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Estimation of genetic diversity in varieties of *Mucuna pruriens* using RAPD

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Abstract

Genetic diversity was estimated in 13 accessions of the otherwise self pollinated *Mucuna pruriens* (L.) DC. (velvetbean) comprising varieties *pruriens* and *utilis* collected from tropical humid forest using 15 RAPD primers. Similarity index value of 0.68 based on Nei and Li's similarity coefficient indicated high degree of genetic variability. Analysis of various genetic diversity indices like total heterozygosity, Nei's gene diversity, percentage of polymorphic loci, expected and observed number of alleles and Shannon index strongly suggests that variety *pruriens* is genetically more diverse than variety *utilis*. Chemical analysis with respect to 3,4-dihydroxy-L-phenylalanine (L-DOPA) content showed uniform distribution. Cluster analysis showed grouping of accessions into two major clusters and tendency of accessions of variety *pruriens* to group according to their geographical locations. Bootstrap analysis confirmed the robustness of the phenogram. The putative hybrid MMP6 with relatively low similarity value index and low L-DOPA content was promising as food or fodder.

Additional key words: *Fabaceae*, L-DOPA, velvetbean.

Introduction

The genus *Mucuna* is comprised of about 100 species of annual and perennial legumes, including the annual velvetbean (Buckles 1995). *Mucuna* is a leguminous climbing plant with long, slender branches and lanceolate leaves on hairy petioles with large white flowers. They grow in clusters of two or three, with a bluish purple, butterfly-shaped corolla. The pods or legumes are shaped like violin sound holes and contain four to six seeds. They are of rich dark brown colour, thickly covered with stiff hairs and cause intense itching (pruritis) if they come in contact with human skin.

Mucuna pruriens, commonly known as velvetbean or cow-itch, is a self pollinated tropical legume known for its medicinal properties and reported to be indigenous to India and China. At least three varieties of *M. pruriens* are known to exist *M. pruriens* (L.)DC, *M. pruriens* (L.)DC var *pruriens* and *M. pruriens* var. *utilis* (Wall *ex* Wight) Baker *ex* Burck are distributed in parts of India. It tolerates a wide range of soil acidity and moisture. Other physical properties like high nitrogen fixing capability, aggressive growth habit and high productivity of vegetative matter make it an excellent soil-improving crop, pasture crop, green manure cover crop, source of food and weed controller (Duggar 1989).

Mucunas as a whole are an important source of the toxic compound 3,4-dihydroxy-L-phenylalanine (L-DOPA), hallucinogenic tryptamines and antinutritional factors such as phenols and tannins (Siddhuraju *et al*. 1996, Ravindran and Ravindran 1988). Distribution of L-DOPA in the species shows wide variation in different parts of the plant. It is reported to be 0.15 % in dried leaves and pods, 0.49 % in stem and highest concentration is seen in raw seed where it ranges from 4.47 to 5.39 % (Bell and Janzen 1971, Duke 1981, Thurtson 1997). Prior to

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Abbreviations: A - observed number of alleles, CTAB - cetyltrimethylammonium bromide; h - Nei's gene diversity; Ht - heterozygosity; I - Shannon index; L-DOPA - 3,4-dihydroxy-L-phenylalanine; Ne - expected number of alleles; P - polymorphic loci; RAPD - random amplified polymorphic DNA
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breeding programmes, it is imperative to assess the range of chemical and genetic variability in natural populations of *Mucuna* and to establish the possible concordance/ discordance relationship between the genetic and chemical profiles. Even otherwise taxonomy of velvetbean is confused with several synonyms at the genus and species levels (Duke 1981) and some designations may be synonymous (Buckles 1995). The problem of taxonomy becomes particularly acute when dealing with the cultivars of the velvet bean. Recently, taxonomists (*e.g.* Wilmot-Dear 1991) have described all cultivars of the velvet bean as *M. pruriens* var *utilis*. It is highly probable that chemical and DNA profiling of *Mucuna pruriens* accessions will solve of these problems and place them on proper taxonomical and phylogenetic perspective apart from identifying genotypes that are less toxic to humans. Hence the present investigation involving to start with, collections from parts of southern India.

