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Nursery performance of American and Chinese chestnuts and backcross generations in commercial tree nurseries

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The American chestnut [*Castanea dentata* (Marsh.) Borkh.] was decimated by an exotic fungus [*Cryphonectria parasitica* (Murr.) Barr] in the early 1900s. Breeding efforts with American and Chinese chestnuts (*C. mollissima* Blume) produced putatively blight-resistant progeny (BC_3F_3) in 2007. We compared two nut size classes for differences in seedling quality of bare-root stock grown in commercial nurseries. We compared the BC_3F_3 generation to parental species and other generations. Nuts in the large size class produced taller trees than nuts in the small size class, but sizing nuts prior to sowing did not reduce variability in nursery seedling size. Results indicate that overall seedling quality could be improved by culling small nuts, but seedling uniformity would only be improved by culling seedlings before planting. We recommend refinement of restoration efforts to match seedling size to site type and planting goals. BC_3F_3 chestnuts differed from Chinese chestnuts in 67% of tests, and were different than American chestnuts in half the tests, indicating not all American traits were recovered in this early phase of seedling development. Family differences within the BC_3F_3 generation were most apparent for mean nut weight, and only one BC_3F_3 family differed from other BC_3F_3 families in seedling growth characteristics.

Introduction

The American chestnut [*Castanea dentata* (Marsh.) Borkh.] was one of the most abundant and important tree species of the eastern deciduous forest of North America for thousands of years, until decimated by chestnut blight, a disease caused by an exotic fungus [*Cryphonectria parasitica* (Murr.) Barr].[1] The fungus probably arrived on imported Japanese chestnut [*C. crenata* Siebold & (Zucc.)] nursery stock in the late 1800s,[2] and spread rapidly, killing mature trees and reducing chestnut populations throughout the natural range to recurring sprouts in the forest understorey by the mid-twentieth century.[1] To date, efforts to restore American chestnut have largely included development of blight-resistant material through traditional breeding techniques with resistant Chinese (*Castanea mollissima* Blume) or Japanese chestnut,[3–6] biological control through hypovirulence of chestnut blight,[7,8] breeding low-to-moderate levels of resistance using pure American parents,[9] and early steps towards genetic transformation.[10,11]

The American Chestnut Foundation (TACF) is a non-profit organization attempting to restore this species using a backcross breeding programme that was first initiated in the mid-1980s, but was based on previous decades of trial and error using various lines of resistance.[5,12] In theory, the first putatively blight-resistant generation, the BC_3F_3 generation, is 94% American chestnut, 6% Chinese chestnut and is predicted to have the desired phenotypic characteristics of the American chestnut parent while maintaining blight resistance of the Chinese chestnut parent.[5] After several decades of breeding work in the early twentieth century that ultimately failed,[12] and then nearly three decades of using a backcross breeding technique, TACF orchards produced sufficient material for field testing of the BC_3F_3 generation in 2007. In addition to assessing blight resistance, a vital research problem in chestnut restoration is to determine whether seedlings will recover American chestnut growth and morphology or if they will carry traits that resemble the Chinese parental species.[13] American chestnuts are the preferred species for growth form and nut production when compared with the Chinese species that has a non-timber form (i.e. low branching with no clear leader, short height growth), less desirable nuts for human consumption and is generally not competitive in a natural forest planting.[3,14,15] Recovery of American genes for desired growth and fruiting characteristics will be important to long-term restoration goals.

Successful restoration of Fagaceae species through artificial regeneration requires improved seedling quality at planting to overcome vegetation competition and animal browse pressure.[16–18] The relationship between seedling quality and seedling response to silvicultural treatments and site quality has only received limited testing with American chestnut,[19–22] despite the fact that American chestnuts have been grown in nurseries and planted since colonial days in the USA.[23] No experiments have tested American chestnut growth in the nursery using pedigreed material in a replicated design, and there has been no research on seedlings from the BC_3F_3 generation, due to lack of availability. A single study has tested the effects of nursery seedling quality on field survival and growth of American chestnut.[22] They found that chestnut could grow to a large size in the nursery (>1 m), had high variability in growth within and among genetic families and also had strong correlations between root-collar diameter (RCD) growth and other growth variables.

We can use previous studies on Fagaceae species, such as oak, *Quercus* L., to make inferences regarding American chestnut response to nursery and silvicultural practices. This assumption is based on the similar growth strategies exhibited by chestnut and oak in greenhouse studies,[24] and the close phylogenetic relationships between the two genera.[25] Most research shows that oak seedling development was positively related to acorn size or weight[26–28] particularly when genetic parameters were controlled.[29,30] However, some research did not support intraspecific relationships between acorn size and seedling height[31] or it showed differences too small to be of practical use.[32]

Seedling quality at planting will affect subsequent field performance, specifically seedlings larger in height and RCD generally outperform smaller seedlings after several years in the field.[33–35] Increasing numbers of chestnuts bred for blight resistance will become available,[5] and understanding how to improve seedling quality at planting will become paramount for successful restoration. Restoration of American chestnut will require planting of the species throughout its native range, a large extent consisting of medium to high-quality sites that will have deer-browsing (*Odocoileus virginianus*) pressures and fast-growing competing vegetation of native species.[36–38] Production of high-quality and uniform seedlings for planting with growth advantages over natural competition and browse pressure will ultimately improve efficiency and success of forthcoming reintroduction efforts. To date, testing seed size effects on nursery seedling development and testing for variation in growth parameters within and among genetic breeding lines of American chestnut and hybrids bred for blight-resistance have not been conducted.

