Using Isozyme Polymorphisms for Identifying and Assessing Genetic Variation in Cultivated Pawpaw [*Asimina triloba* (L.) Dunal]

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ABSTRACT. The utility of isozyme phenotypes for identifying and determining genetic variation in pawpaw cultivars was studied using isoelectric focusing in thin-layer polyacrylamide gels. Based on a sample of 32 clones (cultivars and advanced selections) and 23 enzyme systems, 7 enzymes were found to be polymorphic, involving 9 polymorphic loci [acid phosphatase (ACP), dihydrolipoamide dehydrogenase (DDH), malic enzyme (ME), phosphoglucosomerase (PGI), phosphoglucumutase (PGM), peroxidase (PRX), and shikimate dehydrogenase (SKD)]. Altogether these 9 loci and 32 clones yielded 28 multi-locus isozymic phenotypes useful for cultivar identification; 24 of the 32 clones were uniquely identified. The allozyme variation in these clones has the average of other long-lived woody perennials of widespread geographic range in temperate regions with insect-pollinated outcrossing breeding systems, secondary asexual reproduction, and animal-dispersed seed. Genetic differentiation among these pawpaw clones, measured by Nei's distance, D , was substantial: 496 pairwise comparisons of genetic distance among the 32 clones indicated that they differed on average of $D = 0.068 \pm 0.04$ and ranged from 0 to 0.188. Cluster analysis (UPGMA) produced a most likely division of the 32 clones into 7 groups; however, these groups did not conform to known pedigree relations. Additional polymorphic enzymes are needed for accurate allozyme-based genetic discrimination.

The pawpaw tree produces the largest edible fruit native to the United States and is the only temperate member of the tropical Annonaceae or custard apple family (Darrow, 1975). It has long been appreciated for its delicious, custard-like fruit. Although not a commercially cultivated crop in the United States, the pawpaw has tremendous potential for commercial development and efforts are currently underway to domesticate it. As interest in exotic fruit has increased, under-exploited potentially valuable native fruit, including pawpaw, have attracted enthusiastic attention among the public and horticulturists in the United States and elsewhere. Currently, more than 50 U.S. commercial nurseries market pawpaw seedlings and grafted trees. Recent efforts at collecting germplasm and breeding, led by Kentucky State Univ. (KSU) and the PawPaw Foundation (PPF), have framed current research strategies, which include germplasm collection, variety trials, cultural recommendations, clonal propagation, and breeding commercial cultivars. A comprehensive research program has been active at KSU since 1990, directed toward developing pawpaw as a new commercial tree fruit crop for Kentucky and the United States (Layne, 1996).

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Efforts to domesticate pawpaw began early in the 20th century. Extensive collections of wild selections from many States were assembled by Benjamin Buckman (Farmington, Ill., circa 1900 to 1920), George A. Zimmerman (Linglestown, Pa., 1918 to 1941), and Orland E. White (the Blandy Experimental Farm at Boyce, Va., 1926 to 1955) (Flory, 1958; Popenoe, 1917; Zimmerman, 1941). Since 1900, at least 56 cultivars have been selected and named for propagation (Peterson, 1991). Unfortunately, at least 36 named cultivars dating from 1896 to 1960 have been lost from cultivation through neglect and abandonment of collections and loss of records necessary for identification. Loss of these cultivars has led to a serious erosion of the gene pool of cultivated pawpaws. These cultivars were among the best pawpaw clones that 19th and early 20th century Americans had discovered. However, a resurgence of interest in the pawpaw since 1960 has led to the identification of at least 20 new selections, mostly from the wild (Davis, 1969, 1975; Hickman, 1980; Pape, 1965). New selections have been identified through a cooperative program between the Univ. of Maryland and the PawPaw Foundation, begun in 1981. More than 1300 seedlings (open-pollinated seed) from surviving trees in the Buckman, Zimmerman, Blandy, Hershey, and Allard collections, plus some seedlings from wild trees and named cultivars, were collected and evaluated, resulting in the identification of 18 advanced selections (Peterson, 1986). Currently, 27 cultivars and a few new selections of pawpaw are available in the United States (Layne, 1997).

Of great concern to plant breeders and germplasm custodians is the genetic variation encompassed in cultivars and germplasm collections. For reasons explained below, we suspected that the available pawpaw cultivars and advanced selections might register low in genetic diversity, which would have negative implications for the future domestication of pawpaw and its development as a new crop. The pawpaw is a small deciduous tree that occurs naturally in the understory of forests in lowland drainage basins of

Table 1. The parentage of 32 pawpaw cultivars and advanced selections evaluated in this study².

