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AFLP assessment of genetic variability among velvetbean (*Mucuna* sp.) accessions

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Abstract Velvetbean (*Mucuna* sp.) is a self-pollinated crop classified within the Leguminosae. Using AFLP markers, gene diversity and phenetic relationships were estimated in a collection of 40 velvetbean accessions from cultivated species and different eco-geographic regions. Eleven selective primer combinations generated a total of 508 amplification products. The average number of scorable fragments was 23 per primer combination. A total of 251 polymorphic markers was detected. The polymorphisms obtained ranged from 36% to 61% with an average of 49%. The final phenetic trees were constructed using Nei and Li's coefficient of similarity with UPGMA. Other clustering algorithms were examined and all had high co-phenetic correlations, indicating the goodness of fit for the resulting phylogenetic trees. The phenetic tree as well as principal component analysis (PCA) separated the 40 velvetbean accessions into two main clusters. Bootstrap and Jackknife analyses were completed and their values indicated strong to moderate support for the two main clusters. This grouping confirmed the existing phenological difference with regard to maturity. The high values of the similarity coefficients observed (0.87 to 0.97) imply that the accessions used in this study are similar. The level of genetic variability detected within the velvetbean accessions with AFLP analysis suggests that it is a reliable, efficient, and effective marker technology for determining genetic relationships in velvetbean.

Keywords Velvetbean · *Mucuna* sp. · AFLP · Genetic diversity · Polymorphism · Phenetic

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Introduction

The velvetbean (*Mucuna* sp.), described as a self-pollinated species (Duke 1981), is a tropical legume and classified within the Leguminosae. Originally, velvetbean came from China and eastern India, where it was widely cultivated as a green manure (Burkill 1966; Duke 1981; Wilmot-Dear 1984). Natural out-crossing is rare (Duke 1981). The genus *Mucuna* (Adans) covers perhaps 100 species of annual and perennial legumes, including the annual velvetbean (Buckles 1995). Within the genus, there are found numerous hybrids (Piper and Tracy 1910; Bailey 1947; Burkill 1966). The most commonly cited species include *Mucuna deeringiana* Merrill, *Mucuna utilis* Wallich (Bengal velvetbean), *Mucuna pruriens* (L.)DC, *Mucuna nivea*, *Mucuna hassjoo* (Yokohama velvetbean), *Mucuna aterrima* Holland (Mauritius and Bourbon velvetbean), *Mucuna capitata*, and *Mucuna diabolica* (Duke 1981; Burkill 1966). Sastrapradja et al. (1974) showed the occurrence of n=11 in *Mucuna pruriens* and n=14 in *Mucuna benettii* and *Mucuna gigantea*.

In the Southern United States, the long frost-free season required to produce velvetbean seed initially limited its area of adaptation to Florida and the lower half of the Gulf States (Duggar 1899; Piper and Tracy 1910; McClelland 1919). This limitation was partially overcome when early maturing varieties were selected from Florida velvetbean. Agricultural production began with the discovery of these shorter-season varieties that would mature in most of the southern United States. Thus, the planted acreage expanded rapidly in the early 1900s due to the introduction of the shorter season varieties, recognition of the soil-building attributes of the crop and the demand for livestock feed and grazing. But the most important reason for the rapid increase in velvetbean acreage was the invasion of the boll-weevil (*Anthonomus grandis* Boh.) and the resulting decline in the cotton (*Gossypium hirsutum*) industry of the southern States in early 1900s, making it necessary to change farming systems (McClelland 1919; USDA 1922). Thus, for several years, the velvetbean was an important crop in many of the southern States and its acreage increased yearly until after World War II.

Velvetbean is an example of a successful cover crop. The crop has tremendous potential as a key factor in profitable and sustainable agriculture in the southern United States. In the past, velvetbean has been used as a soil-improving crop, pasture crop, green manure, source of food and for weed control (Duggar 1899). Early in 1896, this plant was used for soil fertilization in *Citrus* orchards in Florida and as a rotation crop with corn (*Zea mays* L.), cotton , and sugarcane (*Saccharum officinarum* L.) in the southern United Sates, as well as a cover crop in *Citrus*, peach (*Prunus persica* L.) and pecan [*Carya illinoinensis* (Wangenh) K. Koch] orchards (Duggar 1899; Bailey 1907; Bort 1909). Like most legumes, velvetbean has the potential to fix atmospheric nitrogen through a symbiotic relationship with soil microorganisms (Buckles 1995). Before reaching maturity, velvetbean sheds significant quantities of leaves and these leaves decay gradually below the actively growing velvetbean (Buckles 1995).

Velvetbean has also been reported to have potential for reducing weed populations, partly by smothering weed competition by rapid growth and partly through protective allelochemicals. After accounting for nematode population effects, studies showed significantly higher yields for cotton, peanut and soybean in rotation with velvetbean, which suggests that the soil-building and weed suppression effects are important in addition to nematode control (Kloepper et al. 1991).

A limited amount of information is available in the literature concerning the genetics and breeding of the crop. The inheritance of flower color, pod hairs, seed coat color, floral biology, and pollination mechanism have been documented. Significant variability has been observed in seed color. Lubis et al. (1980) suggested that multigenic factors were involved in the production of the colors. They also found that two genes designated as R and N were responsible for determining the characteristics of the pod hairs. By artificial hybridization, Lubis et al. (1978) found that the factor controlling purple flower color is dominant over the factor controlling white, and the allelic difference seems to lie at one locus.

