

## ALLOZYME AND RAPD ANALYSIS OF THE GENETIC DIVERSITY AND GEOGRAPHIC VARIATION IN WILD POPULATIONS OF THE AMERICAN CHESTNUT (FAGACEAE)<sup>1</sup>

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Genetic variation among 12 populations of the American chestnut (*Castanea dentata*) was investigated. Population genetic parameters estimated from allozyme variation suggest that *C. dentata* at both the population and species level has narrow genetic diversity as compared to other species in the genus. Average expected heterozygosity was relatively low for the population collected in the Black Rock Mountain State Park, Georgia ( $H_e = 0.096 \pm 0.035$ ), and high for the population in east central Alabama ( $H_e = 0.196 \pm 0.048$ ). Partitioning of the genetic diversity based on 18 isozyme loci showed that ~10% of the allozyme diversity resided among populations. Cluster analysis using unweighted pair-group method using arithmetic averages of Rogers' genetic distance and principal components analysis based on allele frequencies of both isozyme and RAPD loci revealed four groups: the southernmost population, south-central Appalachian populations, north-central Appalachian populations, and northern Appalachian populations. Based on results presented in this study, a conservation strategy and several recommendations related to the backcross breeding aimed at restoring *C. dentata* are discussed.

**Key words:** allozyme variation; *Castanea dentata*; conservation; Fagaceae; geographic variation; genetic diversity; population structure; random amplified polymorphic DNA (RAPD).

The American chestnut (*Castanea dentata* [Marsh.] Brokh) was once a dominant species in the eastern deciduous forest for ~2000 yr before chestnut blight (caused by *Cryphonectria parasitica* [Murrill] Barr) arrived on the North American continent near the turn of the century (Davis, 1981). Palynological studies indicated that *C. dentata* was present in the southern Appalachian region 15 000 yr ago, and in the northern Appalachian region 5000 yr ago and arrived in Connecticut 2000 yr ago (Delcourt et al., 1980; Davis, 1981). Prior to blight, the native range of the American chestnut extended from southern Maine southward to Georgia, Alabama, and Mississippi and westward to southern Michigan, Indiana, and Tennessee. Every fourth tree in the central Appalachian forest was a chestnut (Saucier, 1973). The species was commercially important at one time for its outstanding timber quality and abundant nut production. In 1904 chestnut blight, a fungal disease, was first observed in New York State, apparently introduced from importation of Japanese chestnut (Anagnostakis, 1992). The disease spread rapidly and reduced the entire species to a minor understory shrub within 50 yr. Prolific stump sprouting has enabled the American chestnut to persist over most

of its native range during the past 40 yr. The gene pool of the species still exists, but it could face serious erosion in the future as old root systems fail to produce sprouts and perish. The species is threatened and a conservation plan is needed for the American chestnut.

The genus *Castanea* comprises seven species (Rutter, Miller, and Payne, 1990). Four species are native to Asia: Chinese chestnut (*C. mollissima* Bl.), Seguin chestnut (*C. seguinii* Dode.), and Henry chestnut (*C. henryi* Rehd. & Wils.) on mainland China and Japanese chestnut (*C. crenata* Sieb. & Zucc.) on the Japanese islands and Korean peninsula. Two species are native to North America: the American chestnut and chinkapin (*C. pumila* Mill.). European chestnut (*C. sativa* Mill.) is native to southern Europe and western Asia. All species are diploid ( $2n = 2X = 24$ ) and hybridize freely, but interspecific  $F_1$ 's usually suffer from low seed germination and male sterility (Rutter, Miller, and Payne, 1990). Blight resistance among the species differs. The American and the European species are susceptible, whereas the Asian species are blight resistant (Graves, 1950; Huang et al., 1996). The levels of susceptibility of the European and American species differ, and the American chestnut is the most susceptible species (Graves, 1950). Several recent studies on allozyme diversity suggest that the American chestnut has the lowest levels of genetic diversity among species in the genus (Villani et al., 1991; Huang, Dane, and Norton, 1994a).

Starting as early as the 1920s, considerable breeding efforts were carried out by the U.S. Department of Agriculture (USDA) in an attempt to save the American chestnut. Unfortunately, the breeding programs failed to produce a desirable timber-type American chestnut with

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blight resistance (Burnham, Rutter, and French, 1986), and the USDA programs were abandoned in the 1960s. In the early 1980s, Burnham (1981, 1982) critically reviewed previous breeding programs and concluded that the traditional backcross method, used successfully in crop breeding, offered a more promising approach to the problem. Burnham, Rutter, and French (1986) proposed a backcross breeding program designed to introgress the blight resistance of the Chinese chestnut into the American chestnut. The backcross program for restoration of the American chestnut is based on two crucial assumptions: (1) blight resistance exists in the Chinese chestnut (*C. mollissima*), is heritable, and at least partially dominant (Clapper, 1952) and (2) blight resistance is under oligogenic control (presumably two genes; Burnham, 1981). Kubisiak et al. (1997) constructed a genetic linkage map for *Castanea* species using an  $F_2$  population derived from an interspecific cross between the American and Chinese chestnut and detected three regions that appear to be conditioning resistance to *Cryphonectria parasitica* ( $P < 0.001$ ). An aggressive backcross breeding program led by the American Chestnut Foundation (ACF), a philanthropic organization established to restore the American chestnut, has recently reached  $BC_3$  populations (Hebard, 1996). In theory, introgression of two or three genes conferring blight resistance should be relatively straightforward, however, it will be a far more complicated task to successfully apply backcross breeding to a widespread forest species with a natural range spanning five United States climate zones and 20 states. A well-designed strategy for capturing as much genetic variation as possible from the recurrent parents becomes an essential factor that will affect the recovery of the gene pool of the American chestnut and its adaptability and desirable timber qualities, and ultimately will determine the success of the program. Allard (1960) suggested that when applying backcross methods to an unimproved species, the genetic variation of the recurrent parent must be sampled using many individuals from different source populations. If the species being improved has a wide geographic range, a program should be considered for each major region. Unfortunately, information on genetic diversity and geographic variation in American chestnut populations does not exist. Evaluation of genetic variation in the species is the first step towards addressing this concern.

