Genetic Analysis of 11 Polymorphic Isozyme Loci in Chestnut Species and Characterization of Chestnut Cultivars by Multi-locus Allozyme Genotypes

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Abstract. Allozyme polymorphism in chestnut (*Castanea*) species was investigated using isoelectric focusing in thin-layer polyacrylamide slab gels. Genetic analysis of the progenies of intraspecific crosses and interspecific F_2 s and backcrosses (BC_1 s) allowed the verification of 11 polymorphic isozyme loci from 11 enzyme systems. The following loci were defined: *Acp*, *Adh*, *Est-1*, *Est-2*, *Est-5*, *Me*, *Prx-1*, *Prx-2*, *Prx-3*, *Skd-3*, and *Skd-4*. All polymorphic loci behaved as single-locus Mendelian genes. *Skd* showed unique species specificity. *Skd-1* and *Skd-2* were unique to the American chestnut (*C. dentata* Borkh.) and the European chestnut (*C. sativa* Mill.), whereas *Skd-3* and *Skd-4* were unique to the Chinese chestnut (*C. mollissima* Bl.) and the Japanese chestnut (*C. crenata* Sieb.). Linkage analysis revealed linkage for three pairs of loci: *Skd-3/Skd-4*, *Est-1/Est-2*, and *Est-5/Prx-1*. The single-tree progeny method was used successfully for isozyme genetic analysis. Forty-seven chestnut cultivars in six chestnut species were characterized using 12 isozyme loci and can be unambiguously identified by 12 multi-locus genotypes. The interspecific and geographic relationships among species were also discussed.

The genus *Castanea* includes several commercially important chestnut species that are widely grown from the subtropic to temperate zones around the world. The current American (Am) chestnut (*C. dentata*) breeding program has focused on successive backcrosses of the Am x Chinese (Ch) chestnut (*C. mollissima*) hybrids to the Am chestnuts for chestnut blight resistance (Burnham, 1988). This has resulted in increased public interest in chestnut breeding, collecting germplasm, and evaluating genetic variation between chestnut species (Cochran, 1990; Rutter et al., 1990). However, in contrast to other woody plant species, there have been few investigations into the genetics of these species, mainly because of their long juvenile stage. To date, only nine simply inherited morphological traits have been reported, and it has been difficult to use these as genetic markers in breeding programs (Hebard, 1992; Jaynes, 1975).

Allozymes have been used extensively in other fruit and nut species as genetic markers for identifying cultivars (Cerezo et al., 1989; Degani et al., 1990; Jarret and Litz, 1986; Weeden and Lamb, 1985), confirming hybridity (Anderson et al., 1991; Torres et al., 1982), measuring genetic diversity of plant populations (Hamrick, 1989), verifying taxonomic relationships (DeWald et al., 1992), constructing linkage maps (Bernatzky and Tanksley, 1986; Tanksley and Rick, 1980b), and performing many other aspects of plant breeding programs (Tanksley and Orton, 1983). However, no allozyme genetic studies have been reported in Ch and Am chestnuts.

The purpose of the present study was to 1) establish allozyme markers for *C. mollissima* and *C. dentata* by progeny tests of intra- and interspecific crosses and 2) assign the allozyme multilocus genotypes to 47 cultivars and currently available germplasm selections to aid parental selections in chestnut breeding programs.

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Materials and Methods

Plant material. Families derived from intra- and interspecific crosses were used for genetic studies. At the intraspecific level, ≈ 200 plants were initially surveyed for allozyme polymorphism using 20 enzyme systems. Twenty-five widely grown Ch chestnut cultivars and 30 seguin chestnut (*C. seguinii* Dode.) selections from the China chestnut germplasm plantations, 20 Am chestnut selections, 10 Japanese chestnut (*C. crenata*) cultivars from Japan and Korea, and a few European chestnut (*C. sativa*) seedlings were included in the survey. Progenies obtained from six controlled crosses of Ch chestnut cultivars were used for genetic analysis of allozymes. Additionally, the single-tree progeny method of Gillet (1991) was used to test allozyme inheritance because of the high degree of self-incompatibility of chestnut species and availability of large progeny numbers. Open-pollinated seeds were randomly harvested from a parental tree.

For interspecific studies, F_2 and BC_1 families of Am and Ch chestnuts from the research farm of the American Chestnut Foundation in Virginia were used. The F_2 family was derived from an Am x Ch F_1 sib cross and the BC_1 families were derived from BC_1 s to Am (Hebard, 1992).

Electrophoresis and isozyme staining. Most isozyme assays of the intraspecific crosses were conducted on mature seed tissue, while dormant winter buds were used for F_2 and BC_1 families of interspecific crosses. Because of poor resolution in seed extracts, peroxidase assays were always conducted on dormant winter buds. Tissue specificity and subcellular location of all isozymes were determined using leaves of different developmental stages, catkins, roots, summer vegetative buds, and pollen. The best isozyme definition was obtained in seed tissue and winter dormant buds, and these tissues gave identical phenotypes. Seed tissue or buds (≈ 50 mg) were homogenized by hand in a prechilled mortar and pestle with cold extraction buffer [1 mg tissue/5 to 10 μ l buffer #3 (Wendel and Weeden, 1989) modified slightly by reducing bovine serum albumin to 0.025% (w/v)]. Crude extracts were centrifuged for 5 min at $5000\times g$ in a centrifuge (5415C Eppendorf; Brinkmann Instruments, Westbury, N.Y.). A 1-mm² Whatman no. 3 filter paper was soaked in the supernatant and used as sample for

isoelectric focusing. The high phenolic content of the chestnut tissue made visualization of certain enzyme systems in some tissues impossible.