The taxonomy of velvetbean is confused with several synonyms at the genus and species levels (Duke 1981) and some designations may be synonymous (Buckles 1995). Burkill (1966) recorded *M. nivea* as being synonymous with *Mucuna cochinchinensis* and *Mucuna lyonii* (Awang et al. 1997). Accessions are described only in terms of where they were grown (e.g., *Mucuna* sp. var. Ghana, *M*. sp. var. IRZ, etc.) or by the many popular names under which they came to be known in various places, such as *M. cochinchinensis* in SE Asia or *M. deeringiana* in Florida. It is difficult to rule out the possibility that the name given to a cultivar is representative of its genotype. Extensive exchange of seeds over the years probably led to many names being given to the same cultivar by different people, according to the area. On the opposite side, it is also highly plausible that cultivars given the same name in two or more areas might in fact be different original stock or germplasm. In the late 1800s, velvetbean was incorrectly named *Dolichos multiforus* by McCarthy (Bort 1909). Bailey (1907) re-named it *Mucuna pruriens* var. *utilis*. For 2 years, the Florida velvetbean was referred to as *M. utilis*, a species originally described by Wallich. Bort (1909) characterized the plant and showed that both Florida velvetbean and *M. utilis* (initially described by Wallich) have marked morphological differences in pods and seeds. As a result, the Florida velvetbean was re-named *Stizolobium deeringianum*. *Stizolobium* was first reported by Browne in 1736 to describe the cowitch plant in Jamaica, commonly known in the United States as *M. pruriens* (Bort 1909). This apparent confusion of the two genera for the same plant ended when Prain in 1897 pointed out the morphological differences between the two genera, especially in the shape of the hilum (Bort 1909). The genus *Stizolobium* was used to distinguish velvetbean from perennial *Mucuna* sp., but this distinction was not maintained (Bailey 1947; Burkill 1966). *Stizolobium* was considered a synonym, and all its species were classified in the genus *Mucuna*. Later, the Florida velvetbean was referred to as *M. deeringianum* (Small 1933). Numerous varieties of *M. pruriens* were treated as separate species. Kay (1979) suggested that only two species of *Mucuna* are commonly grown; the true velvetbean, *M. pruriens* var. *utilis*, which has medium-sized seeds, and the horse bean, *Mucuna sloanei*, which has larger seeds and an extremely hard seed coat. A number of taxa that were formerly considered separate species are now considered merely varieties of *M. pruriens*, namely, *Mucuna aterrima*, *M. cochinchinensis*, *M. hassjoo*, *M. nivea* and *M. utilis* (Wilmot-Dear 1984). Because of the confusion surrounding the taxonomy of velvetbean, it is necessary to conduct research at the species level as well as to assess the phenetic relationships among accessions prior to any breeding program. The wide geographical and climatic distribution of velvetbean is likely to reflect a tremendous genetic diversity in velvetbean, which needs to be estimated before any cultivar development program. To the best of our knowledge, there has been no research conducted on the genetic diversity and relationships among velvetbean using molecular markers. This study was undertaken to investigate the genetic diversity in velvetbean and determine the relatedness among accessions.

The introduction of amplified fragment length polymorphism (AFLP) as a technique for precision genotyping circumvents many of the limitations of previous fingerprinting techniques (Zabeau and Vos 1993; Vos et al.1995). AFLPs have proven to be extremely proficient in revealing diversity at the species levels and provide an effective means of covering an area of the genome in a single assay (Karp and Edwards 1995). AFLPs were reported to detect a large number of genetic loci in soybean (*Glycine max*) (Powell et al. 1996) and barley (*Hordeum vulgare* L.) (Russell et al. 1997) as compared to other molecular techniques. Thus, AFLP has the potential to screen a large number of genetic loci per experiment (Ellis et al. 1997). Aggarwal et al. (1999) reported that the results of various tests done to check the robustness of the pheno-

gram/estimates of phylogeny, clearly establish that the polymorphism revealed by AFLP is not only abundant but also stable and statistically reliable. The AFLP method has been used to assess genetic diversity in soybean (Maughan et al. 1996), neem (*Azadirachta indica)* (Singh et al. 1999), lentil (*Lens* sp.) (Sharma et al. 1996), sunflower (*Helianthus annuus* L.) (Hongtrakul et al. 1997), tea (*Camellia sinensis* (L.) O. Kuntze) (Paul et al. 1997), and barley (Russell et al. 1997).

Materials and methods

Plant material

Forty accessions of *Mucuna* sp. were obtained from various sources, including the United States Department of Agriculture (USDA) germplasm collection in Griffin, Ga., the Auburn University (AU) collection in Auburn, Ala., composed of landrace accessions from southeastern U.S. farmers, and the Center for Cover Crops Information and Seed Exchange (CIEPCA) in Africa (Table 1). These accessions originated from various eco-geographical regions and are limited to cultivated species.