The purposes of the present investigation were three-fold: (1) to determine and partition the genetic diversity of wild *C. dentata* populations and to determine whether levels of the genetic diversity attributed to the vulnerability of *C. dentata* to chestnut blight; (2) to detect whether there is any geographical pattern of diversity differentiation and allelic frequency distribution along the native range; and (3) to apply this information to conservation strategies of the extant gene pool of the remnant wild populations and provide guidelines for the selection of recurrent parents for use in the backcross breeding program.

## MATERIALS AND METHODS

**Plant material**—During 1994 and 1995, 12 populations of *C. dentata* were sampled from across the native range, along Appalachia from

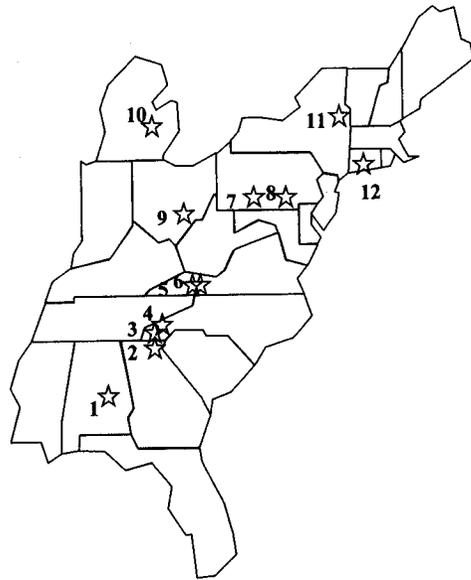


Fig. 1. Range map and geographical distribution of 12 *C. dentata* populations sampled. (1) AL—Macon County, Alabama; (2) GA—Black Rock Mountain State Park, Georgia; (3) NC-1—Wallace Branch, Nantahala National Forest, North Carolina; (4) NC-2—Thomas Ridge, Great Smoky Mountain National Park, North Carolina; (5) VA-1—Glade-2, Smyth County, Virginia; (6) VA-2—Plummer-1, Smyth County, Virginia; (7) PA-1—York County, Pennsylvania; (8) PA-2—southern Pennsylvania; (9) OH—Central-eastern Ohio; (10) MI—Southeastern Michigan; (11) NY—Troy, New York; (12) CT—Essex, Connecticut.

Alabama up to central New York (Fig. 1). For electrophoresis, 1-yr-old twigs with mature buds were collected from sprouts of large stumps of regenerated coppices, remnants of the original blight-killed trees. Each population consisted of sprouts from 16 to 30 trees except the population from Connecticut in which only 11 trees were sampled. Twigs were kept in zip-lock bags at 4°C until electrophoresis. For random amplified polymorphic DNA (RAPD) analysis, DNA extraction and polymerase chain reactions (PCR) were tested using cotyledons, buds, and young and mature leaves. Cotyledonary tissues (diploid) yielded the highest quantity and quality of DNA and produced the most consistent and reproducible RAPD banding patterns. As a result, only four populations that either were producing adequate quantities of seed or purified DNA from leaves (by additional purification steps) were included in the RAPD analysis.

**Allozyme analysis**—Enzyme were extracted from mature buds of 1-yr-old twigs. The extraction procedure was as described by Huang, Dane, and Norton (1994a). The isoelectric focusing polyacrylamide slab gel system of pH 4–9 described by Mulcahy et al. (1981) was used in this study because of its high resolution. Gels were assayed for acid phosphatase (ACP; EC [Enzyme Commission] 3.1.3.2), formate dehydrogenase (FDH; EC 1.2.1.2), glutamate dehydrogenase (GDH; EC 1.4.1.2), isocitrate dehydrogenase (IDH; EC 1.1.1.41), malate dehydrogenase (MDH; EC 1.1.1.37), malic enzyme (ME; EC 1.1.1.40), peroxidase (PRX; EC 1.11.1.7), phosphoglucomutase (PGM; EC 5.4.2.2), phosphoglucoisomerase (PGI; EC 5.3.1.3), shikimate dehydrogenase (SKD; EC 1.1.1.25), and triosephosphate isomerase (TPI; EC 5.3.1.1). Gels were stained as described by Wendel and Weeden (1989) except that only one-third of the original volume of stain solutions was used. Eighteen loci were scored: *Acp-1*, *Acp-2*, *Acp-3*, *Est-2*, *Est-5*, *Fdh*, *Gdh*, *Idh*, *Mdh-2*, *Me*, *Pgi-2*, *Pgm*, *Prx-1*, *Prx-2*, *Prx-3*, *Skd-1*, *Skd-2*, and *Tpi*. Genetic analysis and allele designation of those loci have previously been described by Huang, Dane, and Norton (1994b).

