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Seed and seedling characteristics of hybrid chestnuts (*Castanea* spp.) derived from a backcross blight-resistance breeding program

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Abstract
American chestnut (*Castanea dentata*) was a foundation species in the eastern United States until chestnut blight (*Cryphonectria parasitica*) infestation resulted in range-wide catastrophic reduction. Since 1983, The American Chestnut Foundation (TACF) has engaged in a breeding program aimed at restoring *C. dentata* to the wild. The primary goal has been to introduce blight resistance from *Castanea mollissima* while recovering a *C. dentata* phenotype via successive backcrosses. To diversify the genetic base, state chapters of TACF have been producing BC_3 and BC_4 (third and fourth backcross) lines using *C. dentata* from across its native range. This study focuses on morphology and chemistry of seeds, and morphology and early growth of seedlings derived from BC_3 and BC_4 trees selected for blight resistance in state chapters. Our primary comparisons were among backcross, pure *C. mollissima,* and pure *C. dentata* lines. Seed traits included a suite of morphological characters known to differ between *C. dentata* and *C. mollissima*, as well as dry matter, total carbohydrate, sugar, protein, lipid, and phenolic content. Seedling traits included variables such as stem basal diameter and height, leaf variables such as number and area, and relative growth in key parameters such as stem volume. *C. mollissima* lines tended to differ significantly from *C. dentata* and backcross lines in most parameters, while *C. dentata* and backcross types tended to overlap broadly in traits. These results suggest that seed and seedling characteristics of backcross hybrids studied here are likely to be sufficiently similar to *C. dentata* for use in restoration programs.

Keywords *Castanea dentata* · *Castanea mollissima* · Species restoration · Backcross breeding · Disease resistance · Genetics

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Introduction

As of the late nineteenth century, American chestnut, *Castanea dentata* (Marsh.) Borkh. (Fagaceae; hereafter AM chestnut) flourished across eastern North America and provided rural residents with timber and a cash crop, at the same time being a valuable food resource for wild animals due to a consistent and abundant production of nuts (Anagnostakis 1987; Wang et al. 2013). Chestnut blight, caused by *Cryphonectria parasitica* (Murrill) Barr (Cryphonectriaceae), was introduced to AM chestnuts in or before 1905, apparently having been imported on Japanese/Korean chestnut (*C. crenata* Siebold & Zucc.) and/or Chinese chestnut (*C. mollissima* Blume [hereafter CH chestnut]; Milgroom and Cortesi 2004). After entering the bark via air-borne spores, cankers spread quickly in susceptible trees, and eventually girdle and kill the stem. The portion of the stem below the canker often remains alive and basal sprouting may occur following infection (Griffin et al. 1983). Because of the lack of resistance in AM chestnut, by 1950 the disease had spread throughout its native range, top-killing approximately four billion trees (Jacobs et al. 2013). Currently, 431 ± 30.2 million AM chestnuts remain, but the vast majority (360 ± 22 million) are small (< 2.5 cm in diameter; Dalgleish et al. 2015). These sprouts, along with far lower numbers of large long-term surviving AM chestnut trees, provide germplasm resources for projects designed to restore AM chestnut as a functional component of U.S. forests.

Many strategies to develop resistance to the chestnut blight have been advanced, including biocontrol using hypovirulent strains (Milgroom and Cortesi 2004), cross-breeding surviving AM chestnuts (Griffin et al. 1983), genetic modification of AM chestnut (Zhang et al. 2013; Steiner et al. 2016), and hybridization with Asian chestnuts (Hebard 2005; Steiner et al. 2016). The American Chestnut Foundation (TACF) has focused since 1983 on a backcross breeding approach aimed at producing blight-resistant hybrids with phenotypic features of AM chestnut (Hebard 2012). In recent years, state chapters of TACF have also established backcross programs with the intent of generating regionally adapted backcross hybrids for restoration in their own regions (Fitzsimmons et al. 2012).

In the TACF backcross program, blight resistance genes are introduced to AM chestnut via crosses with CH chestnut (Burnham et al. 1986). Starting with these F_1 hybrids, AM chestnut traits are recovered through a series of backcrosses with AM chestnuts. Backcross trees are selected to advance to the next generation based upon assessments of blight-resistance and morphology. In third generation backcross (BC_3) trees, the AM chestnut complement of the genome should on average be 94% (15/16th AM chestnut and 1/16th CH chestnut) and fourth backcross (BC_4) trees 97% (31/32th AM chestnut and 1/32th CH chestnut). The theoretical model (based upon two or three independently segregating, incompletely dominant genes) that formed the basis for the TACF backcross program predicted that backcross trees should harbor intermediate levels of blight resistance (Burnham et al. 1986). Theoretically, full resistance is to be recovered by intercrossing BC_3 (or BC_4) trees to produce BC_3F_2 (BC_4F_2) trees, a small percentage of which may be genetically fixed for blight resistance. Selected BC_3F_2 (BC_4F_2) trees are inter-crossed to produce BC_3F_3 (BC_4F_3) offspring for restoration to natural sites. The theoretical model underpinning this approach is being assessed via developing molecular and classical genetic analyses (Georgi et al. 2015; Westbrook 2016; Westbrook et al. 2019a). The final intercrossing steps (beyond BC_3 or BC_4) are not intended to further alter the percentage of AM chestnut genes but merely to provide an opportunity for sets of blight-resistance genes to become homozygous. A parallel breeding program has been initiated in the southeast U.S. directed towards selection in *C. dentata/C.mollissima* hybrids for resistance to root rot caused by

Phytophthora cinnamomi Rands (Jeffers et al. 2012). Recent data from the TACF backcross program suggest that BC_3 trees may not achieve the high level of blight resistance originally hypothesized (Westbrook et al 2019a). Nevertheless, the BC_3 generation remains a critical component of early restoration work involving blight resistance (Clark et al. 2019). The BC_3/BC_4 generations also represent important sources of *P. cinnamomi* resistance for the TACF breeding program (Westbrook et al. 2019b, c).