The isoelectric focusing polyacrylamide slab-gel procedure of Mulcahy et al. (1981) was used in this study because of its high resolution and high sample number (≈ 100 samples per run). Serva ampholytes with a pH of 4 to 9 were used. Gels were assayed for acid phosphatase (ACP; EC 3.1.3.2), alcohol dehydrogenase (ADH; EC 1.1.1.1), aminopeptidase (AMP; EC 3.4.11.1), aspartate aminotransferase (AAT; EC 2.6.1.1), catalase (CAT; EC 1.11.1.6),

endopeptidase (ENP; EC 3.4.-.-), esterase (EST; EC 3.1.1), formate dehydrogenase (FDH; EC 1.2.1.2), β -galactosidase (GAL; EC 3.2.1.23), glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49), glutamate dehydrogenase (GDH; EC 1.4.1.2), isocitrate dehydrogenase (IDH; EC 1.1.1.42), lactate dehydrogenase (LDH; EC 1.1.1.27), malate dehydrogenase (MDH; EC 1.1.1.37), malic enzyme (ME; EC 1.1.1.40), menadione reductase (MNR; EC 1.6.99.-), peroxidase (PRX; EC 1.11.1.7), 6-phosphogluconate dehydrogenase (PGD; EC 1.1.1.44), shikimate dehydrogenase (SKD; EC 1.1.1.25), and superoxide dismutase (SOD; EC 1.15.1.1).

Fig. 1. Allele and locus designation and phenotypic segregation in six polymorphic enzymes in chestnut. Anode is toward the top.

Staining recipes were those of Wendel and Weeden (1989) and Pasteur et al. (1988), with minor pH and ingredient concentration modifications.

Allozyme designation and data analysis. The genetic control of isozyme loci was postulated based on the phenotypic banding patterns and the segregation ratios of the controlled crosses. The loci for a given multiple-locus enzyme system were designated sequentially by number starting with 1 for the most anodal locus; at each locus, the alleles were designated sequentially by letter starting with *a* for the most anodal allozyme. An allele lacking staining activity was designated with *n* (null). Chi-square tests were used to determine the goodness-of-fit of segregating loci to expected single-locus Mendelian segregation ratios. In case of a two-class segregation ratio, chi-squares were calculated using Yates' correction (Yates, 1934). Linkage analyses were performed by the maximum likelihood method using the computer program LINKAGE-1 (Suiter et al., 1983).

Results and Discussion

General aspects of allozyme polymorphism. Of the 20 enzyme systems used in our preliminary survey, 11 gave high-resolution banding patterns and were further investigated in the families generated from controlled crosses or in single-tree progenies. The remaining nine enzymes did not provide scorable intensity or acceptable resolution and were excluded: AAT, AMP, CAT, ENP, GAL, G6PDH, LDH, MNR, and PGD. Of the 11 systems resolved, 7 were polymorphic. A considerable level of polymorphism was detected in the material examined. Representative banding patterns and allele and locus designation for all but one of the seven enzyme systems are shown in Fig. 1. MDH was not included because of its complex zymogram patterns. The genetic basis of this enzyme system could not be determined from the populations available in this study. Fifteen loci specifying twenty-eight alleles were identified, including four monomorphic enzymes. Six polymorphic enzymes provided an average of 2.18 alleles per locus and 1.83 loci per enzyme.

Monomorphic enzymes. Four enzymes were monomorphic. FDH, GDH, and SOD were invariant and each showed a single band in all chestnut populations. IDH displayed two intense bands with a weakly stained band occasionally, but no variation was detected in the chestnut populations.

Polymorphic enzymes. Variable, well-resolved banding patterns were observed for six enzyme systems (Fig. 1). Segregation data were obtained for eleven loci. Genetic interpretation of alleles for each locus was based on four types of populations derived from open-pollination of the single-tree progeny, the controlled crosses within Ch chestnut, and F_2 and BC_1 populations derived from interspecific hybridization between Am and Ch chestnut (Tables 1–4). The inheritance of each locus is discussed below.

ACP. Several regions of ACP isozymes were resolved in the gels, but only the most cathodal region was variable (Fig. 1). Segregating families derived from controlled crosses and single-tree progenies demonstrated that the ACP phenotypes observed at this region are encoded by a single Mendelian locus with two alleles in chestnut (Tables 1–4). The observed ACP phenotypes consisted of single or double bands, a result that points to a monomeric enzyme structure for ACP in chestnut species. This was also found in other plant species (Kahler, 1983; Weeden and Robinson, 1986; Weeden et al., 1988).

ADH. Zymograms for ADH revealed only one activity zone (Fig. 1), designated as locus *Adh*. One intense band at pI 3.8 (5 mm from origin) or the complete absence of this band at the same

position (null allele) was observed. The data obtained from 10 families derived from intraspecific crosses and single-tree progenies and one F_2 family from an interspecific cross were tested for their goodness of fit to the expected (presence-absence) segregation ratios. No significant deviations from the expected values were found (Tables 1–3). The segregation data only substantiate the presence of a putative null allele and do not indicate a quaternary enzyme structure. Therefore, two alleles are tentatively assigned at the *Adh* locus: allele *Adh-a* coding for the presence of the band and a null allele *Adh-n* coding for the absence of the band. The presence-absence mode was present in mature seeds and dormant winter buds of chestnut. The presence-absence banding patterns coded by an active and null allele have also been reported for peroxidase (Brewbaker et al., 1985; Rick et al., 1974), esterase (Tanksley, 1984), and β -galactosidase (Weeden and Marx, 1987). ADH has been demonstrated to be a dimeric enzyme in European chestnut and many other plants (Malvolti and Fineschi, 1987), but Torres (1974) reported an *Adh* locus with an early (*E*) allele that behaved as a presence-absence isozyme in mature sunflower seeds. Although two ADH loci are usually reported in diploid plants (Arus and Orton, 1984; Suiter, 1988; Wendel and Parks, 1982), a second ADH locus was not revealed in the present study. This could be due to the use of a gel system with a pH different from that used by Malvolti and Fineschi (1987) for European chestnut. Gomes et al. (1982) indicated that ADH-2 and ADH-3 activity could be greatly inhibited at pH 6.