**RAPD analysis**—Genomic DNA was extracted from 0.2 g of cotyledons using a CTAB-based procedure (Weising et al., 1995). Genomic DNA extracted from 0.2 g of leaf tissue was further purified using GENECLEAN II (BIO 101 Inc, Vista, CA). PCR reactions were carried out in a 25  $\mu$ L volume containing 10 mmol/L Tris-HCl; 1.5 mmol/L  $MgCl_2$ ; 50 mmol/L KCl (pH 8.3), 0.2 mmol/L of each dNTP (Boehringer Mannheim, Indianapolis, IN), 5 pmol/L decamer oligonucleotide primer, 10 ng genomic DNA, and 1 unit of Taq DNA polymerase (Boehringer Mannheim). DNA amplifications were performed in a Perkin Elmer 2400 DNA thermal cycler programmed as follows: 10 min at 94°C; 35 cycles of 45 sec at 94°C, 45 sec at 36°C, 2 min at 72°C, followed by 5 min at 72°C. Amplification products were analyzed by electrophoresis on 1.5% agarose gels, detected by staining with ethidium bromide. The gels were photographed under UV light with Polaroid film 667. A 100-bp DNA ladder (GibcoBRL) was used as molecular size marker. Twenty-two RAPD loci, randomly chosen from the chestnut recombinational linkage map (Kubisiak et al., 1997), were scored: A07<sub>1600</sub>, A11<sub>1100</sub>, A18<sub>0650</sub>, E02<sub>0700</sub>, F16<sub>1050</sub>, G17<sub>0525</sub>, G17<sub>1300</sub>, G17<sub>1350</sub>, G19<sub>0525</sub>, 110<sub>1050</sub>, 153<sub>1200</sub>, 153<sub>1900</sub>, 271<sub>0700</sub>, 271<sub>1650</sub>, 411<sub>0500</sub>, 423<sub>1150</sub>, 423<sub>1700</sub>, 531<sub>0800</sub>, 557<sub>0650</sub>, 557<sub>0900</sub>, 551<sub>0900</sub>, and 551<sub>2400</sub>.

**Data analysis**—Allele frequencies for isozyme loci were estimated (Nei, 1987). Prior to estimating allele frequency for the dominant RAPD loci, Hardy-Weinberg equilibrium was tested using the entire allozyme data set. Provided that Hardy-Weinberg equilibrium existed, allele frequencies ( $p$  and  $q$ ) for RAPD loci were estimated using the homozygous null genotypes  $\sqrt{q^2} = q$  and  $p = 1 - q$  (since  $p + q = 1$ ). A set of measures of intra- and interpopulation genetic statistics were generated using a computer program developed by Loveness and Schnable (Godt and Hamrick, 1993) and BIOSYS-1 (Swofford and Selander, 1981) including: the percentage of polymorphic loci ( $P$ ), the mean number of alleles per locus ( $A$ ), effective number of alleles per locus ( $A_e$ ), observed heterozygosity ( $H_o$ ), Hardy-Weinberg expected heterozygosity ( $H_e$ ), total genetic diversity ( $H_T$ ), genetic diversity within population ( $H_s$ ), genetic diversity between populations ( $D_{st}$ ), the relative magnitude of genetic differentiation among populations ( $G_{st} = D_{st}/H_T$ ) (Nei, 1978), and a  $\chi^2$  test for heterogeneity of allele frequencies among populations.  $F$  statistics were also used including the fixation index of individuals within populations ( $F_{IS}$ ), the fixation index with respect to the total population ( $F_{IT}$ ), and the proportion of genetic differentiation ( $F_{ST}$ ) (Wright, 1978). Nei's (1978) genetic distance and identity, as well as Rogers' modified genetic distance (Wright, 1978) were calculated for all pairwise combinations of populations. A dendrogram was constructed based on the matrix of Rogers' distance using unweighted pair-group method using arithmetic average (UPGMA). The principal components analysis (PCA) was performed based on the allele frequency data of populations using PC-SAS for Windows (SAS, 1989).

## RESULTS

Of 18 isozyme loci investigated, 38 alleles were detected at 14 polymorphic loci across the 12 populations (a total of 42 alleles were detected when monomorphic loci were included). The expected genotype frequencies at all loci, and in all populations, conformed to Hardy-Weinberg expectations except for *Prx-3* and *Acp-3*. A significant excess of heterozygotes was detected at these two loci in eight and four of the 12 populations, respectively (data not shown). Contingency  $\chi^2$  analyses for heterogeneity of allele frequencies across populations were significant at nine of the 14 polymorphic loci (Table 1). A south-north cline was observed in allele frequencies of one species-specific locus, *Skd-2*, along the Appalachian axis (*Skd-2* frequency = 2.654–0.0473 latitude,  $P < 0.05$ ). *Skd-1* and *Skd-2* with two alleles each are isozyme

loci unique to North American *Castanea* species (Huang, Dane, and Norton, 1994b).

Genetic variability among the 12 populations is presented in Table 2. For the species, the percentage polymorphic loci  $P = 77.8$ , mean number of alleles per locus  $A = 2.33$ , effective number of alleles per locus  $A_e = 1.24$  and gene diversity  $H_e = 0.167$ . Averaged across populations, the number of polymorphic loci  $P = 59.7$  (ranging from 44.4 to 72.2), mean number of alleles per locus  $A = 1.69$  (ranging from 1.50 to 1.89), effective number of alleles per locus  $A_e = 1.24$  (ranging from 1.15–1.33), and gene diversity  $H_e = 0.151$  (Table 2). Expected heterozygosity ranged from  $0.096 \pm 0.035$  for the population collected in the Black Rock Mountain State Park, Georgia, to  $0.196 \pm 0.035$  for the population in east-central Alabama. Significant differences in expected heterozygosity were found between the southernmost population (AL) and populations collected in the southern Appalachia (GA and NC). Genetic variability measures examined using the 22 RAPD markers showed that a relatively higher genetic diversity exists in the southernmost population with declines toward the northernmost population (Table 2). Partitioning of population genetic diversity based on the 14 polymorphic isozyme loci showed that ~90% of the allozyme diversity resided within populations. This result was reflected by Nei's  $G_{st}$  (0.110), which measures the proportion of the total genetic diversity partitioned among populations (Table 3).

