Available online at www.sciencedirect.com
SCIENCE DIRECT®

Forest Ecology and Management 223 (2006) 439–447

Forest Ecology and Management

www.elsevier.com/locate/foreco

Recovery of American chestnut characteristics following hybridization and backcross breeding to restore blight-ravaged *Castanea dentata*

Matthew Diskin[a], Kim C. Steiner[a,*], Frederick V. Hebard[b]

[a] *School of Forest Resources, The Pennsylvania State University, 104 Ferguson Building, University Park, PA 16802-4302, USA*
[b] *The American Chestnut Foundation, 14005 Glenbrook Avenue, Meadowview, VA 24361, USA*

Received 15 August 2005; received in revised form 2 December 2005; accepted 5 December 2005

Abstract

Morphological features of leaves and twigs of American chestnut, Chinese chestnut, their F_1 hybrid, and three successive generations of backcrosses between hybrid populations and American chestnut were examined to determine rate of recovery of the American chestnut morphology after hybridization to capture Chinese chestnut genes for blight resistance. In aggregate morphology, as measured by a composite index of species identity (ISI), 96% of trees in the third backcross generation (BC_3) resembled American chestnut and were distinctly different from Chinese chestnut. The majority of BC_3 trees also differed from Chinese chestnut in every individual characteristic measured for this study. Thus, recovery of American chestnut characteristics is largely achieved after three generations of backcrossing. If progeny of the BC_3 hybrids can be made homozygous for blight-resistance alleles, as expected, and if the trees equally resemble American chestnut in important ecological attributes, then backcross breeding appears to be a workable strategy for restoring this species as a important component of eastern U.S. forests.

Keywords: *Castanea dentata*; *Castanea mollissima*; Species restoration; Breeding; Disease resistance; Genetics

1. Introduction

American chestnut (*Castanea dentata* [Marsh.] Borkh.) was one of the dominant tree species in the eastern United States before it was reduced to its current, remnant population by the chestnut blight fungus (*Cryphonectria parasitica* [Murrill] Barr) (Russell, 1987). American chestnut was also unusually significant to the culture and economy of the region in which it grew, and the appearance and spread of the chestnut blight caused great alarm (Davis, 2003; Lutts, 2004). Efforts to control the spread of the disease and discover or breed blight-resistant trees began early in the 20th century soon after discovery of the disease (Beattie and Diller, 1954). Control efforts failed, and no tree-like, blight-resistant cultivar ever came of the early breeding programs, which continued under different leadership even into the 1960s (Burnham, 1987; Diller and Clapper, 1965). Beginning in the 1970s, research on hypovirulent strains of the fungus showed promise of controlling the blight (Van Alfen et al., 1975; Elliston, 1981). However, 3 decades later success with this method remains largely confined to therapeutic treatment of individual cankers, and biological control at the population level has failed almost completely (Milgroom and Cortesi, 2004).

Against this historical backdrop, Burnham and colleagues proposed a plan in the early 1980s to breed a blight-resistant chestnut population through backcross breeding, a substantially different approach than was used by earlier breeders (Burnham, 1981; Burnham et al., 1986). This plan became the foundation for the chestnut breeding program of the non-profit organization The American Chestnut Foundation (TACF). The objective of the backcross breeding program proposed by Burnham and employed by TACF is to produce chestnut trees for species restoration that are indistinguishable from American chestnut in every aspect except susceptibility to blight. Blight resistance is introduced to American chestnut through a cross with the blight-resistant Chinese chestnut (*C. mollissima* Blume), and American chestnut characteristics are subsequently recovered through a series of backcrosses (to American chestnut parents) that reduce the Chinese complement of alleles by an average value of one-half per generation.

* Corresponding author. Tel.: +1 814 865 9351; fax: +1 814 865 3725.
E-mail address: kcs@psu.edu (K.C. Steiner).

0378-1127/$ – see front matter
doi:10.1016/j.foreco.2005.12.022

The third backcross generation is the final backcross step (as currently planned) in TACF's backcross breeding program, and part of the B_3–F_2 generation[1] has been planted and is undergoing selection for resistance (Hebard, 2005). These trees began producing putatively blight-resistant B_3–F_3 seed in 2005. The American complement of the genome should average 94% for third-generation, backcross hybrid progeny (and all descendants of those progeny), but this proportion could be smaller if alleles for resistance are carried in large linkage groups, and it could be larger if selection is practiced against Chinese alleles other than those for resistance. Furthermore, nonadditive gene effects at phenotypically critical loci could disproportionately skew the appearance of a population toward "Chinese" or "American" characteristics.

Because the ultimate goal of this breeding program is ecological restoration of the species (not merely the creation of a timber-type chestnut suitable for planting in eastern North America), a critical question is how well the hybrid population matches American chestnut in its morphological characteristics and, ultimately, ecological behavior. The answer to this question will play a critical role in the decisions by many government and private entities to participate or not in this restoration effort. The objectives of this study were to: (1) quantitatively describe the morphology of American chestnut and Chinese chestnut, their first-generation hybrid, and first, second, and third generation backcross hybrids; and (2) evaluate the degree to which the American chestnut phenotype is recovered through backcross breeding.

2. Materials and methods

The samples for this study were collected from The American Chestnut Foundation's Glenn C. Price Research Farm in Meadowview, Virginia. Samples were taken from American and Chinese chestnuts, their first-generation hybrids, and first-, second-, and third-generation backcross hybrids (Table 1). The population of American chestnuts represented the open-pollinated progenies of seven chestnuts growing wild in Smyth County, Virginia. The population of Chinese chestnuts was composed of two unique pedigrees derived from controlled pollinations between two different sets of Chinese parents. Neither the American nor Chinese chestnuts sampled here were used as parent trees in any of the latter hybrid generations measured in this study.