EST. The best resolution and intensity of EST were obtained in seed tissue and dormant winter buds (Fig. 1). Four well-resolved zones of esterase activity were detected in seeds and dormant buds; three zones in young leaves, catkins, and bark; and two in summer buds. Mature leaves showed very little EST activity. Tissue and substrate specificity also have been demonstrated in apple (Manganaris and Alston, 1992). It seems that at least five loci regulate EST expression in chestnut. Both α - and β -naphthyl acetate were used in the staining solution. Zone 1 was comprised of two loci: *Est-1* (β -naphthyl-specific red phenotype) and *Est-2* (α -naphthyl-specific black phenotype). Allele *b* at locus 1 overlapped with allele *a* at locus 2. Fortunately, the *Est-1* red phenotypes could be reliably distinguished from the *Est-2* black phenotypes. The inheritance of the *a* and *b* alleles at the *Est-1* and *Est-2* locus was studied in 10 families derived from intraspecific crosses and single-tree progenies (Tables 1 and 2). The alleles at each locus encoded monomeric proteins and displayed codominant expression. Expected segregation ratios were observed in the BC_1 and F_2 interspecific families (Tables 3 and 4) for the *Est-1* locus, but a significant deviation from the expected ratio was found at the *Est-2* locus in one F_2 and one BC_1 family. Segregation ratios were skewed in favor of the heterozygote genotype class.

EST-3 (zone 2) and EST-4 (zone 3) were also polymorphic. Two variants could be detected for each isozyme, but inconsistent banding expression and poor staining resolution made it difficult to score these two loci, and they were consequently excluded. The segregation analysis for EST-5 (zone 4) was similar to that of *Est-1* and *Est-2* in three BC_1 families and one F_2 family in that a single *Est-5* locus with two alleles (*a* and *b*) controlled monomeric enzymes (Tables 3 and 4). A third allele *c* with a 2-mm mobility from the *b* allele was detected only in single-tree progenies (Table 1) and can be attributed to the pollen source. Monomeric and dimeric structures of esterase have been reported in some other plants (Tanksley and Rick, 1980a; Tanksley, 1984; Weeden and Marx, 1987). Our data suggest that simple monogenic inheritance of these three esterases in chestnut species may be valuable in genetic studies and chestnut breeding programs.

Table 1. Genetic analysis of 11 polymorphic isozyme loci using single-tree progeny in *Castanea mollissima*.

Locus	Maternal tree ^z		Progeny genotype				Expected progeny relationship ^y	χ^2	
	Name	Genotype	Total	aa	ab	bb			bc
<i>Acp</i>	1	bb	20	0	12	8			
	2	ab	17	3	10	4	$N_{ab} = N_{aa} + N_{bb}$	0.24 ^{NS}	
	3	ab	24	4	11	9	$N_{ab} = N_{aa} + N_{bb}$	0.04 ^{NS}	
	4	bb	24	0	8	16			
	5	ab	24	5	9	10	$N_{ab} = N_{aa} + N_{bb}$	1.04 ^{NS}	
<i>Est-1</i>	1	ab	20	3	11	6	$N_{ab} = N_{aa} + N_{bb}$	0.05 ^{NS}	
	2	bb	17	0	9	8			
	3	ab	24	4	11	9	$N_{ab} = N_{aa} + N_{bb}$	0.04 ^{NS}	
	4	ab	24	6	15	3	$N_{ab} = N_{aa} + N_{bb}$	1.04 ^{NS}	
	5	ab	24	4	13	7	$N_{ab} = N_{aa} + N_{bb}$	0.04 ^{NS}	
<i>Est-2</i>	1	ab	20	6	9	5	$N_{ab} = N_{aa} + N_{bb}$	0.05 ^{NS}	
	2	bb	17	0	9	8			
	3	ab	24	6	14	4	$N_{ab} = N_{aa} + N_{bb}$	0.38 ^{NS}	
	4	ab	24	2	14	8	$N_{ab} = N_{aa} + N_{bb}$	0.38 ^{NS}	
	5	ab	24	1	13	10	$N_{ab} = N_{aa} + N_{bb}$	0.04 ^{NS}	
<i>Est-5</i>	1	bb	20	1	6	6	8		
	2	bb	17	1	7	6	4		
	3	bb	24	0	9	11	4		
	4	bb	24	0	2	13	9		
	5	bb	24	0	9	12	3		
<i>Me</i>	1	bb	20	0	4	16			
	2	bc	17	0	0	9	8	$N_{bc} = N_{bb} + N_{cc}$	0.00 ^{NS}
	3	bb	22	0	6	16			
	4	bb	22	0	0	22			
	5	bb	24	0	0	24			
<i>Prx-1</i>	1	ab	24	6	14	4	$N_{ab} = N_{aa} + N_{bb}$	0.38 ^{NS}	
	2	aa	24	8	16	0			
	3	ab	24	8	15	1	$N_{ab} = N_{aa} + N_{bb}$	1.04 ^{NS}	
	4	aa	24	10	14	0			
	5	aa	24	7	17	0			
<i>Prx-2</i>	1	ab	24	7	13	4	$N_{ab} = N_{aa} + N_{bb}$	0.04 ^{NS}	
	2	ab	24	8	10	6	$N_{ab} = N_{aa} + N_{bb}$	0.38 ^{NS}	
	3	ab	24	6	16	2	$N_{ab} = N_{aa} + N_{bb}$	2.04 ^{NS}	
	4	ab	24	8	12	4	$N_{ab} = N_{aa} + N_{bb}$	0.00 ^{NS}	
	5	ab	24	14	7	3	$N_{ab} = N_{aa} + N_{bb}$	3.38 ^{NS}	
<i>Prx-3</i>	1	ab	24	0	8	16	$N_{ab} = N_{aa} + N_{bb}$	2.04 ^{NS}	
	2	ab	24	0	15	9	$N_{ab} = N_{aa} + N_{bb}$	1.04 ^{NS}	
	3	bb	24	0	4	20			
	4	ab	24	0	11	13	$N_{ab} = N_{aa} + N_{bb}$	0.04 ^{NS}	
	5	bb	24	0	18	6			
<i>Skd-3</i>	1	bb	20	0	17	3			
	2	aa	17	9	8	0			
	3	ab	24	1	16	7	$N_{ab} = N_{aa} + N_{bb}$	2.04 ^{NS}	
	4	ab	24	6	11	7	$N_{ab} = N_{aa} + N_{bb}$	0.04 ^{NS}	
	5	ab	24	6	14	4	$N_{ab} = N_{aa} + N_{bb}$	0.38 ^{NS}	
<i>Skd-4</i>	1	bb	20	0	17	3			
	2	aa	17	9	8	0			
	3	ab	24	3	18	3	$N_{ab} = N_{aa} + N_{bb}$	5.04 [*]	
	4	ab	24	8	8	8	$N_{ab} = N_{aa} + N_{bb}$	2.04 ^{NS}	
	5	ab	24	8	11	5	$N_{ab} = N_{aa} + N_{bb}$	0.04 ^{NS}	
<i>Adh</i>				a ₋	oo				
	1	a ₋	20	16	4		$N_{a_{-}} \geq N_{oo}$		
	2	a ₋	17	11	6		$N_{a_{-}} \geq N_{oo}$		
	3	a ₋	24	16	8		$N_{a_{-}} \geq N_{oo}$		
	4	a ₋	22	13	9		$N_{a_{-}} \geq N_{oo}$		
	5	oo	24	7	17				