Name	Parentage
PPF ^y 1-7-1	Open-pollinated seedling of 'Overleese'
PPF 1-16	Open-pollinated seedling of 'Sunflower'
PPF 2-9	Open-pollinated seedling of 'Davis'
PPF 2-10	Open-pollinated seedling of BEF ^x -30
PPF 2-49	Open-pollinated seedling of GAZ ^w -VA
PPF 2-54	Open-pollinated seedling of GAZ-VA
PPF 3-11	Open-pollinated seedling of BEF-33
PPF 3-21	Open-pollinated seedling of BEF-43
PPF 4-2	Open-pollinated seedling of BEF-53
PPF 7-90	Open-pollinated seedling of RS ^z -2
PPF 8-20	Open-pollinated seedling of 'Sunflower'
PPF 8-58	Open-pollinated seedling of BEF-30
PPF 9-47	Open-pollinated seedling of BEF-49
PPF 9-58	Open-pollinated seedling of BEF-50
PPF 10-35	Open-pollinated seedling of BEF-49
PPF 11-5	Open-pollinated seedling of BEF-53
PPF 11-13	Open-pollinated seedling of BEF-53
Fulton	Wild seedling from Fulton, Ill.
Middletown	Wild seedling from Middletown, Ohio
Mitchell	Wild seedling from Iuka, Ill.
NC-1	'Davis' female x 'Overleese' male
Overleese	Cultivated seedling (open-pollinated) from Rushville, Ind.
PA-Golden	Second generation open-pollinated seedling from G.A. Zimmerman
Prolific	Cultivated seedling (open-pollinated) from Bellevue, Mich.
Rebecca's Gold	Open-pollinated seed from Corwin Davis of Bellevue, Mich.
SAA-Zimmerman	Open-pollinated seedling of 'Zimmerman'
Sunflower	Wild seedling from Chanute, Kan.
Taylor	Wild seedling from Eaton Rapids, Mich.
Taytwo	Wild seedling from Eaton Rapids, Mich.
Well's	Cultivated seedling (open-pollinated) from Salem, Ind.
Wilson	Wild seedling from Cumberland, Ky.
Zimmerman	Open-pollinated seed from G.A. Zimmerman

²More details about named cultivars occur in Layne (1997).

^yPPF = PawPaw Foundation orchards; numerous 'wild' selections from the remnant collections of Allard, Blandy, Buckman, Hershey, Schlaanstone, and Zimmerman, plus some from truly wild trees and some from named cultivars; assembled by R. Neal Peterson and Harry J. Swartz at the Univ. of Maryland experiment stations, Keedysville and Queenstown, Md.

^xBEF = Blandy Experimental Farm collection; numerous wild selections plus much of Zimmerman's collection, donated posthumously; assembled by Orland E. White and staff of the Blandy Experimental Farm, Boyce, Va., from 1926 to 1955.

^wGAZ = George A. Zimmerman collection; containing most, if not all, of the named cultivars of the time, plus numerous wild selections and interspecific hybrids; assembled by George A. Zimmerman of Linglestown, Pa., from circa 1920 to 1940.

^zRS = Ray Schlaanstone collection; material descending from Zimmerman's collection via John Hershey; assembled by Ray Schlaanstone of West Chester, Pa., date uncertain, circa 1960?

streams and rivers where it can form extensive clonal thickets because of its pronounced habit of root suckering (Willson and Schemske, 1980). The strongly protogynous flowers are mainly fly or beetle pollinated (Faegri and van der Pijl, 1971; Zimmerman, 1941). Fruit production is low and seed dispersal and germination are generally poor in the wild (Lagrange and Tramer, 1985; Norman et al., 1992; Willson and Schemske, 1980). Given these facts about the pawpaw's reproductive biology, it is plausible that within-population genetic variation may be low.

On the other hand, species whose populations are distributed over a wide geographic range, such as *Asimina triloba*, may maintain significant genetic variation between populations (Hamrick and Godt, 1989; Rogstad et al., 1991). The reproductive biology of *Asimina* is characterized by inefficient cross-pollination, scarce insect pollinators, poor fruit and seed production, and poor germination of selfed seeds (Norman and Clayton, 1986; Norman et al., 1992; Walker, 1971; Willson and Schemske, 1980).

Because pawpaw is an outcrosser (Willson and Schemske, 1980), seed reproduction must have played an important role in extending the species range and in adapting to environmental and ecological changes through genetic recombination (Peterson, 1991). However, the benefits of rootsuckering are not inconsiderable, including the ability to search the adjacent living space for better soil, water, and light and then to colonize those spaces and the ability of a clone to expand its territory and the quantity of resources at its disposal and thereby increase its size and the ability of a colony to buffer risk by sharing water and nutrients and by surviving disasters that destroy a portion of the colony (Willson and Schemske, 1980). The combined influence of sexual reproduction, clonality, and natural selection should have created, molded, and maintained much genetic variation in *A. triloba*.