All American chestnut parent trees used in the hybrid generations were the plantation-grown progenies of open-pollinated trees growing wild in the mountains of Virginia, except that one (the American parent of the BC_1 generation) was itself a tree growing wild. Each hybrid generation's lineage is unique in that no two hybrid generations have parent trees in common in their pedigrees, and no early hybrid generation was used in the pedigree of a later hybrid generation measured in this study.

Table 1
Population description and sample sizes

Population	Age since planting (years)	Twig sample size (# of trees)	Leaf sample size (# of trees)	Complete sample size (# of trees)
American	2	50	48	48
Chinese	3	49	45	45
F_1	6	50	50	49
BC_1	4	60	54	51
BC_2	4	45	40	39
BC_3	3	49	48	47

All plantations are located at The American Chestnut Foundation's Glenn C. Price Research Farm in Meadowview, Virginia.

Twelve pedigrees of first-generation hybrids were sampled. These trees were the progenies of nine Chinese chestnut mother trees and 12 American chestnut father trees. All first-generation backcross trees were the progenies of a single American chestnut tree crossed with a single first-generation hybrid tree. Three pedigrees composed the population of second-generation backcross trees. The same first-generation backcross tree was used in each pedigree, but a different American chestnut parent was used in each cross. The population of third-generation backcross trees measured for this study comprised the progenies of a single second-generation backcross tree and a single American chestnut tree. (These populations and pedigrees are being used in the TACF breeding program, but

Table 2
Morphological differences between American chestnut and Chinese chestnut with respect to the variables measured in this study

Organ	Variable	Chinese chestnut	American chestnut
Leaf	Shape	Ovate	Oblong-lanceolate
	Apex shape	Obtuse	Acuminate
	Base shape	Rounded to obtuse	Cuneate to acute
	Margin	Coarsely serrate; teeth not pronounced, never curving inward	Coarsely serrate; teeth pronounced, often curving inward
	Interveinal surface	Pubescent	Essentially glabrous
	Veinal surface	Pubescent	Essentially glabrous
Stipule	Size and shape	Large: 5–10 mm at base, tapering to a point; triangular	Small: 1–2 mm broad at base; slender from base to tip
Twig	Color	Tan or pea green	Reddish brown to brownish green
	Surface	Pubescent	Essentially glabrous
	Lenticels	Large: 0.5 mm	Small: 0.1 mm
	Diameter	Stout	Slender
Bud	Color	Tan to dull brown	Reddish brown
	Shape	Rounded, almost as wide as long	Cylindrical, almost twice as long as wide
	Tip shape	Flat	Pointed
	Pitch angle[a]	Appressed to stem	Divergent from stem
	Yaw angle[b]	Parallel to stem	Divergent from stem

[a] Position of the bud relative to the stem when the bud is viewed laterally (leaf scar at 90° to viewer).
[b] Position of the bud relative to the stem from the abaxial side of the bud (leaf scar facing viewer).

[1] The B_1 (first backcross hybrid) generation was produced by crossing the F_1 interspecific hybrid with American chestnut. The B_3 generation was produced by three successive backcrosses to American chestnut, and the B_3–F_2 generation is produced by intercrossing selected B_3 trees.

they represent only a minor part of all pedigrees that will be used to produce the B_3–F_2 generation.)

Two samples were collected from each tree: (1) bud and twig characteristics were measured on twig samples collected before bud-burst on April 7–9, 2003; and (2) leaf and stipule characteristics were measured on leaf samples collected after the leaves had expanded on May 11–15, 2003. Twig samples were obtained by clipping one 20-cm section from the terminal portion of a lateral branch from each tree. The samples were kept frozen to prevent them from drying during the duration of the measurement process, which occurred April 17–20, 2003. Leaf samples were collected from the same trees by clipping the new growth of a lateral branch bearing, typically, about five leaves. Branches growing in full sun were selected because leaves growing in the shade may lack diagnostic leaf hairs, even in the case of Chinese chestnut. The leaf samples were pressed and later measured throughout the month of September 2003.

Table 1 describes the parent populations and sample populations measured in this study. Leaf samples could not be collected from 24 of the trees that provided twig samples, and six trees were used for leaf samples but not twig samples. A ‘‘complete sample’’ consists of a tree for which both a twig sample and leaf sample was taken. The twig sample size or the leaf sample size is the sample size used in the appropriate univariate analyses. For the multivariate analysis, the complete sample size was used.

Because the purpose of this study was to compare hybrids and backcross hybrids with the parental species, it was important to select characteristics for measurement that are known to discriminate between American and Chinese chestnuts. Table 2 shows those characteristics that were used in this study based upon detailed, unpublished descriptions provided by Goldman and Hebard. Other characteristics, such as those based on fruits, late-summer leaf color, leaf wax, and leaf thickness, could not be used because of either their seasonality or the