We used experimental material from several genetic families within American and Chinese parental species and within various breeding generations, including the first putatively blight-resistant generation produced by TACF (BC_3F_3). It is also important to identify genetic differences within the BC_3F_3 generation as a way to maximize growth potential of the species, as has been shown with other hardwoods,[39] and to identify families that are retaining undesirable Chinese characteristics. The genetic material used in this study was limited due to the relatively low numbers of nut-producing mother trees at TACF orchards. Despite the limitations in available genetic material, the nuts used in this study reflect 100 years of chestnut breeding efforts by the United States Department of Agriculture, the Connecticut Agricultural Experiment Station and the TACF[3,5,12,40] to produce blight-resistant American chestnuts. This study represents the first examination of morphological differences within and among families of the BC_3F_3 generation and the first to compare the BC_3F_3 generation to the parental species and other less advanced generations.

The goal of this study was to characterize high-quality chestnut seedlings that have been bred for blight resistance and to examine cultural practices that will improve seedling quality and reduce seedling variability prior to planting in the field. Our objectives were to test chestnut breeding material in commercial forest nurseries to: (1) to determine effect of nut size class on nursery seedling quality, (2) test for similarities and differences in growth characteristics among the different breeding generations and parental species and (3) to test for growth differences among genetic families of the BC_3F_3 generation.

Materials and methods

Experimental material

The experimental material for this study came from TACF's research orchards in Meadowview, VA, USA.[5] Nuts were collected in September 2007 and in September 2008, stored for ~4 months in slightly damp sphagnum moss at 3°C in closed plastic bags, 2 ml in thickness and sowed on 16 January 2008 and 29 January 2009. For this study, a genetic family is defined as progeny from a single open-pollinated mother tree in the orchard. We assume that progeny are a result of crosses between female (known genetic identity) and male (unknown genetic identity) parents within the respective orchard; we recognize that pollen from outside the orchard could contaminate mother trees, but pollen contamination events are probably rare because seed orchards of each generation are isolated.[12] Hereafter, the term breeding generation refers to the specific series of crosses and intercrosses using the blight-susceptible American chestnut and blight-resistant Chinese chestnut, and parental species refers to the American or Chinese chestnuts. For this study, we used 24 families from two parental species and four breeding generations (Table 1).[5] One Chinese, two BC_1F_3, two BC_2F_3 and one BC_3F_3 families (D1) had adequate seed availability during both years of seed collection; the remaining 18 families produced nuts for only 1 year of seed collection (Table 1).

We separated nuts from each family into two equally divided size classes, large and small, by visually assessing the nut size. We split each size class into two equally divided replications by number and weight to the nearest 0.1 g. The 2007 seed crop was sown at the Georgia Forestry Commission's Flint River Nursery near Byromville, GA, USA. The 2008 seed crop was similarly sown at the East Tennessee State Nursery in Delano, TN, USA. Hereafter, seedlings will be referred to by the US postal abbreviation for the nursery in which they were grown [Georgia nursery (GA) or Tennessee nursery (TN].

Experimental design

We used a nested, split-plot treatment arrangement to determine whether nursery (or year nuts were collected), generation/parental species, genetic family and nut size class affected nut weight and seedling growth. We used a randomized complete block design with sampling to arrange the experimental units for each nursery. Nursery was a fixed treatment factor, and generation was a fixed treatment factor nested within nursery because different generations were sown at each nursery. Family was a fixed whole plot factor nested within generation because we used different families within each generation. The nut size class was a fixed split-plot factor within family. Individual nuts or

Table 1. Number of nuts sown and seedlings lifted for each breeding generation/parental species and family at each nursery and year of nut collection.

	GA nursery (nuts collected 2007)					TN nursery (nuts collected 2008)				
Parental species or generation	Family	Number of nuts sown	Number of seedlings	Number of replications of nut size class seed lots	Survival (%)	Family	Number of nuts sown	Number of seedlings	Number of replications of nut size class seed lots	Survival (%)
100% American	GMNew	153	146	2	95	Bell Hollow	152	135	2	89
	PL1S	151	140	2	93	High Knob	148	99	2	67
	Towers1	51	51	1	100	Plummer2	48	44	1	92
100% Chinese	CD	277	192	2	69	CD	132	0	0	0
BC_1F_3 (75% American)	NB1	149	140	2	94	NB1	98	56	1	57
	NB35	151	139	2	92	NB35	108	56	1	52
BC_2F_3 (88% American)	SA330	158	135	2	85	SA330	149	103	2	69
	SA417	152	127	2	84	SA417	150	86	2	57
BC_3F_2 (94% American)						CH283	152	93	2	61
						CH526	102	31	1	30
BC_3F_3 (94% American)	D1	51	42	1	82	D1	150	93	2	62
	D2	152	91	2	60	D6	146	25	1	17
	D3	49	44	1	90	D7	50	46	1	92
	D4	148	131	2	89	D8	98	58	1	59
	D5	147	119	2	81	D9	150	108	2	72
						D10	100	63	2	63
						D11	99	23	1	23

nursery seedlings within the nut size class seed lot were samples. All nut size class treatments within each family were replicated twice, except for some families that could only have one replication due to lack of available material (Table 1). Each of the two replications was grouped into a block to account for environmental differences within the nursery seed beds (e.g. slope of the ground, distance from watering source). Nursery was analysed as a fixed effect in this model because we did not choose nursery from a random sample of available nurseries. We chose them based on our experiences with these two particular nurseries using protocols that favoured growth of high-quality hardwood seedlings for planting.[41,42] A total of 46 seed lots, each representing a distinctive generation/parental species, family, nut size class and replication/block were sown by hand at the GA nursery. Forty-eight seed lots were similarly sown at the TN nursery.

