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Isozyme and RAPD variation among and within hemp dogbane (*Apocynum cannabinum*) populations

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Clonal individuals from 16 hemp dogbane populations with phenotypic variation were analyzed using isozyme and randomly amplified polymorphic DNA (RAPD) analysis. Plants originated from populations in Michigan and Illinois. Three known *Apocynum* species, spreading dogbane, hemp dogbane, and prairie dogbane, were evaluated. Genetic distance among populations was more pronounced with isozyme analysis compared to RAPD analysis. The combined isozyme and RAPD analysis data separated spreading dogbane from all other plants analyzed. Genetic variation was present among the 16 hemp dogbane populations, but was less than expected based on the phenotypic variation present among the collections. The short genetic distance between the 16 hemp dogbane collections and the three *Apocynum* species suggests that variation among populations of hemp dogbane may be from outcrossing with other closely related *Apocynum* species. Isozyme and RAPD analyses were also conducted on plants from two populations in Michigan to determine the level of genetic variation among plants within the same population. Genetic analysis revealed that one population was entirely clonal, while the other population was a mixture of clonal and segregating plants.

Nomenclature: Hemp dogbane, *Apocynum cannabinum* L. APPCA; prairie dogbane, *Apocynum sibericum* Jacq. APCVE; spreading dogbane, *Apocynum androsaemifolium* L. APCAN.

Key words: Genetic variation, polymorphism, isozyme analysis, RAPD analysis, APPCA, APCVE, APCAN.

Hemp dogbane, a perennial weed native to the United States, is widely distributed in areas of the eastern and midwestern states (Anonymous 1970). Hemp dogbane can grow in dense patches within a crop, causing significant yield loss (Schultz and Burnside 1979). The ability of hemp dogbane to grow in diverse environments (Balbach 1965) suggests it is either genetically diverse or highly adaptable. Genetic variation within hemp dogbane could come from its ability to cross with other *Apocynum* species (Anderson 1936; Balbach 1965; Woodson 1930). Hemp dogbane is also apparently self-sterile (Anderson 1936; Balbach 1965; Woodson 1930), and cross-pollination is likely frequent (Anderson 1936). Hemp dogbane morphology can be strongly influenced by soil type and fertility level (Robinson and Jeffery 1972). However, even when grown in a common environment, plants collected from geographically distinct populations exhibited variability in growth and morphology (Ransom et al. 1998). Variation in morphological traits can have limited use in understanding population genetics because they are often limited in number, can be influenced by environmental conditions, and are often inherited by multiple genes (Nissen et al. 1995).

Nissen et al. (1995) recently described two molecular analysis techniques and their application to weed science. Isozyme and randomly amplified polymorphic DNA (RAPD) analyses are two techniques that are used to examine genetic variation among and within plant species. These techniques can be more powerful than morphological markers because they allow the detection of heterogeneity among plants that appear morphologically identical (Anonymous 1991). These techniques can also be used to quantify the level of genetic variation present among plants with different phenotypes. Isozyme analysis has been used to examine genetic variation among and within populations of yellow nutsedge (*Cyperus esculentus* L.) (Horak and Holt 1986; Horak et al. 1987), broom snakeweed [*Gutierrezia sarothrae* (Pursh) Britt. and Rusby] (Hou and Sterling 1995), purple loosestrife (*Lythrum salicaria* L.) (Strefeler et al. 1996), common lambsquarters (*Chenopodium album* L.) (Mouemar and Gasquez 1983), velvetleaf (*Abutilon theophrasti* Medikus.), jimsonweed (*Datura stramonium* L.), wild proso millet (*Panicum miliaceum* L.), johnsongrass [*Sorghum halepense* (L.) Pers.], and giant foxtail (*Setaria faberi* Herrm.) (Warwick 1990). Isozyme analysis has also been employed for the study of pollen flow in common milkweed (*Asclepias syriaca* L.) (Shore 1993). Considerable information about the genetic relationships among *Conyza* species (Thébaud and Abbott 1995) and among the *Setaria* species (Wang et al. 1995a, 1995b) and within the *Daucus carota* (St. Pierre et al. 1990) and *Carex pachystachya* (Whitkus 1992) complexes has been generated utilizing isozyme analysis. Some weaknesses of isozyme analysis are that a limited number of loci can be sampled and allozyme variation may be too low for analysis of genetic diversity within and among plant populations (Nissen et al. 1995).