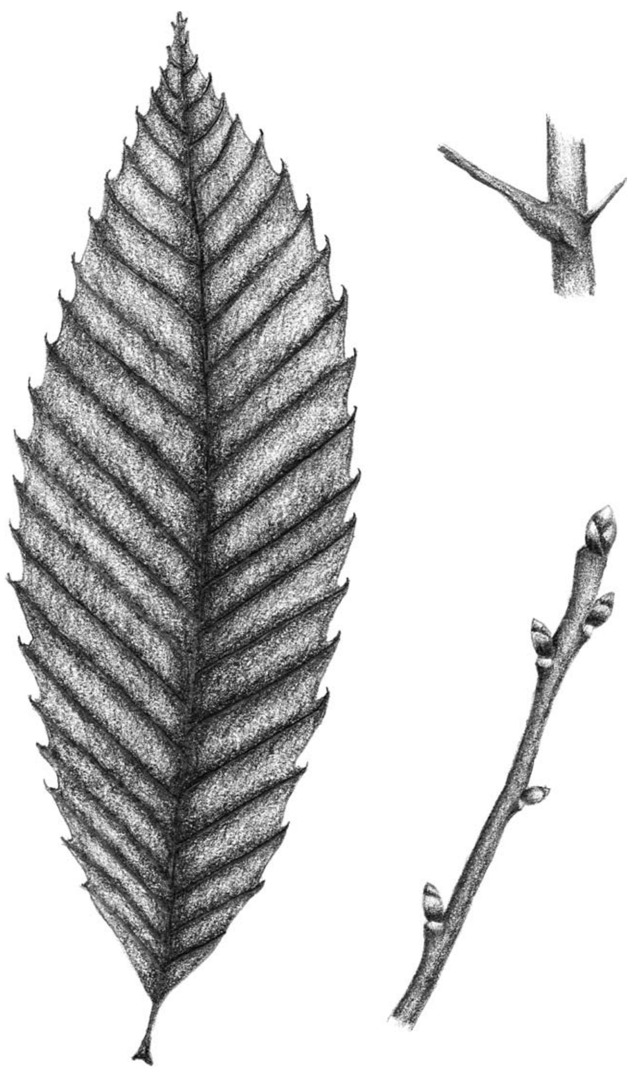

Fig. 1. American chestnut leaf (left), stipule (upper right), and twig (lower right) morphology. The leaf is oblong-lanceolate in shape, with an acuminate tip and cuneate to acute base. The margin is coarsely serrate, with pronounced teeth that often curve inward towards the margin. The surfaces of the leaf are essentially glabrous. Stipules are small and slender from tip to base. The twig is slender with a surface that is essentially glabrous. Buds are cylindrical with pointed tips. The bud is divergent from the stem in both pitch and yaw angle.

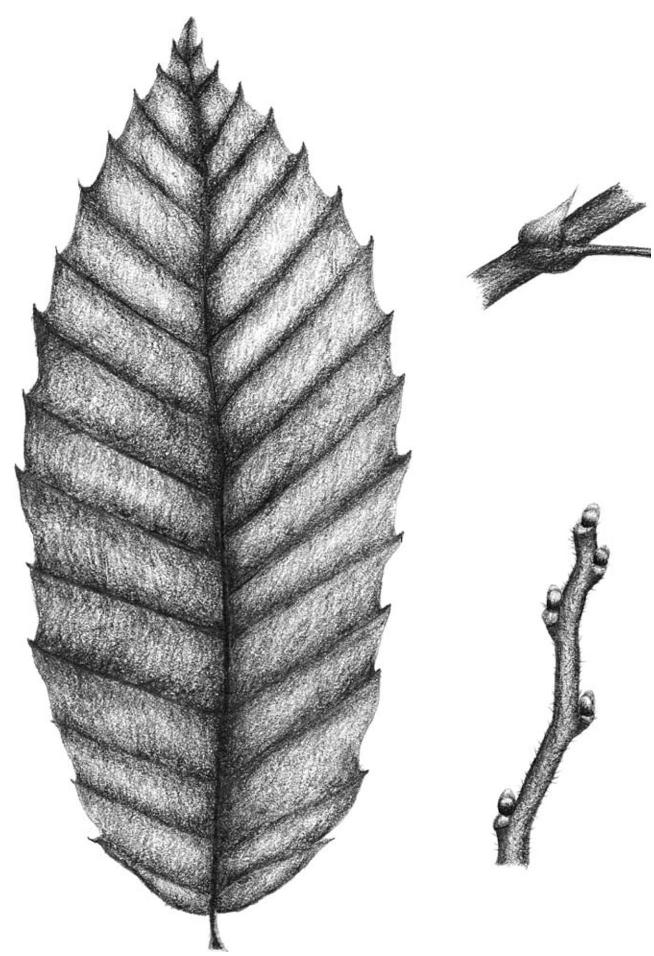

Fig. 2. Chinese chestnut leaf (left), stipule (upper right), and twig (lower right) morphology. The leaf is ovate in shape, with an obtuse tip and rounded to obtuse base. The margin is coarsely serrate, but the teeth are not as pronounced as in American chestnut, and never curve inward towards the margin. It could not be illustrated here, but the surfaces of the leaf are pubescent. Stipules are large at the base and taper to a point. The twig is stout with a pubescent surface. Buds are rounded with flat tips. The bud is appressed to the stem in pitch angle and parallel to the stem in yaw angle.

procedures used for preserving leaf samples. As will be seen in the results section, the characteristics measured in this study were sufficient to distinguish between American and Chinese chestnut with certainty, and therefore may be taken as definitive diagnostic characteristics for these two species of chestnut. Figs. 1 and 2 illustrate some of the differences between American and Chinese chestnuts with respect to leaf, twig, and stipule characteristics.

Morphometric variables were defined to describe the morphology of each generation with respect to the diagnostic differences between American and Chinese chestnut described in Table 2. Table 3 lists the variables used for analysis in this study. Ordinal scoring systems were developed for non-numeric characteristics by examining the parental species to identify extreme values for the variable of interest. Intermediate categories were developed to reflect the number of different grades that could be reliably distinguished between the extreme scores based on visual examination. Fig. 3 illustrates some of the variable measurements.