Another source of genetic variation in *A. triloba* may occur where the southern extremities of its range overlaps the ranges of the southern *Asimina* species. It is known that the eight *Asimina*

species readily hybridize (Kral, 1960; McDaniel, 1970; R.N. Peterson, unpublished data) and, hence, opportunity exists for the introgression of genes from southern *Asimina*, primarily *A. parviflora* into *A. triloba*. Bowden (1948) reported that seven species [*A. pygmaea* (Bartr.) Dunal was not included in his study] are diploid and have 18 chromosomes ($2N = 2X = 18$). Although Bowden (1949) found triploids in *A. triloba*, at a frequency $<2.5\%$, fertile triploids have not been reported.

Identifying pawpaw phenotypes and genotypes would be very useful to pawpaw breeding and development. Isozymes have been used extensively in many tree fruit breeding programs as genetic markers for identifying cultivars such as in lychee and apple (Degani et al., 1995; Weeden and Lamb, 1985), confirming hybridity (Anderson et al., 1991), marker-assisted selection (Manganaris et al., 1994; Yu and Davis, 1995), and performing many other aspects of plant breeding programs, such as selecting donor and recipient parents and monitoring backcross progeny (Tanksley and Orton, 1983). However, no attempts have been made to use isozymes to investigate any of these areas for pawpaw. The purpose of the present study is to establish the isozyme variability in 32 cultivars and advanced selections to 1) identify clonal pawpaw cultivars, and 2) understand the genetic relationships between the different clones in anticipation that they constitute an important part of the germplasm base of future pawpaw improvement efforts.

Materials and Methods

One-year-old dormant shoots were collected from December 1995 through February 1996 from 15 cultivars and 17 advanced selections grown at the KSU research farm and at the PawPaw Foundation orchards located at the Univ. of Maryland experiment stations at Keedysville and Queenstown, Md. (Table 1). These represented most of cultivated pawpaw resources available at the time the study began (Layne, 1997). Enzymes were extracted from the dormant buds using an extraction procedure described by Huang et al. (1994). To achieve higher resolution, the isoelectric focusing polyacrylamide slab gel system (pH 4–9) described by Mulcahy et al. (1981) was used. Gels were stained according to Wendel and Weeden (1989) with the minor modification that only one-third of the original volume of stain solutions was used. All samples were run a minimum of two times to verify reproducibility. The enzymes assayed were acid phosphatase (ACP; EC 3.1.3.2), alcohol dehydrogenase (ADH; EC 1.1.1.1), aconitate hydratase (ACO; EC 4.2.1.3), adenylate kinase (ADK; EC 2.7.4.3), dihydrolipoamide dehydrogenase (DDH; EC 1.8.1.4), esterase (EST; EC 3.1.1.-), formate dehydrogenase (FDH; EC 1.2.1.2), fructose-bisphosphate aldolase (FBA; EC 4.1.2.13), fructose-bisphosphatase (FBP; EC 3.1.3.11), glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49), glutamate dehydrogenase (GDH; EC 1.4.1.2), glyceraldehyde-3-phosphate dehydrogenase (G3PDH;

Table 2. Isozymic phenotypes of 32 pawpaw cultivars and advanced selections for 9 polymorphic isozymes.