Laboratory and field methods

Prior to sowing, we recorded the weight of each individual nut that was to be sown to the nearest 0.1 g. Nuts had not yet germinated at the time of sowing at both nurseries. At both nurseries, chestnuts were sown at a density of 65 per m^2 in prepared seed beds consisting of four rows, 15 cm apart. To reduce variability in available sunlight reaching each seedling, only the inner two rows were sown with chestnuts and the outer two rows were sown with white oak (*Quercus alba* L.) for the 2007 nut crop and with Nuttall oak (*Q. nuttallii* Palmer) for the 2008 nut crop. Seed lots were separated by 0.8 m. Chestnuts and acorns were covered with ~3 cm of pine bark mulch immediately after sowing.

Nursery beds were fertilized from May through August of each year according to prescriptions developed by Kormanik *et al.*[41] and irrigated as needed. At each nursery, the seedlings were grown for one growing season and lifted in the following February. Seedlings were undercut to a depth of 30 cm and lifted using a Fobro machine lifter. In May 2009 at the East Tennessee Nursery, we noticed a complete lack of germination in all of the Chinese seed lots, which we attribute to seed desiccation. We also noticed symptoms of disease caused by *Phytophthora cinnamomi* in portions of the nursery beds for seedlings at the East Tennessee Nursery in June 2009. This fungal pathogen is widespread in the southeastern USA, and can destroy chestnut nursery seedlings and chestnut plantings in years with conducive weather and/or site conditions.[22,43,44] Symptoms included chlorosis or wilting of leaves with associated black legions on root systems.[43] All seedling beds were subsequently treated with a fungicide, aluminium tris(*O*-ethyl phosphonate) (Aliette WDG), at a rate of 2.2 kg ha^{-1} in early June 2009 to control for the disease. Nursery personnel destroyed a few diseased trees within the bed during the growing season. Less than 10 trees in total were removed and impacts on the overall study were judged to be minimal.

After lifting, we measured seedlings for total height (nearest 1 cm) from the root collar to the top of the tallest terminal bud. The root collar is defined as the transition zone between the above-ground and below-ground portion of the stem at the ground-line of the seedling. We measured RCD (nearest 0.1 mm), using digital calipers, and we counted the number of first-order lateral roots (FOLRs). An FOLR is defined as a lateral root stemming from the main tap root that is at least 1 mm at the proximal end. The same individual counted roots on all seedlings from both nurseries to reduce bias in FOLR counts. Measuring the proximal end of each lateral root to ensure it meets the minimum size requirement of 1 mm is impractical; therefore, the FOLR counts can be subjective if different individuals assess the root systems.

We noted whether seedlings lacked a primary tap root, a morphological characteristic that results in a non-typical root system. We also noted whether seedlings had stem forks in order to test genetic and nut size class effects of this phenotypic characteristic. A fork was defined as a lateral stem beginning at or near the root collar and extending at least half the length of the main stem.

Statistical analysis

We did not include diseased or damaged seedlings in any statistical analyses, and we excluded seedlings with missing tap roots in statistical analysis for the number of FOLR. We conducted *t*-tests to determine whether pre-treatment differences existed between replications in mean nut weight for each size class within a family. We did not include the TN nursery Chinese nuts or seedlings in the analysis due to complete lack of germination of this family. We used mixed model ANOVA[45] and DanDA.sas macros[46] to compare treatment means, and tests of significance were reported at $\alpha \leq 0.05$, unless otherwise noted. Degrees of freedom were adjusted using the Kenward–Roger method. Normality and equal variance assumptions of residuals were tested using the Shapiro–Wilk test for normality and by examining plots of residuals. Square-root transformations were used and unequal variance was added to the model using the REPEATED statement when needed. A likelihood ratio test was used to test whether the unequal variance model was justified. For all analysis of variance models, we computed comparisons among the least-squares means using Tukey's mean separation method if main effects or interactions were significant.

Pearson correlation coefficients (PROC CORR) were computed among height, RCD, number of FOLR, occurrence of missing tap root and occurrence of stem forking.

We used indicator variable regression (PROC GLM) to determine whether the mean nut weight of the seed lot could be used to predict seedling height, RCD and the number of FOLR. We ran three regression models for each dependent variable to determine whether breeding generation/parental species, nursery and nut size class affected the relationship between nut weight and the dependent variable.

Logistic regressions (PROC LOGISTIC) were used to determine whether the probability of having a missing tap root and stem fork was influenced by nursery, generation/parental species or nut size class. Significant predictor variables were selected for inclusion in the final model and a Hosmer–Lemeshow goodness-of-fit test was used to test whether the logistic regression model accurately described the data.

Results

Survival and nut weight

Pre-sowing differences between replications in nut weight were not significant ($P \geq 0.10$), demonstrating that bias did not occur in splitting nut size classes into two replications. All families except CH526, D6 and D11 had >50% survival (Table 1). Chinese seed lots at the TN nursery did not germinate, so they were not included in the estimates of survival. Seedlings at the GA nursery had 87% survival, and seedlings at the TN nursery had 57% survival overall. American families had the best overall survival at both nurseries; BC_3F_3 families D6 and D11 had the lowest survival.