It is important to test the hypothesis that disease resistance and AM chestnut morphological and physiological traits can be found in backcross trees, particularly in trees emanating from state chapter programs that rely principally upon citizen scientists. For restoration, phenotype traits of backcross trees should exhibit the same range of variation as wild-type AM chestnuts. In this study, we assess seed and young seedling characteristics of nuts from BC_3 and BC_4 trees originating in state TACF chapters—trees that have been selected for blight resistance. Very little is known about the seed chemistry of AM chestnut and only a little more for the relatively better-studied European chestnut (*C. sativa* Mill.) and Asian species. One common finding is that the sugar profile of chestnuts tends to be dominated by sucrose, with glucose and fructose at very low levels (cf., Senter et al. 1994). It has commonly been reported that AM chestnuts are "sweeter" in taste than Asian and European species (Rosengarten 2004), although Senter et al. (1994) found only marginally higher sugars in AM chestnut in comparison with CH and European chestnuts. AM and CH chestnuts can be distinguished by a variety of nut morphologic traits including mass and dimensions (higher in CH chestnut), and relative length of the arms of the "starfish" shaped vascular scar within the hilum, relative length of the stigma and style, and surface trichome density (all higher in AM; The American Chestnut Foundation 2018). Regarding seedlings, some field studies using BC_3 generation trees have demonstrated relatively minor differences in comparison with AM chestnuts (Clark et al. 2012; Knapp et al. 2014; Clark et al. 2016; Pinchot et al. 2017; see Skousen et al. 2018 for an exception). Backcross trees used in these studies were open-pollinated offspring of parents that had not been selected for blight resistance (early products of unculled BC_3F_2 orchards). Linkage between blight-resistant genes and genes affecting seedling morphology and physiology could produce results that are different for backcross trees selected for blight resistance. In this study, we focus on nut morphological traits, embryo nutrient and phenolic content, and early growth characteristics of nursery-grown seedlings (the time-frame when maternal effects related to seed reserves should most strongly affect offspring morphology and growth). We predicted that: (1) AM and CH chestnuts would be routinely distinguishable in nut and seedling morphology, embryo chemistry, and early seedling growth, and (2) Blight-resistant BC_3 and BC_4 trees would produce nuts whose morphology, seed chemistry, and seedling morphology and growth is indistinguishable from AM chestnut.

Materials and methods

Study site and genetic material

TACF backcross breeding orchards were established by TACF state chapters at a variety of locations (Supplement 1). Trees planted at these sites were BC_3 or BC_4 trees, bred using BC_2 or BC_3 Graves/Mahogany-source trees at TACF's Meadowview, VA research farm as either male or female parents. Breeding was done via hand-pollination using pollen collected from wild AM trees (backcross trees as female parents) or pollen collected from

backcross trees (wild AM trees as female parents). AM chestnuts used in the breeding program came from wild trees located in the state corresponding to the orchard location (Georgia, Kentucky, Pennsylvania, or Tennessee), and species identification was confirmed by consensus of TACF experts.

Blight resistance testing of BC_3 and BC_4 trees was conducted using similar methods at all orchard sites. Description of the inoculation process and evaluation of cankers can be found in Cipollini et al. (2017). Trees with favorable blight resistance were then assessed for AM chestnut phenotype based upon a battery of leaf, stem and bud traits. Selected BC_3/BC_4 trees were maintained within each backcross orchard. Non-selected trees were culled or emasculated, and the selected survivors were permitted to open-pollinate to produce BC_3F_2 or BC_4F_2 offspring. These were the focal nuts evaluated in this study; 23 BC_3 (includes one BC_3F_3 line) and 9 BC_4 lines (Supplement 1). To help control for genetic lineage, no more than two trees from the same parental cross were included in any chemical or physical comparison. We also included seedlots from wild-type AM chestnut (N = 16) and CH chestnuts (N = 8).

Seed harvest and physical analyses

For physical and chemical analysis, seeds were harvested from trees in fall of 2015 and 2017 (Supplement 1). In 2015, nuts (N = 10–25 per line; N = 3 AM, 4 BC_4, 4 BC_3, and 4 CH) were shucked from husks and surface-sanitized with Sanidate 5.0 (9 g/L) for 5 min. Nuts were air-dried briefly, packed in plastic zip-lock bags, and held for ~ 30 days at 4 °C until further processing (this period was selected to optimize after-ripening that affects sugar profiles of chestnut seeds; Chenlo et al. 2010). Each nut was weighed to the nearest 0.001 g, and length (L), widest width (W), and narrowest depth (D) measured to nearest 0.01 mm (see Supplement 2 for a key to all traits assessed in this paper). Seed volume (SVOL) was calculated from the average radius: SVOL = (4/3) * 3.1416 * ((L/2 + W/2 + D/2)/3). Subsamples of 5–10 seeds from selected lines were peeled to remove nut and seed coats, chopped into small particles, weighed to nearest 0.001 g, placed into drying oven at 60 °C, dried to constant mass, and reweighed. Dry matter content (g/g wet mass) was calculated as mass (g) of dried sample/mass (g) of wet sample. Duplicate sub-samples were prepared for chemical analysis by freezing chopped embryo particles to − 80 °C and lyophilizing. Dried samples were milled to pass a 40 mil mesh screen and stored at − 20 °C. Residual water in lyophilized samples was estimated by loss of mass upon drying of ~ 0.2 g subsamples at 60 °C for 24 h, and was taken into account when estimating chemical content.

Nut morphology

In the fall of 2017, individual nuts from 11 AM, 3 BC_4, 15 BC_3, and 7 CH lines (N ~ 25 per line) were measured to the nearest 0.01 mm as follows:

1. L, W, D, and SVOL of each nut, as for 2015 samples.
2. Width (HW) and height (HH) of the hilum scar.
3. Length of the longest "arm" of the starfish shaped vascular scar within the hilum (AL).
4. Length of the stigma plus style (SL).