Statistical analysis of the data was performed using SAS version 8.02 (SAS Institute and Inc., 1999). Analysis of variance (ANOVA) was used to test for significant differences between populations for the continuous variables after performing transformations to homogenize variances. Means were separated using Duncan's multiple range test (Ott, 1992). Brown and Forsythe's test using the absolute deviations from group medians was used to test for homogeneity of variance (Brown and Forsythe, 1974). No transformations were

Table 3
Leaf, twig, and bud characteristics measured in this study

Organ	Variable label	Description	Unit or scale
Leaf	Blade length[a]	–	mm
	Blade width[a]	–	mm
	Distance to maximum width	Distance from leaf base to position of maximum width	mm
	Relative length	Ratio of leaf length to leaf width	–
	Tooth length	Distance between teeth[b]	mm
	Tooth depth	Distance from leaf margin perpendicular to tooth edge[b]	mm
	Leaf length to tooth length ratio	–	–
	Leaf width to tooth depth ratio	–	–
	Tooth hooking	Tooth curvature toward leaf margin	1 (present) or 2 (absent)
Leaf	Apex shape	–	1 (strongly acuminate), . . ., 4 (obtuse)
	Base shape	–	1 (strongly cuneate), . . ., 7 (strongly rounded)
	Interveinal hairs	Presence or absence of interveinal hairs on the abaxial surface	1 (absent) or 2 (present)
	Veinal hair density	Density of hairs on abaxial midrib	1 (low), . . ., 3 (high)
Stipule	Size	Size and shape of the stipule	1 (small), . . ., 3 (large)[c]
Twig	Color	–	1 (red), . . ., 4 (green)[d]
	Hair density	Density of hairs on twig	1 (none), . . ., 4 (high)
	Diameter	Twig diameter measured at the third internode	mm
	Lenticel width	–	mm
Bud	Length	–	mm
	Width	–	mm
	Relative length	Ratio of bud length to bud width	–
	Color	–	1 (red), . . ., 4 (tan)[e]
	Tip shape	–	1 (pointed) or 2 (flat)
	Appression	Distance that the bud is appressed against the stem[b]	mm
	Pitch angle	Angle of bud axis to stem axis when bud is viewed laterally (leaf scar at 90° to viewer)[b]	degrees (°)
	Yaw angle	Angle of bud axis to stem axis when bud is viewed from abaxial side (leaf scar facing viewer)[b]	degrees (°)

[a] Variable not used in analysis.
[b] See Fig. 3 for an illustration of this measurement.
[c] Small stipules were less than 1 mm wide at the base; intermediate stipules were between 1 and 4 mm wide at the base; large stipules were greater than 4 mm wide at the base.
[d] Intermediate character states for twig color were: 2 (greenish red) and 3 (reddish green).
[e] Intermediate character states for bud color were: 2 (intermediate red) and 3 (intermediate tan).

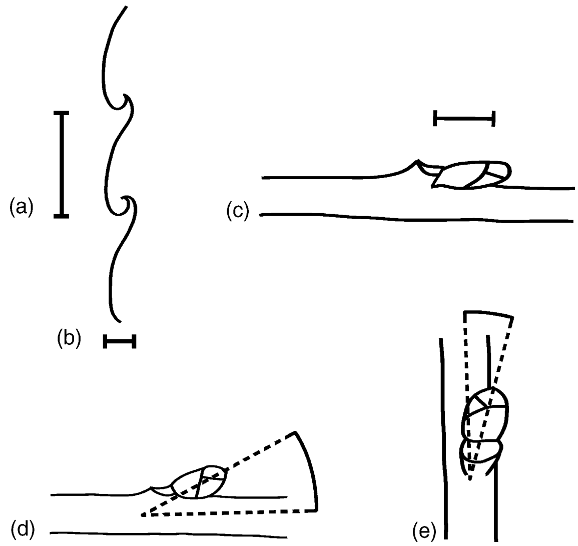

Fig. 3. Illustrations of the variables tooth length (a), tooth depth (b), bud appression (c), bud pitch angle (d), and bud yaw angle (e).

necessary for the distance to maximum width, tooth depth, and twig diameter variables. To homogenize variance in the other variables, the following transformations were performed: the variables lenticel width, bud width, bud relative length, and bud appression were transformed by the square root; the leaf length to tooth length ratio variable was transformed by the fourth root; and the bud length variable was transformed by $1/x$. In the remaining cases where transformations did not homogenize variances (leaf length, leaf width, leaf relative length, tooth length, leaf width to tooth depth ratio, bud pitch angle, and bud yaw angle), t-tests with unequal variances and sample sizes were used to test the significance of differences between populations.

Means for each population were calculated for each of the ordinal variables. Frequencies for each character state within each population were calculated for each ordinal variable. Fisher's exact test was used to test for significant associations between the populations and their frequencies for the ordinal variable character states (Agresti, 2002).

Principal components analysis was performed on a combined dataset of all the individual variables indicated in Table 3 in order to simplify description of the aggregate morphology of each population. By definition, the first principal component captured the most variation between populations (51%), and because each variable was intentionally chosen to ordinate the two species at either end of a single scale, the first principal component captured all of the useful variation in the combined dataset for the purposes of this study. The first principal component scores were then transformed to a scale of 0–1.0 to serve as an index of species identity (ISI). Mean ISI scores were calculated for each population, and t-tests were used to test for significant differences between population means.

Expected mean ISI scores were calculated for each hybrid generation using parental species' means as a starting point and assuming additive gene effects. Thus, the first hybrid generation's expected mean ISI is exactly halfway between the observed means of the parental species, and the expected mean ISI of each succeeding backcross generation is exactly halfway between the previous generation's expected mean and American chestnut's observed mean. t-tests were used to test for significant differences between the observed and expected mean ISI scores.

