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H. Huang · F. Dane · J.D. Norton

Allozyme diversity in Chinese, Seguin and American chestnut (*Castanea* spp.)

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Abstract Allozyme genetic variability in three chestnut (*Castanea*) species was investigated using 19 loci from ten enzyme systems. G-tests of heterogeneity of isozymic allele distribution showed significant differences between the three species at 15 of the 19 loci, and between the 13 *C. mollissima* populations at 13 of the 19 loci examined. *C. mollissima* was found to possess a significantly-higher value of mean gene heterozygosity (H=0.3050±0.0419), the percentage of polymorphic loci (P=84.21%) and the average number of alleles per locus (A=2.05), than any other species in the *Castanea* section *Eucastanon*. When the genetic variability of populations of *C. mollissima* from four regions in China was investigated, the population from the Changjiang river region showed a markedly higher mean gene heterozygosity (H=0.3480±0.0436) than populations from the other regions. Genetic relationships among the four regions were assessed by Nei's genetic identity I and standard genetic distance D. An approximately-identical distance between the population from the Changjiang river region and populations from the three other regions was observed, while populations from the latter regions showed almost the same genetic distance from each other. These data, when considered with information existing prior to this study, contribute to an understanding of the possible origin and progenitor of the chestnut species.

Key words *C. dentata* · *C. mollissima* · *C. seguinii* · Gene heterozygosity · Genetic distance

Introduction

The genus *Castanea* section *Eucastanon* is composed of five chestnut species, which are generally recognized as true chestnuts characterized by three nuts per bur (Camus 1929; Rutter et al. 1990). The species are widely distributed in the temperate zones of the Northern Hemisphere: the American chestnut (*C. dentata* Borkh.) in North America, the European chestnut (*C. sativa* Mill.) in Europe and Asia Minor, the Chinese chestnut (*C. mollissima* Bl.) and the Seguin chestnut (*C. seguinii* Dode.) in China, and the Japanese chestnut (*C. crenata* Sieb.) in Japan and Korea (Jaynes 1975). Little information on the origin, domestication events, and the relationship of congeneric species in the genus *Castanea*, is presently available (Villani 1991 a). Jaynes (1975) hypothesized that the chestnut originated in China and that the native Chinese chestnut species, *C. mollissima* or *C. seguinii*, would be true progenitor(s), giving rise to the American chestnut in North America by eastward migration and to the European chestnut in Europe by westward extension. Recent analysis of European chestnut populations demonstrated that the allozyme genetic variability in populations from East Turkey was higher than that in populations from West Turkey, Italy and other Mediterranean regions (Villani 1991 b). A reduction in genetic variability, which can be predicted by the estimation of Nei's gene heterozygosity or other parameters (Nei 1978,1987), is considered to be a consequence of migration and domestication of the original species (Nei and Chakraborty 1975; Doebley 1989). Based on this theory, the progenitor(s) *C. mollissima* and or *C. seguinii* should possess a higher allozyme heterozygosity than the descendant species, *C. sativa* and *C. dentata*.

Since *C. mollissima, C. seguinii* and *C. dentata* have not been subjected to an analysis of their allozyme genetic variability, the present study will consider populations from these three species only. The Chinese chestnut is indigenous to a large area of China, ranging from the far north of the Jilin province (North 41°29′) to the tropical region of the Hainan province (North 18°31′). More than 300 cultivars have been recognized, which can be divided into five distinct regional groups based on important horticultural traits and regions of origin (Zhang 1987). *C. seguinii* and *C. mollissima* are sympatrically distributed in low-altitude hilly regions of east-central China, where *C. seguinii* has been treated as a wild species. The American chestnut,

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H. Huang (✉) · F. Dane · J.D. Norton
Department of Horticulture, Auburn University, Auburn, AL 36849, USA

which once accounted for more than 25% of the native eastern hardwood forest, has been almost completely destroyed by chestnut blight, caused by the Asian fungus *Cryphonectria parasitica* (Murr). Current breeding efforts toward restoration of the American chestnut depend primarily on the successful transfer of blight resistance from blight-resistant Chinese species to currently-preserved or surviving American chestnut trees (Burnham 1988). A thorough evaluation of the genetic diversity in these species should generate useful genetic information needed for chestnut breeding programs and for germplasm conservation. The purpose of this study was to answer the following questions: (1) does *C. mollissima* or *C. seguinii* possess a higher allozyme genetic variability than *C. sativa* and *C. dentata* ?, (2) where would the possible center of origin of *C. mollissima* or *C. seguinii* be located ?, and (3) does the uniform susceptibility to chestnut blight in *C. dentata* reflect the low genetic variability of the entire species?