RAPD analysis involves the amplification of small sequences of target DNA using random primers. As a DNA-based marker, the strength of RAPD analysis is the ability to assess genetic variation throughout the genome and generate a nearly limitless number of characters for evaluation (Nissen et al. 1995). This makes RAPD analysis a powerful tool for assessing genetic variation within and among weed

TABLE 1. County and state of origin of hemp dogbane collections.

Collection		Collection area	
Number	Code	County	State
1	M-101	Shiawassee	Michigan
2	M-102	Huron	Michigan
3	M-105	Arenac	Michigan
4	M-108	Cass	Michigan
5	M-109	Monroe	Michigan
6	M-112	Kalamazoo	Michigan
7	M-118	Allegan	Michigan
8	M-124	Tuscola	Michigan
9	I-102	Jackson	Illinois
10	I-103	Adams	Illinois
11	I-107	Woodford	Illinois
12	I-109	Iroquois	Illinois
13	I-115	LaSalle	Illinois
14	I-116	LaSalle	Illinois
15	I-122	Livingston	Illinois
16	I-124	Randolph	Illinois

populations. RAPD analysis has been used to analyze different populations of leafy spurge (*Euphorbia esula* L.) and may be important for determining the susceptibility of leafy spurge populations to biological control agents (Nissen et al. 1995). RAPD analysis has also been used to study relationships among populations of cultivated and weedy eggplant (*Solanum melongena* L.) species (Karihaloo et al. 1995) and to examine genetic variation among and within populations of wild mustard (*Sinapsis arvensis* L.) (Moodie et al. 1997). RAPD analysis provides a powerful tool for studying all aspects of weed taxonomy and population genetics.

Both isozyme and RAPD analyses are useful tools in studying genetic differences in plants. Each analysis has strengths and weaknesses. Isozyme markers are codominant; therefore, data from isozyme analysis are more powerful. However, isozyme analysis is limited in the number of loci that can be tested. RAPD markers are dominant; therefore, there is less information per band. However, more loci can be tested with RAPD analysis.

The objectives of this study were to use both isozyme and RAPD analysis: (1) to determine the level of genetic variation among phenotypically variable populations of hemp dogbane, and (2) to examine the genetic variation present within geographically distinct hemp dogbane populations.

Materials and Methods

Among Population Variation

Clonal plants from 16 hemp dogbane populations evaluated in a common garden experiment were used to establish plants in the greenhouse. Plants were collected from populations in Illinois and Michigan (Table 1). Large differences in growth and morphology were observed among the 16 populations when they were grown in a common garden (Ransom et al. 1998).

For both isozyme and RAPD analysis, the youngest fully expanded leaves were collected and used as a tissue source. Representative plants of three *Apocynum* species obtained from the Beal Botanical garden at Michigan State University, East Lansing, MI, were subjected to isozyme and RAPD analysis alongside plants from the 16 populations. The representative species were spreading dogbane, hemp dogbane, and prairie dogbane.

Within Population Variation

To examine the level of genetic variation present within hemp dogbane populations, 25 plants were randomly collected from each of two geographically distinct populations of hemp dogbane in Michigan. One population was from Shiawassee County and the other from Kalamazoo County, MI. Plants were grown in the greenhouse, and leaf tissue was used to conduct isozyme and RAPD analyses.