Materials and methods

Sampling sites and collection of plant material: Total of 13 accessions of *Mucuna pruriens* comprising 6 accessions each of var. *pruriens* and var. *utilis* along with a putative hybrid were collected from the windward side of the Western Ghats in Southern India (Table 1). While Palode in the extreme south receive both south west and north east monsoon, Kottayam and Malappuram are wetted only by south west monsoon rain. The distance between collection sites in the tropical humid forests is 87 - 284 km and within each site accessions were collected at a distance of 0.5 to 2 km.

Table 1. Accessions of *Mucuna pruriens* sampled in the present study.

Accession No	Variety	Locality	Latitude
PMP-01	*pruriens*	Palode	8° 36′ N
PMP-02	*pruriens*	Palode	8° 36′ N
PMP-03	*pruriens*	Palode	8° 36′ N
KMP-04	*pruriens*	Kottayam	9° 15′ N
MMP-05	*utilis*	Malappuram	11° 06′ N
MMP-06	hybrid	Malappuram	11° 06′ N
MMP-07	*pruriens*	Malappuram	11° 06′ N
MMP-08	*pruriens*	Malappuram	11° 06′ N
PMP-09	*utilis*	Palode	8° 36′ N
PMP-10	*utilis*	Palode	8° 36′ N
PMP-11	*utilis*	Palode	8° 36′ N
KMP-12	*utilis*	Kottayam	9° 15′ N
KMP-13	*utilis*	Kottayam	9° 15′ N

Genomic DNA isolation and RAPD: Total genomic DNA from the young leaves was isolated following the modified Murray and Thompson (1980) method using cetyltrimethylammonium bromide (CTAB). After ethanol precipitation DNA was resuspended in 0.1 cm^3 of 1× TE buffer (pH 8.0). The DNA was quantified spectrophotometrically by taking the absorbance at 260 nm. RAPD assay was carried out in 0.025 cm^3 reaction mixture containing 0.2 mM dNTP's, 10mM Tris-HCl, 1.5mM $MgCl_2$, 50 mM KCl, 0.1 % Triton X-100, 1.0 U Taq DNA polymerase (*Finnzymes*, Helsinki, Finland), 15 pmol primers from Kit 'P' and Kit 'C' (*Operon Technologies*, Alameda, USA, and *IDT,* Coralville, USA) and 50 ng of genomic DNA. The mixture was overlaid with 0.020 cm^3 mineral oil and amplification performed in a thermal cycler (*Perkin Elmer 480*, Norwalk, USA). After the initial cycle of 2 min at 94 °C, 2 min at 36 °C and 2 min at 72 °C, 38 cycles of 1 min at 94 °C, 1 min at 36 °C and 2 min at 72 °C were performed. The last cycle was followed by 7 min extension at 72 °C. Reaction mixture wherein template DNA replaced by distilled water was used as negative control. Amplified products were resolved in 1.4 % agarose gel (1x TBE) followed by EtBr staining.

Genetic data analysis: Amplification with each random primer was repeated 3 times and those primers that produced reproducible and consistent bands were selected for data generation. Reproducible RAPD products were scored against the presence or absence of a fragment. Dice coefficient of similarity defined as 2a/2a+u, where 'a' is the number of positive matches and 'u' the number of non-matches was computed using the *WINDIST* software (Yap and Nelson 1996). The scored binary matrix was analyzed for the construction of phenogram and determination of confidence limits by bootstrap analysis using the *WINBOOT* software (Yap and Nelson 1996). Genetic variation between the two varieties was analyzed for various parameters. The genotype and allelic frequency data were used to compute the genetic diversity indices *i.e.* % of polymorphic loci (P), observed number of alleles (A), expected number of alleles (Ne), Shannon index of gene diversity (I) and Nei's gene diversity (h) at the population level using the statistical package *POPGENE 1.31* (Yeh *et al.* 1999). The populations from which the samples taken for the present analysis were assumed to be in Hardy-Weinberg equilibrium implying that the population is at random mating. Based on the above assumption, bands were scored and estimation of heterozygosity (Ht) was done according to the formula: $Ht = 1 - \sum Pi^2$, where Pi is the frequency of the i^{th} allele in the population.

Estimation of L-DOPA content: Extraction of L-DOPA was done following the standard procedure of Brain (1976). L-DOPA concentrations were estimated with reference to standard (*Sigma-Aldrich*, St. Louis, USA) by UV spectrophotometry.

