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Comparative Evaluation of Genetic Diversity among Indian *Mucuna* Species Using Morphometric, Biochemical and Molecular Approaches

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Abstract: Genetic improvement of *Mucuna* presents promising future prospects in view of their multiple uses such as cover crop, green manure, feed and food crop. All the species are also characterized by the presence of L-Dopa (L-3, 4-dihydorxyphenylalanine), a well-known non-protein amino acid that acts as a precursor to the neurotransmitter dopamine, used in the treatment of Parkinson's disease. Though India is one of the natural centres of origin, very little information is available on genetic variation present in the sub-continent. In this paper we describe the results of comparative evaluation of genetic diversity among 18 *Mucuna* accessions belonging to four species carried out using morphometric, biochemical, isozyme and RAPD approaches. The UPGMA dendrogram based on RAPD results grouped 18 accessions in 3 distinct clusters; two of them separating wild and cultivated forms of *M. pruriens* and the third one placing perennial types distinctly. The results were in consensus with morphometric analysis of *M. pruriens* based on taxonomic distances. Isozyme study, though failed to discern infra-species grouping, revealed a few important diagnostic markers at species level that is of predictive value in taxon delimitation programs. None of the data however sustained an earlier held view that var. *hirsuta* is unique from var. *pruriens* inside Velvetbean group. The results obtained from different markers were positively correlated with r value >0.50.The L-Dopa content among the accessions ranged from 1.09 to 2.85%. All the diversity estimates confirmed good variability among the studied accessions proving their utility in breeding programs.

Key words: Diversity · Isozyme · Morphometry · *Mucuna* spp · RAPD · Taxonomy

INTRODUCTION

Genus *Mucuna* belongs to the family Fabaceae and includes 150 species of annual and perennial legumes of pantropical distribution. Some of its species, prominently members of *M. pruriens* (Velvetbean) exhibits important agronomic potentials such as: 20-30% protein in the seed; yield of seed upto 2.4 t/ha/yr; biomass and dry mass accumulation of 20-30 t and 7-9 t/ha/yr; thrives well in dry farming and low soil fertility conditions; exhibits resistance against wide ranging diseases [1-3], possess allelopathic activities [4] and show anti-nematode properties [5].

Cultivation of *M. pruriens* as a green manure cover crop (GMCC) has a long history in temperate regions, especially in USA [2, 6]. In recent times recognizing its potential as alternative human food and/or ruminant feed, extensive efforts were made to breed improved varieties to promote its cultivation among the nutrition-deprived parts of Asia and Sub-Saharan Africa [2, 7]. The idea appears to have gained backing from the point that such a use was already in practice among the native tribes of these regions, who consumed it as minor food unique to their cultural habits [2]. Bressani [8] compared the nutritional and anti-nutritional potentials of Velvetbean vis-à-vis other edible legumes and established their parallels, both in quality as well as quantity parameters. However, the genus as a whole is characterized by the presence of unique anti-physiological substance L-Dopa at bizarrely high levels (1-5%) [9], which, when consumed as food is known to induce serious side effects such as nausea, anorexia, vomiting etc. in human beings [10] and intestinal ailments such as chronic ulcers among the ruminant animals [11]. Past experiences have shown higher content

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of L-Dopa as the major bottleneck to be addressed before popularization of *Mucuna* among the farmers field and any efforts, therefore, to breed improved varieties of velvetbean should start with wide ranging efforts to address this. Interestingly, L-Dopa - a non-essential amino acid has also attracted significant pharmaceutical attention as precursor of the neurotransmitter drug dopamine used in the treatment of Parkinson's disease [12].

Originally *Mucuna* is a native of China and Eastern India from where its cultivation spread to other regions of the world creating newer popular production niches. In India, it is represented by nine species [13] of which five are endemic to Western Ghats and Eastern Himalayan regions and the only annual species *M. pruriens* shows ubiquitous distribution. Rich genetic diversity coupled with wide ranging traditional knowledge offers great scope for improvement of *Mucuna* in the sub-continent. However, owing to fact that the beneficial attributes of *Mucuna* was never popularized in India, very little effort has gone into its improvement either through conventional breeding or by biotechnological methods. Lack of such primary information as: diversity and variability, relationship between the species, key germplasm and breeding behavior etc. have seriously constrained the effective utilization of *Mucuna* genetic resources in the country. On the other hand, even the taxonomy of the genus is confused with several synonyms at the species and the varietal levels rendering authentic identification of the taxa difficult [13]. In many such cases of other plant species, availability of the diverse marker systems with proven efficacy in plant breeding and conservation programs has greatly facilitated addressing these issues [14, 15, 16 and 17].