Nei's genetic identity and distance (1978) based on allozyme allele frequencies were estimated for all 66 pairwise comparisons among the populations (Table 4). The mean identity for pairwise comparisons of the 12 *C. dentata* populations was 0.972, ranging from 0.902 to 1.000. These estimates are typical for conspecific populations (Crawford, 1989). Nei's genetic identity and distance were also estimated using RAPD marker allele frequencies, showing similar results (Table 5). Genetic relationships among the populations were further examined by UPGMA using Rogers' genetic distance and by PCA. The UPGMA dendrogram revealed four groups: the southernmost population (AL), south-central Appalachian populations (GA, NC-1, NC-2, VA-1, VA-2, including OH and MI), north-central Appalachian populations (PA-1, PA-2), and northern Appalachian populations (NY, CT), based on allele frequencies of both isozyme and RAPD loci (Fig. 2a, b). The relationships suggested by PCA based on allozymes are similar to those implied by UPGMA. The first three components, which accounted for 60% of the total variation, divided the 12 populations into the same four groups (Fig. 3).

## DISCUSSION

**Genetic diversity in *C. dentata***—The American chestnut species has not recovered from the devastating effects of fungal colonization by *C. parasitica*. For nearly a century, pathologists from around the world have applied their expertises to combat the disease, but their efforts to date have not been successful. On the other hand, little research has been conducted to investigate the genetic base involved in host plant susceptibility or resistance, and the underlying genetics of blight resistance is still poorly understood. Furthermore, the genetic vulnerability

TABLE 1. Allozyme allele frequencies and contingency  $\chi^2$  analysis of heterogeneity of allele frequencies in 12 American chestnut populations.<sup>a</sup>

Locus and allele	Population												$\chi^2$
	AL	GA	NC-1	NC-2	VA-1	VA-2	PA-1	PA-2	OH	MI	NY	CT	
<i>Pgm</i>													119.22** <sup>b</sup>
<i>a</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.120	0.190	0.129	0.000	0.406	0.273	
<i>b</i>	1.000	1.000	1.000	1.000	1.000	1.000	0.880	0.762	0.871	1.000	0.594	0.727	
<i>c</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.048	0.000	0.000	0.000	0.000	
<i>Pgi-2</i>													142.64**
<i>a</i>	0.000	0.000	0.024	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
<i>b</i>	0.000	0.000	0.000	0.017	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000	
<i>c</i>	0.550	1.000	0.929	0.966	0.971	0.911	0.960	0.952	0.952	0.950	0.875	0.909	
<i>d</i>	0.000	0.000	0.024	0.000	0.000	0.018	0.040	0.024	0.032	0.025	0.000	0.000	
<i>e</i>	0.450	0.000	0.024	0.017	0.029	0.054	0.000	0.024	0.016	0.025	0.125	0.091	
<i>Tpi</i>													24.01*
<i>a</i>	0.175	0.000	0.071	0.086	0.000	0.054	0.000	0.119	0.016	0.075	0.094	0.045	
<i>b</i>	0.825	1.000	0.929	0.914	1.000	0.946	1.000	0.881	0.984	0.925	0.906	0.955	
<i>Mdh-2</i>													163.44**
<i>a</i>	0.225	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
<i>b</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.020	0.000	0.000	0.000	0.000	0.000	
<i>c</i>	0.775	0.976	1.000	0.931	1.000	1.000	0.960	0.976	1.000	1.000	0.938	1.000	
<i>d</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.020	0.024	0.000	0.000	0.063	0.000	
<i>e</i>	0.000	0.024	0.000	0.069	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
<i>Skd-1</i>													99.64**
<i>a</i>	0.250	0.214	0.262	0.276	0.412	0.250	0.520	0.929	0.500	0.475	0.031	0.591	
<i>b</i>	0.750	0.786	0.738	0.724	0.588	0.750	0.480	0.071	0.500	0.525	0.969	0.409	
<i>Skd-2</i>													169.55**
<i>a</i>	1.000	1.000	1.000	0.948	0.941	0.839	0.980	0.929	0.823	0.925	0.313	0.364	
<i>b</i>	0.000	0.000	0.000	0.052	0.059	0.161	0.020	0.071	0.177	0.075	0.688	0.636	
<i>Prx-1</i>													14.90 <sup>NS</sup>
<i>a</i>	1.000	1.000	1.000	1.000	1.000	1.000	0.960	1.000	0.968	1.000	1.000	1.000	
<i>b</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.040	0.000	0.032	0.000	0.000	0.000	
<i>Prx-2</i>													17.52 <sup>NS</sup>
<i>a</i>	1.000	1.000	1.000	0.983	0.941	1.000	0.960	1.000	1.000	1.000	1.000	1.000	
<i>b</i>	0.000	0.000	0.000	0.017	0.059	0.000	0.040	0.000	0.000	0.000	0.000	0.000	
<i>Prx-3</i>													26.35 <sup>NS</sup>
<i>a</i>	0.500	0.500	0.500	0.500	0.500	0.500	0.260	0.333	0.500	0.500	0.656	0.409	
<i>b</i>	0.500	0.500	0.500	0.500	0.500	0.500	0.720	0.643	0.500	0.500	0.344	0.591	
<i>c</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.020	0.024	0.000	0.000	0.000	0.000	
<i>Est-2</i>													37.72*
<i>a</i>	0.300	0.119	0.119	0.086	0.176	0.125	0.220	0.214	0.145	0.175	0.094	0.273	
<i>b</i>	0.700	0.833	0.881	0.914	0.824	0.875	0.780	0.786	0.855	0.825	0.906	0.727	
<i>c</i>	0.000	0.048	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
<i>Est-5</i>													32.77**
<i>a</i>	0.125	0.071	0.024	0.034	0.147	0.268	0.020	0.143	0.048	0.100	0.063	0.091	
<i>b</i>	0.875	0.929	0.976	0.966	0.853	0.732	0.980	0.857	0.952	0.900	0.938	0.909	
<i>Acp-1</i>													18.77 <sup>NS</sup>
<i>a</i>	0.325	0.143	0.143	0.207	0.176	0.036	0.140	0.190	0.161	0.225	0.281	0.136	
<i>b</i>	0.675	0.857	0.857	0.793	0.824	0.964	0.860	0.810	0.839	0.775	0.719	0.864	
<i>Acp-2</i>													13.53 <sup>NS</sup>
<i>a</i>	0.775	0.952	0.929	0.931	0.824	0.857	0.940	0.905	0.919	0.900	0.906	0.864	
<i>b</i>	0.225	0.048	0.071	0.069	0.176	0.143	0.060	0.095	0.081	0.100	0.094	0.136	
<i>Acp-3</i>													127.01**
<i>a</i>	0.000	0.000	0.000	0.034	0.206	0.143	0.480	0.405	0.032	0.100	0.188	0.091	
<i>b</i>	0.950	0.952	0.952	0.776	0.735	0.768	0.520	0.571	0.903	0.850	0.719	0.909	
<i>c</i>	0.050	0.048	0.048	0.190	0.059	0.089	0.000	0.024	0.065	0.050	0.094	0.000	

