been prescreened for PRR resistance were planted in earlier years than our prescreened hybrid seedlings, a statistical analysis of these data would not have been appropriate. However, the fact that 70% of our nursery-screened hybrids were alive after nearly an entire growing season in a P. cinnamomi-positive orchard does suggest that our nursery trials were effective in reducing the number of susceptible seedlings planted in the field.

One of the major goals of the American chestnut restoration effort is to produce a population of advanced backcross trees with the chestnut blight resistance of Castanea mollissima, but with all other phenotypic traits of Castanea dentata. Researchers have pursued this goal in earnest since the early 1980s (Burnham, 1988); however, the need for host resistance to PRR in these populations only became apparent in 2003 (Jeffers et al., 2009). A population of trees with resistance to both chestnut blight and PRR would facilitate successful plantings of Castanea dentata-type hybrids in the southern portion of the American chestnut’s distribution, where PRR-induced plant failures have been most common (Jeffers et al., 2009).

As a first step toward producing populations of PRR-resistant plants for the American Chestnut Foundation’s breeding program in the southeastern United States, we have generated PRR-resistant seedlings in hybrid families derived from multiple sources of resistance. Although hybrid derivatives of Castanea mollissima ‘Nanking’ have been assessed previously for PRR resistance (Zhebentyayeva et al., 2014), our study represents the first attempt to identify PRR resistance in descendents of the following Castanea mollissima cultivars: Amy, Byron, Gideon, Lindstrom 99, Payne, and Petersburg. Of the progeny challenged with P. cinnamomi in our study, seedlings derived from the Castanea mollissima cultivars Amy, Byron, Gideon, Nanking, and Petersburg are still alive and growing in a P. cinnamomi-positive orchard as of Nov. 2018. In addition to the host resistance to PRR identified here, the cultivar grandparents of...
the hybrid progeny also have some desirable agronomic and culinary traits, including high average nut mass, high yield, and superior taste (Metaxas, 2013). Consequently, the hybrid progeny screened here may also be of interest to plant breeders with a focus on chestnut as a nut crop for the southern United States.

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