Materials and methods

Plant material

Seeds of 13 traditional cultivars of *C. mollissima*, representing four regional cultivar groups, were obtained from the China National Chestnut Germplasm Plantation and Hubei Academy of Agricultural Science. Each population consisted of 25–38 seeds selected from each isolated cultivar block by bulk pollination of five to ten individual trees. Three populations were chosen from the Northern regional cultivar group, five populations from the Changjiang river regional group, one from the Southwest regional group, and four populations from the Southeast regional group. This represents the entire range of the diverse germplasm of *C. mollissima*. Most of these traditional cultivars were technically open-pollinated land races with known uniformity in nut size (Liu et al. 1988). Four seguin populations, composed of 25 seeds each, were collected from their region of origin in three counties of the Hubei Province, China. Fifty bulked samples (seeds or seedlings) of *C. dentata* were collected from different locations in Wisconsin, Iowa, and Alabama, USA.

Allozyme analysis

The electrophoretic assays were conducted on seed tissues, except for peroxidase assays which were conducted on winter dormant buds (Huang et al. 1993). The isoelectric focusing polyacrylamide slab-gel system described by Mulcahy et al. (1981) was used, with minor modifications. Four gels and approximately 100 samples were operated simultaneously. Serva ampholytes with a pH range of 4–9 were used. Gels were assayed for ten enzyme systems: acid phosphatase (ACP; EC 31.3.3), alcohol dehydrogenase (ADH; EC 1.1.1.1), esterase (EST; EC 3.1.1.), format dehydrogenase (FDH; EC 1.2.1.2), glutamate dehydrogenase (GDH; EC 1.4.1.2.), isocitrate dehydrogenase (IDH; EC 1.1.1.42), malate dehydrogenase (MDH; EC 1.1.1.37), malic enzyme (ME; EC 1.1.1.40), peroxidase (PRX; EC 1.11.1.7) and shikimate dehydrogenase (SKD; EC 1.1.1.25). Staining protocols were those of Wendel and Weeden (1989) and Pasteur et al. (1988) with minor modifications for pH and ingredient concentrations. The following 19 allozyme loci could be resolved: *Acp-1, Acp-2, Acp-3, Adh-1, Est-1, Est-2, Est-5, Idh-1, Mdh-1, Me-1, Skd-1, Skd-2, Skd-3, Skd-4, Fdh-1, Gdh-1, Prx-1, Prx-2, Prx-3*. These loci were found to be inherited as single Mendelian genes as described by Huang et al.(1993).

Statistical analysis

Allele frequencies for each population were estimated using the formula $Xi=(2\,n_{ii}+\sum n_{ij})\,/\,(2N)_{(i\neq j)}$ (Nei 1987), in which n_{ii} is the number of individuals with genotype A_iA_i; n_{ij} is the number of individuals with genotype A_iA_j; and N is the total number of individuals in the population. In addition, allele frequencies were calculated for each region and species so that the data could be analyzed and summarized for regions, and species, respectively. The following indices were used to evaluate the genetic variation and relationships among populations, regions and species: (1) the allelic distributions at each isozyme locus, which were tested for heterogeneity using the G-test, based on the likelihood ratio principle (Sokal and Rohlf 1981); (2) the average number of alleles per locus (A), the percent polymorphic loci (P) (95% criterion), Nei's gene diversity defined as $h=2n(1-\sum x_i^2)/(2n-1)$, and the average gene diversity (H) which were all estimated across loci with the standard error; and (3) Nei's standard genetic distance (D) and identity (I) which are defined as $D=-\log_e I$ and $I=[J_{xy}/(J_xJ_y)^{1/2}]$, where J_x, J_y and J_{xy} are the average of $j_x=\sum x_i^2$, $j_y=\sum y_i^2$ and $j_{xy}=\sum x_iy_i$, respectively (Nei 1987). The computations were performed using the Statistics Analysis System (SAS Institute Inc. 1985). The programs were written by Dr. J. Williams, Research Data Analysis of Auburn University, Alabama.