From the above values, we calculated the following parameters:

1. Hilum area (HA): HA = 3.1416 * (HW/2) * (HH/2).
2. Hilum area/surface area ratio (HASA): HASA = HA/SA.
3. Hilum width/seed width ratio (HWSW): HWSW = HW/SW.
4. Arm length/hilum width ratio (ALHW): ALHW = AL/HW
5. Stigma/style length to seed length ratio (SLL): SLL = SL/L.
6. Surface area: While the surface area of a general ellipsoid cannot be expressed exactly by a mathematical function, we used the following approximate formula to calculate nut surface area (Michon 2018): $SA \sim 4 * 3/1415 * [(L^p * W^p + L^p * D^p + W^p * D^p)/3]^{\{1\backslash p\}}$, where p = 1.6075.

Using a hand lens, hairiness (HRS) of the upper half of each nut was visually estimated on a scale of 0% (no hairs visible) to 100% (no nut surface visible).

Nut samples from 2017 (N = 9 AM, 8 BC_3, and 5 CH) were prepared for chemical analysis using the same methods for 2015 samples.

Embryo nutrient analyses

Using dried, milled samples from 2015 and 2017, we measured the following constituents:

1. Total nonstructural carbohydrates (TNC) and total sugars using the anthrone method (Yemm and Willis 1954; Spiro 1966) following amylase digestion of ~ 0.01 g samples for TNC (Smith 1981) and 80% methanol extraction of ~ 0.05 g samples for total sugars (Li et al. 1985). Results were expressed as % dry mass soluble starch equivalents for TNC (starch standards) and % dry mass sucrose equivalents for sugars (sucrose standards).
2. Total lipids using gravimetric analysis of ~ 0.2 g samples extracted for 16 h with petroleum ether using a Soxhlet apparatus (AOAC International 2012). Results were expressed as % dry mass crude fat content.
3. Total proteins using the Bradford assay (Jones et al. 1989) using 0.1 N NaOH extraction of ~ 0.02 g samples. Results were expressed as % dry mass bovine serum albumin equivalents (BSA standards).
4. Total phenolics using the Prussian Blue assay (Budini et al. 1980) following acidified methanol extraction of ~ 0.05 g samples. Results were expressed as mg/g catecholic acid equivalents (catecholic acid standards).
5. Sucrose and glucose (2015 samples only) using the Sigma-Aldrich SCA20 sucrose assay (Sigma-Aldrich.com, St. Louis, MO) following water extraction of ~ 0.3 g samples. This assay allows for the simultaneous estimation of free glucose as well as glucose released enzymatically from sucrose. Previous analysis of sugars in chestnuts suggest that sucrose dominates the sugar profile, with glucose and fructose at much lower levels and with other sugars in trace amounts; e.g., Senter et al. 1994; De Vasconcelos et al. 2010; Hernández Suárez et al. 2012).

For each of the above assays, duplicate samples were run and spectrophotometric measurements were made using a Spectronic 20 D + (Thermo Scientific, Waltham, MA) set to the appropriate wavelength for each analysis. Analyses were repeated one additional time if the first two replicates differed by > 5%.

Seedling measurements

Replicate seedlots from fall 2015 (N = 3 AM, 9 BC_3, 3 BC_4, and 4 CH) were stored in moist, sterile peat moss at 4 °C in plastic bags. Seeds were planted on 4 February 2016 in D40 pots (Steuwe and Sons, Inc., Tangent, OR) in media comprised of 1:1 coarse vermiculite:Pro Mix BX (Premier Horticulture, Quakertown, PA). Seedlings were moved outdoors on 14 March 2016 and maintained under 50% neutral shade cloth under ambient conditions.

On 4 April 2016, 10 seedlings randomly selected from each line were measured for leaf number (LN), stem height (HT; cm), and basal diameter (BD; mm) of the main stem (measured at the soil surface). From these measurements, we calculated basal area ($BA = \pi * (BD/2)^2$), stem height-to-basal area ratio (HTBA = HT/BA), and stem volume estimated as a cone (VOL = BA * HT)/3). The largest leaf on each seedling was measured for length (LL; cm) and width (LW; cm) at the midpoint on the leaf axis. These measurements were used to calculate leaf area as an ellipse ($LA = \pi * ((LL/2) * (LW/2))^2$ and leaf length-to-width ratio (LLLW = LL/LW).

One week later (11 April 2016), measurements were taken on the same seedlings. For each parameter P, relative growth rate (RGR) per time unit was calculated as (lnP2 − lnP1)/(t2 − t1). For logistical reasons (seedlings needed to be transplanted to a remote field site on 12 April 2016), there was a relatively short interval (1 week) between successive measurements. We therefore repeated the growth experiment with additional lines collected in fall 2016 (3 AM, 3 BC_4, and 6 BC_3 lines; N = 20 per line), planted in late January 2017, and moved outdoors in mid-March 2017. These seedlings was measured at two periods (17 April and 23 May 2017; 36 day growth period) and morphological/growth parameters calculated as in 2016.

Statistics

Means for variables were compared among genetic types using a series of one-way ANOVAs (hereafter ANOVA; Statistix 10; Analytical Software 2013). Significant ANOVAs were followed by Tukey's HSD All Pairwise Comparisons ($\alpha = 0.05$; hereafter Tukey's tests) comparing genetic types. Non-parametric Kruskal–Wallis (hereafter K–W) tests were used for analysis of embryo chemical data. For these analyses, data were available on only two or three replicate analyses per line, and dependent variables could not be transformed to fit requirements of equal variance and normality for parametric tests. Significant K–W results were followed by Dunn's All Pairwise Comparisons ($\alpha = 0.05$; hereafter Dunn's tests) comparing genetic types. We used genetic type (CH, AM, etc.) as the independent variable and conducted ANOVA or K–W tests using the mean value for each genetic line. For lines that had both seed and seedling traits measured, we also regressed mean SVOL on average seed size (either dry mass or VOL) to assess this relationship.

Because dependent variables tended to be strongly correlated, we also used Principle Components Analysis (PCA) to generate a smaller number of uncorrelated variables (PC variables) for parametric analysis using ANOVA. We conducted ANOVA/Tukey's tests comparing PC scores among genetic lines or types for the first two principal components from each experiment. As with the original variables, we used mean values for each genetic line and genetic type as the independent variable. Nested ANOVA (genetic lines nested within genetic type) might have been appropriate for the

basic data structure. However, differences in the number of genetic lines within genetic type (unbalanced design) renders calculation and interpretation of F values from nested ANOVA problematic (McDonald 2014). In our analyses, ANOVA using genetic lines and genetic type gave qualitatively similar results, so for simplicity we present the latter.