^zMaternal tree: 1 = Cropper; 2 = Leader; 3 = Homestead; 4 = AU-60; 5 = AU-17.^yN = total number of progeny genotype.^{NS,*} Nonsignificant or significant at $P = 0.05$ or 0.01 , respectively.

Table 2. Single-locus segregation and chi-square test of 11 polymorphic loci in intraspecific progenies of *Castanea mollissima*.

Locus	Crosses ^z	Parental genotypes	Progeny				Expected ratio	χ^2	
			Total	aa	ab	bb			bc
<i>Acp</i>	1	ab x bb	20	---	10	10	1:1	0.000 ^{NS}	
	2	bb x bb	19	---	19	---			
	3	ab x bb	24	---	8	16	1:1	2.042 ^{NS}	
	4	bb x ab	19	---	7	12	1:1	0.842 ^{NS}	
	5	ab x bb	36	---	22	14	1:1	1.361 ^{NS}	
<i>Est-1</i>	1	ab x ab	20	3	10	7	1:2:1	1.600 ^{NS}	
	2	bb x ab	19	5	9	5	1:2:1	0.053 ^{NS}	
	3	bb x ab	24	---	12	12	1:1	0.000 ^{NS}	
	4	ab x bb	19	---	9	10	1:1	0.000 ^{NS}	
	5	ab x ab	36	6	20	10	1:2:1	1.333 ^{NS}	
<i>Est-2</i>	1	ab x ab	20	7	11	2	1:2:1	2.700 ^{NS}	
	2	ab x ab	19	4	10	5	1:2:1	0.157 ^{NS}	
	3	bb x ab	24	---	10	14	1:1	0.375 ^{NS}	
	4	ab x bb	19	---	9	10	1:1	0.000 ^{NS}	
	5	ab x ab	36	9	19	8	1:2:1	0.167 ^{NS}	
<i>Est-5</i>	1	bb x bb	20	---	---	20			
	2	bb x bb	19	---	---	19			
	3	bb x bb	24	---	---	24			
	4	bb x bb	19	---	---	19			
	5	bb x bb	36	---	---	36			
<i>Me</i>	1	bb x bb	20	---	---	20			
	2	bb x bb	19	---	---	19			
	3	bc x bb	24	---	---	15	9	1:1	1.042 ^{NS}
	4	bb x bc	19	---	---	13	6	1:1	1.895 ^{NS}
	5	bb x bb	36	---	---	36			
<i>Prx-1</i>	1	ab x ab	24	8	14	2	1:2:1	3.667 ^{NS}	
	2	ab x aa	24	16	8	---	1:1	2.042 ^{NS}	
	3	aa x aa	24	24	---	---			
	4	ab x aa	24	14	10	---	1:1	0.375 ^{NS}	
	5	ab x aa	24	13	11	---	1:1	0.042 ^{NS}	
<i>Prx-2</i>	1	ab x ab	24	5	15	4	1:2:1	1.583 ^{NS}	
	2	ab x ab	24	8	13	3	1:2:1	2.249 ^{NS}	
	3	ab x ab	24	4	16	4	1:2:1	2.667 ^{NS}	
	4	ab x ab	24	6	15	3	1:2:1	2.250 ^{NS}	
	5	ab x ab	24	5	12	7	1:2:1	0.333 ^{NS}	
<i>Prx-3</i>	1	bb x ab	24	---	8	16	1:1	2.042 ^{NS}	
	2	ab x ab	24	10	10	4	1:2:1	3.667 ^{NS}	
	3	ab x ab	24	5	12	7	1:2:1	0.333 ^{NS}	
	4	ab x ab	24	4	10	10	1:2:1	3.667 ^{NS}	
	5	bb x ab	24	---	9	15	1:1	1.042 ^{NS}	
<i>Skd-3</i>	1	ab x bb	20	---	17	3	1:1	8.450 ^{**}	
	2	bb x ab	19	---	9	10	1:1	0.000 ^{NS}	
	3	aa x ab	24	15	9	---	1:1	1.042 ^{NS}	
	4	bb x aa	19	---	9	---			
	5	ab x ab	36	3	18	15	1:2:1	8.000 [*]	
<i>Skd-4</i>	1	ab x bb	20	---	9	11	1:1	0.050 ^{NS}	
	2	bb x ab	19	---	8	11	1:1	0.211 ^{NS}	
	3	aa x ab	24	11	13	---	1:1	0.042 ^{NS}	
	4	bb x aa	19	---	19	---			
	5	ab x ab	36	12	6	18	1:2:1	18.000 ^{**}	
<i>Adh</i>				a_	oo				
	1	ao x ao	20	16	4		3:1	0.067 ^{NS}	
	2	ao x ao	19	15	4		3:1	0.018 ^{NS}	
	3	ao x ao	24	20	4		3:1	0.500 ^{NS}	
	4	ao x ao	19	15	4		3:1	0.018 ^{NS}	
	5	ao x ao	36	25	11		3:1	0.333 ^{NS}	