The nut weight was highly variable, ranging from 1.0 to 17.7 g (Table 2). All main effects and their interactions for the nut weight were significant sources of variation (Table 3), indicating that we were successful at visually distinguishing two size classes of nuts that differed in weight, and that generation/parental species and family differences existed. Nuts in the large size class weighed 1.2–2.0 g more than nuts in the small size class, for the 2008 and 2007 nut collections, respectively (Table 2). Interactions were because small nut size classes of the Chinese parent and BC_1F_3 generations/families had heavier

Table 2. Descriptive statistics (standard errors in parentheses) of nut weight and seedling growth characteristics and least-squares mean differences between nut size classes and nursery/year of nut collection.

	Nut size class				Nut size class			
	GA nursery (nuts collected 2007)				TN nursery (nuts collected 2008)			
	Overall mean	Range	Large	Small	Overall mean	Range	Large	Small
Nut weight (g)[1]	6.0 (0.03)A	1.2–17.7	7.0 (0.04)a	5.0 (0.04)b	3.9 (0.02)B	1.0–9.8	4.5 (0.03)a	3.3 (0.02)b
Height (cm)	92 (0.09)A	17–198	96 (0.09)a	91 (0.09)b	135 (0.09)B	10–262	137 (0.09)a	130 (0.09)b
RCD (mm)	13.4 (0.3)A	3.2–30.0	13.8 (0.19)a	13.1 (0.19)a	16.3 (0.3)B	2.0–31.4	16.7 (0.38)a	15.9 (0.39)a
FOLR number	18 (0.005)A	0–48	18 (0.006)a	18 (0.006)a	15 (0.006)B	0–47	15 (0.008)a	14 (0.008)a
Missing tap root (percent)[2]	13 (0.82)	–	14 (1.24)	11 (1.19)	13 (1.01)	–	13 (1.39)	13 (1.5)
Stem forking (percent)	12 (0.82)	–	13 (1.20)	10 (1.13)	5 (0.63)	–	4 (0.81)	6 (1.00)

Means followed by same upper case letter are not significantly different between nurseries. Means followed by same lower case letter are not significantly different between nut size classes within a nursery/year of nut collection.
[1]Least-squares means were calculated for nut weight, height, RCD, and FOLR number.
[2]Raw means were calculated for missing tap root and stem forking.

Table 3. Analysis of variance used to determine differences among nurseries, breeding generations/parental species, genetic families, nut size class and their interactions for nut weight, height, RCD and number of FOLRs.

Source of variation	Nut weight		Height		RCD		Number of FOLR	
	F-statistics	*P*-value	*F*-statistics	*P*-value	*F*-statistics	*P*-value	*F*-statistics	*P*-value
Nursery	3077.44	<0.001	21.19	0.044	56.36	0.017	18.47	0.050
Generation (nursery)[1]	1191.13	<0.001	30.60	0.027	4.53	0.005	1.45	0.250
Family [generation (nursery)]	294.12	<0.001	3.00	0.015	1.75	0.131	1.48	0.216
Size(nursery)	1074.30	<0.001	8.74	0.003	30.50	0.074	0.99	0.393
Size × generation(nursery)	65.16	<0.001	0.84	0.584	0.58	0.781	1.51	0.227
Size × family[generation (nursery)]	8.83	<0.001	1.72	0.132	0.91	0.582	1.42	0.238

[1]Generation denotes both breeding generation and parental species.

mean nut weights than large nut size classes of other generations/families (Figure 1).

Nuts from the Chinese and BC_1F_3 generations had significantly heavier weights and were more variable in weight compared with other generations (Table 4). Nuts from the BC_3F_3 generation were different in weight than the American parental species, but the trend depended on the year seed was collected. The BC_3F_3 generation had significantly lower weight than both parental species and all generations for nuts collected in 2007, but had the second highest weight compared with other generations and parental species, including the American parent, for nuts collected in 2008. All generations and parental species with two or more families exhibited family differences in the mean nut weight.

Approximately 57% of the variation in seedling height could be explained by nut weight and its interaction with nursery ($P<0.001$), but the slope of this regression line for the TN seedlings was not significantly different from zero. Using the GA nursery data, the regression model predicted an increase of 3 cm height for every 1 g increase in the mean nut weight ($P < 0.0001$, $R^2 = 0.57$, Figure 2a). The mean nut weight could not be used to explain variation in nursery seedling height when using generation/parental species or nut size class as an interaction term in the regression models. None of the regression models had a significant relationship between the mean nut weight and RCD. Slope interaction terms between mean nut weight and nursery, generation/parental species, and size class were not significant in explaining the variation in the number of FOLR. Pooled data for the mean nut weight, however, did explain the variation in the number of FOLR (Figure 2b; $P = 0.0002$). For every 2 g increase in the mean nut weight, seedlings were predicted to gain one FOLR, although this model had low predictive power ($R^2 = 0.14$).

Seedling growth and morphology

Seedlings were highly variable in size, ranging from 10 to 262 cm in height, from 2.0 to 31.4 mm in RCD and from 0 to 48 in FOLR number (Table 2). The strongest correlation coefficients were between height and RCD, and correlations between height and number of FOLR and between RCD and FOLR number were slightly lower (Table 5). For the TN seedlings, height and RCD