Cultivars or advanced selections	PGI-2	PRX-4	SKD	PGM	PRX-1	PRX-3	ACP-2	ME	DDH-2	Multi-locus phenotype ID
PPF 2-49	bb	aa	aa	aa	ab	aa	aa	bb	bb	1
PPF 3-21	bb	aa	aa	aa	ab	aa	ab	bb	ab	2
PPF 3-11	bb	aa	aa	aa	ab	aa	ab	bb	ab	2
Taytwo	bb	aa	aa	aa	ab	aa	ab	ab	bb	3
PPF 4-2	bb	aa	aa	aa	ab	ab	aa	bb	bb	4
PPF 7-90	bb	aa	aa	aa	ab	ab	ab	bb	bb	5
PPF 1-7-1	bb	aa	aa	aa	ab	ab	aa	bb	bc	6
PPF 9-47	bb	aa	aa	aa	ab	ab	aa	bb	bc	6
PPF 11-13	bb	aa	aa	ab	ab	aa	ab	bb	bc	7
PA Golden	bb	aa	aa	ab	ab	ab	ab	bb	bb	8
Sunflower	bb	aa	aa	ab	ab	ab	ab	bb	bb	8
Mitchell	bb	aa	aa	ab	aa	ab	ab	bb	bb	9
Rebecca's Gold	bb	aa	aa	ab	aa	ab	ab	bb	bb	9
Overleese	bb	aa	aa	ab	ab	ab	ab	bb	bc	10
PPF 2-10	bb	aa	aa	ab	ab	ab	ab	bb	ab	11
PPF 1-16	bb	aa	ab	ab	ab	ab	ab	bb	bc	12
NC-1	bb	aa	ab	aa	ab	aa	aa	bb	bb	13
Fulton	bb	aa	ab	aa	ab	ab	ab	bb	bb	14
SAA-Zimmerman	bb	aa	ab	aa	ab	n ²	ab	bc	bb	15
Zimmerman	bb	aa	ab	aa	n	n	ab	bc	bb	16
Prolific	bb	aa	ab	aa	n	n	ab	ab	bb	17
PPF 2-9	bb	aa	ab	aa	ab	aa	ab	bc	bb	18
PPF 11-5	bb	aa	ab	ab	ab	ab	ab	bc	ab	19
PPF 8-58	bb	aa	ab	ab	ab	ab	ab	bb	ab	20
Well's	bb	ab	ab	aa	ab	ab	aa	bb	bb	21
Middletown	bb	ab	aa	aa	ab	aa	ab	bc	bb	22
Taylor	ac	aa	aa	aa	aa	ab	ab	bb	ab	23
Wilson	ac	aa	aa	aa	ab	ab	aa	bc	ab	24
PPF 8-20	ac	aa	aa	aa	ab	aa	aa	bb	bb	25
PPF 2-54	bb	aa	aa	aa	ab	ab	aa	bc	bb	26
PPF 9-58	bb	aa	ab	ab	ab	aa	aa	bb	ab	27
PPF 10-35	bb	aa	aa	aa	ab	ab	aa	bb	ab	28

²n = Null allele phenotype.

Fig. 1. Typical isozyme phenotypes of nine polymorphic loci in cultivated pawpaw collection. The letters correspond to those listed in Table 2. The anode is at the top of the figure.

EC 1.2.1.12), hexokinase (HEX; EC 2.7.1.1), isocitrate dehydrogenase (IDH; EC 1.1.1.41), malate dehydrogenase (MDH; EC 1.1.1.37), malic enzyme (ME; EC 1.1.1.40), mannose phosphate isomerase (MPI; EC 5.3.1.8), peroxidase (PRX; EC 1.11.1.7), phosphoglucomutase (PGM; EC 5.4.2.2), phosphogluconate dehydrogenase (PGD; EC 1.1.1.44), phosphoglucose isomerase (PGI; EC 5.3.1.3), shikimate dehydrogenase (SKD; EC 1.1.1.25), and triosephosphate isomerase (TPI; EC 5.3.1.1).

All isozyme loci and allele assignments were designated tentatively, because no controlled crosses were available to study banding pattern segregation in pawpaw. For most enzymes, however, genetic control and quaternary structures are known well enough from previous studies of other plant species for genetic inferences to be made from the banding patterns we observed (Murphy et al., 1990; Wendel and Weeden, 1989). Loci for a given multiple-locus enzyme system were designated sequentially by numbers starting with 1 for the most anodal locus; letters were assigned to different alleles in each locus, starting with 'a' for the most anodal allele (Table 2, Fig. 1); an allele lacking staining activity was designated 'n' for a null allele. The combination of isozyme phenotypic differences across the polymorphic loci was defined as a multi-locus ID for cultivar identification. Genetic variation in this cultivated pawpaw collection was evaluated using standard genetic statistics, including the mean gene heterozygosity, H_{es} (unbiased estimate, Nei, 1978); the percent polymorphic loci, P_s (proportion of polymorphic loci, 0.95 criterion); the average alleles per locus, A_s (including monomorphic loci as well as polymorphic loci); and the effective alleles per locus, A_{es} (calculated as $1/[1 - H_{es}]$). The level of genetic similarity among cultivars was

determined by Nei's genetic distance, D (Nei, 1972). A dendrogram was constructed from the distance matrix using UPGMA

Table 3. Comparison of genetic variation of pawpaw with plant species having the same characteristics^z.