Results

Nut morphology, 2015 samples

In 2015, nuts differed significantly among genetic types in L, W, D, wet and dry mass and SVOL. ANOVA results for each of these highly correlated variables were qualitatively similar (all $F_{3,11}$ values > 19 using log-transformed data; all P values < 0.001), so we show only results for SVOL (Fig. 1). Average SVOL was about three times higher for CH lines in comparison with AM and backcross lines. As expected, AM and backcross types tended to have much smaller dimensions, masses and volumes in comparison with CH types.

The first axis of the PCA using morphological variables explained > 87% of the overall variation (see Supplement 3 for detailed results for these and all other PCA analyses), rendering consideration of other PCA axes needless. This axis (PC1) was negatively associated with size and mass variables (most strongly with masses and SVOL; dimensional variables less so). ANOVA using PC1 scores (Fig. 1) showed two groups (AM=BC_4=BC_3, and BC_4=BC_3=CH). While this analysis did not segregate CH and backcross types statistically, the magnitude of the difference between backcross and CH types was pronounced in comparison with the magnitude of the difference between backcross and AM types.

Embryo chemistry, 2015 and 2017 samples

Embryo dry matter, sucrose, and glucose from 2015 samples averaged 0.55 g/g, 5.6%, and 0.4% respectively. While sucrose and glucose did not differ among types according to Dunn's tests, dry matter differed significantly between AM and CH types, both of which overlapped with backcross types (Table 1).

Embryo chemistry from combined 2015 and 2017 samples varied among genetic types, particularly for lipids, which ranged from ~ 11 to 15% for AM and backcross types to only ~ 2.6% for CH types (Table 2). AM, BC_4, and BC_3 types did not differ significantly in any chemical constituent. Protein content showed some suggestion of an intermediate value for backcross lines in that two statistical groups were recognized (CH=BC_3=BC_4, and BC_3=BC_4=AM). Most likely a result of a trade-off with lipid content, CH lines tended to be higher in TNC, starch and sugars, although the only statistically significant difference was that TNC was greater for CH than for BC_3 types.

The first two axes of the PCA using embryo chemical variables explained ~ 65% of the overall variation (~ 39% for PC1 and ~ 26% for PC2). PC1 scores differed significantly among genetic types based upon ANOVA (Fig. 2), although PC2 scores did not. AM, BC_4, and BC_3 types formed a homogenous group on PC1 (high values) that differed significantly from CH types. PC1 scores related positively to lipid and protein content, and negatively to sugar and starch content.

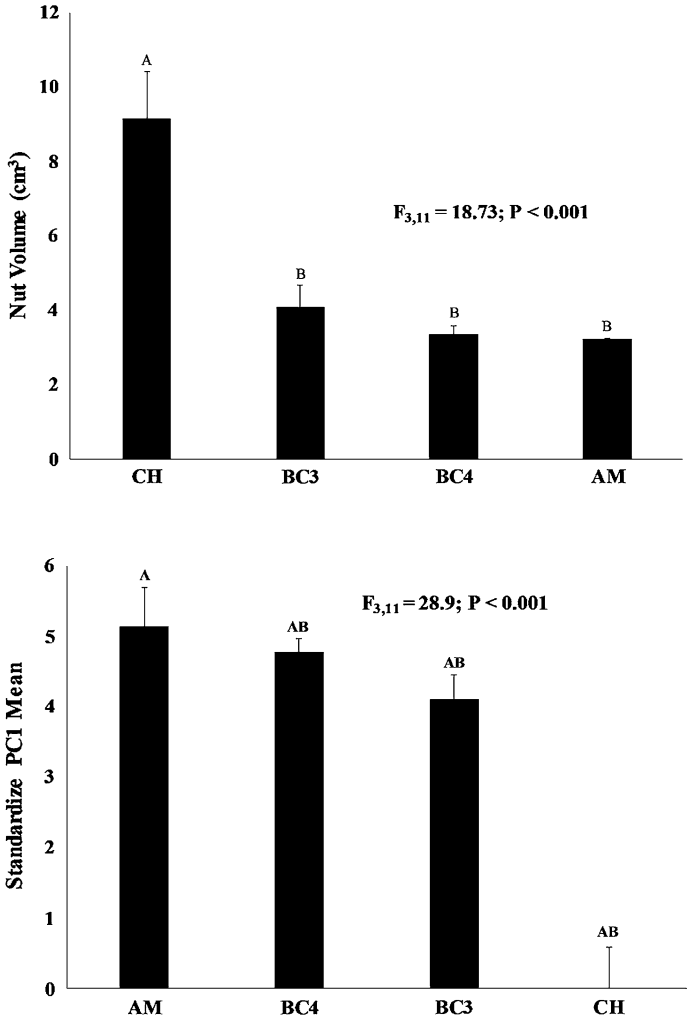

Fig. 1 Mean (±S.E.) nut volume (SVOL; upper figure) and PC1 scores (standardized by subtracting the minimum; lower figure) for nut morphological data collected from 2015 samples (N = 4 CH, 4 BC_3, 4 BC_4, and 3 AM lines). Identical letters denote genetic types that did not differ statistically ($P > 0.05$) based upon Tukey's tests following one way ANOVA

Nut morphology 2017 samples

Similar to results from 2015 samples, nuts from CH types tended to have higher overall dimensions in comparison with AM, BC_4, and BC_3 types (Table 3). Significant differences were detected among genetic types for all morphological variables except HASA, AL, and ALHW. For variables that differed significantly among genetic types, in no case did BC_3 or BC_4 types differ from AM types. Probably due low sample sizes for the BC_4 type (N = 3 lines), BC_4 lines could not be distinguished from CH types for several variables (e.g., L, HW, HH, and HWSW). Again, focusing on variables that differed significantly among

Table 1 Embryo dry matter, sucrose, and glucose content and K–W results for samples collected in 2015 (N=4 CH, 4 BC_3, 4 BC_4, and 3 AM lines)