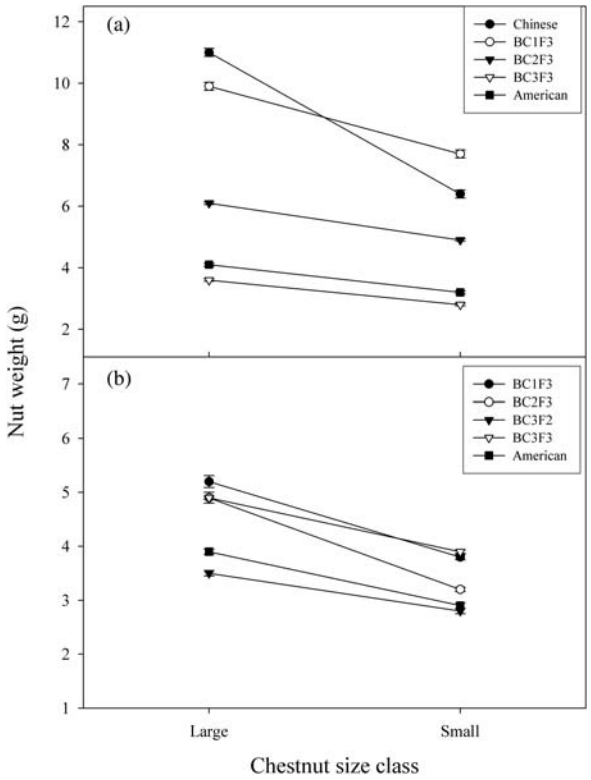

Figure 1 Nut weight and standard error bars of each generation/parental species and nut size class for the GA nursery (a) and the TN nursery (b).

had weak and negative correlations with missing tap root occurrence, and height had a weak and negative correlation with stem forking. For both nurseries, stem forking had weak, but positive relationships with missing tap root and with the FOLR number.

Interactions with nut size class were not significant for generation/parental species or family effects for any seedling growth variable (Table 3). Large nut size classes had 5–7 cm taller heights than small nut size classes, depending on nursery (Table 2). Large and small nut size classes had similar standard errors and ranges in seedling heights, and the largest seedling in the study originated from a nut classified as small (Figure 3). RCD and number of FOLR were not affected by nut size class, but RCD differences were approaching significance (Table 3).

Generation/parental species affected height and RCD growth, but not number of FOLR (Table 3). Rankings of generation/parental species depended on the nursery in which the seedlings grew (Table 4). The BC_3F_3 generation had smaller heights compared with the American parent at the TN nursery. At the GA nursery, however, all generation/parental species had similar heights to the American parent, except the BC_1F_3 generation, which was taller. At the GA nursery, the BC_3F_3 generation and the American parent were smaller in RCD than the Chinese and BC_1F_3 generation seedlings. American chestnuts and BC_3F_3 generations were similar in RCD at both nurseries.

Family differences were significant for height, but not for RCD or number of FOLR (Table 3). BC_3F_3 families did not have significant differences in height growth at the GA nursery (Table 4). The largest family difference at the TN nursery for any generation/parental species was between BC_3F_3 families D10 (168 cm) and D6 (79 cm). In fact, D6 was smaller in height than every other BC_3F_3 family at the TN nursery, and no other family differences within this generation was significant at either nursery. Family distributions of height indicated that uniformity within a family was similar for both large and small size nut classes (Figure 3). Although the interaction between nut size class and family was not significant, it did appear that some families had more trees with taller heights in small nut size classes than in large nut size classes (Figure 3, D9 family).

In the logistic regression analysis, the nut size class did not affect the chances in stem forks or missing tap roots, and this term was removed from the final regression model. The model fit was good according to the Hosmer–Lemeshow goodness-of-fit test statistic ($P > 0.30$ for stem forks and $P > 0.14$ for missing tap roots). Chinese chestnut and BC_1F_3 generation seedlings had the highest odds of stem forking and missing tap roots compared with the BC_3F_3 generation seedlings (Table 6). American chestnut seedlings had significantly lower odds than BC_3F_3 generation seedlings of stem forks (1.9 times) and missing tap root (3.1 times). If the model was conducted using Chinese chestnut seedlings as the missing dummy variable, all other generation/parental species had significantly lower odds of having stem forks, and all other generation/parental species, except BC_1F_3 had significantly lower odds of having a missing tap root.

Discussion

Seedling quality was high for both nurseries according to hardwood standards developed for planting in even-aged silvicultural harvests.[47,48] In this study, culling smaller sized nuts prior to sowing and/or culling smaller sized seedlings after lifting would have further improved the overall seedling quality. Specific recommendations on minimum nut size for each parental species and breeding generation cannot be made without confirmation and refinement of our findings using additional families grown at several nurseries.

The number of FOLR tended to increase linearly with increasing nut weight, as indicated by the regression models; however, the large nut size class did not have more roots than the small nut size class. Undercutting roots at lifting may have artificially minimized differences between the two nut size classes; however, if we assume small and large size nuts produce similar root system architecture (e.g. one size class would not produce more roots below the point of undercutting than the other size class), then our lack of differences may be real. Most studies had positive relationships between seed size/weight and root development in Fagaceae species,[26,27,31] but this relationship has not been well studied for chestnut. More controls for seed moisture and seed damage may help refine the relationship between nut size and root development.[31] Improvement in root biomass for this species warrants further investigation because a well-developed root system can improve a seedling's ability to withstand drought during the early years of establishment and the seedling's ability to survive under shade.[28,49,50] We propose that improvement in root biomass of chestnut species may only be achieved through nut grading on a weight basis, which may be impractical for large-scale reforestation efforts.

Table 4. Least-squares means differences (standard error in parentheses) in nut weight, height and RCD for each breeding generation/parental species and genetic family within each nursery/year of nut collection.