Isozyme Analysis

For isozyme analysis, the first fully expanded leaves were collected and placed between moist paper towels. Fresh leaf tissue was crushed in 125 μl of extraction buffer (75 mM Tris-HCl buffer, pH 7.5, 50 g/L polyvinylpyrrolidone-40, and 0.2% v/v 2-mercaptoethanol [14 mM]) using a chilled plexiglass pestle and 12-sample porcelain plates. The extract was absorbed onto two 3- by 9-mm filter paper[1] wicks and immediately placed in a −20 C refrigerator. Two gel buffer systems were used to resolve isozyme banding patterns: a pH 5.7 histidine-citrate gel and a pH 8.3 lithium-borate gel (Wendel and Weeden 1989). The gels consisted of 10.5% potato starch. The pH 5.7 gel was electrophoresed at 35 mA for 5 h, and the pH 8.3 gel was electrophoresed at 50 mA for 4 h. Gels were run in a 4 C cooler with ice bags placed on them to prevent heat buildup. Following electrophoresis, gels were removed from the gel trays and sliced horizontally into four slices. Each slice was assayed with a different enzyme-specific stain. Phosphoglucose isomerase (PGI), phosphogluconate dehydrogenase (6-PGDH), malate dehydrogenase (MDH), and aconitase (ACO) enzyme systems were assayed using the histidine-citrate pH 5.7 buffer system, while phosphoglucomutase (PGM), aminoaspartate transaminase (GOT), diaphorase (DIA), peroxidase (PRX), and triose phosphate isomerase (TPI) were resolved with the lithium-borate pH 8.3 buffer system. Enzyme-staining techniques are described by Vallejos (1983). Once the stains were added, gels were incubated until banding patterns had adequately developed. Enzyme activity was stopped by rinsing the gels twice with water and fixing in 50% ethanol. Isozyme gels were repeated. Gels were evaluated, and banding patterns were interpreted.

RAPD Analysis

For RAPD analysis, young leaf tissue was collected from plants in the greenhouse, frozen at −80 C, and freeze dried using a speed-vac. DNA was extracted from 200 mg of freeze-dried leaf material using a CTAB DNA extraction described by Dellaporta et al. (1983). The extraction was modified by adding a DNA purification procedure described by Li et al. (1994). DNA quantities were determined by DNA fluorometry and by visual comparison with known quantities of λ DNA on an agarose gel stained with EtBr (0.5 μg ml^{-1}). DNA concentrations were adjusted to less than 100 ng μl^{-1} prior to use for RAPD analysis. Amplification was performed in a 25-μl reaction containing approximately 50 ng of genomic plant DNA template, 10 mM

TABLE 2. Enzyme systems and RAPD primers used for analysis of hemp dogbane populations.

Isozyme analysis			RAPD analysis		
Enzyme	Number of loci	Number that are polymorphic	Primer	Number of bands	Number that are polymorphic
ACO	1	0	OPA-01	3	0
DIA	1	1	OPA-02	5	1
GOT	1	0	OPA-03	4	1
MDH	2	1	OPA-09	4	2
6-PGDH	2	2	OPA-10	3	0
PGI	1	1	OPA-11	2	1
PGM	1	1	OPA-12	5	0
PRX	1	0	OPA-13	6	0
TPI	2	1	OPA-14	5	3
Total	12	7	OPA-15	3	0
			OPA-16	5	0
			OPA-18	2	1
			Total	47	9

Tris-HCl, pH 8.3, 10 mM KCl, dATP, dCTP, dGTP, and dTTP (200 μM each), 5 mM $MgCl_2$, 2 μM random primer, and stoffel fragment enzyme[2] (two units 100 μl^{-1}). The primers used were 10-base primers from random primer kits[3] A, E, I, and X. The reactions were incubated in a DNA thermal cycler[4] with one cycle of 94 C for 2 min; 40 cycles of 92 C for 1 min, 15 s; 37 C for 1 min, 15 s; and 72 C for 2 min, followed by one cycle of 72 C for 5 min. Following amplification, 25 μl of each reaction was loaded on a 2% agarose gel with 250 ng of Hind III digested λ DNA loaded in the first lane as a size standard. Amplified fragments were separated at 50 V for 5 h in 1× Tris-acetate (TAE) buffer (4 mM Tris-acetate, 1 mM EDTA). Gels were stained with EtBr (0.5 $\mu g\ ml^{-1}$), allowing DNA fragments to be visualized on an ultraviolet transilluminator. RAPD analyses were repeated for reactions producing polymorphic amplification products. Repeatable bands were rated as present or absent for all collections analyzed.