^zCross 1 = Homestead x Cropper; 2 = Cropper x AU-60; 3 = Leader x AU-60; 4 = Cropper x Leader; 5 = Homestead x AU-60.NS, *, ** Nonsignificant or significant at $P = 0.05$ or 0.01 respectively.

Table 3. Single-locus segregation and chi-square test of nine polymorphic loci in the F₂ family from the interspecific cross *Castanea mollissima* × *C. dentata*, R4T52 × R4T31.

Locus	Parental genotype	Progeny genotypes				Expected ratio	χ^2
		Total	aa	ab	bb		
<i>Acp</i>	ab × ab	63	10	32	21	1:2:1	3.857 ^{NS}
<i>Est-1</i>	bb × ab	63	---	38	25	1:1	2.286 ^{NS}
<i>Est-2</i>	aa × ab	60	17	43	---	1:1	10.417 ^{**}
<i>Est-5</i>	ab × ab	63	13	34	16	1:2:1	0.683 ^{NS}
<i>Me</i>	ab × ab	63	13	20	30	1:2:1	17.571 ^{**}
<i>Prx-1</i>	ab × ab	63	19	33	11	1:2:1	2.175 ^{NS}
<i>Prx-2</i>	ab × ab	63	18	30	15	1:2:1	0.428 ^{NS}
<i>Prx-3</i>	bb × ab	63	---	22	41	1:1	5.143 [*]
			a_	oo			
<i>Adh</i>	a_ × oo	48	25	23		1:1	0.021 ^{NS}

^{NS}, ^{*}, ^{**} Nonsignificant or significant at $P = 0.05$ or 0.01 respectively.

Table 4. Single-locus segregation and chi-square test of eight polymorphic loci in BC₁ families from the interspecific cross of *Castanea mollissima* × *C. dentata*.

Locus	Crosses ^z	Parental Genotype	Progeny genotypes				Expected ratio	χ^2
			Total	aa	ab	bb		
<i>Acp</i>	2	bb × ab	27	---	9	18	1:1	2.370 ^{NS}
	3	bb × ab	25	---	16	9	1:1	1.440 ^{NS}
	4	bb × ab	50	---	24	26	1:1	0.020 ^{NS}
<i>Est-1</i>	2	bb × ab	27	---	15	12	1:1	0.148 ^{NS}
	3	bb × ab	25	---	12	13	1:1	0.000 ^{NS}
	4	bb × ab	50	---	22	28	1:1	0.500 ^{NS}
<i>Est-2</i>	2	aa × ab	27	7	20	---	1:1	5.294 [*]
	3	bb × aa	25	---	25	---		
	4	aa × ab	50	20	30	---	1:1	1.620 ^{NS}
<i>Est-5</i>	2	bb × ab	27	---	10	17	1:1	1.333 ^{NS}
	3	ab × ab	25	5	14	6	1:2:1	0.267 ^{NS}
	4	aa × ab	50	23	27	---	1:1	0.180 ^{NS}
<i>Me</i>	2	bb × ab	27	---	11	16	1:1	0.593 ^{NS}
	3	bb × ab	25	---	10	15	1:1	0.640 ^{NS}
	4	bb × ab	50	---	30	20	1:1	1.620 ^{NS}
<i>Prx-1</i>	2	aa × ab	27	14	13	---	1:1	3.278 ^{NS}
	3	aa × ab	25	24	1	---	1:1	19.360 ^{**}
	4	aa × ab	50	22	28	---	1:1	0.500 ^{NS}
<i>Prx-2</i>	2	aa × ab	27	13	14	---	1:1	0.000 ^{NS}
	3	ab × ab	25	15	5	5	1:2:1	22.500 ^{**}
	4	aa × ab	50	21	29	---	1:1	0.980 ^{NS}
<i>Prx-3</i>	2	bb × ab	27	---	14	13	1:1	0.000 ^{NS}
	3	ab × bb	25	---	18	7	1:1	4.000 [*]
	4	bb × ab	50	---	27	23	1:1	0.180 ^{NS}

^zCrosses: 2 = American × R4T31; 3 = QAI × R4T52; 4 = American × Nanking.

^{NS}, ^{*}, ^{**} Nonsignificant or significant at $P = 0.05$ or 0.01 respectively.

ME. ME exhibited only one activity zone. Its one- vs. five-banded phenotype can be interpreted as the expression of a tetrameric enzyme. The genetic control was best described by the action of a single Mendelian locus (*Me*) consisting of three codominant alleles *a*, *b*, and *c*, because *a* and *b* alleles segregated normally in the BC₁ families (Table 4) and the *b* and *c* alleles segregated normally in three intraspecifically derived families (Tables 1 and 2). Although a significant deviation was observed in the F₂ family (Table 3), this could be considered as sampling error or preferential segregation in the F₂ family of an interspecific cross. ME behaves electrophoretically as a tetrameric protein with genotype *Me-aa* and *Me-bb* for the homozygote and *Me-ab* for the heterozygote (Fig. 1). ME has been reported to be tetrameric in apple (Weeden and Lamb, 1987) but monomeric in citrus (Torres et al., 1985).