Species characteristics	P_s^y	A_s	A_{es}	H_{es}
Life form: long-lived woody perennial	64.7 ± 2.7	2.19 ± 0.09	1.24 ± 0.02	0.177 ± 0.010
Regional distribution: widespread	58.9 ± 3.1	2.29 ± 0.16	1.31 ± 0.03	0.202 ± 0.015
Geographic range: temperate	48.5 ± 1.5	1.91 ± 0.05	1.21 ± 0.01	0.146 ± 0.00
Breeding system: outcrossing-animal	50.1 ± 2.0	1.99 ± 0.07	1.24 ± 0.02	0.167 ± 0.010
Seed dispersal: animal ingested	45.7 ± 3.9	1.69 ± 0.08	1.25 ± 0.03	0.176 ± 0.019
Mode of reproduction: sexual and asexual	43.8 ± 3.7	1.69 ± 0.08	1.20 ± 0.03	0.138 ± 0.016
Average of all characteristics	51.2 ± 8.2	1.96 ± 0.25	1.24 ± 0.04	0.168 ± 0.023
Cultivated pawpaw	44.4	1.80 ± 0.20	1.19	0.166 ± 0.048
California cherimoya ^x	73.3	2.40 ± 0.30	1.41	0.330 ± 0.064
California and Spanish cherimoya ^w	44.8	1.70 ± 0.20	1.22	0.183 ± 0.044

^zData from Hamrick and Godt, 1989.

^y P_s , A_s , A_{es} , and H_{es} correspond to the percent polymorphic loci, the average alleles per locus, the effective alleles per locus, and the mean gene heterozygosity, respectively.

^xCalculated from the data in Table 1 and included other three monomorphic loci, published by Ellstrand and Lee (1987).

^wCalculated from the data in Table 2 and included 16 other monomorphic loci, published by Pascual et al. (1993).

cluster analysis (unweighted pair-group method using arithmetic average). Computations were performed using the BIOSYS-1 program (Swofford and Selander, 1981) based on 18 putative isozyme loci.

Results and Discussion

ISOZYME POLYMORPHISMS. Of the 23 enzyme systems examined in this study, 11 produced high-resolution banding patterns. Three enzymes (EST, MDH, and PGD) resolved poorly and inconsistently and, hence, were excluded from further study. Of the 11 highly resolving enzymes, 4 (ADK, FDH, GDH and TPI) were monomorphic and fixed as a single band (one allele) in all 32 clones. Polymorphisms were observed for the other 7 enzymes (ACP, DDH, ME, PGI, PGM, PRX, and SKD) (Fig. 1). Eighteen

loci were tentatively designated: nine monomorphic loci—*Adk*, *Acp-1*, *Acp-3*, *Ddh-1*, *Fdh*, *Gdh*, *Pgi-1*, *Prx-2*, and *Tpi*—and nine polymorphic loci—*Acp-2*, *Ddh-2*, *Me*, *Pgi-2*, *Pgm*, *Prx-1*, *Prx-3*, *Prx-4*, and *Skd*. The nine polymorphic loci, which are informative for cultivar identification, are presented in Table 2.

Examination of zymograms revealed only one polymorphic zone for DDH, PGM, and SKD, consisting of either single- or double-banded patterns (Fig. 1). ACP exhibited three zones of activity, but only ACP-2 showed variability among the cultivars and two banding patterns were observed. For PRX, three polymorphic zones—PRX-1, PRX-3, and PRX-4—were found, each having single- or double-banded patterns (Fig. 1). The observed banding patterns of these enzymes combined with the knowledge that the quaternary structures of ACP, DDH, PGM, PRX, and SKD are monomeric in many plant species (Murphy et al., 1990; Wendel

Table 4. Nei's genetic distance among 32 pawpaw cultivars and advanced selections evaluated in this study.