The aim of the present study is to characterize eighteen accessions of *Mucuna* belonging to four species using morphometric, isozyme, RAPD and biochemical approaches.

MATERIALS AND METHODS

Species and Populations Sampled: The experimental material consisted of 18 *Mucuna* accessions belonging to wild and cultivated forms. These included 7 accessions obtained from the National Bureau of Plant Genetic Resources (NBPGR), New Delhi and 11 collected from diverse geographical locations across India including wild (Table 1).

Morphometric Analysis: Fifteen accessions belonging to *M. pruriens* were analyzed for variability in 26 morphometric traits (Table 2) based on descriptor and the data was subjected to one-way analyses of variance (ANOVA) using Tukey- Kramer HSD test. Perennial accessions were omitted from the analysis owing to indefinite growth habit and possible extremes in data scores. Jaccard's similarity coefficient values for each pair-wise comparison between accessions were calculated and a similarity coefficient matrix was constructed.

Table 1: *Mucuna* accessions used in the study

Name of the Accession	Accession Number	Place of collection	Latitude and longitude
M. pruriens var. *utilis*	500102KA	Karnataka	-
M. pruriens var. *utilis*	500101KA	Karnataka	-
M. pruriens var. *utilis*	500108KA	Hunasamaranahalli, Karnataka	13°4' N, 77° 62' E
M. pruriens var. *pruriens*	500112KA	Mysore, Karnataka	12°18' N, 76° 42' E
M. pruriens var. *pruriens*	500113MH	Triambakeshwar, Maharashtra	20°00' N, 73° 77' E
M. pruriens var. *hirsuta*	500109KA	Shimoga, Karnataka	13°56' N, 75° 38' E
M. pruriens var. *hirsuta*	500115TN	Raw drug vendor, Madurai	-
M. gigantea	500104KL	Waynad, Kerala	11°60' N, 76° 08' E
M. monosperma	500103AN	Andaman Islands, Bay of Bengal	11°61' N, 92° 72' E
M. bracteata	500124KL	Seed company, Kerala	-
M. pruriens var. *hirsuta*	500123KL	Seed company, Kerala	-
M. pruriens var. *utilis*	IC385925	NBPGR, Dhangadih, Dumka, Jharkand	24°26' N, 87° 24' E
M. pruriens var. *utilis*	IC385928	NBPGR	-
M. pruriens var. *utilis*	IC185926	NBPGR	-
M. pruriens var. *utilis*	IC385841	NBPGR, Pakud, Jharkand	24°62' N, 87° 84' E
M. pruriens var. *utilis*	IC369144	NBPGR, Latehar, Jharkand	23°74' N, 84° 50' E
M. pruriens var. *utilis*	IC385926	NBPGR, Dhangadih, Dumka, Jharkand	24°26' N, 87° 24' E
M. pruriens var. *pruriens*	IC265577	NBPGR, Njeezhoor, Kottayam, Kerala	9°58' N, 76° 52' E

Table 2: Maximum and minimum measures for different morphometric traits among 15 *Mucuna pruriens* accessions

