Isozyme Polymorphisms in Chinese Chestnut Cultivars

Hong Wen Huang, J.D. Norton, G.E. Boyhan and R.B. Abrahams

Department of Horticulture, Auburn University, Auburn, AL 36849, USA

ABSTRACT. Chinese chestnut is one of the most genetically diverse species among *Castanea* spp. In China, more than 300 local cultivars have been recognized and divided into 6 distinct regional cultivar groups based on their original distribution and horticulturally important traits. However, less than 50 cultivars are commercially grown for nut production. The objectives of this study were to determine the level of isozyme polymorphisms in C. *mollissima* and evaluate the possibility of isozyme banding patterns for cultivar identification. Four enzyme systems (ACP, AAT, EST, PRX) were studied in 22 widely grown cultivars that represent four regional cultivar groups and 1 colonized population, using polyacrylamide gel isoelectric focusing of bud extracts. No clear zymograms of AAT were obtained in this study (data excluded). High level of isozyme polymorphisms of three enzyme systems were found on these 22 Chinese chestnut cultivars. There were 20, 15 and 13 phenotypic banding patterns observed for EST, PRX and ACP, respectively. Overall, 20 of 22 Chinese chestnut cultivars could be identified by one or more phenotypic banding patterns.

Chinese chestnut, *Castanea mollissima* Bl., is the most important *Castanea* species and has been responsible for about one-third of chestnut production in world commerce (1). C *mollissima* is native to large areas of China, ranging from the far north of Jilin province (north 41° 29') to the tropical region of Hainan province (North 18° 31'), and from 50-2,800 m above sea level. More than 300 local cultivars have been recognized but less than 50 cultivars are commercially grown for nut production in China (2, 8). These cultivars are divided into six distinct regional cultivar groups based on important horticultural traits and the origins of regional distribution (8). Variation within and between these groups is very high for virtually every morphological and physiological characters investigated (4, 8). However, some of these characters are highly influenced by the environment. Furthermore, cultivar introductions between different regions by chestnut growers were undertaken for centuries before the establishment of cultivar designations. Occasionally the same cultivar with different names is grown in different regions, and different cultivars with the same name are grown in the same location. Isozyme phenotypes for cultivar identification have been developed in many fruit and nut tree crops (6). The objectives of this preliminary study were to determine the level of isozyme polymorphisms in *C. mollissima* and evaluate the possibility of isozyme banding patterns for cultivar identification.

MATERIALS AND METHODS

Cultivars. Twenty-two clonal Chinese chestnut cultivars were used in this study: fourteen were traditional cultivars obtained from the China National Chestnut Germplasm Plantation; three are widely grown cultivars in Hubei province; and, five are named varieties or selected breeding lines developed by Auburn University (Table 1). Plant tissue from all cultivars was taken from 1- or 2-yr-old grafted trees and grown in 3-gallon pots under irrigation in the Auburn University horticulture greenhouse complex.

Tissue and Extraction Buffer. Mature buds were collected from current shoot growth. Five buds of each sample were ground in 125 ul of the extraction buffer recommended by Wendel and Weeden (7), containing 100 mM Tris-HC1 (pH 7.5), 7% sucrose (w/v), 10% PVP-40 (w/v), 14 mM mercaptoethanol (0.1% v/v), 250 mM ascorbic acid, 20 mM diethyldithiocarbamate, 25 mM Bovine serum albumin/100 ml (reduced rate), 20 mM sodium metabisulfite and 200 mM sodium tetraborate. Sample preparation was performed in an ice bath and ground tissue was centrifuged at 5000 rpm for 5 min. One-mm #3 filter paper was used to wick the supernatant for electrophoresis.

Gel Isoelectrocfocusing. The electric focusing polyacrylamide gel procedures described by Mulcahy (3) were used in this experiment. Two pieces of slides of polyacrylamide gels were made of 720µl acrylamide and bisacrylamide stock (4.75 g acrylamide + 0.25 g bisacrylamide in 10 ml H20), 720/41 ammonium persulfate solution (0.025 g/10 ml H20). The gels were formed on 75 x 50 x 1 mm microscope slides pre-treated with Silane A174 to promote gel adhesion. The positive electrode buffer was 0.025 M aspartic acid and 0.025 M glutamic acid in water, and the negative electrode buffer was 0.025 M arginine and 0.025 M lysine in 2.0 M ethylenediamine. Electrofocusing was conducted in an ice bath and run at 50, 100, 200, 300, and 400 volts for 15 min each, respectively.