Nursery and year of nut collection	Generation or parental species	Nut weight mean	Height mean	RCD mean	Family	Nut weight mean	Height mean
GA, 2007	Chinese	8.7 (0.09)A	100 (0.22)DEF	15.3 (0.74)BCD	CD	8.7 (0.09)B	100 (0.22)GHIJ
GA, 2007	BC1F3	8.8 (0.09)A	104 (0.15)DE	14.1 (0.55)CD	NB1	8.6 (0.13)B	102 (0.22)GHIJ
GA, 2007					NB35	9.1 (0.13)A	105 (0.22)FGHIJ
GA, 2007	BC2F3	5.5 (0.03)B	90 (0.15)EF	13.5 (0.55)DE	SA330	4.7 (0.04)G	92 (0.10)HIJKL
GA, 2007					SA417	6.3 (0.04)C	87 (0.22)IJKL
GA, 2007	BC3F3	3.2 (0.03)H	91 (0.12)EF	12.5 (0.44)EF	D1	2.6 (0.08)N	94 (0.41)GHIJKL
GA, 2007					D2	3.4 (0.04)K	92 (0.23)HIJKL
GA, 2007					D3	3.7 (0.08)J	103 (0.41)EFGHIJK
GA, 2007					D4	3.7 (0.05)J	89 (0.22)IJKL
GA, 2007					D5	2.5 (0.05)N	78 (0.22)KL
GA, 2007	American	3.6 (0.03)F	82 (0.14)F	11.8 (0.53)F	GMNew	5.0 (0.04)F	94 (0.22)HIJK
GA, 2007					PL1S	3.3 (0.04)K	88 (0.22)IJKL
GA, 2007					Towers1	2.6 (0.08)N	67 (0.40)L
TN, 2008	BC1F3	4.5 (0.06)C	114 (0.24)CDE	14.7 (0.79)BCD	NB1	5.2 (0.06)E	116 (0.40)CDEFGHI
TN, 2008					NB35	3.9 (0.11)IJ	113 (0.40)DEFGHI
TN, 2008	BC2F3	4.0 (0.05)E	144 (0.15)AB	17.5 (0.58)A	SA330	3.9 (0.04)IJ	163 (0.23)A
TN, 2008					SA417	4.2 (0.10)H	125 (0.24)BCDEFG
TN, 2008	BC3F2	3.2 (0.04)H	127 (0.20)BCD	15.7 (0.74)ABC	CH283	3.0 (0.04)L	135 (0.23)ABCDEF
TN, 2008					CH526	3.3 (0.05)K	118 (0.43)BCDEFGHI
TN, 2008	BC3F3	4.4 (0.02)D	133 (0.12)BC	16.1 (0.44)AB	D1	2.8 (0.04)M	145 (0.23)ABCD
TN, 2008					D6	3.9 (0.05)I	79 (0.35)JKL
TN, 2008					D7	6.2 (0.08)C	154 (0.41)ABC
TN, 2008					D8	5.7 (0.06)D	143 (0.40)ABCDE
TN, 2008					D9	4.3 (0.04)H	127 (0.23)BCDEFG
TN, 2008					D10	3.5 (0.05)K	168 (0.39)A
TN, 2008					D11	4.2 (0.06)H	127(0.52)ABCDEFGH
TN, 2008	American	3.4 (0.03)G	151 (0.14)A	17.4 (0.55)A	Bell Hollow	4.2 (0.04)H	137 (0.22)ABCDE
TN, 2008					High Knob	2.5 (0.05)N	153 (0.23)AB
TN, 2008					Plummer2	3.4 (0.08)K	164 (0.41)A

Means followed by same letter within each breeding generation and family are not significantly different.

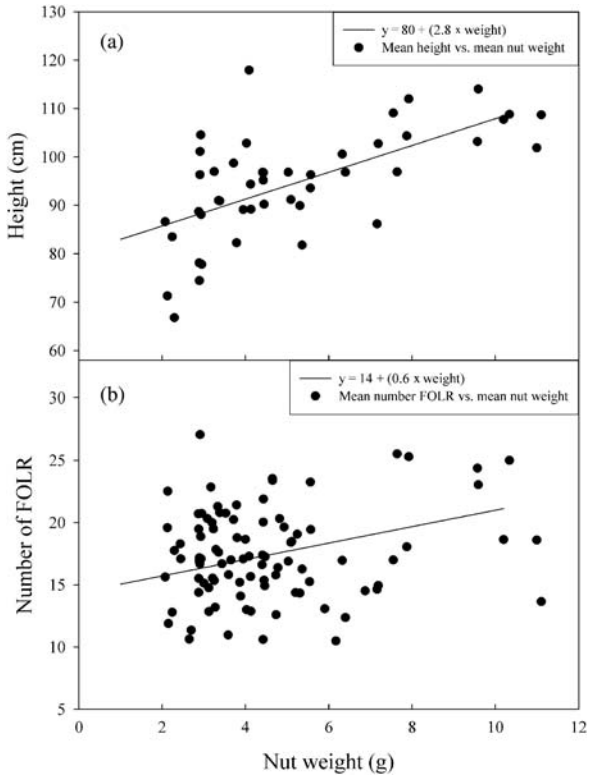

Figure 2 Linear regression of mean seedling height versus mean nut weight for GA nursery seedlings (a) and of mean seedling FOLR number versus mean nut weight for all seed lots (b).

Table 5. Pearson correlation coefficients for chestnut height, RCD, number of FOLRs and occurrence of missing tap root (MTR) and stem forking for each nursery.