For both isozyme and RAPD analysis, genetic distance between collections and species was determined using Nei's distance formula (Nei 1972). Dendograms were created using the unweighted pair group method with arithmetic averages (UPGMA) cluster analysis. All genetic distance calculations and dendograms were made using NTSYS-pc version 1.7 software (Rohlf 1992).

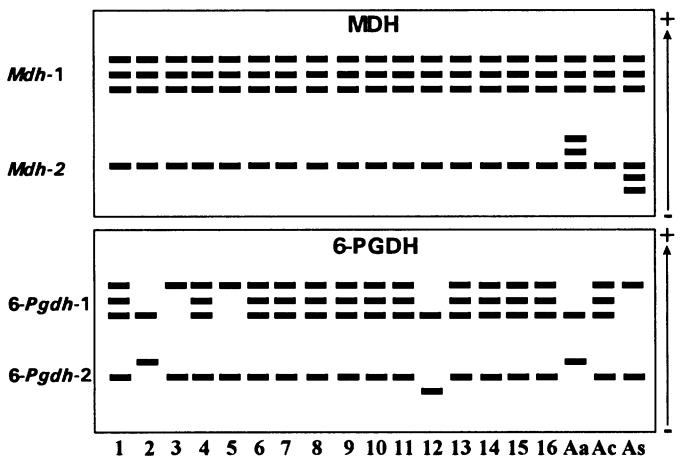

FIGURE 1. Schematic diagram of isozyme patterns for MDH and PGDH for 16 populations of hemp dogbane (1 to 16) and three *Apocynum* species. Representative species are Aa, *Apocynum androsaemifolium*; Ac, *Apocynum cannabinum*; and As, *Apocynum sibericum*. The origin (−) is at the bottom and the front (+) at the top of both electrophoretic diagrams.

Results and Discussion

Variation Among Populations

The nine enzyme systems assayed revealed 12 isozyme loci, seven of which were polymorphic (Table 2). *Mdh*-2, *Pgi*-1, and *Tpi*-2 were polymorphic only among the three representative *Apocynum* species, but not among the 16 collections of hemp dogbane. Polymorphism was present among the 16 hemp dogbane collections for some of the remaining isozyme loci. The three *Apocynum* species had distinct electrophoretic patterns for *Mdh*-2 and 6-*Pgdh*-1, while polymorphic patterns were apparent among the 16 hemp dogbane collections for 6-*Pgdh*-1 and 6-*Pgdh*-2 (Figure 1). Genetic distance among the 16 populations as calculated from the isozyme data ranged from 0 to 0.302, while distance among the three different species ranged from 0.118 to 0.423 (data not shown).

For RAPD analysis, amplification was affected by the random 10-base primer used, and some primers failed to amplify DNA bands. Of the 15 primers tested, 12 primers amplified bands for evaluation. Amplified DNA fragments ranged in size from 200 to 1,500 base pairs. Amplification was inconsistent for collections 3, 13, and 16, with half of the amplified bands absent for these collections. The 12 primers generated 47 bands, and nine bands were polymorphic (Table 2). Figure 2 shows RAPD gels with polymorphic bands present among hemp dogbane populations for DNA sequences amplified with OPI-14 and OPI-18 primers. Polymorphic fragments amplified with OPI-14 are indicated by arrows labeled a, b, and c in gel A. Collection 3 does not have fragment a, but it is the only collection with fragment b. Collection 4 does not have fragment a or b, while collection 5 has fragment a and not b. All plants analyzed have fragment c, except for spreading dogbane. For RAPD analysis with OPI-18 (gel B), one polymorphic fragment (d) was amplified. Fragment d was amplified in only five of the collections.