		Maximum		Minimum	
Sl. No.	Morphological traits	Accessions Number	Trait score	Accessions Number	Trait score
1.	Days to emergence	500108KA	8 days	500115TN:	5 days
2.	Terminal leaf length (cm)	500108KA	12.84±2.13	500115TN	7.08±1.323
3.	Adjacent leaf length (cm)	500108KA	12.48±6.44	500115TN	7.03±1.41
4.	Terminal leaf width (cm)	500108KA	8.56±1.53	500113MH	3.46±0.577
5.	Adjacent leaf width (cm)	500108KA	7.83±1.41	500113MH	3.48±0.00
6.	Petiole length (cm)	500108KA	10.93±2.65	IC385841	1.40±0.69
7.	Days to flowering	500112KA	163	IC471870	52
8.	Inflorescence length (cm)	500113MH	93.00±6.82	IC369144	2.20±4.16
9.	No. of flowers per inflorescence	500113MH	86.75±9.73	IC471870	4.50±1.50
10.	Flower length (cm)	500130KA	5.18±0.05	500159TN	4.15±0.04
11.	Pedicel length (cm)	500113MH	0.70±0.12	500130KA	0.30±0.04
12.	Innermost stamen length (cm)	IC185926	4.53±0.11	500113MH	3.80±0.071
13.	Outer stamen length (cm)	IC185926	4.65±0.02	500110KA	3.40±0.071
14.	Outermost stamen length (cm)	500108KA	4.20±0.08	500130KA	3.40±0.212
15.	Length of ovary (cm)	500159TN	1.15±0.112	500110KA	0.60±0.071
16.	Length of style (cm)	IC185926	3.95±0.166	500130KA	2.88±0.18
17.	Days to first mature pods	500112KA	185	IC385928	65
18.	Pod length (cm)	IC385928	16.10±0.30	500130KA	7.90
19.	Pod width (cm)	IC471870	2.55±0.05	500130KA	1.43±0.13
20.	No. of pods per cluster	500109KA	28.0±4.98	IC471870	4.0±1.414
21.	No. of pods per plant	500113MH	340	IC385841	41.3
22.	Days to maturity	500112KA	221	IC385928	95
23.	Seed length (mm)	IC385926	18.12±3.04	500115TN	9.27±1.17
24.	Seed width (mm)	IC385841	14.0±1.237	500115TN	6.6±0.35
25.	Seed thickness (mm)	IC385841	11.04±0.392	500113MH	3.83±0.61
26.	Weight of 100 seeds (g)	IC385841	200.01±6.096	500123KL	21.90±1.78

This was subjected to unweighted pair-group average analysis (UPGMA) [18] to generate dendrogram using average linkage procedure and cophenetic correlation factor [19] was also calculated. All these computations were carried out using NYSYS-pc version 2.0 [20]. Robustness of the clustering was tested using 1000 resampling with WINBOOT software [21].

Isozyme Method: Eight isozyme systems; alcohol dehydrogenase (ADH), glucose-6-phosphate dehydrogenase (G6PDH), esterase (EST), polyphenol oxidase (PPO), peroxidase (POX), shikimic dehydrogenase (SKDH), superoxide dismutase (SOD) and catalase (CAT) were assayed. Fresh young leaves from at least 10 plants of each accession (except perennial species) were bulked separately and used in enzyme analysis. Enzymes were isolated by homogenizing 150 mg of leaf tissue in a buffer containing: 0.1 M Tris-HCl (pH-7.2), 5% sucrose, 0.5% polyvinyl pyrrolidone (PVP) and 10 mM 2-Mercaptoethanol. The samples were centrifuged at 15,000 rpm for 15 min and the supernatant was collected and stored at -20°C. The proteins were separated by polyacrylamide gels run in tris-glycine (pH 6.8) buffer at 20 mA for about 2.0-2.5 h at 4°C and stained as described by [22, 23].

RAPD Amplification: Genomic DNA isolated using CTAB method [24] were screened with fifty selected decamer primers purchased from Operon Technologies (USA) for RAPD analysis.

Amplification reactions for RAPD analyses were used in a final volume of 25 μl containing 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, pH 8.3), 0.2 mM dNTPs, 15 pmol primer, 50 ng of template DNA and 1.0 U of *Taq* polymerase (New England Biolabs, USA). Reactions were performed in a PTC-200™ thermocycler (MJ Research Inc. USA).

PCR amplification was performed with the following conditions: initial extended step of denaturation at 94°C for 2 min followed by 38 cycles of denaturation at 94°C for 1 min, primer annealing at 36°C for 1 min and elongation at 72°C for 1 min. The 38th cycle was followed

by an extended primer extension step at 72°C for 7 min. PCR products were analyzed using 1.8% agarose gel electrophoresis and visualized with 0.5 ng/µl ethidium bromide staining. The sizes of the fragments were estimated based on 1kb DNA ladder (MBI Fermentas) and photographed and archived using Gel Documentation System (Bio-RAD, USA).