	GA nursery	TN nursery
Height–RCD	0.80[1]	0.79[1]
RCD–FOLR	0.56[1]	0.67[1]
FOLR–height	0.57[1]	0.58[1]
Height–MTR	0.01	−0.15[1]
Height–Fork	0.04	−0.09[2]
RCD–MTR	0.02	−0.13[1]
RCD–Fork	0.01	−0.04
FOLR–Fork	0.22[1]	0.07[2]
MTR–Fork	0.09[1]	0.09[2]

[1]Coefficient was statistically significant ($P \leq 0.0001$).
[2]Coefficient was statistically significant ($P \leq 0.05$).

Our results are in agreement with research conducted on Chinese chestnut that found seedling height differed among nut size classes that were visually distinguished.[51] Oak species have also shown positive relationships between height and seed size,[27,28,30,32] but this relationship has not been previously tested in *Castanea* species of North America. Since chestnut has been shown to have an intermediate shade tolerance, with the best growth in full-sun conditions,[20,49,52] successful field establishment will require seedlings to remain competitive with the fastest growing natural vegetation on site. Additionally, hardwood seedlings that are already taller than deer browse height (~1.3 m) will have a significant advantage over small seedlings if planted in areas with high deer populations.[17] The preliminary results of chestnut test plantings using seedlings from this study indicate deer preferred to browse on shorter seedlings.[53]

The results concurred with past studies of northern red oak (*Quercus rubra* L.) that found seed weight can affect nursery height and this relationship appears to be under genetic control.[29,30] Too few families were studied to calculate genetic gains or heritability in this study, but a previous study noted that the nut size in F_1 hybrid chestnuts was heritable.[3] Similar to our results, Detlefsen and Ruth[16] found that parental trees of American chestnut were visually different in nut size and shape, and they found that first-generation hybrids with Japanese chestnuts had the largest variation. Our results indicate that genetic family will have the strongest effect on nut weight and, to a lesser extent, seedling height. Identification of BC_3F_3 families with superior growth was not possible in this study due to too few differences among families within this generation and too few families studied. Family differences may be inconsequential at this early stage, compared with differences due to seed sizes or other unknown elevation or geographic influences.[29,30]

Small nut size may be partially dominant over large nut size in F_1 hybrid chestnuts,[3] and nut size may be controlled by multiple genes.[54] A potential problem could arise if selecting for larger size nuts in the B_3F_3 generation leads to indirectly selecting for seedlings that have retained other Chinese chestnut morphological traits. Studies have not been conducted in determining whether nut size/weight is linked to other genes found in Chinese chestnut (e.g. blight resistance, non-timber form).

Increasing seedling uniformity at planting is desired in addition to improving overall seedling size; if seedlings can be grown to a uniformly adequate size, then predictions of planting success and planting efficiency will increase. Our results indicate seedlings would not have been more uniform if small-sized nuts were culled prior to sowing, but the overall height would have increased. In previous studies, the within-family growth variation was high for chestnut and oak species grown in commercial nurseries,[22,30,42] and variation in bulked seed where genotype was unknown was also high.[31] Thus, improving nursery production efficiency by culling seed instead of culling seedlings does not seem possible for American chestnut or blight-resistant hybrids unless better controls for seed moisture loss and seed damage can be instituted.

Due to the large variation in seedling size at lifting, we recommend that seedlings are graded into different size classes based on RCD after lifting. Visually separating seedlings using RCD has been proposed in other studies involving oak and chestnut, and is easily transferable to management in the nursery.[35,42]Chestnuts from advanced breeding generations are highly valued due to decades of breeding work and research,[3,5,12] and culling seedlings may be undesirable. Managers can match the seedling size to site quality and goals of the planting. For example, the largest seedlings can be planted on the most competitive sites or on sites where early nut production is desired.[18] Limited

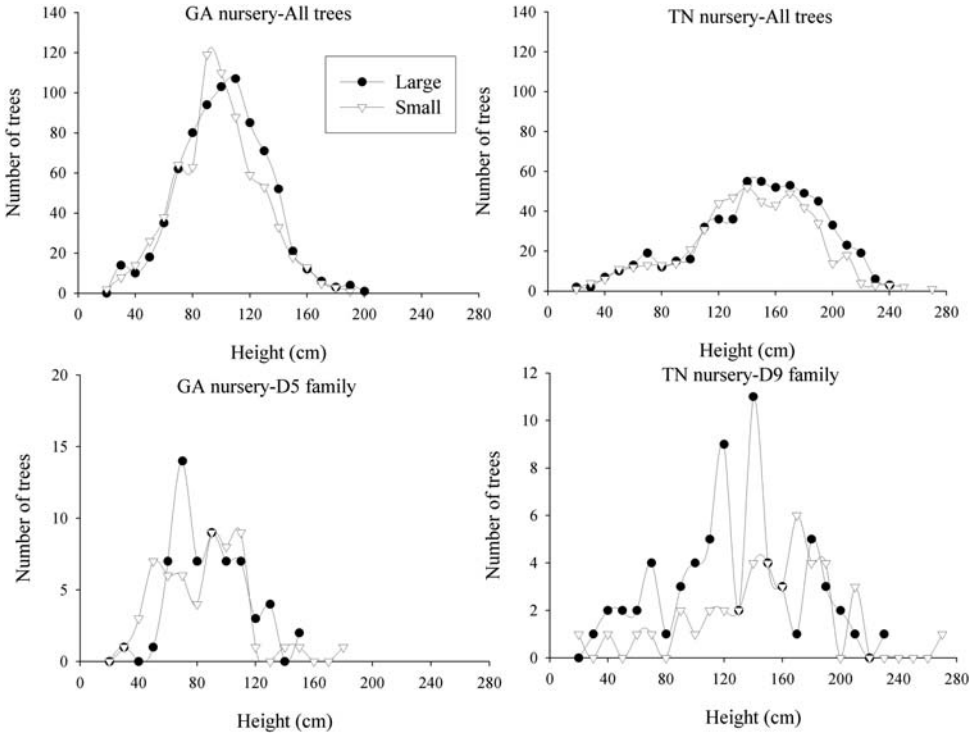

Figure 3 Height distribution for each nut size class for all seedlings from each nursery and for seedlings in families D4 and D9.