Genetic distance values were less than observed with isozyme analysis. For the RAPD data, genetic distance values

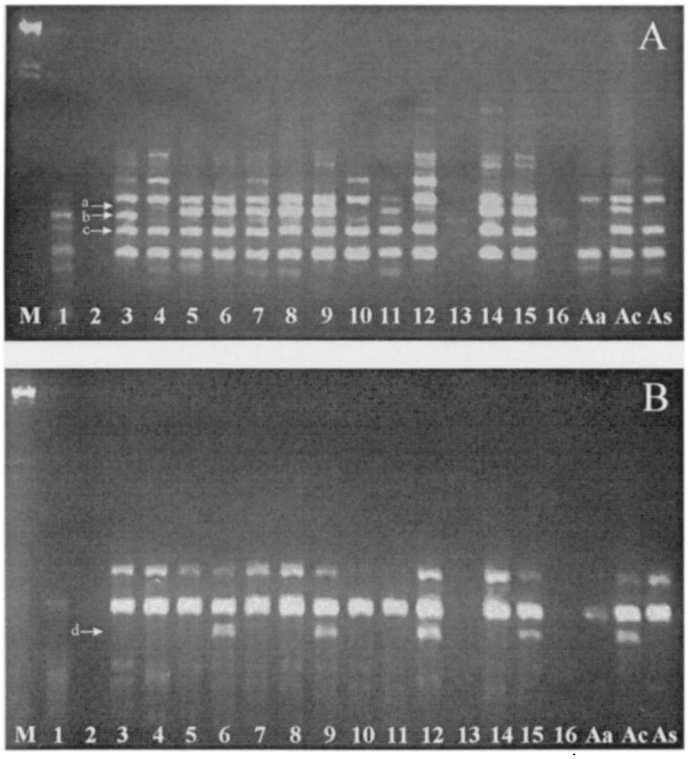

FIGURE 2. Genomic DNA fragments from 16 populations of hemp dogbane (1 to 16) and three *Apocynum* species amplified with (A) OPI-14, and (B) OPI-18 primers. Representative species are Aa, *Apocynum androsaemifolium*; Ac, *Apocynum cannabinum*; and As, *Apocynum sibericum*. Arrows labeled with a, b, and c in OPI-14 (gel A) and d in OPI-18 (gel B) represent amplified DNA fragments that are polymorphic among plants.

among the 16 populations of hemp dogbane ranged from 0 to 0.091, and distance among the three species ranged from 0.055 to 0.187 (data not shown). Genetic distance was generally smaller between each of the 16 hemp dogbane collections and the three species than among the three *Apocynum* species. Because similar trends were observed with genetic distances calculated from the isozyme and the RAPD data, the data were combined and used to calculate genetic distance values (Table 3). The genetic distance among the 16 collections was less than 0.1, except between collection number 2 and a total of nine of the other hemp dogbane collections. The genetic distance between the 16 collections and spreading dogbane was generally greater than 0.1. A dendogram (Figure 3) for the 16 populations of hemp dogbane and the three *Apocynum* species was created from the genetic distance matrix using UPGMA cluster analysis. Several branches are present on the dendogram, with spreading dogbane and collection 2 separating from the other plants analyzed. Collection 12 is isolated on a branch as is prairie dogbane, while the hemp dogbane specimen from the botanical garden and the other hemp dogbane populations fall under the same broad cluster. Based on the morphological variation observed in the common garden experiment (Ransom et al. 1998), more genetic distance among the 16 populations of hemp dogbane was expected. However, genetic variation is present among the hemp dogbane collections examined, and the clustering of the hemp dogbane collections with the three *Apocynum* species suggests that some hemp dogbane populations may be closely related to other *Apocynum* species. Variability among hemp dogbane populations may be partially due to outcrossing with other closely related species. Anderson (1936) suggested that hemp dogbane and spreading dogbane are capable of cross-pollinating. These two species are found throughout the entire range of the genus (Woodson 1930). The lack of genetic distance between the hemp dogbane collections and prairie dogbane is not surprising since taxonomic separation of these species is viewed with skepticism (Voss 1996). The genetic distance grouping of the 16 collections of hemp dogbane do not correlate with the geographic location from which the plants originated or with differences observed for morphological characteristics. Genetic analysis of hemp dogbane using iso-

TABLE 3. Matrix of Nei's (1972) coefficients of genetic distance for RAPD and isozyme data for 16 collections of hemp dogbane and three *Apocynum* species.