Dry matter[a] (g/g)				Sucrose[b] (% dry mass)			Glucose[b] (% dry mass)		
Type	Mean	SD		Mean	SD		Mean	SD	
CH	0.531	0.014	A	5.60	1.60	A	0.19	0.33	A
BC_3	0.553	0.011	AB	5.90	1.20	A	0.36	0.35	A
BC_4	0.553	0.031	AB	4.60	1.40	A	0.61	0.49	A
AM	0.605	0.07	B	6.50	1.40	A	0.51	0.48	A
	K–W	7.79		K–W	3.64		K–W	4.66	
	P value	0.025		*P* value	0.332		*P* value	0.207	

Identical letters denote genetic types that did not differ significantly ($P \geq 0.05$) based upon Dunn's tests. For this table and all subsequent tables, genetic types are listed in approximate ascending order of *C. dentata* parentage and key to genetic types can be found in Supplement 1

[a] N = 1 bulk sample per genetic line

[b] N = 2 replicates from a single bulk sample per genetic line

Table 2 Embryo chemical data and results of K–W tests for samples collected in 2015 and 2017 (N = 10 CH, 12 BC_3, 4 BC_4, and 12 AM lines)

Lipids (% dry mass)				Proteins (% dry mass)			Phenolics (mg/g dry mass)		
Type	Mean	SD		Mean	SD		Mean	SD	
CH	2.6%	0.7%	A	2.7%	0.8%	B	6.6	1.8	A
BC_3	13.6%	3.6%	B	3.4%	1.0%	AB	7.6	2.3	A
BC_4	13.6%	1.2%	B	3.1%	0.7%	AB	9.2	2.1	A
AM	15.1%	2.8%	B	4.0%	0.6%	A	6.8	0.9	A
	K–W	21.3		K–W	11.1		K–W	6.89	
	P value	< 0.001		*P* value	< 0.006		*P* value	0.069	
TNC (% dry mass)				Starch (% dry mass)			Total sugars (% dry mass)		
Type	Mean	SD		Mean	SD		Mean	SD	
CH	87.0%	9.8%	A	78.3%	9.9%	A	8.8%	1.9%	A
BC_3	76.6%	7.1%	B	69.1%	5.4%	A	7.6%	1.2%	A
BC_4	76.7%	8.2%	AB	69.4%	7.0%	A	7.3%	0.7%	A
AM	78.2%	8.0%	AB	71.3%	7.3%	A	6.9%	1.7%	A
	K–W	11.1		K–W	4.02		K–W	5.41	
	P value	0.008		*P* value	0.266		*P* value	0.142	

Identical letters denote groups that did not differ significantly ($P > 0.05$) based upon Dunn's tests

types, in only one case (HWSW) did the AM and BC_3 type not differ significantly from the CH type.

The first two vectors of the PCA analysis for nut morphological variables explained ~ 64% of the overall variation (~ 48% and ~ 16% for PC1 and PC2, respectively).

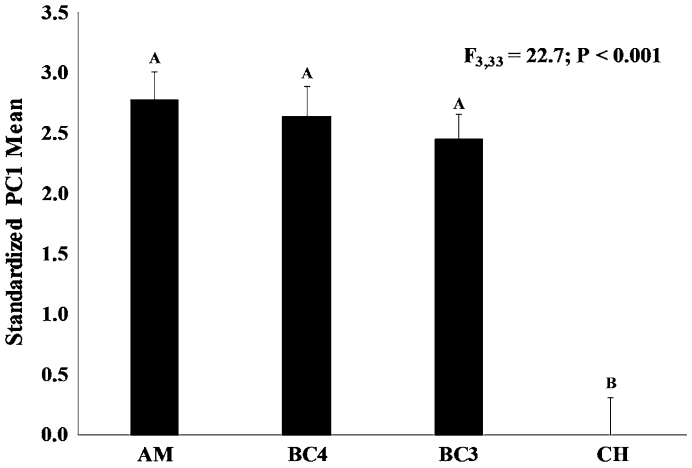

Fig. 2 Mean (±S.E.) PC1 scores (standardized by subtracting the minimum) for genetic types based upon PCA using embryo chemical variables for 2015 and 2017 samples combined (N = 12 AM, 12 BC_3, 4 BC_4, and 10 CH lines). Identical letters denote genetic types that did not differ significantly ($P > 0.05$) based upon Tukey's tests following one-way ANOVA

Scores on PC1 but not PC2 varied among genetic types based upon ANOVA, so we show results only for PC1. PC1 scores were positively related with SLL, HRS, SL, ALHW, and HWSW (AM chestnut-like traits), and negatively related with variables related to overall nut size (CH chestnut-like traits). On PC1, Tukey's tests distinguished CH from BC_3, BC_4, and AM types; Fig. 3). This suggests that overall nut morphology of backcross and AM lines was indistinguishable.

Seedling morphology and growth 2015 samples

For seedlings grown in 2016, significant ANOVA results were found only for only one leaf variable (LN) and for four stem variables (BD, BA, HT, and VOL; Table 4). For LN, BD, BA, and VOL, Tukey's tests showed CH types to differ significantly from backcross and AM types. For two variables, relationships were complex, with Tukey's tests showing two groups for LA (CH=BC_3=BC_4; CH=BC_4=AM) and three groups for HT (CH; BC_3=BC_4; BC_4=AM). So, of the variables studied, only HT showed a suggestion of intermediate values for BC_3 types. That said, mean HT for CH types was about 50% taller than BC_3 types (32.3 vs. 19.7 cm), whereas BC_3, BC_4 and AM types differed proportionately less (19.3, 17.4, and 14.9 cm, respectively).

PCA using seedling morphological and growth variables from 2016 samples showed considerable variation, with only ~ 65% of the overall variation explained by the first three PC axes (~ 32%, ~ 17%, and ~ 15% for PC1, PC2, and PC3, respectively). Nonetheless, ANOVA showed significant differences among genetic types for both PC1 and PC2. In both cases, Tukey's tests showed CH types to differ significantly from backcross and AM types (Fig. 4). Scores on the PC1 axis were positively related to stem size variables and negatively with relative growth variables and HTBA ratio. Scores on the PC2 axis were positively related with LL and LLLW ratio, and negatively with HT, LN, HTBA ratio, and relative growth parameters other than RGRLN.