Three of four enzymes were resolved for this study. Staining solutions for acid phosphatase (ACP; E.C. 3.1.1.2.), esterase (EST; E.C.3.1.1.), peroxidase (PRX;

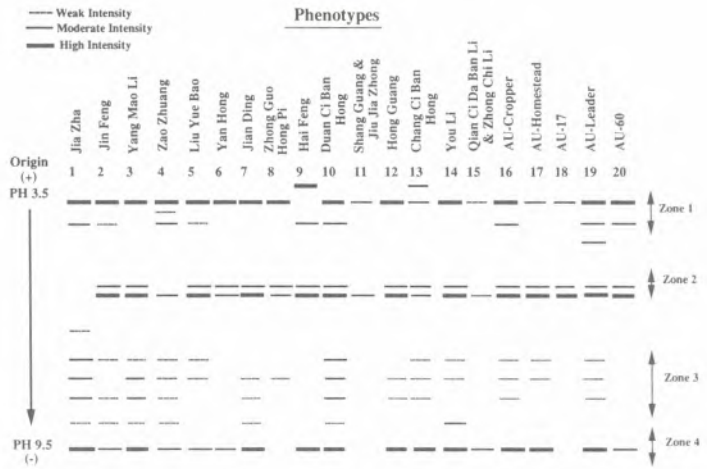

Figure 1. Schematic illustration of EST phenotypic banding patterns in Chinese chestnut cultivars.

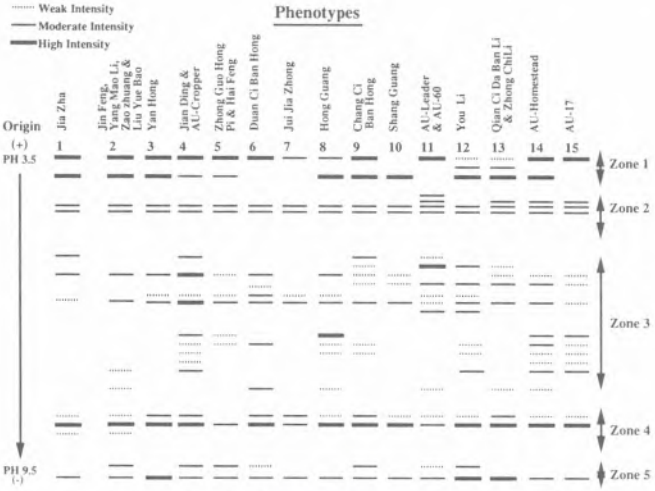

Figure 2. Schematic illustration of PRX phenotypic banding patterns in Chinese chestnut cultivars.

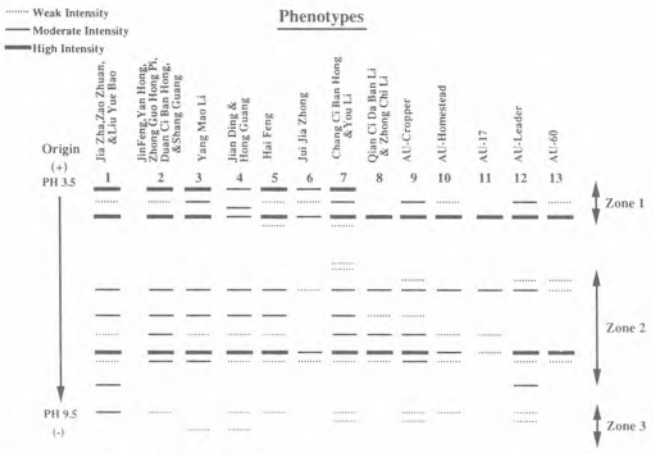

Figure 3. Schematic illustration of ACP phenotypic banding patterns in Chinese chestnut cultivars.

E.C.1.11.1.7), and aspartate aminotransferase (AAT; E.C.2.6.1.1) were used as described by Wendel and Weeden (7). No clear zymograms of AAT were obtained; therefore, AAT is not discussed.