	Hemp dogbane collections[a]																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Aa	Ac
2	0.140																	
3	0.030	0.137																
4	0.052	0.066	0.039															
5	0.064	0.112	0.032	0.022														
6	0.065	0.066	0.051	0.011	0.011													
7	0.038	0.112	0.038	0.034	0.022	0.022												
8	0.058	0.078	0.038	0.017	0.006	0.005	0.016											
9	0.045	0.102	0.032	0.028	0.011	0.016	0.005	0.005										
10	0.040	0.095	0.033	0.012	0.029	0.023	0.023	0.023	0.023									
11	0.032	0.141	0.060	0.052	0.040	0.040	0.017	0.034	0.023	0.042								
12	0.091	0.066	0.084	0.045	0.045	0.033	0.044	0.027	0.033	0.052	0.063							
13	0.026	0.102	0.008	0.025	0.017	0.025	0.008	0.008	0.000	0.017	0.034	0.051						
14	0.064	0.066	0.051	0.011	0.011	0.000	0.022	0.005	0.016	0.023	0.040	0.033	0.025					
15	0.040	0.118	0.041	0.035	0.023	0.023	0.000	0.017	0.006	0.024	0.018	0.047	0.009	0.023				
16	0.070	0.114	0.028	0.049	0.029	0.047	0.047	0.019	0.019	0.031	0.060	0.066	0.019	0.047	0.053			
Aa	0.186	0.053	0.156	0.114	0.156	0.130	0.130	0.130	0.130	0.136	0.152	0.119	0.130	0.130	0.098	0.152		
Ac	0.049	0.086	0.042	0.018	0.012	0.006	0.006	0.006	0.006	0.031	0.024	0.036	0.009	0.006	0.006	0.022	0.130	
As	0.05	0.196	0.045	0.033	0.039	0.045	0.045	0.045	0.045	0.020	0.040	0.092	0.054	0.045	0.049	0.050	0.195	0.055

[a] Abbreviations: Aa, *Apocynum androsaemifolium* L.; Ac, *Apocynum cannabinum* L.; As, *Apocynum sibericum* Jacq.

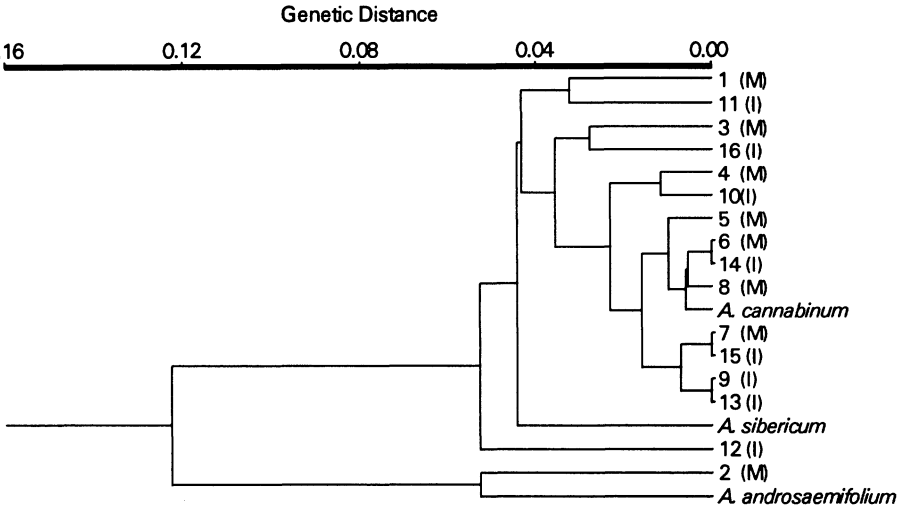

FIGURE 3. Dendogram based on Nei's genetic distance for 16 collections (1 to 16) of hemp dogbane and three *Apocynum* species generated by UPGMA cluster analysis. Collection numbers are followed by an (M) if they were collected in Michigan and an (I) if collected in Illinois.