Table 3 Nut morphological data and ANOVA results for samples collected fall 2017 (N = 7 CH, 15 BC_3, 3 BC_4, and 11 AM lines)

Cross type	L	W	D	SVOL	SA	HW	HH	HA	HASA
CH	27.28	30.30	23.12	10,554	2007	23.00	16.73	299.3	0.151
	3.45	4.62	4.61	3336	436	4.88	3.746	80.92	0.036
	A	A	A	A	A	A	A	A	A
BC_3	20.99	21.20	13.66	3606	979	17.50	11.14	154.5	0.156
	2.75	2.55	2.16	1288	234	1.97	3.58	50.0	0.024
	B	B	B	B	B	B	B	B	A
BC_4	23.31	23.90	14.54	4887	1200	21.41	12.24	207.5	0.178
	6.51	0.77	0.16	1936	281	1.3	0.257	17.3	0.041
	AB	B	B	B	B	AB	AB	B	A
AM	20.28	20.99	14.20	3515	961	18.24	10.82	157.6	0.163
	3.19	2.63	3.15	1333	236	2.99	2.64	49.8	0.039
	B	B	B	B	B	B	B	B	A
$F_{3,32}$	9.8	22.2	33.0	50.7	39.2	8.66	7.42	18.1	1.0
P value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.406

Cross type	HWSW	AL	ALHW	HRS	SL	SLL
CH	0.760	4.19	0.190	8.64	6.07	0.224
	0.104	1.46	0.075	8.4	5.22	0.192
	A	AB	A	A	A	A
BC_3	0.832	4.70	0.272	56.9	15.3	0.724
	0.076	1.01	0.061	15.39	4.15	0.131
	AB	B	A	B	B	B
BC_4	0.894	7.55	0.355	49.27	14.27	0.641
	0.044	1.51	0.081	44	3.8	0.248
	B	A	A	B	B	B
AM	0.867	5.36	0.304	43.29	13.37	0.658
	0.090	1.95	0.128	20.13	4.24	0.188
	B	AB	A	B	B	B
$F_{3,32}$	5.02	2.73	1.83	11.74	10.0	21.94
P value	0.006	0.060	0.162	< 0.001	< 0.001	< 0.001

Results are means and standard deviations. ANOVA was conducted using mean values for each genetic line. Identical letters within a column denote genetic types that did not differ significantly ($P \geq 0.05$) based upon Tukey's tests. See Supplement 2 for variable definitions

For lines for which both seed and seedling traits were measured, we regressed seedling stem volume (VOL) on dry seed mass (Fig. 5). A power function showed a very strong relationship ($R^2 > 90$) between these variables, and the 10 backcross/AM lines nested in a cluster apart from the four CH lines.

Seedling morphology and growth 2016 samples

For seedlings grown in 2017, ANOVA did not detect any significant differences among BC_3, BC_4, and AM lines for any leaf or stem trait ($F_{2,9}$ ranged from 0.1 for LW to 3.0 for

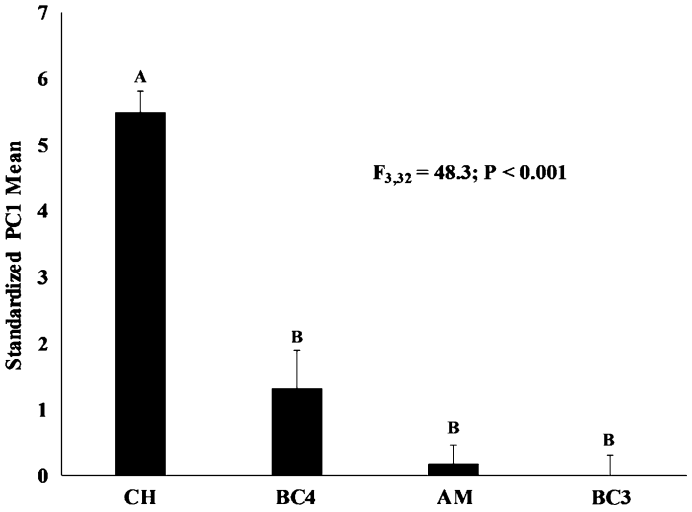

Fig. 3 Mean (±S.E.) Mean PC1 scores (standardized by subtracting the minimum) for genetic types based upon PCA using nut morphological variables for 2017 samples (N = 7 CH, 3 BC_4, 11 AM, and 15 BC_3 lines). Identical letters denote genetic types that did not differ significantly ($P > 0.05$) based upon Tukey's tests following one-way ANOVA

LLLW; $P > 0.05$ for all variables). As for seedlings grown in 2016, there was considerable overall variation; in this case, the first three PC axis explained only 68% of the overall variation (~34%, ~19%, and ~15% for PC1, PC2, and PC3, respectively). As with the original variables, ANOVA could not detect significant differences among genetic types for either PC1 or PC2 scores ($F_{2,9} < 2.6$, $P > 0.05$ for both PC variables).

Only six genetic lines had both seed and seedling traits measured in 2016/2017 (too few to determine a valid statistical relationship). Nevertheless, a graph of the relationship between seed volume (SVOL) and seedling stem volume (VOL) was at least suggestive of an influence of seed size on seedling size (Fig. 5).

Discussion

Our results support the prediction that seeds and seedlings of blight-resistant BC_3 and BC_4 trees show a combination of phenotypic traits that commonly differ from CH chestnut, but cannot be routinely distinguished from those of AM chestnut. Selected BC_3 parent trees at Berry College (Supplement 1) had been previously shown to exhibit adult morphological characteristics indistinguishable from those of AM chestnut (Cipollini et al. 2017). It should be noted that most of the BC_3F_2 and BC_4F_2 seeds used in this study are not expected to carry the full complement of blight-resistance genes of the parent trees; the theoretical framework suggests that only a fraction of the offspring might carry significant blight resistance (Burnham et al. 1986).