Cultivars or advanced selections	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1 PPF 2-49																
2 PPF 3-21	0.029															
3 PPF 3-11	0.029	0.000														
4 Taytwo	0.029	0.031	0.031													
5 PPF 4-2	0.014	0.046	0.046	0.046												
6 PPF 7-90	0.029	0.031	0.031	0.031	0.015											
7 PPF 1-7	0.029	0.047	0.047	0.063	0.015	0.031										
8 PPF 9-47	0.029	0.047	0.047	0.063	0.015	0.031	0.000									
9 PPF 11-13	0.045	0.031	0.031	0.047	0.062	0.047	0.047	0.047								
10 PA Golden	0.045	0.047	0.047	0.047	0.030	0.015	0.047	0.047	0.032							
11 Sunflower	0.045	0.047	0.047	0.047	0.030	0.015	0.047	0.047	0.032	0.000						
12 Mitchell	0.060	0.063	0.063	0.063	0.046	0.031	0.063	0.063	0.047	0.015	0.015					
13 Rebecca's Gold	0.060	0.063	0.063	0.063	0.046	0.031	0.063	0.063	0.047	0.015	0.015	0.000				
14 Overleese	0.061	0.048	0.048	0.064	0.046	0.031	0.031	0.031	0.016	0.016	0.016	0.031	0.031			
15 PPF 2-10	0.061	0.031	0.031	0.064	0.046	0.031	0.048	0.048	0.032	0.016	0.032	0.031	0.031	0.016		
16 PPF 1-16	0.077	0.064	0.064	0.082	0.063	0.048	0.048	0.048	0.032	0.032	0.062	0.048	0.048	0.016	0.033	
17 NC-1	0.014	0.046	0.046	0.046	0.030	0.046	0.046	0.046	0.062	0.062	0.032	0.077	0.077	0.079	0.079	0.063
18 Fulton	0.045	0.047	0.047	0.047	0.030	0.015	0.047	0.047	0.065	0.032	0.098	0.047	0.047	0.049	0.049	0.032
19 SAA-Zimmerman	0.109	0.114	0.114	0.097	0.095	0.080	0.114	0.114	0.134	0.098	0.098	0.114	0.114	0.118	0.118	0.101
20 Zimmerman	0.159	0.164	0.164	0.147	0.144	0.129	0.164	0.164	0.185	0.149	0.149	0.164	0.164	0.169	0.169	0.153
21 Prolific	0.159	0.164	0.164	0.129	0.144	0.129	0.164	0.164	0.185	0.149	0.149	0.164	0.164	0.169	0.169	0.153
22 PPF 2-9	0.045	0.047	0.047	0.031	0.062	0.047	0.080	0.080	0.065	0.065	0.065	0.080	0.080	0.083	0.083	0.066
23 PPF 11-5	0.094	0.065	0.065	0.082	0.080	0.065	0.082	0.082	0.067	0.049	0.049	0.065	0.065	0.051	0.033	0.034
24 PPF 8-58	0.077	0.048	0.048	0.082	0.063	0.048	0.064	0.064	0.049	0.032	0.032	0.048	0.048	0.033	0.016	0.017
25 Well's	0.045	0.080	0.080	0.080	0.030	0.047	0.047	0.047	0.098	0.065	0.065	0.080	0.080	0.083	0.083	0.066
26 Middletown	0.045	0.047	0.047	0.031	0.062	0.047	0.080	0.080	0.065	0.065	0.065	0.080	0.080	0.083	0.083	0.101
27 Taylor	0.077	0.047	0.047	0.080	0.062	0.047	0.063	0.063	0.081	0.065	0.065	0.047	0.047	0.065	0.049	0.084
28 Wilson	0.061	0.064	0.064	0.081	0.046	0.064	0.048	0.048	0.100	0.083	0.083	0.098	0.098	0.084	0.067	0.103
29 PPF 8-20	0.014	0.046	0.046	0.046	0.030	0.046	0.046	0.046	0.062	0.062	0.062	0.077	0.077	0.079	0.079	0.096
30 PPF 2-54	0.029	0.063	0.063	0.047	0.015	0.031	0.031	0.031	0.080	0.047	0.047	0.063	0.063	0.064	0.064	0.082
31 PPF 9-58	0.045	0.047	0.047	0.080	0.062	0.080	0.063	0.063	0.048	0.065	0.065	0.080	0.080	0.065	0.049	0.049
32 PPF 10-35	0.029	0.031	0.031	0.063	0.015	0.031	0.015	0.015	0.063	0.047	0.047	0.063	0.063	0.048	0.031	0.064
17 NC-1	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
18 Fulton	0.030															
19 SAA-Zimmerman	0.095	0.065														
20 Zimmerman	0.144	0.114	0.047													
21 Prolific	0.144	0.114	0.063	0.015												
22 PPF 2-9	0.030	0.032	0.065	0.114	0.131											
23 PPF 11-5	0.080	0.049	0.084	0.136	0.155	0.049										
24 PPF 8-58	0.063	0.032	0.101	0.153	0.153	0.066	0.017									
25 Well's	0.030	0.032	0.098	0.149	0.149	0.065	0.084	0.066								
26 Middletown	0.062	0.065	0.134	0.149	0.167	0.032	0.084	0.101	0.065							
27 Taylor	0.095	0.065	0.090	0.185	0.185	0.098	0.052	0.066	0.098	0.098						
28 Wilson	0.079	0.083	0.118	0.169	0.188	0.083	0.068	0.085	0.083	0.083	0.049					
29 PPF 8-20	0.030	0.062	0.129	0.179	0.179	0.062	0.115	0.096	0.062	0.062	0.062	0.046				
30 PPF 2-54	0.046	0.047	0.080	0.129	0.147	0.047	0.065	0.082	0.047	0.047	0.080	0.031	0.046			
31 PPF 9-58	0.030	0.065	0.134	0.185	0.185	0.065	0.049	0.032	0.065	0.098	0.098	0.083	0.062	0.080		
32 PPF 10-35	0.046	0.047	0.114	0.164	0.164	0.080	0.065	0.048	0.047	0.080	0.047	0.031	0.046	0.031	0.047	

and Weeden, 1989) allowed us to tentatively assign seven polymorphic (*Acp-2*, *Ddh-2*, *Pgm*, *Prx-1*, *Prx-3*, *Prx-4*, and *Skd*) and four monomorphic (*Acp-1*, *Acp-3*, *Ddh-1*, *Prx-2*) isozyme loci for pawpaw (Table 2, Fig. 1).