RAPD and Isozyme Data Analysis: Polymorphic bands were considered as binary characters and scored as '1' for presence and '0' for absence. The scores were then converted to binary matrix for analysis by the Numerical Taxonomy and Multivariate Analysis System, (NTSYS PC) software version 2.0 [20]. The data were analyzed with the SIMQUAL option on the basis of Jaccard's coefficients to generate genetic similarity matrix [25]. This was run on Sequential Agglomerative Hierarchical and Nested (SAHN) clustering [18] using Unweighted Pair Group Method with Arithmetic Average (UPGMA) algorithm [19] to generate a dendrogram. The confidence limits of the obtained trees were ascertained using WINBOOT software [21] with 1000 iterations. The MXCOMP subroutine was used to calculate a cophenetic correlation matrix between the similarity and original matrix to measure the goodness of fit. The genotype and allelic frequency data were used to compute the genetic diversity indices such as: 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 statistical package POPGENE 1.31 [26]. The populations from which the samples are taken for the present study 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: $Ht = 1 - \Sigma Pi^2$, where Pi is the frequency of the i^{th} allele in the population. Analysis of molecular variance (AMOVA) was applied on Euclidean distance matrix to partition the genetic variation. AMOVA was done using computer software Genalex 6 [27].

L-Dopa Analysis: Seeds from at least 10 plants of each accession (except perennial species) were bulked separately and used for L-Dopa analysis. From the powdered seeds, L-Dopa was extracted following modified [28] method. Initial defatting was carried out as follows: To a gram of fine seed powder, 10 ml of petroleum ether was added and vortexed for 48 h on a rotary shaker at 80 rpm and the resultant meal was dried at room temperature. For L-Dopa isolation, 100 mg of this defatted sample was taken with 5 ml of 0.1 N HCl and stirred constantly for 10 min at room temperature before incubating in boiling water bath with constant shaking for 1 h. To this, 10ml of 80% ethanol with 0.1% ascorbic acid was added and mixed thoroughly for 10 min and the mixture was then centrifuged at 4000 rpm for 15 min. For each sample, the above extraction step was repeated twice and each time the supernatants were collected separately. Later, the supernatants from each sample were pooled individually; made upto 100 ml and then filtered. L-Dopa concentration was determined by measuring the UV absorption of the supernatant at 283 nm. Each sample was analyzed in quadruplicates and the concentration of L-Dopa was represented as percentage composition. All the data were subjected to analyses of variance (ANOVA) using Tukey-Kramer HSD test.

RESULTS

Morphometric Analysis: The results of morphometric analysis showed considerable variability for inflorescence, pod and seed characters ($P<0.0001$) and less variability for leaf characters. The maximum and minimum measures for different characters are shown in Table 2. Jaccard's pair-wise similarity estimates between genotypes indicated good genetic diversity (Similarity index, SI, 50.6%) within *M. pruriens* accessions; while among its subgroups, variety *pruriens* was found to be more diverse (SI 54%) than var. *utilis* (SI 71%) . UPGMA analysis (Fig. 1) placed these accessions into two major clusters with var. *pruriens* and var. *hirsuta* grouping together in cluster-I, while var. *utilis* accessions separated distinctly in cluster-II. Grouping of former two accessions in the same cluster indicated close similarity between them at genetic level. The data on genetic diversity indices *viz.* 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 based on morphometric data are shown in Table 3.

Isozyme and L-Dopa Analysis: Of the 8 isoenzymes assayed, 4 were detected (PPO, POX, EST and SKDH) in *Mucuna* species. They produced totally 16 marker loci of which 14 were polymorphic with a polymorphic index of 87.5%. Some diagnostic markers were recorded mainly to distinguish annual and perennial accessions. PPO produced eight marker loci, of which except one all others were monomorphic to *M. pruriens*. This single extra locus found in 500113MH is otherwise seen only in perennial accessions and its appearance in this annual plant needs