PRX. Three main polymorphic activity zones were observed for PRX (Fig. 1). Single- and double-banded phenotypes were observed at zone-1 and zone-3 and suggest the monomeric and codominant nature of two functional loci: *Prx-1* and *Prx-3*. Intraspecific and interspecific crosses between one- and two-banded parents yielded offspring with two phenotypic classes (similar to the parental classes) in a 1:1 ratio. A normal 1:2:1 segregation ratio was found when crosses were made between two-banded parents (Tables 1–4). No significant deviation from the expected segregation ratio was found in 10 intraspecific crosses and single-tree progenies and most interspecifically derived BC₁ or F₂ families, except one F₂ and one BC₁ family with marginal significance at the 5% level at the *Prx-3* locus and one BC₁ family with 1% significance at the *Prx-1* locus. This could be considered

due to chance alone because there was no consistent trend observed among other families. It can be concluded that *Prx-1* and *Prx-3* act as a single locus in Mendelian fashion with two active *a* and *b* alleles at each locus (Fig. 1). *Prx-2* exhibited one- or two-doublet phenotypic patterns. Segregation analysis for *Prx-2* (Tables 1–4), based on monogenic inheritance patterns, supports the hypothesis that a single locus codes for this monomeric doublet isozyme. Two alleles were detected at *Prx-2*, with allele *a* coding for an anodal doublet and allele *b* coding for a cathodal doublet. The heterozygote genotype *Prx-2-ab* expressed both doublets. PRX is one of most extensively investigated enzyme systems. In maize, as many as 13 peroxidases have been specified (Brewbaker et al., 1985). PRX has been reported to be monomeric in most plants (Garcia et al., 1982; Quiros and McHale, 1985; Quiros and Morgan, 1981; Rick et al., 1974; Torres, 1980; Weeden and Marx, 1987; Weeden and Robinson, 1986; Weeden et al., 1988) and frequently to be expressed as presence-absence phenotype (Brewbaker et al., 1985), doublet (Berg and Wijsman, 1982), or triplet (Hirano and Nagunuma, 1979). However, PRX has not been investigated extensively in chestnut species. Sawano et al. (1984) concluded, based on their study of three chestnut species, that there was no special differentiation of peroxidase isozymes among *Castanea* spp. Anagnostakis (1991) reported that an unspecified PRX anodal locus, which behaved as a monomeric isozyme, could be equivalent to the *Prx-1* in this study. It seemed, based on our germplasm survey, that *a* and *b* alleles at *Prx-1* have almost equal gene frequencies in *C. mollissima*, but that the *a* allele is predominant in *C. dentata* and the *b* allele is predominant in *C. seguinii*. Intraspecific and interspecific hybridization could generate sufficient polymorphism at this locus for it to be used as a genetic marker.

SKD. This enzyme exhibited a unique species-specific zymogram for *C. mollissima* and *C. dentata*. Two relatively anodal SKD activity zones were observed and designated as SKD-1 and SKD-2 for *C. dentata*. Either single- or double-banded patterns were detected at both zones. The two bands at the SKD-1 have pI values of 4.9 and 5.1 (10 and 11 mm from origin), respectively, whereas the two bands at SKD-2 have pI values of 5.4 and 5.6 (13 and 14 mm from origin). SKD-1 and SKD-2 seemed to be regulated by two separate single loci, tentatively designated as *Skd-1* and *Skd-2*, with alleles *a* and *b* at each locus governing monomeric enzymes. Because *C. dentata* is almost extinct, sufficient progenies from controlled crosses within pure *C. dentata* can hardly be obtained and allelic segregation at the *Skd-1* and *Skd-2* locus cannot be analyzed in this species.

In *C. mollissima*, two different zones cathodal to SKD-2 were observed. One zone, designated as SKD-3, showed two different bands of pI 5.6 (14 mm) and 5.8 (15 mm). The other zone, SKD-

4, showed two bands of pI 6.0 (16 mm) and 6.2 (17 mm) (Fig. 1). The most-anodal band at SKD-3 overlapped with the most cathodal band of SKD-2 at pI 5.6 (14 mm from origin). Genetic analysis was carried out in families of controlled crosses and single-tree progeny (Tables 1 and 2). Segregation data supported the monogenic inheritance of monomeric enzymes SKD-3 and SKD-4 in *C. mollissima*. SKD-3 and SKD-4 are governed by two loci, *Skd-3* and *Skd-4*. SKD has been known to be a regular monomeric enzyme, and one or two loci are usually reported in diploid plant species (Navot and Zamir, 1986; Quiros and McHale, 1985; Weeden and Gottlieb, 1980; Weeden and Robinson, 1986). Although monomeric doublet phenotypes have been found in pepper (Tanksley, 1984), celery (Arus and Orton, 1984), and maize (Wendel et al., 1988), the clear banding phenotypes resolved by the high-resolution isoelectric focusing system used in this study suggested that SKD was expressed as an one- or two-banded monomeric enzyme in chestnut species, even if the bands are closely spaced (≈ 1 mm apart). The observed data also suggest a linkage between *Skd-3* and *Skd-4*, because high frequencies of the same genotypes have occurred at both loci and significant deviations from the expected ratios were found in the controlled crosses and single-tree progenies (Tables 1 and 2).

In *C. mollissima* $\times$ *C. dentata* hybrids, the F_1 possessed the isozyme banding pattern of both parent species and displayed hybrid phenotypic patterns with four zones. The F_2 population segregated into three isozyme phenotype pattern classes only: *C. dentata* with SKD-1, -2; hybrid with SKD-1,-2,-3,-4, and *C. mollissima* with SKD-3, -4. This phenotypic segregation in the F_2 , based on a 1:2:1 Mendelian segregation ratio was significantly skewed in favor of *C. mollissima* phenotypes. However, three BC_1 families segregated normally into two classes to fit the expected 1:1 ratio. Chi-square tests do not detect heterogeneity or significant deviations from the totals of all families. The overall data suggest that the SKD phenotypic patterns are inherited in a Mendelian fashion.