Results

RAPD polymorphism and genetic variation: A total of 15 random primers were used for the estimation of inter- and intravarietal variation in *Mucuna pruriens* (Table 2). Out of 101 products generated, 91 were found to be polymorphic (90.1 % polymorphism). On an average, the primers generated 7.2 products and 6.5 polymorphism per primer. The number of products generated by these arbitrary 10-mer primers was found to range from 4 (C76) to 12 (OPP B). The number of amplicons so generated could be arbitrarily grouped under 2-band classes- 4-6 and 8-12. Lower number of bands was generated by primers - OPP 13, C67, 72, 73, 74, 75, 76 and 77 while higher number of products was by primers OPP 09, OPP 11, C68, C69, 70 and 71. Though there were primers OPP 09, C66, 71, 72, 73, 75 and 76 that produced 100 % polymorphism, not a single primer could reveal 100 % monomorphism across the accessions. The similarity matrix developed using the *WINDIST* software showed that similarity index ranges from 0.39 to 0.90 with mean value of 0.68 thereby suggesting high levels of genetic variability in the species (Table 3). At the intravarietal level, extent of variability was more in variety *pruriens* as similarity index is from 0.53 to 0.82 (mean value 0.70) whereas in variety *utilis* the similarity index ranged from 0.72 to 0.92 with mean value of 0.82.

Table 2. List of primers and their sequence used for RAPD analysis.

Primers	Primer sequence 5'→3'	Number of bands	Number of polymorphic bands
OPP 09	GTGGTCCGCA	12	12
OPP 11	AACGCGTCGG	9	8
OPP 13	GGAGTGCCTC	4	4
C66	GAACGGACTC	8	8
C67	GTCCCGACGA	5	3
C68	TGGACCGGTG	9	7
C69	CTCACCGTCC	8	7
C70	TGTCTGGGTG	8	6
C71	AAAGCTGCGG	8	8
C72	TGTCATCCCC	5	5
C73	AAGCCTCGTC	5	5
C74	TGCGTGCTTG	5	4
C75	GACGGATCAG	6	6
C76	CACACTCCAG	4	3
C77	TTCCCCCCAG	5	5

Table 3. Similarity matrix of *Mucuna pruriens* varieties analyzed based on Dice's coefficient.

PMP1	1.00												
PMP2	0.72	1.00											
PMP3	0.80	0.68	1.00										
KMP4	0.66	0.55	0.60	1.00									
MMP5	0.67	0.53	0.68	0.70	1.00								
MMP6	0.56	0.65	0.51	0.46	0.56	1.00							
MMP7	0.61	0.60	0.67	0.64	0.70	0.72	1.00						
MMP8	0.55	0.55	0.64	0.58	0.68	0.62	0.80	1.00					
PMP9	0.64	0.54	0.63	0.75	0.70	0.56	0.59	0.65	1.00				
PMP10	0.69	0.52	0.69	0.76	0.78	0.55	0.67	0.69	0.86	1.00			
PMP11	0.69	0.48	0.63	0.90	0.71	0.39	0.60	0.58	0.71	0.80	1.00		
KMP12	0.70	0.47	0.71	0.79	0.74	0.45	0.60	0.65	0.74	0.81	0.84	1.00	
KMP13	0.65	0.48	0.64	0.74	0.73	0.49	0.66	0.76	0.72	0.80	0.79	0.89	1.00
	PMP1	PMP2	PMP3	KMP4	MMP5	MMP6	MMP7	MMP8	PMP9	PMP10	PMP11	KMP12	KMP13

Cluster analysis: The accessions of *Mucuna* clustered broadly under two major groups with subgrouping in cluster II (Fig. 1). Accessions of variety *pruriens* followed a definite pattern of geographical affiliation while those of *utilis* tend to group together though they were collected from geographically distant regions. All accessions of variety *pruriens* collected from southern part of Kerala (Palode) grouped as Cluster I while those from northern Kerala (Malappuram) clustered separately as cluster IIa. Accessions of variety *utilis* collected from all the three regions along with an out grouped member of *pruriens* (KMP 4 from Kottayam) formed cluster II.

Genetic diversity and heterozygosity: Total heterozygosity for *Mucuna pruriens* was 0.44 whereas within the varieties *pruriens* and *utilis* it was 0.46 and 0.43 respectively (Table 4). Ht value indicates that variation in var *pruriens* is higher to var *utilis*. This observation was ascertained by other diversity indices like percentage of polymorphic loci, Nei's genetic diversity, Shanon Index and expected and observed number of alleles.