Results and discussion

Allelic isozyme variation

A total of 39 alleles at 19 isozyme loci could be identified in the 333 individual chestnut trees analyzed. As indicated by the highly-significant G-values (Table 1), a high degree of difference in allele frequency distribution was observed at each locus between, as well as within, chestnut species. Significant differences in allele frequency distribution were observed at 15 loci in interspecific comparisons, at 13 loci among the 13 *C. mollissima* populations, but at only five loci when comparing the four *C. mollissima* regions. This suggests that the allozyme variation of *C. mollissima*

Table 1 G-tests for heterogeneity of allelic distribution at 19 allozyme loci in three collections of chestnut

Locus	Heterogeneity					
	C. mollissima populations	df	*C. mollissima* regions	df	*Castanea* spp.	df
Acp-1	12.341 NS[a]	12	1.211 NS	3	7.454 *	2
Acp-2	29.329 **	12	13.005 **	3	100.598 **	2
Acp-3	99.382 **	24	21.031 **	6	74.639 **	4
Adh-1	31.218 **	12	2.705 NS	3	24.029 **	2
Est-1	131.906 **	12	21.153 **	3	8.584 **	2
Est-2	20.659 NS	24	7.566 NS	6	7.758 NS	4
Est-5	115.555 **	24	17.328 **	6	60.668 **	4
Idh-1	33.656 **	12	18.377 **	3	8.872 *	2
Mdh-1	0.000 NS	12	0.000 NS	3	8.022 *	2
Me -1	50.963 **	12	7.205 NS	3	262.278 **	4
Skd-1	0.000 NS	12	0.000 NS	3	>300.000 **	4
Skd-2	0.000 NS	12	0.000 NS	3	>300.000 **	4
Skd-3	46.550 **	12	6.116 NS	3	>300.000 **	4
Skd-4	13.561 NS	12	2.137 NS	3	>300.000 **	4
Fdh-1	21.674 *	12	3.054 NS	3	0.167 NS	2
Gdh-1	23.184 *	12	6.954 NS	3	1.440 NS	2
Prx-1	89.076 **	12	4.889 NS	3	0.707 NS	2
Prx-2	29.136 **	12	1.750 NS	3	239.001 **	2
Prx-3	66.062 **	24	1.251 NS	6	81.908 **	2

[a] NS, *, **, not significant, significant at $P=0.05$, or at $P=0.01$, respectively.

is retained mostly within the local populations. Four loci (*Skd-1, 2, 3* and *4*) were important in distinguishing *C. dentata* from *C. mollissima* and *C. seguinii*. *Skd-1*^{n} and *Skd-2*^{n} were fixed in *C. mollissima and C. seguinii*, while *Skd-3*^{n} and *Skd-4*^{n} were fixed in *C. dentata*. *C. mollissima* could be distinguished from *C. seguinii* by allele frequency differences at the *Acp-3, Me-1*, and *Prx-2* loci, while allele frequency differences at *Gdh-1* and *Prx-1* separated *C. seguinii* from *C. dentata* (data not shown).