RESULTS AND DISCUSSION

C. mollissima is the most genetically diverse species in the genus, *Castanea* (8), and the 22 cultivars represented four Chinese chestnut regional cultivar groups and one colonized population. As anticipated, a high level of isozyme polymorphism of all three enzyme systems was found on the 22 Chinese chestnut cultivars examined (Table 1).

Table 1. Isozyme phenotypes for 22 Chinese chestnut cultivars.

Cultivar Groups	Cultivar	Phenotypes EST	PRX	ACP
Changjiang River Region (China)	Jia Zha	1	1	1
	Yang Mao Li	3	2	3
	Zao Zhuang	4	2	1
	Liu Yue Bao	5	2	1
	Jian Ding	7	4	4
	Jiu Jia Zhong	11	7	6
	You Li	14	12	7
	Qian Ci Da Ban Li	15	13	8
	Zhong Ci Li	15	13	8
Northern Region (China)	Jin Feng	2	2	2
	Yan Hong	6	3	2
	Hai Feng	9	5	5
	Hong Guang	12	8	4
Southeast Region (China)	Duan Ci Ban Hong	10	6	2
	Chang Ci Ban Hong	13	9	7
	Shang Guang	11	10	2
Southwest Region (China)	Zhong Guo Hong Pi	8	5	2
Auburn University (USA)	AU-Cropper	16	4	9
	AU-Homestead	17	14	10
	AU-17	18	15	11
	AU-Leader	19	11	12
	AU-60	20	11	13

Esterase. Four zones of activity were observed in the gels for this enzyme (Figure 1). Zone 4 (37 mm) might be monomorphic. Zone 3, distributed between 24 mm to 31 mm region, was the most polymorphic one, varying with 3 to 5 bands. Twenty phenotypic banding patterns were observed among the 22 cultivars.

Peroxidase. Five zones of activity occurred in peroxidase (Figure 2). A total of 17 bands, varying from 9 to 17, were distinguishable. This result similarly agreed with that in previous work on *C. crenata* Sieb. and Zucc. by Sawano et al. (5). However, they only visualized 10 bands on *C. crenata*. Zone 2, distributed at 9 mm, had two monomorphic 2 bands in most of the cultivars, except four of the cultivars had 1 or 2 extra bands. Zones 1, 4, and 5, found at 3-5 mm, 34 mm and 39-40 mm, respectively, were the regions with high intensity bands and relatively little variation. They might be more useful for evaluating Chinese chestnut cultivar groups and verifying the cultivars between cultivar groups. Although there were more bands and variations in zone 3 (11-30 mm), most of the bands were weak in intensity and much more time is needed to interpret these data in genetically diverse large populations. Fifteen phenotypic banding patterns of PRX were exhibited among the 22 Chinese chestnut cultivars (Figure 2).

Acid Phosphatase. The best resolution in the present study was for ACP (Figure 3). Three zones were observed for this enzyme and all of them were polymorphic among 22 Chinese chestnut cultivars. Thirteen phenotypic banding patterns were found (Figure 3). Combining our previous studies on ACP phenotypic banding patterns by the extraction of dormant buds, phloem, and young leaves, this enzyme probably could be quite useful for chestnut cultivar identification and germplasm research.

Overall, 20 of 22 Chinese chestnut cultivars could be identified by one or more phenotypic banding pattern (Table 2). Only two cultivars, Qian Ci Da Ban Li and Zhong Chi Li, could not be separated by these three enzyme systems; they are major cultivars that originated in Hubei province, People's Republic of China. Their morphological characters are very similar and it is very difficult to distinguish them even by nut morphological characteristics. Zhang claimed that they were the same clonal cultivars by examining all of the possible morphological characters (L.T. Zhang, personal communication). This is probably true based on the evidence of the present study. Some cultivars with the same phenotypic banding pattern in one enzyme system were observed. This usually occurred in the same cultivar group. For instance, Jia Zha, Zao Zhuang, and Liu Yue Bao with the same ACP phenotype 1 belong to Chang Jiang River Regional c.v group; Yang Mao Li, Zao Zhuang, and Liu Yue Bao with the same PRX phenotype 2 belong to the same regional c.v group. These results suggest that the cultivar group classification is valid to a large degree, although cultivars from different groups with the same enzyme phenotype were observed. Further research in this area might provide more information on the origins and evolution of *Castanea* spp.

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