DNA extraction

Genomic DNA was extracted from leaves according to the modified CTAB method of Doyle and Doyle (1990). Approximately 0.5 g of ground leaves were incubated with 2× CTAB buffer for 20 min. A volume of 20 µl of beta-mercaptoethanol was added to the tube and then incubated at 60–65°C for 20 min. The tube was then placed on a rotator and mixed for 15 to 20 min. An equal volume of SEVAG (24-chloroform:1-isoamyl alcohol) (950 µl) was added to the tube. After centrifugation at 12,000 rpm for 2 min, the aqueous (top) phase was removed and placed into a 1.5-ml tube. A volume of the 1:2 ratio of the aqueous (top) phase and –20°C isopropanol was mixed gently to precipitate the DNA. The pellet formed after

Table 1 Accessions of velvetbean (*Mucuna* sp.) used for AFLP analysis. The full accession names are listed in this table. In the text these are abbreviated by replacing the code given by the authors

Taxon	Plant name	Accession number	Code	Donor[b]	Origin
Mucuna sp.	None	PI 227479	PI227479	USDA, ARS	Costa Rica
Mucuna sp.	Somerset[a]	PI 344047		USDA, ARS	Zimbabwe
Mucuna sp.	None	PI 337098	PI337098	USDA, ARS	Brazil
Mucuna sp.	None	PI 364362	PI364362	USDA, ARS	Mozambique
Mucuna sp.	Branco	PI 365411	PI365411	USDA, ARS	Mozambique
Mucuna sp.	Osccola	PI 365414	PI365414	USDA, ARS	Mozambique
Mucuna sp.	Verde Radio	PI 365415	PI365415	USDA, ARS	Mozambique
Mucuna sp.	None	PI 365573	PI365573	USDA, ARS	Brazil
Mucuna sp.	None	PI 366024	PI366024	USDA, ARS	Brazil
Mucuna sp.	African yellow	PI 383272	PI383272	USDA, ARS	USA
Mucuna pruriens	var. deeringiena		Deeringiena	CIEPCA	Brazil
Mucuna pruriens	var. cochinchinensis		Cochinchinensis	CIEPCA	Singapore
Mucuna pruriens	var. utilis		Utilis	CIEPCA	Nigeria
Mucuna sp.	var. rajada		Rajada	CIEPCA	Brazil
Mucuna sp.	var. Ghana		Ghana	CIEPCA	Ghana
Mucuna sp.	var. jaspada		Jaspada	CIEPCA	Brazil
Mucuna sp.	var. Georgia		Georgia	CIEPCA	Cimmyt (Mex.)
Mucuna sp.	var. IRZ		IRZ	CIEPCA	IITA
Mucuna sp.	var. veracruz-speckled[a]			CIEPCA	Cimmyt (Mex.)
Mucuna sp.	var. veracruz-white		19.W	CIEPCA	Cimmyt (Mex.)
Mucuna sp.	*Mucuna* sp. var. preta		Preta	CIEPCA	Brazil
Mucuna sp.	Mexican (Chiapas)S.		21.S	AU	Chiapas (Mex.)
Mucuna sp.	Mexican (Chiapas)B.		21.B	AU	Chiapas (Mex.)
Mucuna sp.	Mexican (Chiapas)W.		21.W	AU	Chiapas (Mex.)
Mucuna sp.	USA (AL)-S.		22.S	AU	USA
Mucuna sp.	USA (AL)-B.		22.B	AU	USA
Mucuna sp.	USA (AL)-W.		22.W	AU	USA
Mucuna sp.	Edgar Farm (AL)S.		23.S	AU	USA
Mucuna sp.	Edgar Farm (AL)B.		23.B	AU	USA
Mucuna sp.	Edgar Farm (AL)W.		23.W	AU	USA
Mucuna sp.	90 day runner-S.		24.S	AU	USA
Mucuna sp.	90 day runner-B.		24.B	AU	USA
Mucuna sp.	90 day runner-W.		24.W	AU	USA
Mucuna sp.	Belle Mina (AL)L.S.		25.LS	AU	USA
Mucuna sp.	Belle Mina (AL)L.B.		25.L B	AU	USA
Mucuna sp.	Belle Mina (AL)S-1		25.S1	AU	USA
Mucuna sp.	Belle Mina (AL)S-2		25.S2	AU	USA
Mucuna sp.	Belle Mina (AL)S-3		25.S3	AU	USA
Mucuna sp.	Belle Mina (AL)S-4		25.S4	AU	USA
Mucuna sp.	Belle Mina (AL)S-5		25.S5	AU	USA
Mucuna sp.	Belle Mina (AL)S-6		25.S6	AU	USA
Mucuna sp.	Belle Mina (AL)S-7		25.S7	AU	USA

[a] These accessions did not germinate
[b] USDA, ARS: United States Development of Agriculture, Agricultural Research Service; CIEPCA: Centre d'information et d'échange sur les Plantes de Couverture en Afrique; AU: Auburn University