Genetic variability

Genetic diversity statistics, calculated for each isozyme locus of *C. mollissima, C. seguinii* and *C. dentata*, are given in Table 2. The t-test of significance for the expected gene heterozygosity (h) revealed significant differences between *C. mollissima* and *C. dentata* at 12 loci; *Acp-1, 3, Adh-1, Est-2, 5, Idh-1, Skd-3, 4, Fdh-1, Gdh-1, Prx-1, 2,* and between *C. mollissima* and *C. seguinii* at nine isozyme loci. *C. mollissima* showed the highest value of mean heterozygosity H (0.3050), the percent of polymorphic loci P (0.8421), and the average number of alleles per locus A (2.05), of all the chestnut species. The mean heterozygosity (H) of *C. mollissima* is significantly higher than that of *C. dentata* and *C. seguinii*. However, no significant differences in H, P and A were found between *C. seguinii* and *C. dentata*. Hamrick and Loveless (1989) reported values of P of 60.9% and H of 0.211 for 16 tropical tree species. *C. mollissima* obviously shows higher gene diversity statistic values than the tropical tree species, while values of *C.seguinii* and *C. dentata* closely resemble those of other tree species. Villani et al. (1991 a, b) reported values of P ranging from 46% to 54%, of A ranging from 1.46 to 1.77, and of H ranging from 0.16 to 0.25, for European chestnut (*C. sativa*) populations in Italy, and an average value of P of 0.8269 and H of 0.287 for Turkish *C. sativa* populations. Results from the present study suggest that *C. mollissima* possesses a higher degree of genetic variability than all other species investigated in the *Castanea* section *Eucastanon*.

The American chestnut, *C. dentata*, has occasionally been cited as an example of a species without much genetic variability because of its uniform susceptibility to chestnut blight (Rutter et al. 1990). The genetic diversity statistics of this species showed relatively high values of H (0.1830±0.0394), P (0.6317) and A (1.74) (Table 2), compared to other related species. Manos and Fairbrothers (1987) reported that six northeastern American red oaks (*Quercus* spp.) had values of H ranging from 0.058 to 0.124, with P ranging from 0.22 to 0.35 and A ranging from 1.25 to 1.50. As predicted by Jaynes (1975), the uniform blight susceptibility of American chestnut probably only implies an evolutionary event in which *C. denata* lost its blight-resistance (a characteristic present in its progenitor *C. mollissima*) when it evolved on the American continent in the absence of the chestnut blight fungus. It does not reflect the overall diverse genetic composition of this species.

Table 2 Genetic diversity statistics at 19 allozyme loci for three chestnut species: Expected gene heterozygosity (h) estimated at each locus; H=mean gene heterozygosity, P=percentage of polymorphic loci, A=average number of alleles per locus

Locus	*mollissima* (N=220)	*seguinii* (N=63)	*dentata* (N=50)
Acp-1	0.4919±0.0065	0.3697±0.0397	0.2344± 0.0359
Acp-2	0.4018±0.0182	0.3960±0.0445	0.3788±0.0433
Acp-3	0.4315±0.0187	0.2071±0.0385	0.3445±0.0358
Adh-1	0.3625±0.0213	0.3334±0.0511	0.0000±0.0000
Est-1	0.4350±0.0190	0.3810±0.0210	0.5028±0.0066
Est-2	0.4005±0.0198	0.3960±0.0445	0.3031±0.0095
Est-5	0.5328±0.0211	0.3732±0.0474	0.3971±0.0201
Idh-1	0.3545±0.0301	0.0000±0.0000	0.1994±0.0489
Mdh-1	0.0000±0.0000	0.1105±0.0446	0.0000±0.0000
Me -1	0.2893±0.0275	0.2258±0.0536	0.2209±0.0496
Skd-1	0.0000±0.0000	0.0000±0.0000	0.2531±0.0408
Skd-2	0.0000±0.0000	0.0000±0.0000	0.1175±0.0422
Skd-3	0.4560±0.0083	0.2616±0.0387	0.0000±0.0000
Skd-4	0.3589±0.0682	0.0000±0.0000	0.0000±0.0000
Fdh-1	0.0501±0.0233	0.0000±0.0000	0.0000±0.0000
Gdh-1	0.1032±0.0183	0.0000±0.0000	0.0000±0.0000
Prx-1	0.4760±0.0104	0.1901±0.0443	0.1175±0.0421
Prx-2	0.2368±0.1870	0.2787±0.0244	0.0000±0.0000
Prx-3	0.4138±0.0253	0.3394±0.0597	0.4084±0.0535
H	0.3050±0.0419	0.2033±0.0368	0.1830±0.0394
P	0.8421	0.6842	0.6317
A	2.05	1.74	1.74