3. Results

3.1. Morphology of the parental species

All characteristics were useful in distinguishing between American and Chinese chestnut. American chestnut was significantly different from Chinese chestnut for every continuous variable (Table 4), and character state frequencies differed significantly between American and Chinese chestnut for every ordinal variable (Table 5). For most ordinal variables, American and Chinese chestnut trees always had different scores. For all variables except leaf apex shape, the overlap

Table 4
Population means and standard errors (in parentheses) for continuous variables

Variable	Chinese chestnut	F_1	BC_1	BC_2	BC_3	American chestnut
Leaf relative length	2.32^{a} (0.244)	2.55^{b} (0.041)	2.94^{c} (0.050)	3.19^{d} (0.047)	2.95^{c} (0.041)	3.00^{c} (0.049)
Distance to maximum width	76.71^{a} (2.630)	$66.12^{b,c}$ (3.551)	$61.28^{b,c}$ (2.951)	68.95^{b} (2.544)	50.52^{d} (2.035)	58.60^{c} (2.158)
Tooth length	14.71^{a} (0.542)	9.76^{b} (0.326)	7.64^{d} (0.373)	8.70^{c} (0.341)	7.39^{d} (0.261)	7.29^{d} (0.273)
Tooth depth	1.66^{a} (0.173)	2.32^{b} (0.119)	2.20^{b} (0.118)	2.35^{b} (0.116)	2.14^{b} (0.076)	2.21^{b} (0.109)
Leaf length to tooth length ratio	10.89^{a} (0.314)	15.32^{b} (0.445)	18.58^{c} (0.535)	17.89^{c} (0.526)	15.89^{b} (0.381)	16.12^{b} (0.559)
Leaf width to tooth depth ratio	54.79^{a} (3.769)	27.37^{b} (1.431)	22.53^{c} (0.782)	22.00^{c} (1.267)	18.81^{d} (0.576)	18.23^{d} (0.635)
Lenticel width	0.421^{a} (0.0120)	0.319^{b} (0.0104)	0.254^{c} (0.0085)	0.228^{d} (0.0080)	$0.232^{c,d}$ (0.0068)	0.225^{d} (0.0084)
Twig diameter	3.09^{a} (0.081)	2.53^{b} (0.054)	2.40^{b} (0.076)	2.56^{b} (0.084)	2.77^{c} (0.074)	2.52^{b} (0.078)
Bud length	5.34^{a} (0.126)	4.81^{b} (0.162)	$4.32^{b,c}$ (0.092)	$4.17^{c,d}$ (0.133)	3.68^{d} (0.060)	3.90^{d} (0.106)
Bud width	4.11^{a} (0.085)	3.42^{b} (0.083)	2.92^{c} (0.048)	3.01^{c} (0.066)	3.00^{c} (0.057)	2.68^{d} (0.074)
Bud relative length	$1.31^{a,b}$ (0.031)	$1.40^{c,d}$ (0.033)	1.48^{e} (0.023)	$1.39^{b,c}$ (0.033)	1.23^{a} (0.019)	$1.46^{d,e}$ (0.025)
Bud appression	2.67^{a} (0.087)	2.25^{b} (0.098)	1.63^{c} (0.040)	1.80^{c} (0.073)	1.75^{c} (0.050)	1.43^{d} (0.060)
Bud pitch angle	12.4^{a} (1.17)	24.1^{b} (2.17)	31.3^{c} (1.23)	$28.7^{b,c}$ (1.49)	35.5^{d} (0.95)	38.8^{e} (0.94)
Bud yaw angle	1.5^{a} (0.57)	7.5^{b} (1.06)	20.0^{c} (0.99)	13.8^{d} (1.23)	17.3^{c} (1.16)	20.1^{c} (1.07)

Means within a row not followed by the same superscript letters are statistically different at $P \leq 0.05$.

Table 5
Population means for ordinal variables

Variable	Chinese chestnut	F_1	BC_1	BC_2	BC_3	American chestnut
Tooth hooking	1.02^{a}	1.92^{b}	1.96^{b}	2.00^{b}	2.00^{b}	1.96^{b}
Apex shape	1.22^{a}	2.56^{b}	3.33^{c}	3.52^{c}	3.44^{c}	3.35^{c}
Base shape	2.24^{a}	3.38^{b}	4.11^{c}	4.62^{c}	4.71^{c}	5.73^{d}
Interveinal hairs	1.00^{a}	1.06^{a}	1.89^{b}	1.87^{b}	2.00^{c}	2.00^{c}
Veinal hair density	1.20^{a}	2.50^{b}	2.57^{b}	$2.92^{c,d}$	2.83^{d}	3.00^{c}
Stipule size	1.07^{a}	1.96^{b}	2.96^{c}	2.92^{c}	2.98^{c}	3.00^{c}
Twig color	1.10^{a}	2.28^{b}	3.87^{c}	3.84^{c}	3.98^{c}	3.98^{c}
Twig hair density	1.12^{a}	2.52^{b}	$3.80^{c,d}$	3.69^{c}	$3.92^{d,e}$	3.96^{e}
Bud color	1.16^{a}	3.06^{b}	3.97^{c}	4.00^{c}	4.00^{c}	4.00^{c}
Bud tip shape	1.00^{a}	1.36^{b}	1.95^{c}	$1.84^{c,d}$	1.69^{d}	1.94^{c}

Means within a row not followed by the same superscript letters are statistically different at $P \leq 0.05$.

between species amounted to just 1–3 individual trees of the 45–50 in a population. Frequency distributions for index of species identity (ISI), shown in Fig. 4, clearly differentiate between the aggregate morphologies of American chestnut (mean = 0.85) and Chinese chestnut (mean = 0.11) ($P < 0.001$), with no overlap in scores between the two species.