Table 2 Adapter and +3 primer sequences (5′–3′) used for AFLP analysis

Name	Enzyme	Type	Sequence (5′–3′)
E-0	*Eco*RI	Adapter	CTCGTAGACTGCGTACC CTGACGCATGGTTAA
M-0	*Mse*I	Adapter	GACGATGAGTCCTGAG TACTCAGGACTCAT
E-ACT	*Eco*RI	Primer +3	AGACTGCGTACCAATTCACT
E-AAG	*Eco*RI	Primer +3	AGACTGCGTACCAATTCAAG
E-AGT	*Eco*RI	Primer +3	AGACTGCGTACCAATTCAGT
E-ACG	*Eco*RI	Primer +3	AGACTGCGTACCAATTCACG
M-CTC	*Mse*I	Primer +3	GATGAGTCCTGAGTAACTC
M-CAG	*Mse*I	Primer +3	GATGAGTCCTGAGTAACAG
M-CTG	*Mse*I	Primer +3	GATGAGTCCTGAGTAACTG
M-CTT	*Mse*I	Primer +3	GATGAGTCCTGAGTAACTT
M-CAT	*Mse*I	Primer +3	GATGAGTCCTGAGTAACAT
M-CAA	*Mse*I	Primer +3	GATGAGTCCTGAGTAACAA

centrifugation at 12,000 rpm for 2 min was washed with 750 µl of 75% ethanol. DNA was run out on a ethidium bromide-stained agarose gel. DNA concentration was measured with a versaFluor Fluorometer (Bio-Rad Laboratories, Hercules, Calif.) and compared with the known concentration of Lambda DNA in a 1% agarose gel. Then the DNA was stored at –20°C.

AFLP analysis was performed according to Vos et al. (1995) with slight modifications. AFLP core reagent and starter primer kits were purchased from Life Technology (Gibco BRL, Gatherburg, Md., USA). Adapters and selective primer pairs used and their sequences are listed in Table 2.

Digestion-restriction and ligation of genomic DNA

Approximately 250 ng of genomic DNA was double-digested using restriction enzymes *Eco*RI and *Mse*I. Digestion was carried out in a final volume of 25 µl at 37°C for 2 h, then heated to 70°C for 15 min to inactivate enzymes. *Mse*I and *Eco*RI adapters were subsequently ligated to digested DNA fragments by adding to the digestion 24 µl of adapter ligation solution and 1 µl of T4 DNA ligase. The ligation was incubated for 2 h at 20°C and up to overnight at 4°C. Digested-ligated DNA fragments were used as templates for the first amplification reaction (pre-amplification step prior to selective amplification).

Pre-amplification reaction

Pre-amplification reactions were performed in a 25.5-µl volume containing 2.5 µl of 10× PCR buffer for AFLP (100 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$, 500 mM KCl), 20 µl of pre-amplification primer MixI, 2.5 µl of template DNA and 0.5 µl of Taq DNA polymerase. The PCR amplifications were carried out in a PTC-100 Programmable Thermal Controller (MJ Research, Inc., Watertown, Mass. 02172, USA) using 20 cycles of: 94°C (denaturation) for 30 s, 56°C (annealing) for 60 s, 72°C (extension) for 60 s.

Selective amplification

Selective amplification primers were purchased from Life Technology, Inc. as individual oligonucleotides. A volume of 5.3-µl PCR reaction contained 1 µl of pre-amplification product, 0.3 µl of *Eco*RI, 1 µl of *Mse*I, and 3 µl of TaqMix. The TaqMix for 50 samples contained 30 µl of 10× PCR buffer, 18 µl of $MgCl_2$, 100.5 µl of ddH_2O, and 1.5 µl of Perkin Elmer Taq. This second amplification was carried out by programming a touch-down cycle profile (Don et al. 1991) as follows: 94°C for 30 s, 65°C (–0.7°C/cycle) for 30 s, and 72°C for 60 s during 12 cycles, until reaching the optimal annealing temperature of 56°C. At this temperature, 23 more cycles were achieved to complete the second amplification. The products were held at 4°C.

Gel analysis

Gel electrophoresis was conducted using a Li-Cor Long ReadIR DNA sequencer (Li-Cor Inc., Biotechnology division, Lincoln, Neb., USA). Following the amplification reaction, the PCR products were mixed with 3 µl of formamide stop/loading buffer and denatured at 94°C for 3 min, then chilled immediately to 4°C using a PTC-100 Pelter-effect thermal cycler (MJ Research, Inc.). One microliter of each reaction was loaded onto a 8% Long Ranger gel by using a 8-channel Hamilton syringe (Hamilton Company, Reno, Nev., USA). The gel matrix was prepared with 9.5 g of urea, 2.7 ml 10X TBE, 3.6 ml long-ranger solution, and 7.5 µl ddH_2O. To 23.3 g of gel solution, 150 µl of 10% ammonium persulfate solution (APS) and 15 µl of tetramethylenediamine (TEMED) were added. The gel was pre-run in 1× TBE, freshly prepared from a 10× TBE stock solution. The key electrophoresis parameters include voltage set at 15,000 V, current at 40 mA, power at 25 W, and temperature at 45°C. Square-tooth combs with 48 wells were used.