zyme and RAPD techniques was able to separate spreading dogbane from the other *Apocynum* species in the study. However, the spreading dogbane in this study was an isolated specimen, and further analysis would be required to determine if spreading dogbane can consistently be distinguished from other *Apocynum* species using molecular markers.

Within Population Variation

In determining within population variability, seven enzyme systems were examined including DIA, GOT, MDH, 6-PGDH, PGI, PGM, and TPI. RAPD analysis was conducted with eight random 10-base primers (OPA-02, OPA-04, OPA-05, OPA-15, OPE-07, OPE-18, OPE-20, and OPX-02). No variation was observed with either isozyme or RAPD analysis for the plants collected from the hemp dogbane population in Shiawassee County, MI. In addition, all plants from the Shiawassee population were heterozygous for the 6-*Pgdh*-1 locus, suggesting they are all clones. For plants collected from the Kalamazoo County population, isozyme analysis revealed variation at two loci (*Mdh*-2 and 6-*Pgdh*-1). For RAPD analysis of plants from the Kalamazoo County population, a single plant had two unique amplified bands with OPA-04 and OPE-18. For the two polymorphic isozyme loci, four possible isozyme phenotypes were identified (Table 4). The *Mdh*-2 "fast" allozyme did not segregate in a Mendelian fashion, but a relationship was observed between this locus and the polymorphic 6-*Pgdh*-1 locus.

TABLE 4. Isozyme phenotypes for plants collected within the Kalamazoo County population.

Phenotype	Allelic pattern		Individuals with phenotype
	Mdh-2[a]	6-*Pgdh*-1[b]	
			Number
1	S	FS	7
2	F	FS	2
3	F	F	3
4	F	S	7

[a] F, fast allozyme; S, slow allozyme.
[b] F, "fast" homozygote; S, "slow" homozygote; FS, heterozygote.

Plants with the "slow" allozyme for *Mdh*-2 always were heterozygous for 6-*Pgdh*-1. Plants with a "fast" allozyme for *Mdh*-2 had either the homozygous or heterozygote patterns of 6-*Pgdh*-1 typical of an interbreeding population. This suggests that the seven plants with the phenotype 1 are likely clonal and represent a proportion of the Kalamazoo County population that is clonal. The other plants have segregated for the 6-*Pgdh*-1 locus and likely represent a portion of the population that arose from outcrossing with adjacent clonal populations or arose from more than one clone at the time the population became established.

Understanding the extent of genetic diversity in a population of hemp dogbane may be useful in weed management, especially if the genetic diversity results in plants that respond differently to weed management tactics. Further studies may reveal specific genetic markers correlated with herbicide tolerance, shoot growth rate, and other characteristics that influence effectiveness of control measures. This research also demonstrates the usefulness of the isozyme and RAPD techniques for genetic studies on weed populations. By using two marker systems, researchers can limit the genetic bias that is inherent in each system.

Sources of Materials

[1] Whatman No. 3 filter paper, Whatman International Ltd., Whatman House, St. Leonard's Road, Maidston, England.

[2] Perkin-Elmer PCR reagents, Perkin-Elmer Applied Biosystems Division, 850 Lincoln Centre Drive, Foster City, CA 94404.

[3] Primer kits, Operon Technologies, Inc., 1000 Atlantic Avenue, Suite 108, Alameda, CA 94501.

[4] Perkin-Elmer DNA thermal cycler 480, Perkin-Elmer Applied Biosystems Division, 850 Lincoln Centre Drive, Foster City, CA 94404.

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