3.2. Comparisons among the hybrid generations and parental species

All hybrid populations were typically more-or-less intermediate between the parental species in individual character measurements, and backcross hybrids tended to be more like American chestnut than did the F_1 (Tables 4 and 5). For virtually every continuous and ordinal variable, the F_1 and every backcross generation differed significantly from Chinese chestnut. The F_1 also differed significantly from American

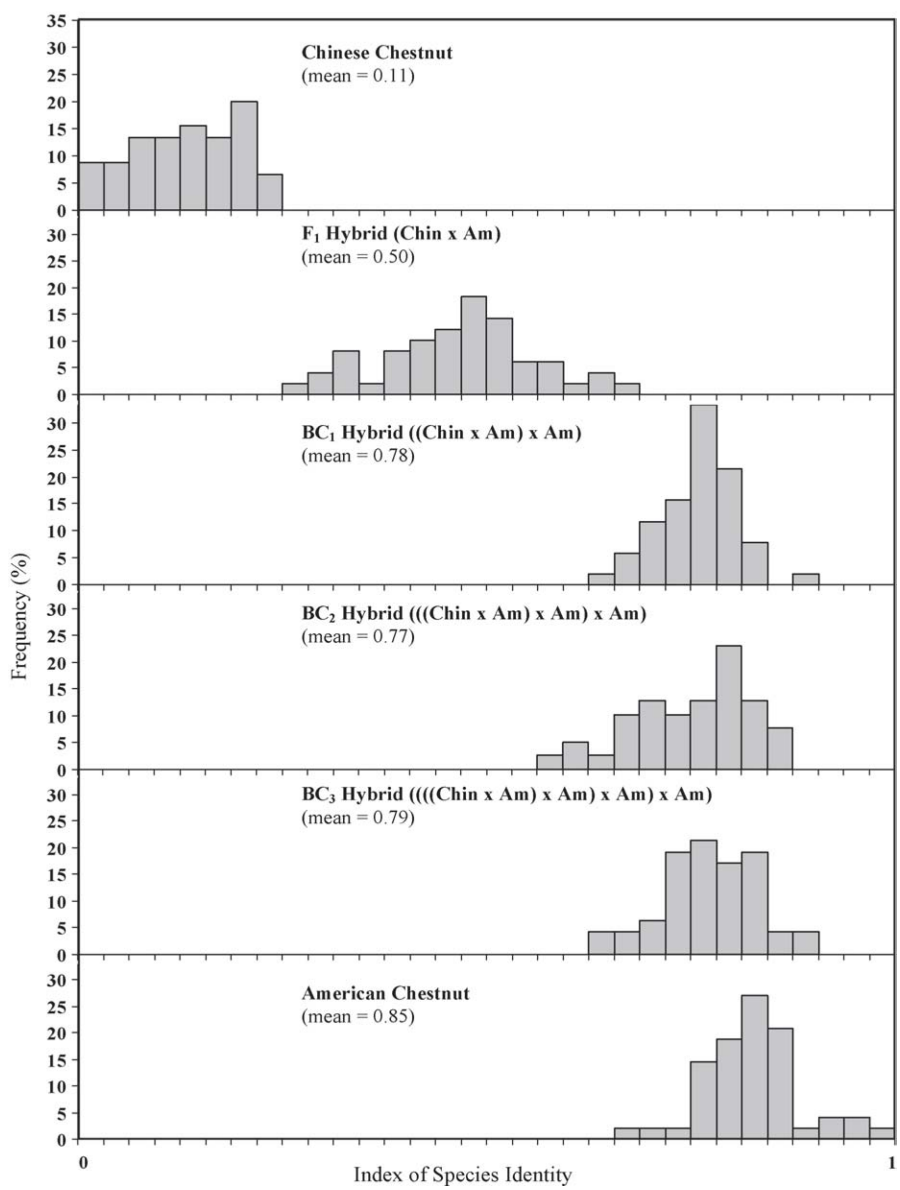

Fig. 4. Frequency distributions in the index of species identity for populations American chestnut, Chinese chestnut, their F_1 hybrid, and 1st to 3rd backcross hybrids.

chestnut in most respects, but backcross generations usually did not. Hybrid generations were significantly ($P \leq 0.05$) ''outside'' the mean scores of parental species for 3 of 24 variables, always in the direction of the American parent: leaf relative length for BC_2, distance to maximum width of leaf for BC_3, and leaf length to tooth length ratio for BC_1 and BC_2. However, these instances could be chance occurrences given that they were the only ones among 192 one-way comparisons between the four hybrid populations and the two species.

The mean ISI of every hybrid population was significantly larger than the mean ISI for Chinese chestnut and significantly smaller than the mean for American chestnut ($P \leq 0.001$) (Fig. 4). The F_1 population (Fig. 4b) was more-or-less exactly intermediate in ISI between the two parental species, having a mean score of 0.50 that is almost identical to an expected value of 0.48 based on observed parental means and assuming additive gene effects.

All three backcross hybrid populations had significantly ($P \leq 0.001$) larger mean ISI values than the F_1, and (as mentioned) significantly smaller mean ISI values than American chestnut. Thus, backcross hybrid populations resembled American chestnut much more than did the first-generation hybrid in their aggregate morphology, but none quite approached the species in mean value (Fig. 4). Assuming additive inheritance, each successive backcross generation should have more closely resembled American chestnut. Observed and expected (based on species' means) mean ISI values for each backcross population were as follows: BC_1, $0.78 > 0.66$ ($P < 0.001$); BC_2, $0.77 \sim 0.75$; and BC_3, $0.79 \sim 0.80$. In other words, the second and third backcross generations behaved approximately as expected, but the first backcross generation was significantly more American than its pedigree suggests. None of the backcross generations differed significantly from another in mean ISI.

ISI frequency distributions (Fig. 4) show that no tree in any hybrid generation fell within the range of values for Chinese chestnut but that every hybrid generation including the F_1 contained at least one tree that fell within the range of values for American chestnut. This was especially true of the backcross generations: 98% of the BC_1 trees, 90% of the BC_2 trees, and 96% of the BC_3 trees fell within the observed range of American chestnut in aggregate morphology.