The main difference in nutritional quality of AM and backcross chestnuts was lipid content, which was nearly an order of magnitude higher than that of CH chestnuts. This difference might explain common perceptions of a relatively "sweeter" flavor for AM versus CH chestnuts (Senter et al. 1994; Anagnostakis and Devin 2004). Lipid content is an important predictor of attractiveness of nut-bearing trees to potential seed dispersers

Table 4 Seedling morphological and growth data and ANOVA results for seed samples collected in fall 2015 and grown in spring 2016 (N = 4 CH, 10 BC_3, 3 BC_4, and 3 AM)

	Leaf variables							Stem variables	
	LN	LL	LW	LA	LLLW	RGRLN	RGRLA	BD	BA
CH	8.9	14.0	5.8	81.9	2.39	−0.003	0.012	3.8	11.3
	1.5	4.1	1.4	35.2	0.36	0.013	0.037	0.5	2.7
	A	A	A	AB	A	A	A	A	A
BC_3	6.1	15.4	5.9	91.9	2.64	−0.004	0.003	3.0	7.5
	1.6	2.6	1.1	27.7	0.34	0.014	0.014	0.5	2.6
	B	A	A	A	A	A	A	B	B
BC_4	6.4	14.4	5.5	79.0	2.67	−0.003	−0.002	3.0	7.0
	1.7	1.8	0.9	18.3	0.38	0.023	0.016	0.3	1.3
	B	A	A	AB	A	A	A	B	B
AM	6.4	12.9	4.8	62.2	2.70	−0.002	0.001	3.0	7.2
	1.2	1.6	0.8	14.6	0.33	0.005	0.012	0.5	2.4
	B	A	A	B	A	A	A	B	B
$F_{3,16}$	8.51	3.50	1.71	3.95	1.86	0.24	2.76	8.25	9.33
P value	0.001	0.041	0.204	0.028	0.177	0.865	0.076	0.002	< 0.001

	Stem variables					
	HT	VOL	HTBA	RGRBA	RGRHT	RGRVOL
CH	32.2	12.2	3.0	0.001	0.006	0.006
	5.4	4.1	0.8	0.037	0.010	0.041
	A	A	A	A	A	A
BC_3	19.7	5.0	2.8	−0.012	0.008	−0.004
	4.6	2.3	0.9	0.045	0.063	0.079
	B	B	A	A	A	A
BC_4	17.4	4.1	2.5	−0.007	0.002	−0.004
	2.7	1.0	0.6	0.028	0.008	0.029
	BC	B	A	A	A	A
AM	14.9	3.6	2.3	−0.017	0.002	−0.015
	2.4	1.5	1.1	0.040	0.005	0.041
	C	B	A	A	A	A
$F_{3,16}$	37.5	54.02	0.25	0.12	0.10	1.33
P value	< 0.001	< 0.001	0.863	0.950	0.959	0.299

Results are means and standard deviations. ANOVAs were calculated using means for each genetic line. Identical letters within a column denote genetic types that did not differ significantly ($P \geq 0.05$) based upon Tukey's tests. See Supplement 2 for variable definitions

(e.g., squirrels; Smith and Follmer 1972; Burke 2013). If backcross chestnuts are to fill the niche formerly occupied by AM chestnut, dispersers and seed predators should perceive and respond to them equally. Our results suggest that this should be the case, if the nutrients we assayed are proximate factors in seed selection. The proximate assays we used may be influenced to some degree by qualitative differences in constituent profiles.

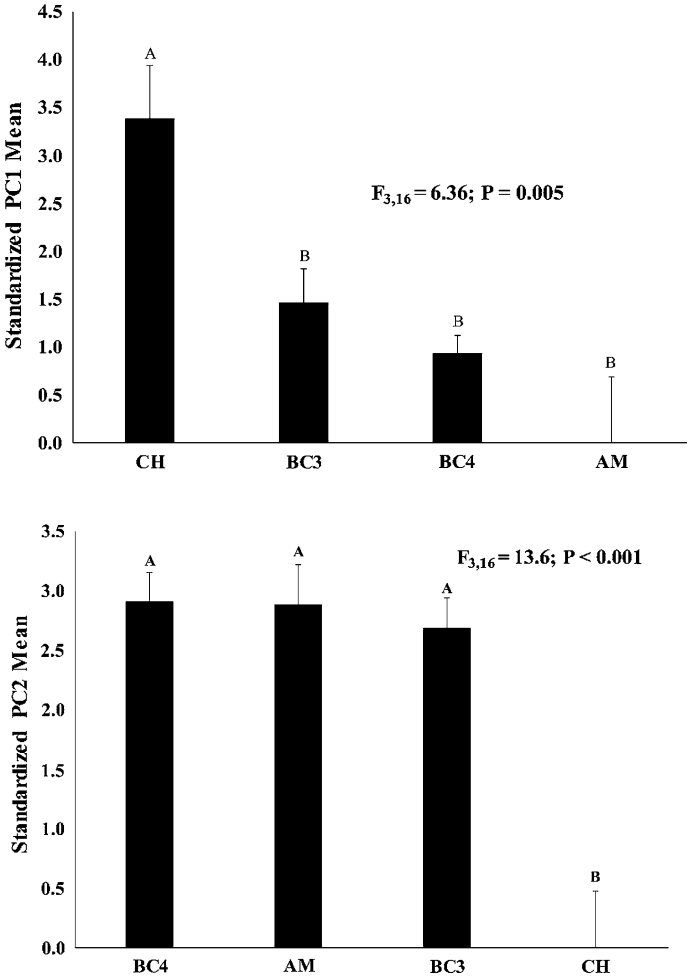

Fig. 4 Mean (±S.E.) PC1 (upper figure) and PC2 (lower figure) scores (standardized by subtracting the minimum) for genetic types based upon PCA for seedling morphological and growth variables for 2015 samples grown in 2016 (N = 4 CH, 9 BC_3, 3 BC_4, and 3 AM lines). Identical letters denote genetic types that did not differ significantly ($P > 0.05$) based upon Tukey's tests following one-way ANOVA

That said, when applied in comparisons of closely related genetic types, they should reflect relative variation in these chemical classes.