<sup>a</sup> Four loci, *Me*, *Gdh*, *Fdh*, and *Idh*, were fixed in the samples examined, i.e., monomorphic loci.

<sup>b</sup> NS, \*, \*\* Nonsignificant or significant at  $P \leq 0.05$  or 0.01, respectively.

of the American chestnut, resulting in its elimination as a predominant canopy species, has been overlooked. No attempt has been made to investigate a possible link between levels of population genetic diversity and genetic vulnerability. Population genetic parameters estimated in this study suggest that *C. dentata*, in general, has average levels of allozyme variation when compared to other plant species with similar ecological and life-history characteristics (Hamrick and Godt, 1989). However, comparisons of such generalized information should be in-

terpreted cautiously (Hamrick and Godt, 1996). In a recent study, Park et al. (1994) pointed out that historical factors may have a more profound influence on determining and partitioning of the genetic diversity in plant species. Historically, oak-chestnut forests (*Quercus-Castanea*) played an important role in the deciduous forests of eastern North America for 8000 yr after the last maximum glaciation, the Wisconsin glacier (Davis, 1983). In the genus *Quercus* low values of  $P = 29.7$ ,  $A = 1.37$ , and  $H_e = 0.081$  were recorded for 17 populations of sev-

TABLE 2. Genetic variability measures for 12 American chestnut populations.<sup>a</sup>

Population	N	P	A	A <sub>e</sub>	H <sub>o</sub>	H <sub>e</sub>
<b>Allozyme variability</b>						
AL	20	55.6	1.56	1.33	0.264 (0.074)	0.196 (0.048)
GA	21	44.4	1.50	1.15	0.135 (0.052)	0.096 (0.035)
NC-1	21	50.0	1.61	1.16	0.146 (0.055)	0.104 (0.035)
NC-2	29	66.7	1.78	1.20	0.169 (0.052)	0.135 (0.037)
VA-1	17	55.6	1.61	1.25	0.183 (0.070)	0.156 (0.043)
VA-2	28	55.6	1.72	1.23	0.185 (0.056)	0.151 (0.041)
PA-1	25	72.2	1.83	1.24	0.142 (0.062)	0.150 (0.041)
PA-2	21	66.7	1.89	1.26	0.175 (0.072)	0.169 (0.041)
OH	31	66.7	1.78	1.23	0.194 (0.054)	0.147 (0.039)
MI	20	55.6	1.67	1.23	0.206 (0.064)	0.146 (0.040)
NY	16	66.7	1.61	1.29	0.222 (0.086)	0.179 (0.043)
CT	11	61.1	1.72	1.30	0.187 (0.095)	0.182 (0.044)
Average	22	59.7 (3.3)	1.69 (0.12)	1.24 (0.05)	0.184 (0.019)	0.151 (0.012)
<b>RAPD variability</b>						
AL	25	86.4	19 (0.1)	1.66	—	0.398 (0.038)
VA-1	27	86.4	1.9 (0.1)	1.62	—	0.382 (0.037)
PA-2	25	77.3	1.8 (0.1)	1.58	—	0.368 (0.044)
NY	28	68.2	1.7 (0.1)	1.44	—	0.305 (0.047)

<sup>a</sup> Standard errors are in parentheses.

en species in New Jersey (Manos and Fairbrothers, 1987); in contrast values of  $P = 62.5 \pm 3.0$ ,  $A = 2.8 \pm 0.21$ ,  $A_e = 1.70 \pm 0.07$  and  $H_e = 0.31 \pm 0.027$  were reported for five populations of two *Quercus* species in Michigan (Hokanson et al., 1993). Within the genus *Castanea*, much higher genetic diversity has been reported for *C. mollissima*, and even for a regional sample of *C. seguinii*, as compared to *C. dentata*. Huang, Dane, and Norton (1994a and Huang's unpublished data) found  $P = 84.2-86.8$ ,  $A = 2.05-2.15$ , and  $H_e = 0.31-0.38$  in *C. mollissima*;  $P = 68.42$ ,  $A = 1.74$  and  $H_e = 0.20$  in *C. seguinii*. Villani et al. (1991) reported  $P = 62.15-82.69$ ,  $A_e = 1.40-1.51$ , and  $H_e = 0.29-0.34$  in *C. sativa*. It is possible that the narrow genetic diversity of the American chestnut as compared to congener species may have contributed to its demise. *Castanea sativa* suffered severe

damages as a result of infection by the chestnut blight fungus during the 1930s, but in recent decades has regenerated due to hypovirulent isolates of the pathogen (*C. parasitica*). However, development of hypovirulent strains of *Cryphonectria parasitica* thus far has not proven successful in the recovery of *C. dentata* (Burnham, Rutter, and French, 1986). This lack of success has been attributed to the presence of more mycelial compatibility groups in North America than in Europe, which has restricted spread of determinants of hypovirulence among isolates of the pathogen (Anagnostakis, Hau, and Kranz, 1986; Melzer et al., 1997).