Table 6. Logistic regression model for probability of stem forking and missing tap roots.

	Stem forking			Missing tap root		
Predictor variable	Parameter estimate	*P*-value	Odds ratio estimate	Parameter estimate	*P*-value	Odds ratio estimate
Intercept	−3.0122	<0.0001		−2.125	<0.0001	
GA nursery	0.5751	0.0009	1.777	−0.3585	0.0068	0.699
Chinese	1.3384	<0.0001	3.813	1.4125	<0.0001	4.106
$BC1F_3$	0.7471	<0.0001	2.111	1.031	<0.0001	2.804
$BC2F_3$	0.0028	0.9898	1.003	0.4473	0.0072	1.564
BC_3F2	−1.799	0.0764	0.165	0.2154	0.4563	1.24
BC_3F_3	0			0		
American	−0.6571	0.0052	0.518	−1.1284	<0.0001	0.324

resource material could also be retained by growing small nuts in separate seed lots with a more intense fertilization regime to improve the seedling size or for planting on different site types.

The results suggested TACF accomplished its goal to reduce the Chinese chestnut contribution in advanced generations. BC_3F_3 generation chestnuts performed differently than Chinese chestnuts in 67% of ANOVA and logistic regression statistical tests. In 5 of 10 statistical tests, however, the BC_3F_3 generation also differed from American chestnut. We would expect some similarities between American and Chinese chestnut species in morphology,[13] but the results do raise some questions regarding recovering gene expression of American characteristics expressed in the backcross generations. Of particular interest is the higher probability of stem forking, a characteristically Chinese chestnut trait,[55] in the BC_3F_3 generation when compared with the American chestnut parent.

Identification of superior generation/parental species and families depended on the nursery, indicating that environmental (e.g. climate, soil, weather) by genetic interactions were apparent in this study. Testing differences between advanced generations and the preferred American parent may be difficult if interactions between generation/parental species and nursery site conditions are real. Inferences on breeding efforts of American chestnut should be made with caution regarding the seedling performance in commercial tree nurseries until more families can be repeatedly tested, and nut viability can

be better identified and controlled. The low amount of replication for each family could also be a contributing factor to the discrepancies in performance between nurseries. Additional replication would be desirable, but is limited by current seed source availability.[5]

A potential, yet untested, explanation for the high amount of variation in survival and growth within family/nut size seed lots could be related to cotyledon damage from weevils (Coleoptera: *Curculionidae*) prior to sowing in the nursery. In northern red oak, weevil-damaged northern red acorns had reduced RCD and stem height.[56] Effects of nut damage to *Castanea* species on subsequent seedling development has not been well studied, but chestnut was highly susceptible to weevil damage in early hybridization studies,[54] and insect damage to American chestnut was widely reported in early forestry reports.[37] If large nut size classes were targeted over smaller size nuts, then we postulate that large nut size class seedlings would have similar ranges in height as small nut size class seedlings (Figure 3); however, larger acorns were not targeted over smaller acorns in a study with northern red oak.[32] Despite these unknown variables, our study is probably representative of conditions in most southern tree orchards and nurseries.

New knowledge was discovered in this study that will require further testing. The differences between the BC_3F_3 generation and the American parent seen at both nurseries do raise some concerns in regards to gene expression of a breeding line that is predicted to maintain blight resistance, but in other regards, behave like the American parent. More testing is needed before definitive differences or similarities can be determined. The relationship between FOLR number and stem forking has never been reported for any hardwood species. We initially suspected that late-season frosts may have caused stem forks, but that theory was eliminated after examining weather data from the nurseries; no late season frosts were reported during the time seedlings would have emerged (mid-April through late May). We suspect that seedlings with stem forks had more leaf area than seedlings with only one primary stem, and more leaf area resulted in a more productive root system.[49]

Another new characteristic we examined was the occurrence of missing tap root. We interpret the occurrence of missing tap roots as a negative characteristic for seedling performance due to its negative relationship with height and RCD in TN nursery seedlings, and that it was related to the Chinese genotype in the logistic regression. Seedlings with missing tap roots are more difficult to plant due to their spreading root system (S.L. Clark and S.E. Schlarbaum, personal observation), and we hypothesize that this characteristic could lead to poor performance in the field. Data are currently being collected from field plantings to investigate this hypothesis. Missing tap root occurrence may be under genetic control, or it could be due to the relationship between the nut size and planting depth. The larger nut that is associated with Chinese chestnut may be more prone to damage of the emerging radical at germination because larger nuts would indirectly be sown at a shallower depth, leaving them more prone to freezing/thawing while overwintering in the beds.

We established a similar nursery study in 2011 and installed 11 experimental plantings since 2009 using material studied in this paper with individual tree identity retained in the field.[53] Results from these studies will be used in reforestation efforts of the National Forest System of the U.S. Department of Agriculture, Forest Service by providing recommendations on cultural practices to improve seedling quality and determine how that translates into field performance. Breeding programmes at TACF and Connecticut Agricultural Experiment Station[5,57] can also use these results to refine breeding efforts and to better understand how phenotypic traits are being recovered in backcross generations.

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Conflict of interest statement

None declared.

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