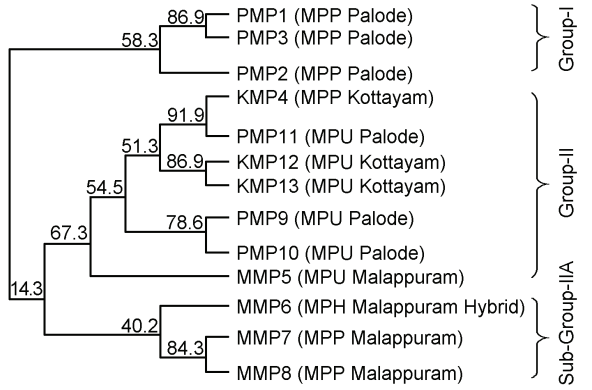

Fig. 1. Phenogram based on UPGMA analysis of *Mucuna pruriens* var. *pruriens* (MPP) and *Mucuna pruriens* var. *utilis* (MPU). Numbers at the fork indicate bootstrap values.

Table 4. Summary of genetic diversity data in *Mucuna* varieties (P - percentage of polymorphic loci, A - observed number of alleles, Ne - expected number of alleles, h - Nei's gene diversity, I - Shannons information index of gene diversity, Ht - total heterozygocity).

	Var. *pruriens*	Var. *utilis*	Total
P	100	66.67	100
A	2.00	1.67	2.00
Ne	1.84	1.57	1.78
h	0.46	0.31	0.43
I	0.65	0.43	0.62
Ht	0.46	0.43	0.44

L-DOPA content: Seeds of all the accessions except the hybrid variety (MMP6) showed no extensive variation in L-DOPA content (2.2 ± 0.45 % d.m.). The accession MMP6 which is a putative hybrid gave relatively low content (1.40 %) of L-DOPA.

Discussion

Morphological variations present in *Mucuna pruriens* with respect to qualitative and quantitative traits like leaf shape and size, variegation, flower colour, size of fruits, number of pods, early and late maturation period of pods, seed colour, hairiness on pods, *etc.*, are so significant that a high degree of taxonomical confusion prevails within the species with several synonyms at the species level. A number of taxa formerly considered separate species are now known to be merely varieties of *Mucuna pruriens* namely *M. aterrima*, *M. cochinchinensis*, *M. hassjoo*, *M. nivea* and *M. utilis*. This confusion in taxonomy and lack of information on pattern and distribution of genetic diversity in the species has in fact impeded the effective utilization of *Mucuna* genetic resources. Therefore, it is imperative to address these issues before an effective breeding programme is initiated for improvement of *M .pruriens* either as a food crop (with low) or as medicinal plant (with high) L-DOPA content.

The RAPD data generated out of the limited number of accessions from the windward side of the Western Ghats in Kerala state of India was sufficient to provide a semblance of diversity available within *M. pruriens*. The genetic similarity value was found to be in the range of 0.39 - 0.89 with mean value of 0.68 suggesting high levels of genetic variation in both the varieties of the species. At the intervarietal level, variety *utilis* with similarity index in the range of 0.69 to 0.92 and mean value of 0.82 was genetically less diverse than variety *pruriens* that possessed an overall similarity index of 0.70 having minimum similarity value of 0.46 between accessions MMP6 and KMP4 and maximum of 0.80 between PMP1 and PMP 3. Cluster analysis based on scoring data using the *WINBOOT* software clearly grouped all the 13 accessions under two major clusters. All the accessions (PMP1, PMP2 and PMP3) of variety *pruriens* from south Kerala except KMP4 clustered as group I supported at > 50 % confidence interval limits while their counterparts from north Kerala grouped separately at > 40 % confidence limits as sub-group IIA at the other end of the phenogram The accessions of variety *utilis* from different geographical regions, however, grouped as single cluster and found placed in between the clusters of variety *pruriens* at confidence interval limits of > 60 %. Grouping of accessions was strongly based on geographical affiliations in the case of *pruriens* as accessions of this variety from the southern and northern regions formed separate clusters flanking the two ends of the phenogram with apparent out grouping of a single accession (KMP4) that clustered with variety utilis. The obvious clustering of accessions of *utilis* from different geographical regions (south, central and northern Kerala) could be attributed to the high genetic similarity index shared by them. Placement of MMP6 at the similarity index of 0.58 with other two closely linked accessions of *pruriens* (MMP7 and MMP8) from the same region may be due to its unique genetic make up as it is a putative hybrid developed between varieties *pruriens* × *utilis.*