Two zones of activity were observed for PGI, and tentatively designated as PGI-1 and PGI-2 (Fig. 1). PGI-1 was monomorphic, having a single band (a single putative allele) in all the cultivars and advanced selections. PGI-2 was polymorphic, consisting of either single- or triple-banded patterns. This typical one- vs. three-banded pattern suggests that PGI is dimeric in pawpaw with three putative alleles (*a*, *b*, and *c*) among the 32 clones (Fig. 1). Additionally, an apparent heterodimeric band located between the two putative loci PGI-1 and PGI-2 was also detected (Fig. 1). TPI exhibited a single-banding pattern in all cultivars except two, where a triple-banded pattern was observed. However, this triple-banded pattern did not show the typical 1x : 2x : 1x intensity of dimeric enzymes. TPI is temporarily treated as a monomorphic locus among the 32 clones. PGI and TPI have been demonstrated

to be dimeric enzymes (Murphy et al., 1990; Wendel and Weeden, 1989); thus, two PGI and one TPI isozyme loci could tentatively be assigned for pawpaw (Table 2, Fig. 1). Three ME banding patterns were observed among the cultivars (Fig. 1). ME exhibited one zone of activity characterized by one- vs. five-banded patterns similar to those reported for apple (Weeden and Lamb, 1987), which suggests that ME is tetrameric in pawpaw.

CULTIVAR IDENTIFICATION. To a large extent, isozyme analysis is a useful tool for identifying pawpaw cultivars; 24 of 32 clones were uniquely identified by combinations of these phenotypes (Table 2). Of the seven polymorphic enzyme systems studied, DDH and ME were the most useful for pawpaw cultivar identification because of three isozyme phenotypes found in each system. The PRX-1 and PRX-3 loci also yielded three phenotypes, but one was due to a null allele. For the other five polymorphic loci (*ACP-2*, *PGI-2*, *PGM*, *PRX-4*, and *SKD*), only two isozymic phenotypes were observed.

Eight clones, arranged in four pairs, were not distinguishable by isozyme phenotype: PPF 3-21 and PPF 3-11; PPF 1-7 and PPF 9-47; 'PA-Golden' and 'Sunflower'; and 'Mitchell' and 'Rebecca's Gold'. Except for PPF 3-21 and PPF 3-11, there is no possibility of a pedigree relationship among them (Table 1). 'PA-Golden'

descended from Zimmerman seed; 'Sunflower' is a wild selection from Kansas; 'Mitchell' is a wild selection from Illinois; and 'Rebecca's Gold' is a cultivated selection from seed from Corwin Davis, whose pawpaw collection did not contain 'Mitchell' (Layne, 1997). It is highly doubtful that any pedigree relationship exists between PPF 1-7 and PPF 9-47. The former numbered selection is a seedling of 'Overleese' and the latter a seedling of BEF-49, which was a specimen in the Blandly collection. The situation with PPF 3-21 and PPF 3-11 is less certain. These two clones originate from mother trees BEF-33 and BEF-43, which were found growing not far apart in the Blandly collection and which may be related. Perhaps PPF 3-21 and PPF 3-11 had the same pollen parent. Two mother-daughter pairs of clones produced isozyme phenotypes indicating that they may be unrelated. 'Sunflower' (mother) and PPF 8-20 (daughter) have PGI-2 phenotypes bb and ac. 'Zimmerman' (mother) and 'SAA-Zimmerman' (daughter) have PRX-1 phenotypes n and ab. The pedigrees of clones PPF 8-20 and 'SAA-Zimmerman' had not been in any doubt up until now. Finally, it is worth noting that 'Taylor' and 'Taytwo', which were always thought to be closely related because as wild selections they were found growing close to each other in the same patch, differ at five of the nine loci.

GENETIC VARIATION AND DIFFERENTIATION AMONG PAWPAW CULTIVARS. Despite its habit of clonal reproduction, the level of allozyme variation in this collection of pawpaws is within the average of the other plant species that are similar in life form (long-lived woody perennial), geographic range (widespread), regional distribution (temperate), breeding system (outcrossing-insect), seed dispersal mechanism (animal-ingested), and mode of reproduction

Fig. 2. UPGMA dendrogram showing genetic similarity among 32 cultivars or advanced selections based on the distance matrix of Table 4.