**Distribution and geographic pattern of genetic diversity in *C. dentata***—The proportion of the genetic diversity found among *C. dentata* populations ( $G_{st} = 0.110$ ; Table 3) was similar to other long-lived woody perennials (0.076), wind-outcrossing species (0.099), and late-successional species (0.101) (Hamrick and Godt, 1989). *Castanea dentata* was also found to have almost the same level of genetic diversity among populations when compared to the congener species *C. sativa* ( $F_{ST} = 0.10$ ) (Pigliucci, Benedettelli, and Villani, 1990). Based on an average for all isozyme loci, *C. dentata* appears to harbor most of its genetic diversity within populations. However, significant differences in  $G_{st}$  were observed from locus to locus with values ranging from 0.028 (*Est-2*) to 0.326 (*Skd-2*) (Table 3). Average gene heterozygosity ( $H_e$ ) was significantly different among the 12 populations. The highest level ( $H_e = 0.196 \pm 0.048$ ) was found in central-east Alabama (Macon County) and the lowest ( $H_e = 0.096 \pm 0.035$ ) was found in the southern Appalachian region (specifically, Black Rock Mountain, Georgia), which might have been the result of genetic drift with loss of individuals occurring through death of stumps and current lack of gene flow among populations. Gene flow among populations was low with  $Nm$  according to the Slatkin method  $Nm_{(S)} = 1.12$ , and  $Nm$  according to Wright  $Nm_{(W)} = 2.23$ . Moderately higher levels of average gene heterozygosity were also observed in the north-

TABLE 3. Genetic variability statistics (Nei, 1987) and Wright's *F* statistics (Wright, 1978) for 20 isozyme loci in the American chestnut populations.

Locus	H <sub>T</sub>	H <sub>S</sub>	G <sub>st</sub>	F <sub>IS</sub>	F <sub>IT</sub>
<i>Pgm</i>	0.152	0.124	0.184	-0.144	0.066
<i>Pgi-2</i>	0.158	0.132	0.163	-0.049	0.123
<i>Tpi</i>	0.112	0.107	0.046	-0.115	-0.063
<i>Mdh-2</i>	0.071	0.063	0.112	-0.093	0.029
<i>Skd-1</i>	0.476	0.385	0.192	-0.230	0.006
<i>Skd-2</i>	0.227	0.153	0.326	-0.004	0.323
<i>Prx-1</i>	0.015	0.015	0.029	-0.038	-0.008
<i>Prx-2</i>	0.019	0.018	0.034	-0.045	-0.010
<i>Prx-3</i>	0.502	0.486	0.032	-0.852	-0.792
<i>Est-2</i>	0.280	0.272	0.028	-0.202	-0.168
<i>Est-5</i>	0.171	0.160	0.063	-0.082	-0.014
<i>Acp-1</i>	0.289	0.278	0.036	-0.202	-0.159
<i>Acp-2</i>	0.186	0.181	0.026	-0.146	-0.116
<i>Acp-3</i>	0.343	0.295	0.138	0.323	0.416
<i>Me</i>	0.000	0.000	0.000	0.000	0.000
<i>Gdh-1</i>	0.000	0.000	0.000	0.000	0.000
<i>Fdh-1</i>	0.000	0.000	0.000	0.000	0.000
<i>Idh</i>	0.000	0.000	0.000	0.000	0.000
Mean	0.167	0.148	0.110	-0.226	-0.091
Mean (Poly)	0.214	0.191	0.101	-0.134	-0.026

TABLE 4. Allozyme estimates of Nei's (1978) unbiased genetic identity (below diagonal) and distance (above diagonal) among 12 American chestnut populations.

Population	1	2	3	4	5	6	7	8	9	10	11	12
1. AL		0.018	0.015	0.018	0.019	0.022	0.042	0.061	0.025	0.017	0.062	0.052
2. GA	0.982		0.000	0.001	0.003	0.005	0.021	0.046	0.006	0.003	0.047	0.037
3. NC-1	0.985	1.000		0.000	0.002	0.005	0.020	0.042	0.004	0.001	0.047	0.034
4. NC-2	0.982	0.999	1.000		0.002	0.005	0.017	0.039	0.005	0.001	0.042	0.034
5. VA-1	0.981	0.997	0.998	0.998		0.001	0.007	0.022	0.002	0.000	0.048	0.026
6. VA-2	0.978	0.995	0.995	0.995	0.999		0.020	0.040	0.008	0.005	0.038	0.027
7. PA-1	0.959	0.979	0.980	0.983	0.993	0.980		0.011	0.014	0.011	0.067	0.034
8. PA-2	0.940	0.955	0.959	0.962	0.979	0.961	0.989		0.021	0.020	0.093	0.031
9. OH	0.975	0.994	0.996	0.995	0.998	0.992	0.986	0.979		0.000	0.039	0.012
10. MI	0.983	0.997	0.999	0.999	1.000	0.995	0.989	0.980	1.000		0.049	0.022
11. NY	0.940	0.955	0.954	0.959	0.954	0.963	0.935	0.912	0.962	0.953		0.025
12. CT	0.950	0.964	0.966	0.967	0.975	0.973	0.967	0.969	0.988	0.978	0.976	