Scoring of AFLPs and data analysis

Each AFLP marker was treated as a unit character and scored as a binary code (1/0). Thus, the matrix values estimating the number of AFLP fragments shared (or not shared) between two accessions has been suggested as an appropriate estimator of relatedness under the assumption that the presence or absence of a discrete character in two or more accessions results from the same genetic changes (Skroch and Neihuis 1992). The 1/0 matrix was used to estimate three different genetic similarity coefficients between accessions i and j according to Nei and Li (1979) [$GS_{NL}=2a/(2a+b+c)$], Sokal and Michener (1958) [$GS_{SM}=(a+d)/(a+b+c+d)$] and Jaccard (1908) [$GS_J=a/(a+b+c)$], where a is the number of bands shared by i and j, b is the number of bands present in i and absent in j, c is the number of bands present in j and absent in i, d is the number of bands absent in i and j, and (a+d) is the number of bands in “matched” (number of bands present and absent in i and j) for the data pooled over all the primer combinations. The resulting distance matrices were subjected to four clustering methods by UPGMA (unweighted pair-group method analysis; Sokal and Michener 1958), WPGMA (weighted pair group method analysis; Sneath and Sokal 1973), complete linkage (Lance and Williams 1967), and single linkage (Lance and Williams 1967). The goodness of fit of the clustering to the data matrix was calculated by the COPH and MXCOMP programs. Principal component analysis (PCA) was performed to visualize the dispersion of the individuals in relation to the first two principal axes of variation. Computations were done using the procedures in the NTSYS-pc statistical package (version 2.0, Rohlf 1998). Gene diversity was estimated according to Nei (1973) using POPGENE (version 1.31, Yeh et al. 1999). The reliability and robustness of the phenograms were tested by bootstrap/jackknife analyses with 10,000 replications to assess branch support using the software PAUP (version 3.1, Swofford 1993). Some workers consider that the confidence limits obtained in both

bootstrap and jackknife must be over 95% in order to consider the grouping of taxa at a branch to be statistically significant (Felsenstein 1985). Others use a lower limit (above 50% or at least 50%) as indicating statistical support for the topology at a node (Highton 1993). In our study we used the lower limits to assess the grouping of taxa to be statistically significant.

Results

AFLP polymorphism and gene diversity

Analysis of the 40 *Mucuna* accessions with 11 AFLP primer pairs identified a total of 508 fragments of which 251 (49%) were polymorphic between two or more accessions. An example of typical AFLP variation for a single AFLP primer pair is shown in Fig. 1. Polymorphic fragments were generated by each of the primer pairs. The average number of fragments detected by an individual primer pair (Table 3) ranged from 28 (for the primer E-AGT/M-CAG) to 70 (E-AAG/M-CAT), thus confirming the high multiplex ratio produced by AFLP markers. The number of polymorphic fragments for each primer pair varied from 10 (for E-AGT/M-CAG) to 34 (for E-AAG/M-CTC) with an average of 23 per primer pair (Table 3). Based on the percentage of polymorphic fragments, different levels of polymorphism ranging from 36% (E-AGT/M-CAG) to 61% (E-AAG/M-CTC) were detected. Gene diversity ranged from 0.10 (for E-AAG/M-CTT) to 0.21 (for E-AAG/M-CAA) with average of 0.15 per primer combination (Table 3). Nei's genetic similarity between the U.S. landraces and the exotic lines ranged from 0.98 (for E-AAG/M-CTG) and 0.87 (for E-AAG/M-CTT) with average of 0.92 per primer combination (Table 3).

AFLP fragment sizes

The size of the AFLP fragments was determined by comparing an AFLP standard marker to AFLP patterns. AFLP fragment sizes ranged from approximately 50 to 400 base pairs (bp). Polymorphic fragments were distributed across the entire size range with the major proportion between 75 and 300 bp. The remainder of the polymorphic fragments were shared among the remaining size range of 50–75 bp and 301 bp and above.

Phenetic analysis

The dendrograms constructed using the three different similarity coefficients (Nei and Li's, Sokal and Michener's, and Jaccard's) and four different clustering methods (UPGMA, WPGMA, complete linkage, and single linkage) were examined (Table 4). These various tests done to evaluate the goodness of fit of the resulting phylogenetic trees revealed the reliability and stability of the inferred relationships. In general, high cophenetic correlation values ranging from 0.87 to 0.91 were obtained where $r>0.9$ indicates a very good fit; $0.8<r<0.9$ indicates a good fit; $r<0.8$ indicates a poor fit. No major variations among the four distance methods or in dendrogram patterns were found. However, the UPGMA always gave higher cophenetic correlation values than the other clustering methods and Nei and Li's coefficient gave higher cophenetic correlation values than either Jaccard's or Sokal and Michener's (Table 4).

To assess the usefulness of AFLPs as phenetic markers, a similarity matrix based on Nei and Li's coefficients was constructed to estimate the level of relatedness among the 40 velvetbean accessions used in the present study. The calculation of Nei and Li's coefficients was