The high levels of polymorphism detected in otherwise self-pollinated accessions of *Mucuna pruriens* may be attributed to the broad genetic base of the species that in the process of speciation might have acquired novel gene combinations for better adaptability in the changing environmental conditions. The observation is consistent with the earlier report on genetic variation analysis in *Mucuna* sp. where genetic similarity index ranged from 0.68 to 1.00 (Capo-chichi *et al.* 2003). Although the high genetic diversity observed in the species is thought to be an indication of the adaptability of the species to different geographical climatic belts, in the present study variation of significant order has been observed within the collection from the humid forests of the Western Ghats which form a distinct phytogeographic block.

The high number of polymorphic products generated by certain primers as discussed above might be attributed to the fact that in RAPD even small divergence between two cultivars can result in distinct patterns as polymorphism may be the result of any of the various reasons such as *a*) single nucleotide change within the primer binding site, *b*) insertion or deletion with the amplified region so that part of the primer binding site in one of the strand is missing, *c*) complete absence of complementary sites, and *d*) the region between the binding sites on opposite strands is beyond the normal amplifiable length.

L-DOPA content analyzed in the accessions showed uniformity in percentage distribution of the active principle with the exception of the hybrid variety collected from north Kerala. The relatively low content of L-DOPA in the hybrid is possibly the reflection of its unique genetic make-up. The present data on percentage distribution of L-DOPA in these tropical accessions however, is not congruent with the previous report on higher L-DOPA synthesis in plants grown at lower latitudes near the equator wherein light and other latitude related factors were assumed to have contributory role on enhanced synthesis of the compound (Lorenzetti *et al*. 1998). Recent studies on regression analysis of L-DOPA content in seeds against latitude revealed that variation in latitude could explain only 8 % of variation in the active principle content (St-Laurent *et al.* 2000). This along with earlier observations on synthesis of L-DOPA as function of irradiance that could be either stimulatory (Wichers 1983) or inhibitory (Brain 1976, Pras *et al.* 1993) to L-DOPA synthesis suggest that factors other than latitude must be largely responsible for differential synthesis of the compound.

Earlier attempt to analyze genetic variation in *M. pruriens* var *utilis* by amplified ITS region of nuclear DNA showed no variation between the accessions of this variety (St-Laurent *et al*. 2000) The non coding spacer region that flanks the much conserved 5.8S gene sequence is known to harbor many hot spots for nucleotide substitution leading to genetic polymorphism in closely related species and at the subspecific level as well (Soltis and Soltis 1998). The ITS sequence analysis approach that successfully resolved phylogenetic and taxonomical issues in many angiosperms in the past (Baldwin 1992, Baldwin *et al*. 1995, Cox *et al.* 1997), however, was not able to detect intraspecific variation in the variety *utilis* thereby suggesting the unsuitability of this region of DNA for genetic differentiation analysis in *utilis* and necessitates other molecular markers for effective resolution of the taxa at the subspecies level. The relatively low degree of genetic variability observed in *utilis* confirm these earlier finding and lend credence to the utility of RAPD in the detection of accession-specific polymorphism in *Mucuna* sp.

As shown in Table 4, the various parameters analyzed shows relatively high genetic diversity in variety *pruriens*. The total heterozygosity for *Mucuna* is 0.44 while for variety *pruriens* and *utilis* it is 0.46 and 0.43, respectively, which are greater than the values reported for other self pollinating legumes of cosmopolitan distribution like *Trigonella caerulea* (0.346) and *Trigonella foenum-graecum* (0.203) by RAPD (Rakhee *et al.* 2004). It is also higher than those reported for tropical cross pollinated tree species like *Gaultheria fragrantissima* (0.17), *Symplocos laurina* (0.24) and *Eurya nitida* (0.18 and 0.27) using ISSR markers (Deshpande *et al.* 2001, Bahuliker *et al.* 2004). In spite of low percentage of polymorphic loci in variety *utilis*, RAPD proved to be an effective molecular marker tool to detect more heterozygosity in this variety. Though the most favored mode of reproduction in *Mucuna pruriens* is by self-pollination, the possibility of out crossing cannot be ruled out as evidenced by the development of hybrid variety.

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