ern Appalachian region ( $H_e = 0.179 \pm 0.043$  at Troy, New York, and  $H_e = 0.182 \pm 0.044$  at Essex, Connecticut). The average gene heterozygosities found in central Appalachian populations were intermediate to those of the southernmost and northern populations. This is contradictory to the general notion that populations at the margin of the species range, in particular northern populations, maintain less genetic diversity than centrally located or southerly populations (Critchfield, 1984; Waller, O'Malley, and Gawler, 1987; Bayer, 1991; Godt and Hamrick, 1993). It has been well documented that many plant species were forced southward to refugia in Gulf Coastal regions and Florida during the Wisconsin glacial maximum 18 000–20 000 yr ago and migrated northward after the glacier's retreat (Davis, 1981, 1983; Pielou, 1991). However, the migration route for *Castanea* (mainly *C. dentata*) remains obscure as few fossil records are available. Wisconsin glacial refugia and subsequent migration for *Castanea* have been hypothesized by Davis (1976, 1981, 1983). Davis (1976) proposed that *Castanea* may have survived on the continental shelf, or at least may have moved from its refuge, using the shelf as migration route, and subsequently migrated from east to west as the glacier receded. It was estimated that ~2000 yr ago, *Castanea* became a dominant species. This hypothesis implies multirefugia and migration routes. However, later reports by Davis (1981, 1983), primarily based on limited palynological data (Delcourt et al., 1980), hypothesized a south–north migration route for *Castanea*. In this study, only one (*Skd-2*) of 14 polymorphic loci showed a clinal trend along the Appalachian axis (Table 1). Zanetto and Kremer (1995) found correlations between allele frequencies and longitude in seven out of eight loci for *Quercus*, indicating a migration pathway on the European continent. Lack of spatial patterning of allele frequencies in both the isozyme and RAPD markers

along the Appalachian axis point more toward the occurrence of multirefugia for *Castanea* prior to the end of the Wisconsin glaciation, one in southerly regions in Alabama and the other on the continental shelf around North Carolina or Virginia.

A strong association was found between genetic distance and geographic distance, particularly among populations along the Appalachian axis (MI excluded). A

TABLE 5. RAPD estimates of Nei's (1978) unbiased genetic identity (below diagonal) and distance (above diagonal) among the American chestnut populations.

Population	1	2	3	4
1. AL		0.183	0.186	0.222
2. VA-1	0.833		0.064	0.109
3. PA-2	0.830	0.938		0.126
4. NY	0.801	0.897	0.882	

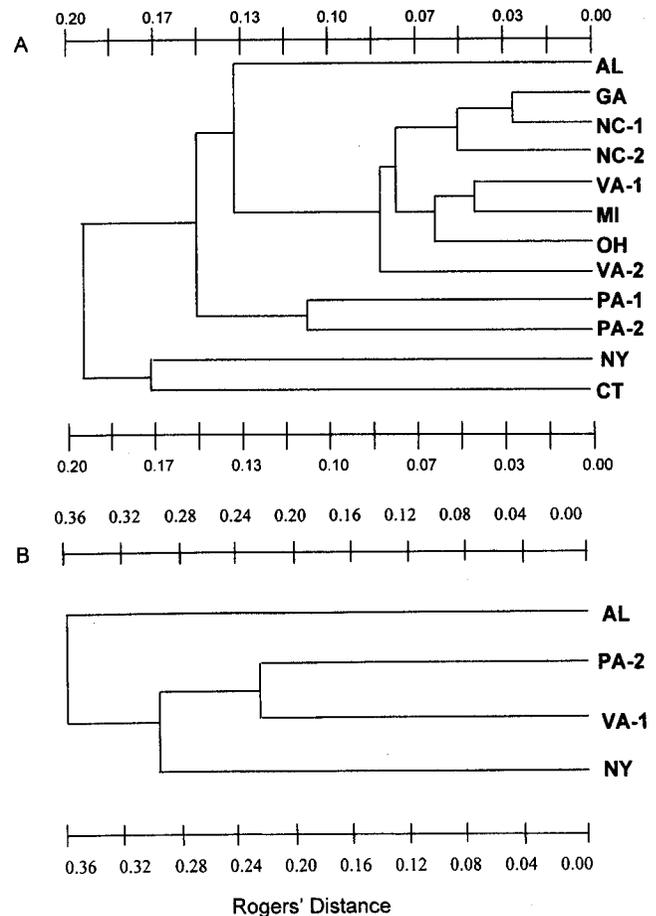


Fig. 2. UPGMA dendrogram of allozyme (A: above) and RAPD (B: below) markers based on modified Rogers' genetic distance (Wright, 1978) between *C. dentata* populations.