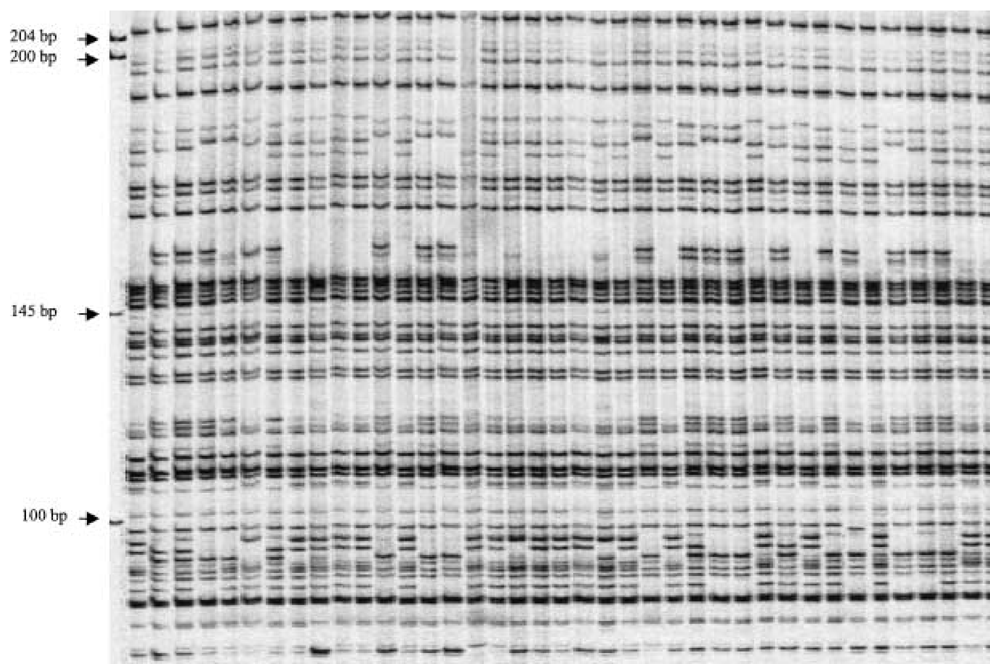

Fig. 1 Autoradiograph displaying AFLP fingerprints detected in 39 velvetbean accessions using primer combination E-AAG/M-CAT

Table 3 Number of total and polymorphic fragments, gene diversity in overall accessions, US landraces, and exotic lines

Primber combination	Total frag-ments	Overall accessions			US landraces			Exotic lines			GS betweem U.S. and exotic[d]
		Pol[a]	% pol[b]	h[c]	Pol	% pol	h	Pol	% pol	h	
E-AAG/M-CAA	55	32	58	0.21	25	45	0.16	32	58	0.23	0.96
E-AAG/M-CAG	35	16	46	0.15	8	23	0.08	16	46	0.15	0.94
E-AAG/M-CAT	70	28	40	0.15	13	19	0.05	28	40	0.14	0.87
E-AAG/M-CTC	56	34	61	0.20	17	30	0.11	33	59	0.18	0.88
E-AAG/M-CTG	46	27	59	0.14	15	33	0.10	26	57	0.15	0.98
E-AAG/M-CTT	53	19	36	0.10	14	26	0.08	19	36	0.09	0.96
E-ACT/M-CAG	44	25	57	0.18	14	32	0.10	24	55	0.18	0.92
E-ACT/M-CAT	42	20	48	0.16	15	36	0.13	16	38	0.13	0.96
E-ACT/M-CTC	37	17	46	0.15	13	35	0.12	13	35	0.11	0.91
E-ACG/M-CAG	42	23	55	0.18	14	33	0.11	22	52	0.17	0.90
E-AGT/M-CAG	28	10	36	0.11	6	21	0.06	9	32	0.11	0.94
Total	508	251	–	–	154	–	–	238	–	–	–
Mean	46	23	49	0.15	14	30	0.10	22	46	0.14	0.92
Number of observations	11	11	11	11	11	11	11	11	22	11	11
Maximum	70	34	61	0.21	25	45	0.16	33	59	0.23	0.98
Minimum	28	10	36	0.10	6	19	0.05	9	32	0.09	0.87
Standard deviation	11.69	7.2	9.3	0.03	4.8	7.57	0.03	7.8	0.33	0.03	0.03

[a] Total number of polymorphic fragments;
[b] Percentage of polymorphic fragments;
[c] Gene diversity;
[d] Genetic similarity between U.S. landraces and exotic lines