Genetic relationship

Standard genetic distances estimated between the four regions of *C. mollissima* and the three chestnut species examined are presented in Table 3. The genetic distance between *C. mollissima* and *C. dentata* was four-fold greater than that between *C. mollissima* and *C. seguinii*. This is in agreement with previous reports on the genetic relationships of many congeneric species (Gottlieb 1981; Giannasi and Crawford 1986). The relatively-low genetic distance between *C. mollissima* and *C. seguinii* could probably be explained by the rapid radiation of continental congeneric species as a result of out-cross pollination, coupled with the sympatric distribution and the cross-compatibility of these two closely-related species, as was demonstrated by Soltis (1985) in the genus *Heuchera*. The genetic distances

Table 3 Nei's genetic identities and standard genetic distance for chestnut *Castanea* sect. *Eucastanon*

	Identities	Distance
Between *mollissima* regions		
Changjiang vs. Northern	0.9778	0.0224
Changjiang vs. Southwest	0.9809	0.0192
Changjiang vs. Southeast	0.9778	0.0224
Northern vs. Southwest	0.9916	0.0084
Northern vs. Southeast	0.9897	0.0103
Southwest vs. Southeast	0.9875	0.0125
Between species		
C. mollissima vs. *C. dentata*	0.5651	0.5708
C. mollissima vs. *C. seguinii*	0.8856	0.1215
C. dentata vs. *C. seguinii*	0.5999	0.5110

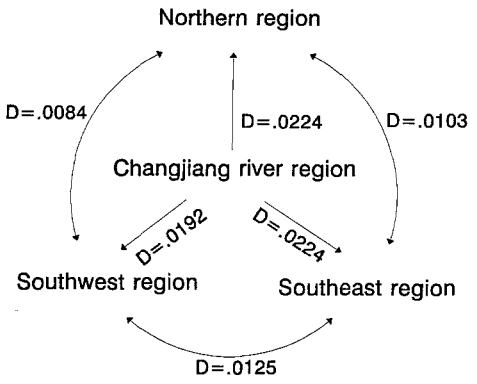

Fig. 1 Genetic relationship of *C. mollissima* in four regions

Table 4 Genetic diversity statistics at 19 allozyme loci of chestnut populations from four regional cultivar groups in *C. mollissima*: expected gene heterozygosity (h) estimated at each locus, H=mean gene heterozygosity, P=percentage of polymorphic loci, A=average number of alleles per locus

Locus	Region			
	Northern	Changjiang	Southwest	Southeast
Acp-1	.5006±.0046	.5132±.0128	.4828±.0310	.4814±.0209
Acp-2	.1663±.0345	.4184±.0334	.2657±.0693	.2103±.0489
Acp-3	.4097±.0285	.5327±.0860	.3623±.0629	.2298±.0515
Adh-1	.3859±.0309	.3926±.0369	.3982±.0574	.2715±.0494
Est-1	.4610±.0202	.4738±.0189	.3824±.0600	.1720±.0469
Est-2	.3689±.0324	.4312±.0313	.3283±.0742	.4332±.0379
Est-3	.2314±.0386	.4131±.0318	.3623±.0629	.2946±.0552
Idh-1	.1222±.0314	.4304±.0314	.2837±.0689	.0701±.0342
Mdh-1	.0000±.0000	.0000±.0000	.0000±.0000	.0000±.0000
Me -1	.1937±.0357	.3201±.0429	.1449±.0625	.0941±.0386
Skd-1	.0000±.0000	.0000±.0000	.0000±.0000	.0000±.0000
Skd-2	.0000±.0000	.0000±.0000	.0000±.0000	.0000±.0000
Skd-3	.2717±.0366	.5128±.0124	.1449±.0625	.3736±.0434
Skd-4	.0528±.0225	.4600±.0431	.1449±.0625	.0569±.0313
Fdh-1	.0000±.0000	.1083±.0213	.0000±.0000	.0511±.0233
Gdh-1	.1028±.0296	.2438±.0421	.0000±.0000	.0298±.0232
Prx-1	.4965±.0082	.5319±.0202	.4164±.0536	.4987±.0111
Prx-2	.1377±.0327	.2882±.0269	.1345±.0611	.1537±.0456
Prx-3	.3473±.0409	.5420±.0215	.3522±.0640	.3914±.0540
H	0.2236±.0409	0.3480±.0436	0.2212±.0385	0.2006±.0393
P	0.7895	0.8421	0.7368	0.8421
A	1.79	1.89	1.68	1.95