The eleven isozyme loci described above are inherited as single Mendelian genes in chestnut species. As neither Am nor Ch chestnuts have been subjected to a systematic investigation of allozyme variation, the results from this study are quite encouraging and can be used to evaluate the overall genetic diversity of the two species for germplasm collection and conservation. It may lead to a saturated gene linkage map and tagging genes of economically important traits (Bernatzky and Tanksley, 1986; Tanksley and Rick, 1980b). More allozyme polymorphism will be discovered by expanding additional enzyme systems and using more-diverse germplasm. The presence of species-predominant alleles, such as *Prx-1-aa* in *C. dentata* and *Prx-1-bb* in *C. seguinii*, and

Table 5. Number of families analyzed by chi-square contingency tests for independent assortment of jointly segregating isozyme loci (above the diagonal) and number of families deviating from independent assortment (below the diagonal) in *Castanea* spp.

Locus	<i>Acp</i>	<i>Adh</i>	<i>Est1</i>	<i>Est2</i>	<i>Est5</i>	<i>Me</i>	<i>Prx1</i>	<i>Prx2</i>	<i>prx3</i>	<i>Skd3</i>	<i>Skd4</i>
<i>Acp</i>		6	7	5	3	4	6	7	6	4	4
<i>Adh</i>	0		5	5	1	3	5	6	5	4	4
<i>Est1</i>	0	1		5	3	5	6	7	8	4	4
<i>Est2</i>	0	1	3		---	2	4	5	5	4	4
<i>Est5</i>	0	0	0	---		4	3	3	3	---	---
<i>Me</i>	0	0	0	0	0		3	3	3	1	1
<i>Prx1</i>	0	0	0	0	2	0		4	4	3	3
<i>Prx2</i>	0	0	0	0	0	0	0		7	4	4
<i>Prx3</i>	0	0	0	0	1	0	0	0		4	4
<i>Skd3</i>	0	0	0	0	---	0	0	0	0		4
<i>Skd4</i>	0	0	0	0	---	0	0	0	0	4	

Table 6. Assignment of multi-locus genotypes for chestnut (*Castanea*) cultivars.

Cultivar	Genotype ²											
	1	2	3	4	5	6	7	8	9	10	11	12
<i>C. mollissima</i>												
Jiazha	ab	a_	ab	ab	ab	bb	aa	bc	oo	oo	ab	aa
Yangmaoli	bc	oo	ab	ab	ab	ab	aa	ab	oo	oo	ab	aa
Zaozhuang	ab	a_	bb	ab	ab	ab	aa	bc	oo	oo	aa	aa
Liuyuebao	bc	a_	ab	ab	aa	bb	aa	bb	oo	oo	aa	aa
Jianding	bc	oo	ab	ab	aa	aa	aa	bc	oo	oo	ab	aa
Jiujiazhong	bc	oo	ab	aa	aa	aa	aa	bb	oo	oo	aa	aa
Youli	bb	oo	ab	aa	aa	bb	ab	bb	oo	oo	ab	ab
Zhongcili	bc	oo	ab	aa	ab	ab	aa	ab	oo	oo	ab	aa
Jinfeng	ab	a_	ab	bb	ac	ab	ab	bb	oo	oo	bb	aa
Yanhong	ab	oo	ab	bb	aa	ab	aa	bb	oo	oo	aa	aa
Hongguang	bc	oo	ab	aa	aa	bb	aa	bc	oo	oo	aa	aa
Duancibanhong	bc	a_	bb	ab	aa	aa	aa	ab	oo	oo	ab	aa
Changcibanhong	bb	oo	ab	bb	aa	ab	aa	bb	oo	oo	aa	aa
Zhongguohongpi	bb	a_	ab	bb	aa	ab	aa	bb	oo	oo	aa	aa
Homestead	ab	a_	ab	bb	bb	ab	ab	bb	oo	oo	ab	ab
Leader	ab	a_	bb	bb	bc	aa	ab	ab	oo	oo	aa	aa
Cropper	bb	a_	ab	bb	bb	ab	ab	ab	oo	oo	bb	bb
AU-17	ab	oo	ab	bb	bb	aa	ab	bb	oo	oo	ab	ab
AU-60	bb	a_	ab	bb	bb	aa	ab	ab	oo	oo	ab	ab
<i>C. dentata</i>												
WS male sterile	oo	oo	bb	aa	oo	aa	aa	bb	bb	aa	oo	oo
WS E1	ab	oo	ab	ab	bb	aa	aa	bb	aa	aa	oo	oo
Hesper	aa	oo	ab	aa	bc	aa	aa	bb	ab	aa	oo	oo
Shinebone	bb	oo	ab	ab	bb	bb	aa	bb	aa	aa	oo	oo
<i>C. crenata</i>												
Tanawa	oo	a_	bc	aa	aa	bb	aa	bc	oo	oo	ab	aa
Ibuki	bb	a_	bc	aa	aa	bb	aa	ab	oo	oo	aa	aa
Toyotawa-wase	bb	a_	ab	ab	aa	bb	aa	bb	oo	oo	aa	aa
Ginyose	oo	a_	cc	bb	aa	bb	aa	bb	oo	oo	ab	aa
Yinhong	bb	a_	cc	aa	aa	ab	aa	bb	oo	oo	ab	ab
Toshan 60	bb	a_	bc	bb	ab	bb	aa	bb	oo	oo	ab	aa
Toshan 9	ab	oo	bc	bb	ab	ab	aa	ab	oo	oo	aa	aa
<i>C. sativa</i>												
Weich	bc	a_	bc	ab	ab	aa	aa	ab	aa	aa	oo	oo
Klepper	cc	oo	bc	aa	ab	oo	aa	ab	aa	aa	oo	oo
<i>C. seguinii</i>												
H91-13	ab	oo	bb	bb	ab	ab	ab	ab	oo	oo	ab	aa
H91-6	ab	oo	bb	ab	ab	bb	aa	bb	oo	oo	ab	aa
H91-9	bb	a_	ab	ab	ab	bb	ab	ab	oo	oo	ab	aa
H91-3	aa	a_	ab	bb	aa	bb	bb	ab	oo	oo	ab	aa
H91-8	aa	a_	ab	bb	ab	bb	bb	ab	oo	oo	ab	ab
H91-4	bb	oo	bb	ab	aa	bb	bb	bb	oo	oo	aa	aa
H91-2	ab	a_	ab	ab	aa	bb	bb	bb	oo	oo	aa	aa
H91-5	bc	a_	ab	ab	aa	bb	ab	ab	oo	oo	ab	aa
H91-7	ac	a_	ab	bb	aa	bb	ab	ab	oo	oo	ab	ab
H91-10	aa	a_	ab	aa	aa	bb	aa	bb	oo	oo	aa	aa
H91-79	bc	oo	ab	aa	aa	bb	bb	bb	oo	oo	aa	aa
<i>C. pumila</i>												
Am4065	bb	oo	ab	ab	ab	aa	aa	bb	ab	aa	oo	oo
Am2348	aa	oo	ab	ab	aa	aa	aa	ab	aa	aa	oo	oo
Am2342	aa	oo	oo	oo	aa	aa	aa	ab	ab	aa	oo	oo
Am3532	ac	oo	ab	ab	aa	aa	aa	bb	aa	ab	oo	oo