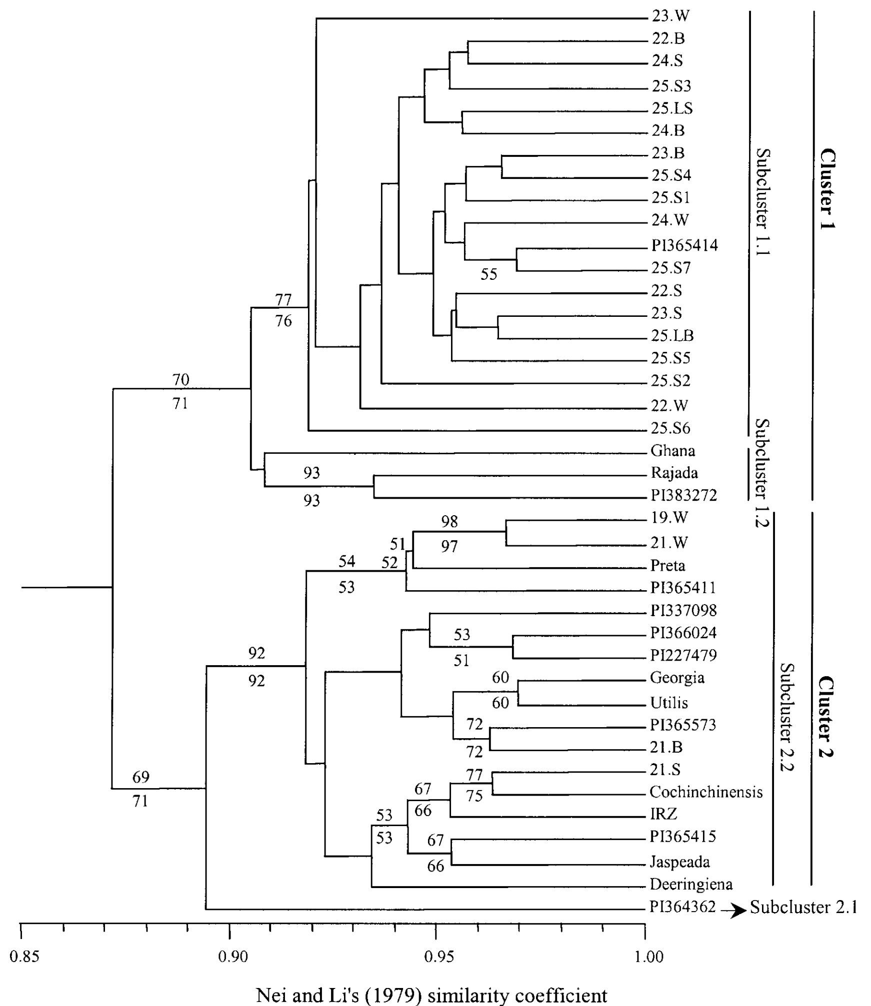

Fig. 2 Phenogram of 40 velvet-bean accessions revealed by UPGMA cluster analysis based on AFLP markers obtained with 11 primer combinations. *Numbers* shown above different branches represent percentage confidence limits obtained in the bootstrap analysis, those below branches are percentage confidence limits in the jack-knife analysis. Branches lacking bootstrap and jackknife values received <50% bootstrap and jackknife supports

Table 4 Comparison of co-phenetic correlation values obtained from three similarity coefficients and four clustering methods used for analyzing the present AFLP data

Clustering method	Similarity coefficients[a]		
	Nei and Li's	Jaccard's	Sokal and Michener's
UPGMA	0.91	0.90	0.90
WPGMA	0.89	0.89	0.90
Single linkage	0.87	0.88	0.88
Complete linkage	0.87	0.87	0.88

[a] Nei and Li (1979), Jaccard (1908), Sokal and Michener (1958)

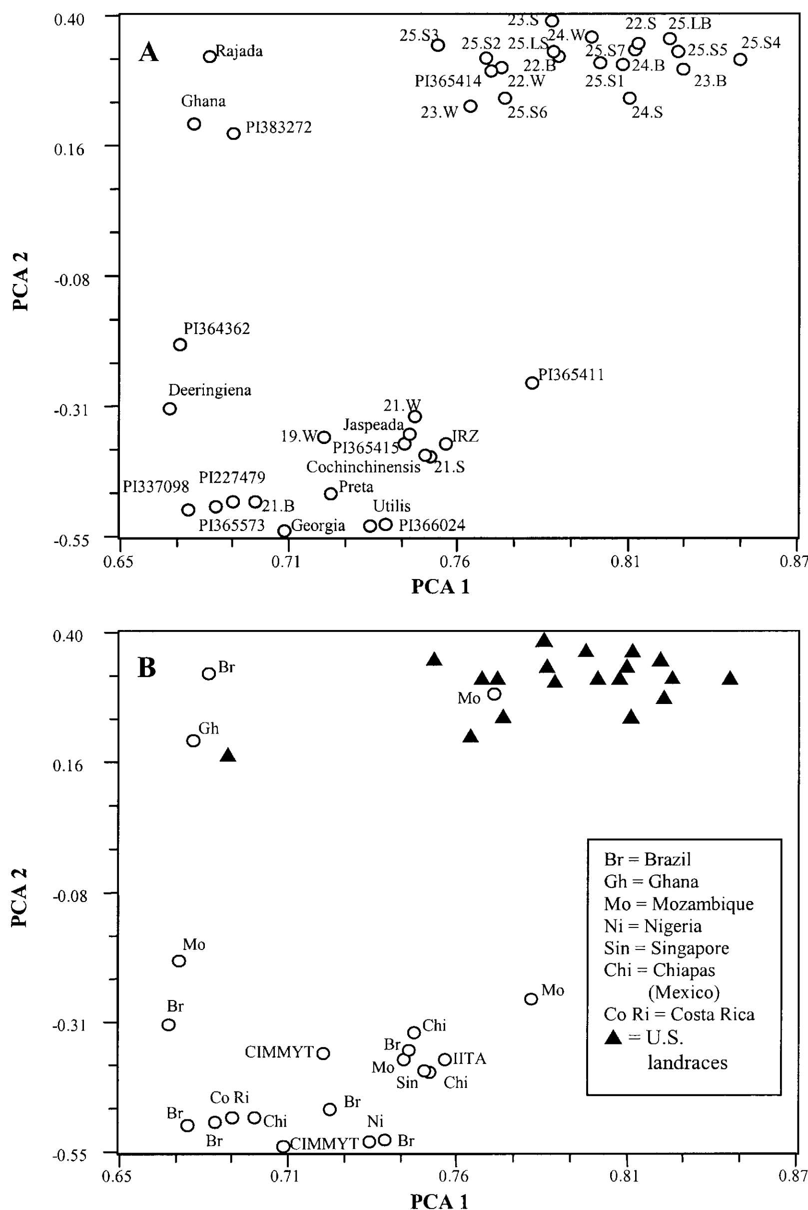

Fig. 3 Scatter-plot of 40 velvetbean accessions with 251 polymorphic AFLP markers showing **A** grouping by accession names, and **B** grouping by geographical distribution