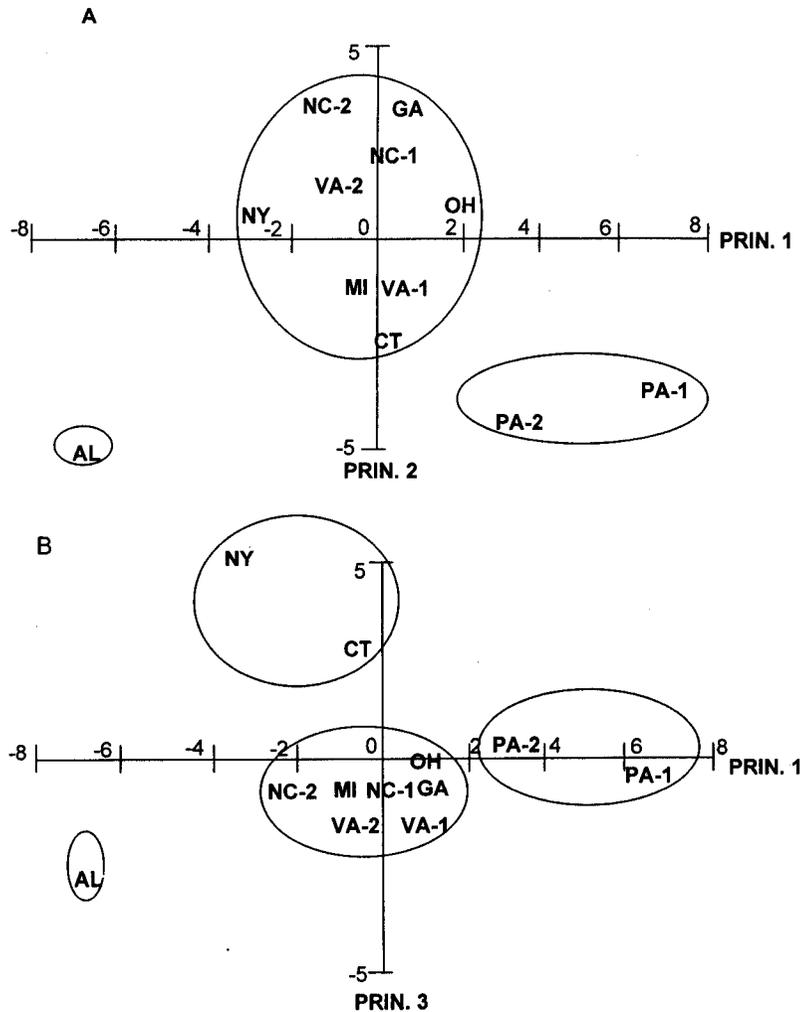


Fig. 3. Principal components analysis of allozyme frequencies of 12 *C. dentata* populations.

negative correlation ( $r = -0.7077$ ,  $P < 0.01$ ) existed between genetic distance and geographic distance, suggesting geographical isolation of *C. dentata* populations. This consideration is reinforced by UPGMA dendrograms (Fig. 2a, 3b) of allozyme and RAPD genetic distance and PCA (Fig. 3). It is clear that the distinction can be made between the southernmost population (AL), the south-central Appalachian populations (GA, NC-1, NC-2, VA-1, VA-2, including MI and OH), north-central Appalachian populations (PA-1, PA-2) and northern Appalachian populations (NY, CT). It is important to note that low mean  $H_e$  and  $G_{st}$  do not imply lower or no difference in genetic composition among the regions, particularly for a widespread species. The importance of regional and local alleles (frequency of private alleles  $p = 0.073$ ) should not be overlooked when a conservation plan or breeding program is considered for restoring the American chestnut. For instance, based on visual inspection of allele frequencies of the 14 polymorphic isozyme loci (Table 1), *Mdh-2a* and *Pgi-2a* are unique alleles only detected in the Alabama and Great Smoky Mountain populations (NC-2), respectively. *Pgm-a* is only associated with northern populations from Pennsylvania to New

York. Allele *Prx-3c* is unique to Pennsylvania populations only. Similar unique allelic distribution can also be found for other alleles, such as *Pgi-2b*, and *Prx-1b*.

**Conservation and breeding considerations for restoration of *C. dentata***—Relatively high levels of genetic diversity existing in southerly populations, presumably one of the refugia and the origin of the genetic diversity prior to postglacial migration, suggest that conservation efforts definitely should consider this a focal point for capturing much of the genetic variation for *C. dentata*. Relict populations in southern region are particularly vulnerable and the number of remnant populations is declining rapidly due to logging disturbance and large-scale reforestation to pine species by the pulp and paper industry. Such isolated relict populations have been disappearing dramatically in the past two decades. In central Alabama, many relict populations are disappearing and resprouting stumps appear to be on the decline (Huang, personal observation). Several southern states have classified *C. dentata* as an endangered species (Kentucky State Nature Preserves Commission, 1996). Relict populations in central Alabama, Mississippi, and Tennessee

need to be more extensively surveyed and evaluated so that appropriate conservation strategies can be implemented. From the consideration of regional differences in genetic composition and the existence of local alleles, it is pertinent that samples be collected from different regions. Based on the results of this study, a conservation program should consider the establishment of at least three regional reservations, southern, central and northern Appalachian regions. Regional programs should also focus on unique morphological types and ecotypes, particularly in terms of tree form, growth rate, timber quality, and other quantitative characters.

Full restoration of the American chestnut to its native habitats should take into consideration many aspects of conservation biology, conservation genetics, population and evolutionary genetics, and plant breeding. Horticulturists and plant breeders have traditionally used only a few genotypes as recurrent parents in backcross programs for cultivar improvement. To date, this has been the case in the breeding program of the American Chestnut Foundation to combine the blight resistance of the Chinese chestnut with the desirable timber-type qualities of the American chestnut (Hebard, 1996). Use of a few recurrent parents might undermine restoration efforts even if blight resistance were successfully transferred from *C. mollissima* and stabilized in BC progenies. Also, more information is needed on the distribution of genetic diversity and on population structure of *C. mollissima*. A cooperative research effort is needed to survey variation in levels of blight resistance in *C. mollissima* in its native range, so that the most highly resistant individuals that encompass all possible major and minor modifying genes conferring blight resistance (Burnham, Rutter, and French, 1986; Kubisiak et al., 1997) are incorporated into the breeding program. The ultimate success of this program will depend upon the survivability and adaptation of backcross populations in natural forest settings and the reestablishment via colonization of *C. dentata* to its native range so that evolutionary processes driven by natural selection can take their course on these populations. Sufficient genetic diversity and maintenance of such genetic diversity must be monitored and evaluated in each subsequent generation. This should provide a clearer understanding of the genetic base associated with successful colonization and could be used to direct and restore the species to its native range.

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