obtained for the four regions of *C. mollissima* varied from 0.0084 for the Northern and Southwest region to 0.0224 for the Changjiang and Northern region as well as for the Changjiang and Southeast region. The results are similar to the range in genetic distances obtained among European chestnut regions in Italy (0.017–0.030) (Villani et al. 1991 a). However, it is interesting to note that the Changjiang river region has almost the same genetic distance in relation to the Northern, Southeast and Southwest regions, while the genetic distances among the Northern, Southeast and Southwest regions are also almost identical to each other (Fig. 1). An estimation of gene heterozygosity among the four regions demonstrated that the Changjiang river region possesses a significantly higher H (0.3480 ± 0.0436) than the other regions. Also, no significant differences in H were found among the three other regions (Table 4). Villani et al.(1991 b) reported H values for the European chestnut of 0.3133 for East Turkey, 0.2611 for West Turkey, 0.2522 for Italy, and 0.2630 for France, indicating a decline in allozyme genetic variability from central Asia to the Mediterranean.

Despite the widespread belief that the chestnut originated in China (Jaynes 1975; Rutter et al. 1990; Villani et al. 1991 b), there is little evidence available to demonstrate which species (*mollissima* or *seguinii* ?) and what region could have possibly given rise to the other chestnut species. A recent allozyme investigation conducted in European chestnuts by Villani et al. (1991 b) provided strong evidence that Eastern Turkey is the center of origin of the European chestnut *C. sativa*. A clear decline in total allozyme genetic variation from East Turkey to West Turkey and thence to Italy was demonstrated in their study. The low genetic variability found in *C. seguinii* in the present study, combined with a previous germplasm survey (Rutter et al. 1990), may well eliminate the seguin chestnut as the progenitor of chestnut species. This leaves *C. mollissima* as the only candidate species as a possible progenitor. The significantly higher genetic variation found in *C. mollissima* supports the *C. mollissima*-progenitor hypothesis, because a derivative species should only contain a portion of the total genetic variability of the progenitor species. Our studies indicate (Fig. 1 and Table 4) that the center of origin of the chestnut is located in the Changjiang river region in South-central China, most likely in the Shennongjia mountain range and surrounding Changjiang river valleys where large forests of wild *C. mollissima* and several so-called living fossil species, such as the dawn redwood *Metasequoia*, were found. Warm climates experienced here during glaciation periods provided an ideal refuge center for many plants and animals (Zhang Litian 1989, personal communication). Considering this, as well as evidence from palynological studies (Zohary and Hopf 1988), the decline in allozyme genetic variation from South-central Asia to Asia Minor and from here to the Mediterranean region of Europe can be explained as follows. While the westward expansion of the chestnut from South-central Asia to Asia Minor and to Europe started before the Wurm glaciation, radiation expansion occurred from the center of origin. During the Wurm glaciation period, European derivatives retreated backwards and survived in East Turkey, giving rise to the current species during the Tertiary (Zohary and Hopf 1988; Villani et al. 1991 a, b). Other Asian derivatives probably retreated back into their original center and a secondary slow dispersion after the glaciation gave rise to several regional groups with almost the same genetic distance from their original region (Fig. 1). However, this speculation can not be considered conclusive from the present data. Further sampling of wild populations in both Asia and Europe is needed to provide more detailed information on the evolutionary events in chestnuts.

The analysis of the genetic diversity in these three species, particularly the regional data for *C. mollissima*, will

allow us to make a better decision in future germplasm collections and conservation.

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