(sexual and asexual) (Table 3) (Hamrick and Godt, 1989). Clonal plant species are usually found to have moderate genetic diversity and unequal richness of genotypes among populations (Ellstrand and Roose, 1987), and this appears true of this cultivated pawpaw collection. For the 9 polymorphic loci and the 32 clones, an average of 3.75 loci were found to be heterozygous, ranging from 1 to 7 loci. It is interesting to compare the results with cherimoya (*Annona cherimola* Mill.), a related nonclonal Annonaceous species that was domesticated in antiquity by pre-Incan people, which is commercially cultivated for its fruit and has been studied much more than pawpaw. The allozyme variation in this cultivated pawpaw collection was found to be lower than that in a group of 16 California cherimoya cultivars, but similar to a group of 21 California and Spanish cultivars (Table 3) (Ellstrand and Lee, 1987; Pascual et al., 1993). Further applications of isozymes as genetic markers to evaluate the wild pawpaw populations over the entire native range would be worthwhile and may provide more accurate information on the genetic diversity of the species.

A total of 496 pair-wise comparisons of Nei's genetic distance D among the 32 clones indicated that they differed on average of $D = 0.068 \pm 0.04$, with differences ranging from 0 to 0.188 (Table 4). In general, intraspecific differentiation measured by Nei's genetic distance is <0.10 (Crawford, 1989) and, thus, an average of $D = 0.068 \pm 0.04$ suggests that the genetic differentiation in this pawpaw collection is within the average range of intraspecific differentiation. However, substantial genetic difference can be found in some pairs of clones (Table 4). For instance, the highest distance found was 0.188 between 'Wilson', a wild selection from the mountains of eastern Kentucky, and 'Prolific', a wild selection from western Michigan (Layne, 1997). Apparently, they were developed in the northern extreme and southern portion of the native range of *A. triloba*, respectively. Lagrange and Tramer (1985) have demonstrated a significant difference in fruit size and productivity between the pawpaws in the northern and southern extremes.

The degree of genetic similarity among the 32 clones was further investigated using UPGMA cluster analysis of Nei's genetic distance. A dendrogram was constructed for the purpose of showing the structure of allozyme similarities among the clones (Fig. 2). Initially, seven clusters were identified for closer examination, based on the cutoff level of $D = 0.05$, which is the modal value of the frequency distribution of the genetic distances among the clones. These seven clusters were then compared to pedigree information on the clones (Table 1). This information is quite limited because exact pedigrees are unknown. Most cultivars were selected and developed by pawpaw enthusiasts with the result that cultivars have been either poorly documented in the literature or not at all. Most were selected from the wild, and the site of origin is the only information available. The situation for the advanced selections is slightly better as the identity of the mother trees is known, which gives rise to the following relationships: mother and daughter ('Overleese' and PPF 1-7; 'Overleese' and 'NC-1'; 'Zimmerman' and 'SAA-Zimmerman'; 'Sunflower' and PPF 8-20; 'Sunflower' and PPF 1-16) and half siblings (PPF 4-2, PPF 11-5 and PPF 11-13; PPF 2-9 and 'NC-1'; PPF 1-16 and PPF 8-20; PPF 9-47 and PPF 10-35; PPF 2-49 and PPF 2-54; 'NC-1' and PPF 1-7). When the dendrogram was examined in light of the pedigrees, it was obvious that the seven clusters separated many of the kinship pairs. In fact, eight pairs were split by cluster boundaries while only five pairs were preserved. A different partition of the data that grouped the 32 clones into three clusters was found to preserve pedigree relationships, based on the cutoff level of $D = 0.07$. However, under that partition, 27 of the 32 clones grouped together

as one cluster; that is, they were not detectably divergent from one another.

Overall, these analyses are encouraging but they do reveal a need for improved means of discriminating genetic differences among pawpaw clones. Four pairs of clones had identical isozyme phenotypes and could not be uniquely identified, and eight out of thirteen kinship pairs were split into different clusters. This result simply manifests a reality that phenotypic variation in pawpaw may not be well reflected in isozymic variation of the 23 enzymes examined here. Additional polymorphic enzymes and DNA markers such as randomly amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) are needed to accurately discriminate *A. triloba* cultivars and to assess more fully *Asimina* genetic variation. Cheap, efficient, reproducible means of identifying phenotypes will be important to pawpaw domestication with numerous applications in germplasm management, collection, and maintenance and in the development of new cultivars. Our intention to evaluate wild populations of *A. triloba* over its native range for presenting an accurate picture of the genetic diversity of the species, and as a guide for future germplasm collection, may well depend on the new genetic markers that are developed.

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