based on the presence or absence of discrete characters (AFLP markers). The similarity matrix was then used to cluster the data using the UPGMA algorithm. The resulting dendrogram constructed by Nei and Li's coefficient and by the UPGMA clustering method (Fig. 2) formed two main clusters. These two clusters were identified at the 87% similarity level. Cluster 1 is supported at 70% and 71% confidence interval limits respectively in the bootstrap (BS) and jackknife (JK) analyses. In cluster 2, the branch is supported at 69% (BS) and 71% (JK) levels. Within cluster 1, two subclusters were identified. Subcluster 1.1 is supported at 77% (BS) and 76% (JK) levels. The branch formed by the accessions "Rajada" and PI383272 in cluster 1 is strongly supported by bootstrap and jackknife values (93% confidence interval limits). In cluster 2, two subclusters were identified. Sub-

cluster 2.2 was supported at 92% confidence interval limits in both (BS) and (JK), and subcluster 2.1 consists of the single accession PI364362. A significant association was found within subcluster 2.2 between the accessions 19.W and 21.W, in which the branch is supported at 98% and 97% confidence interval limits in the (BS) and (JK) analyses, respectively (Fig. 2).

The similarity matrix was also used as input data for principal component analysis (PCA). The first two components explained 71% of the total variation. The scatterplot representation of the PCA showed a clear-cut separation of the 40 accessions in relation to the first two principal axes of variation (Fig. 3). The U.S. landrace accessions formed a separate group including three exotic accessions from Brazil, Ghana, and Mozambique (Fig. 3B).

Discussion

Molecular markers have not been used to evaluate and characterize velvetbean germplasm. The current study was undertaken to measure the level of genetic variability in velvetbean. The different accessions used in this study were from cultivated species. Genetic diversity was evaluated with 11 primer combinations and 251 AFLP fragments were polymorphic. The genetic diversity was greater in the exotic lines compared to that in the U.S. landraces (Table 3), indicating that the exotic lines were more heterogeneous than the U.S. landraces. This may due to the wide range of geographical origins of the exotic lines. When genetic similarity between the U.S. landraces and exotic lines was compared (Table 3) the highest value was obtained with the primer combination E-AAG/M-CTG and the lowest with E-AAG/M-CAT. Thus, different primer combinations produced different levels of genetic similarity.

Cluster analyses of velvetbean accessions using UPGMA and Nei and Li's coefficients, as well as principal component analysis, led to the separation of the accessions into two distinct groups. Clearly, all U.S landraces were clustered together (Subcluster 1.1, Fig. 2). As can be seen, the three exotic accessions in cluster 1 were clearly discernible from the rest. The U.S. landrace accessions may be fewer generations removed from unknown ancestral introductions than the exotic lines. Early reports speculated that mutation is at the origin of the genetic variation observed in the landrace accessions (Coe 1918). Within cluster 2, two separate subclusters are formed (Fig. 2). The accession PI364362 forms a separate group at the 0.89 similarity level. This accession is different from the rest of the group by the color and shape of its pods (data not shown). The similarity coefficients were high (0.89–0.97 and 0.87) respectively within and among the two main clusters, thereby indicating that the accessions used in this study should not be considered as different species. This is in agreement with the results of Wilmot-Dear (1984) who reported that the species name *M. pruriens* (L.) is most commonly utilized today for the cultivated *Mucuna*. A number of taxa that were formerly considered separate species are now regarded merely as varieties of *M. pruriens*, namely, *M. aterrima*, *M. cochinchinensis*, *M. hassjoo*, *M. nivea* and *M. utilis*. Previous reports showed that the range of germplasm being exploited to-date is quite restricted and derives from natives in Central America, especially Honduras, with most accessions being nominally of the *M. pruriens* var. *utilis* type (Kay 1979; Buckles 1995).

The two main clusters based upon AFLP analysis correspond to differences in maturity class (data not shown). Thus, maturity is an important character in differentiating velvetbean accessions. As can be seen, all the exotic accessions are grouped together except the accessions Ghana, Rajada and PI 383272 (Fig. 3B). PCA place these three accessions very near to the U.S. landraces which are temperate types (Fig. 3A). The earliness of the accessions Ghana, Rajada and PI 383272 in the exotic lines may explain their separation.

The AFLP technique is an efficient and useful tool for detecting genetic diversity. AFLP analysis from the present study provided an estimate of genetic relationships in velvetbean accessions that was reliable and consistent. This supports the conclusions of previous studies which recommended AFLPs as efficient, reliable, and useful tools compared to random amplified polymorphic DNAs (RAPDs) and simple sequence repeats (SSRs) (Jones et al. 1997). The results demonstrated that genetic resolution provided by AFLP is amenable to phylogenetic analysis of closed related species.

Our results showed a clear classification between different taxa. The level of genetic variation observed within the U.S. landraces used in this study seems to be low. Accessions from a broader geographical range, especially including the exotic lines, have increased the variation, thereby increasing the scope for developing a breeding program. Future attempts in velvetbean breeding programs should therefore take into consideration our results as these have direct implications in velvetbean improvement programs for the specific cropping systems. We have shown the genetic diversity and established relationships among the velvetbean collection of 40 accessions using AFLP. Future study on genetic diversity in *Mucuna* should include the whole genus of *Mucuna* sp. including the wild related species. This research represents one of the most comprehensive investigations of DNA diversity for velvetbean and is among the first to report on the effectiveness of the AFLP technique for determining genetic relationships in velvetbean.

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