Seed and early seedling morphology and physiology is thought to be influenced by the genetic makeup and environment of the parent plant more so than the genotype of the embryo (González-Rodríguez et al. 2011; Singh et al. 2017). This is partly through maternal effects that affect provisioning of resources to embryos. In our case, difference in size of seedlings was strongly related to differences in seed size. That said, Anagnostakis (2009) and Zang et al. (2016) suggested that pollen source may influence nutritional quality and morphology of *Castanea* nuts. As such, pollen source could potentially affect seed quality and early seedling morphology. In our study, pollinators were of the same genetic type as the maternal tree (e.g., AM X opAM = AM, etc.). The expectation for restoration is that backcross trees will be pollinated by other backcross trees or by wild-type

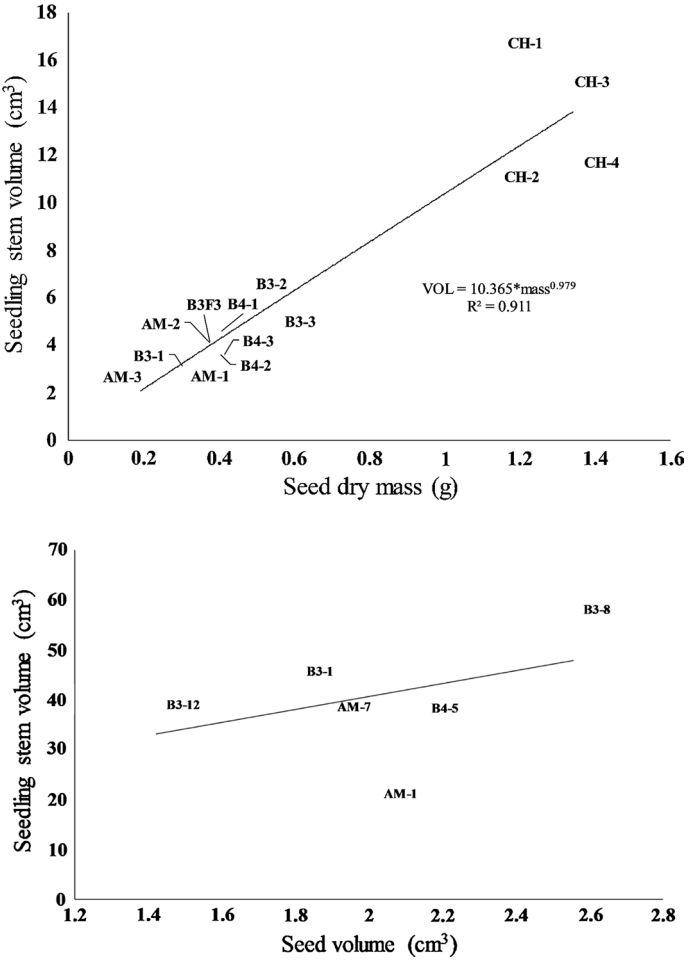

Fig. 5 Relationship between mean seed mass and mean seedling stem volume for genetic lines collected in 2015 and grown in 2016 (upper figure) and relationship between mean seed volume and mean seedling stem volume for genetic lines sampled in 2016 and grown in 2017 (lower figure). For key to genetic lines, see Supplement 1

AM chestnuts. As such, concern over variation among pollen sources strongly influencing nut quality should be minimal.

Because the seedlings used in our 2016 study had to be immediately transported to a distant field site, our estimates of relative growth parameters were compromised by a relatively short period of elapsed time (1 week) between the first and second morphological measurements. In fact, some lines apparently shrank in certain size parameters (e.g., BD) between measurement periods, most likely a product of the hardening up of woody tissue and/or differences in seedling moisture content between measurements. The 2017 experiment spanned a longer time frame (36 days). Neither experiment demonstrated strong or consistent differences among AM, BC_4, and BC_3 lines in morphology and early growth. For example, while BC_3 seedlings seemed to exhibit higher LA than AMs in 2016, this difference was not apparent in 2017 when larger number of replicates were used (10 in 2016,

20 in 2017). Some silvicultural studies suggest culling small seedlings prior to out-planting backcross hybrids (e.g., Clark et al. 2014; Clark et al. 2016). While useful for ensuring high early survival rates in the field, culling to retain only the largest seedlings may carry risks of inadvertently selecting for CH chestnut traits linked to large seedling size. In particular, due to the relationship of seed size to seedling size, this practice could select for greater seed size (a CH-like trait). Culling should therefore probably only be done when seedlings are significantly outside the range of the natural variation of AM chestnuts.

Our results, at the minimum, support the hypothesis that backcross trees selected for blight resistance produce nuts and seedlings that are not reliably distinguishable from those of AM chestnut. These results thus support previous studies showing that the TACF backcross breeding program is accomplishing the goal of bringing blight resistance into trees of an otherwise AM chestnut morphology (Diskin et al. 2006; Cipollini et al. 2017). That said, recent evidence suggests that blight resistance may be quantitative and obtaining fixation for full blight resistance in BC_3 and BC_4 generations will not likely be possible (Westbrook et al. 2019a). It is uncertain, without further field study, whether the current level of blight resistance in these generations is sufficient for restoration (Clark et al. 2019). To obtain acceptable blight resistance through this program, it may be necessary to employ earlier generations (e.g., BC_2) or to utilize particular crosses to achieve acceptable resistance (e.g., selected $F1 \times BC_3$ crosses). If earlier generations are to be used for restoration, studies of nut and seedling traits analogous as those employed in this study ought to confirm that differences from wild-type AM chestnuts are not significant, or if so, are not so pronounced as to be ecologically important. Regardless of the approach to obtaining blight resistance in restoration of AM chestnut, studies of range-wide genetic and ecological variation (and relations among these variables) are paramount. This study was limited to a relatively small number of samples from states with TACF backcross programs. Analysis of larger populations at the landscape level will help inform breeding efforts directed towards strategically diversifying the population of trees incorporated into the TACF breeding program and its companion transgenic program (Westbrook et al. 2019c).

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