3.3. *Specific resemblance of BC_3 hybrids to American chestnut*

Since intercrossed progeny of the BC_3 generation is the current breeding goal for the initial release of blight-resistant seed, a detailed comparison of BC_3 trees with American chestnut seems appropriate. The third backcross generation was not significantly different from American chestnut for eight of the 14 continuous variables measured (Table 4) (leaf relative length, tooth length, tooth depth, leaf length to tooth length ratio, leaf width to tooth depth ratio, lenticel width, bud length, and bud yaw angle). Among the six variables for which the BC_3 was significantly more ''Chinese'' than American chestnut, it was also significantly more ''American'' than Chinese chestnut for five of the those variables (distance to maximum width, twig diameter, bud width, bud relative length, bud appression, and bud pitch angle). In only the variable ''bud relative length'' was the BC_3 significantly different from American chestnut and not significantly different from Chinese chestnut.

A similar pattern held also for the ordinal variables (Table 5). Character state frequencies did not differ significantly between the BC_3 and American chestnut for seven of these 10 variables. For the remaining three variables (leaf base shape, veinal hair density, and bud tip shape), the BC_3 was significantly more ''Chinese'' than American, but also significantly more ''American'' than Chinese chestnut.

4. Discussion

For that portion of the chestnut genome that is not shared between American and Chinese chestnuts, the populations measured for this study should fall on average into a simple progression of the ratio of Chinese to American alleles: 16:0 (Chinese chestnut), 8:8 (F_1), 4:12 (BC_1), 2:14 (BC_2), 1:16 (BC_3), and 0:16 (American chestnut). In truth, of course, Chinese chestnut resembles American chestnut more than just about anything else in the world. But because of our choice of variables, measuring only those that discriminate between Chinese and American chestnuts, the two species had the most distinct and extreme morphologies in the study, as shown by their ISI scores (0.11 and 0.85 for Chinese and American chestnuts, respectively). The morphology of the first-generation hybrids also fit expectations based on genotype, as it was almost exactly intermediate (mean ISI = 0.50) between the parental species. Dilution of the Chinese fraction of the hybrid genome through recurrent backcrossing to American chestnut is a statistical inevitability. However, how well this dilution is reflected in the phenotypes of the backcross hybrid populations depends on other factors, which will be addressed in the following discussion.

There was considerable variation in ISI among individual trees, not just within the hybrid populations but in the parent species as well (Fig. 4). Some of this variation may have been caused by environmental effects or measurement error, but some may have arisen from genetic differences within populations. Each hybrid population used in this study was derived from a rather small number of crosses. This was particularly true of the first and third backcross generations, each of which was produced from crosses between only two trees. Given the possibility that chance selection of parents could have influenced the outcome, it seems remarkable that hybrid populations conformed to expectations as well as they did.

The progression towards American chestnut morphology in the three backcross generations fits expectations based on genotype in a very general way: the first backcross generation is more American than the first-generation hybrids and the morphology of the third backcross generation most closely resembles that of American chestnut. The only somewhat anomalous population in this respect is the BC_1, which was significantly more American than expected (mean ISI of 0.78

versus 0.66). Neither the second backcross generation (mean ISI of 0.77 versus 0.75) nor the third backcross generation (mean ISI of 0.79 versus 0.80) differed significantly from expectations based upon an assumption of completely additive gene action.

The most likely explanation for the unexpectedly "American" nature of the BC_1 trees is that some of the seeds from this cross, the hand transfer of F_1 pollen onto an American chestnut female, were actually fertilized by American chestnut trees that surrounded the female parent. Although the female flowers were always bagged before hand-pollination, contamination from unwanted pollen can occur if the flowers are not bagged soon enough. In the case of the BC_1 population, the contamination would have been in the direction of American chestnut because there were other American chestnut trees, but no Chinese or hybrid chestnut trees, in the vicinity of the female parent. Bagged but unpollinated flowers on other American chestnut trees in the same stand yielded seed that spring, and it has been suspected from the beginning that BC_1 cross is contaminated. In other words, some of the seedlings in this progeny set may look very much like American chestnut because they actually are American chestnut. Unlike the BC_1, contamination from American pollen was very unlikely in the BC_2 and BC_3 controlled pollinations.

The expectation that successive backcross populations should progress steadily in phenotype toward American chestnut is based on the assumption that morphological traits are controlled by a reasonably large number of alleles with additive affects. Unbalanced dominance effects in the direction of either parent species would alter the apparent rate of progression toward recovery of the American genome. Balanced dominance effects would not necessarily alter the mean ISI, but they would tend to increase the phenotypic variance within populations. The fact that the F_1 was almost exactly intermediate in ISI between the two species tends to suggest that any dominance effects were working equally in the directions of both species (the F_1 population was created from a fairly large number of American and Chinese parents and should not exhibit any sampling bias as described above). However, the presence of some dominance effects is suggested by the flattened distribution of ISI for the F_1 population (Fig. 4). It had much the highest variance in ISI of all sample populations.

The results of individual variables indicate that some characteristics were inherited in a dominant fashion in the F_1. For the continuous variables, this conclusion is suggested (though not proven) when the mean for the first hybrid generation was not significantly different from either American chestnut or Chinese chestnut. By this criterion, at least three continuous variables (tooth depth, leaf length to tooth length ratio, and twig diameter) appear to show dominance effects in the F_1, and all are in the direction of American chestnut (Table 4). None of the continuous variables appear to be inherited in a dominant fashion from the Chinese parent. For the ordinal variables, using a similar criterion, one variable (tooth hooking) appears to be controlled by dominant alleles from the American parent, and one variable (interveinal hairs) by dominant alleles from the Chinese parent. Partial dominance in the direction of Chinese chestnut could also be involved in the expression of several other variables. These results tend to agree with those of Hebard (1994), who studied the inheritance of several morphological characteristics in crosses between American and Chinese chestnut. For example, Hebard concluded that the presence of interveinal hairs was controlled by dominant Chinese alleles at a single locus.