²1 = *Acp*; 2 = *Adh*; 3, 4 = *Est-2*; 5 = *Me*; 6, 7, 8 = *Prx-1*, 2, 3; 9, 10, 11, 12 = *Skd-1*, 2, 3, 4.

species-specific allozymes (*Skd*) in different chestnut species can help generate sufficient polymorphism and segregation markers when interspecific hybridizations are made. SKD isozymes are particularly interesting in chestnut species. SKD-1 and SKD-2 are

restricted to Am and European species, while SKD-3 and SKD-4 are restricted to Ch and Japanese species. The existence of this species-specific marker provides a unique tool for verifying interspecific hybrids and detecting species introgression.

The single-tree progeny method (Gillet, 1991) was effective for the genetic analysis of chestnut isozymes. Disturbed segregation ratios ($P < 0.05$) were observed in 5.8% of the controlled crosses and 5.4% of the single-tree progeny. Such a frequency of segregation distortion can be attributed in both cases to chance alone. The single-tree progeny method is a convenient and effective alternative for isozyme genetic analysis in self-incompatible species, particularly in the preliminary stage, when large amounts of material are needed for detecting isozyme polymorphism.

Linkage analysis. Forty-two pairs of loci were tested for independent assortment (Table 5). Three pairs of isozyme loci with significant deviations from random assortment were identified: *Skd-3* with *Skd-4*; *Prx-1* with *Est-5*, and *Est-1* with *Est-2*. A highly significant ($P < 0.01$) linkage between *Skd-3* and *Skd-4* was consistently observed in all four segregating families. A significant linkage was also observed in three of five families between *Est-1* and *Est-2* and in two of three families between *Est-5* and *Prx-1* ($P < 0.05$). In addition, significant departure from independent assortment ($P < 0.05$) occurred between the following pairs of loci: *Est-1* and *Adh*, *Est-2* and *Adh*, and *Prx-3* and *Est-5*, each in one segregating family.

Characterization of chestnut cultivars by multi-locus genotypes. Theoretically, there are n^m multi-locus genotype combinations available for cultivar discrimination, if we refer to m as the number of polymorphic loci and n as the average number of genotypes at each locus. However, the practical number of combinations is less than n^m due to the presence of predominant or most common alleles in certain species or populations (Torres, 1980) and also depends on the basic genome composition and the history of cultivar development. Theoretically, the number of multi-locus genotypic combinations of 11 loci identified above would be $3^{11} = 14,348,907$, if we assume an average of two alleles (producing three genotypes) per locus. This is far more than the currently known number of ≈ 600 chestnut cultivars found worldwide (Liu et al., 1988; Rutter et al., 1990). As indicated in Table 6, 47 chestnut cultivars and selections can be uniquely identified by their multi-locus genotypes using 12 polymorphic isozyme loci. The isozyme genetic polymorphism is quite extensive, which makes multi-locus genotype identification a very powerful tool for identifying chestnut cultivars. The 47 cultivars differed on average at 7.08 loci, ranging from a difference of 1 to 12 loci. The average number of allele differences among intraspecific cultivars was 4.00 ± 0.00 for European chestnuts, 4.17 ± 1.47 for Am chestnuts, 4.17 ± 0.98 for Am chinquapins, 4.87 ± 1.69 for Ch chestnuts, 4.67 ± 1.49 for Japanese chestnuts, and 5.20 ± 1.74 for seguin chestnuts. The Japanese chestnut is closely related to the Ch chestnut with an average allelic difference of 5.58 ± 1.32 , while between Japanese chestnut and seguin a 5.73 ± 1.49 allele difference was observed. Likewise, the European chestnut is closely related to the Am chestnut, as indicated by an allele difference of 6.13 ± 0.64 and a difference of 5.38 ± 1.06 alleles between European chestnut and Am chinquapin.

However, a higher degree of allozyme polymorphism is observed when comparing eastern and western species. On average, the allelic difference between Ch and Am cultivars is 8.70 ± 1.19 , between Ch and European 9.43 ± 1.50 , between Japanese and Am 9.36 ± 0.99 , and between Japanese and European 9.07 ± 1.21 . The results are in accordance with the traditional classification of chestnut species. Interestingly, the European species show more similarity to the Am species than to the Asian species. The Ch chestnut is considered the progenitor of both (Jaynes, 1975), giving rise to the European chestnut by westward extension and to several Am species by eastward migration. The data also suggest

that the analysis of multi-locus isozyme genotypes could be very useful for plant systematics and evolution studies. Further studies are necessary to clarify the interspecific relationships and origin of the species.

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