The apparent mode of inheritance for individual characteristics could be affected by linkages in the backcross generations. In each stage of the breeding process, emphasis is placed on selecting as parents only trees that exhibit the highest possible degree of blight resistance, a Chinese chestnut trait. In fact, no hybrid trees are used in backcrossing that do not show good evidence of carrying the Chinese alleles for resistance. It is believed that these are carried at two or possibly three loci, each on a separate chromosome (Kubisiak et al., 1997). If genes coding for morphological traits are closely linked to the genes coding for blight resistance, then Chinese chestnut morphological traits will be selected for as well, at least until the linkages are broken through recombination, and this would be manifested in our data as an apparent tendency toward dominance of Chinese alleles. However, there is countervailing selection for American morphological type in each backcross generation, and under some circumstances this could involve linkages that mimic dominance on the part of American alleles. We cannot parse these effects with our data, but it is important to understand that backcross breeding does not yield unequivocal progress toward recovery of the recurrent phenotype.

Our data support a conclusion that the American morphology can be essentially recovered by three generations of backcrossing Chinese/American hybrids to American chestnut. Although certain individual American chestnut traits are not fully attained in the BC_3 as a whole, in each case the majority of individuals do resemble American chestnut for that characteristic. In aggregate morphology as measured by ISI, 96% of the BC_3 generation fell within the range of American chestnut and none within the range of Chinese chestnut. In effect, based upon the standard botanical practice of assigning individual plants to one species or another based upon diagnostic morphological characteristics (or, more formally, resemblance to the type specimen from which the species was named), most BC_3 hybrid trees "are" American chestnut. All but a few cannot be morphologically distinguished from American chestnut, at least by the characteristics measured for this study, and the population would especially resemble American chestnut if the least "American" trees were removed from the population, as will occur when producing the BC_3–F_2 population in the actual TACF breeding program.

Of course, it is the goal of the TACF breeding program that BC_3 hybrids resemble Chinese chestnut in one very important characteristic, blight resistance. It remains possible that the hybrids resemble Chinese chestnut in other respects not examined in this study. For example, we did not study growth, form, and many other attributes that have potential ecological or economic relevance. Because hybrid populations will eventually be used to restore chestnut to forests, it will be critical to test whether their ecological behavior sufficiently resembles that of native American chestnut populations (Anonymous, 2004). Also, in addition to purely biological considerations, successful use of BC_3 hybrids to restore chestnut faces important practical hurdles and possibly, on federal lands, questions of policy (Steiner and Carlson, 2005). However, on the important question of whether BC_3 hybrids have the appearance of American chestnut, as Burnham et al. (1986) predicted they would, our results show that they do.

References

Agresti, A., 2002. Categorical Data Analysis, second ed. John Wiley and Sons, NY, 710 pp.

Anonymous, 2004. Guidelines for testing blight-resistant American chestnuts. J. Am. Chestnut Found. 18 (1), 7–11.

Beattie, R.K., Diller, J.D., 1954. Fifty years of chestnut blight in America. J. For. 52, 323–329.

Brown, M.B., Forsythe, A.B., 1974. Robust tests for equality of variances. J. Am. Stat. Assoc. 69, 364–367.

Burnham, C.R., 1981. Blight-resistant American chestnut: there's hope. Plant Dis. 65, 459–460.

Burnham, C.R., 1987. Historical overview of chestnut breeding in the United States. J. Am. Chestnut Found. 2 (1), 9–11.

Burnham, C.R., Rutter, P.A., French, D.W., 1986. Breeding blight-resistant chestnuts. Plant Breed. Rev. 4, 347–397.

Davis, D.E., 2003. Where There Are Mountains: An Environmental History of the Southern Appalachians. University of Georgia Press, 352 pp.

Diller, J.B., Clapper, R.B., 1965. A progress report on attempts to bring back the chestnut tree in the eastern United States, 1954–1964. J. For. 63, 186–188.

Elliston, J.E., 1981. Hypovirulence and chestnut blight research: fighting disease with disease. J. For. 79, 657–660.

Hebard, F.V., 1994. Inheritance of juvenile leaf and stem morphological traits in crosses of Chinese and American chestnut. J. Hered. 85, 440–446.

Hebard, F.V., 2005. The backcross breeding program of The American Chestnut Foundation. In: Carlson, J.E., Steiner, K.C. (Eds.), Restoration of American chestnut to forest lands. Proceedings of Conference and Workshop, May 2004, Asheville, NC. The Pennsylvania State University.

Kubisiak, T.L., Hebard, F.V., Nelson, C.D., Zhang, J., Bernatzky, R., Huang, H., Anagnostakis, S.L., Doudrick, R.L., 1997. Molecular mapping of resistance to blight in an interspecific cross in the genus *Castanea*. Phytopathology 87, 751–759.

Lutts, R.H., 2004. Like manna from God: The American chestnut trade in southwestern Virginia. Environ. Hist. 9, 497–525.

Milgroom, M.G., Cortesi, P., 2004. Biological control of chestnut blight with hypovirulence: a critical analysis. Ann. Rev. Phytopath. 42, 311–338.

Ott, R.L., 1992. An Introduction to Statistical Methods and Data Analysis, fourth ed. Duxbury Press, Belmont, 1051 pp.

Russell, E.W.B., 1987. Pre-blight distribution of *Castanea dentata* (Marsh.) Borkh. Bull. Torrey Bot. Club 114, 183–190.

SAS Institute, Inc., 1999. The SAS System for Windows, version 8.02. Cary, NC.

Steiner, K.C., Carlson, J.E., 2005. Restoration of American chestnut to forest lands. In: Proceedings of a Conference and Workshop. Asheville, NC, May 2004. The Pennsylvania State University.

Van Alfen, N.K., Jaynes, R.A., Anagnostakis, S.L., Day, P.R., 1975. Chestnut blight: biological control by transmissible hypovirulence in *Endothia parasitica*. Science 189, 890–891.