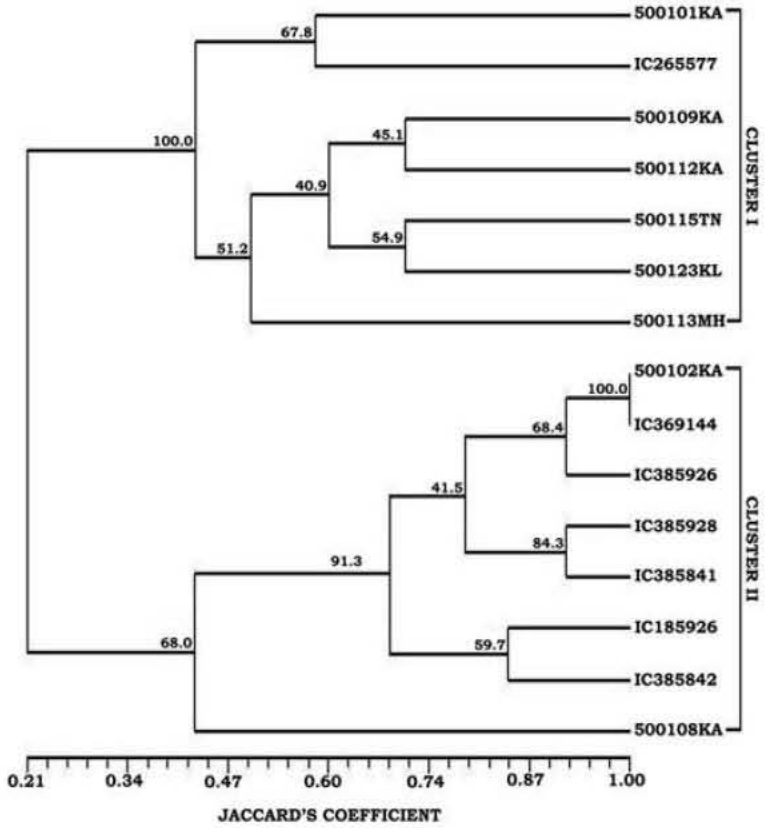

Fig. 1: UPGMA dendrogram based on morphometric data

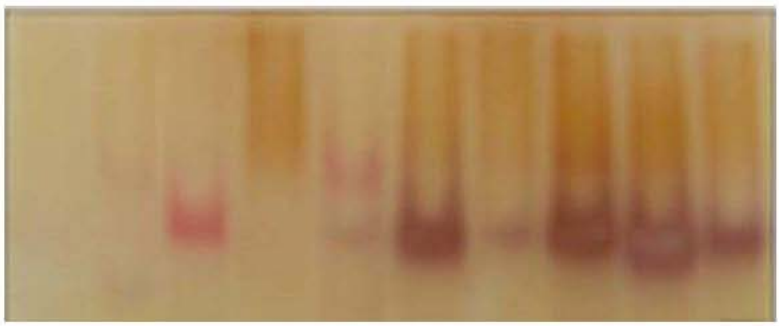

Fig. 2: Esterase Isoenzyme banding profile

Table 3: Genetic diversity estimates based on three used techniques

Diversity indices	Morphology (excluding perennials)	Isozymes	RAPD
Na	1.9808	1.8750	1.8926
Ne	1.6538	1.5269	1.3515
H	0.3687	0.3067	0.2207
I	0.5419	0.4581	0.3507
Ht	0.3571	0.3361	0.2428

(Na - observed number of alleles, Ne - expected number of alleles, h - Nei's gene diversity, I - Shannon's information index of gene diversity, Ht - total heterozygosity)

to be investigated. The Nei's [29] gene diversity with respect to PPO loci ranged from 0.17 to 0.50 with mean at 0.40. Esterase produced two marker loci (Fig. 2) with mean Nei's [29] gene diversity of 0.21. Peroxidase produced 4 loci with Nei's [29] gene diversity ranging from 0.00 to 0.43 with mean at 0.39. Two diagnostic bands were recorded in this, of which one uniquely identified *M. monosperma* while the other was exclusive to perennials species. Interestingly, enzyme Shikimic acid dehydrogenase (SKDH) produced two loci which were specific to annual species. With respect to SKDH, mean Nei's [29] gene diversity is 0.28. The data on all genetic

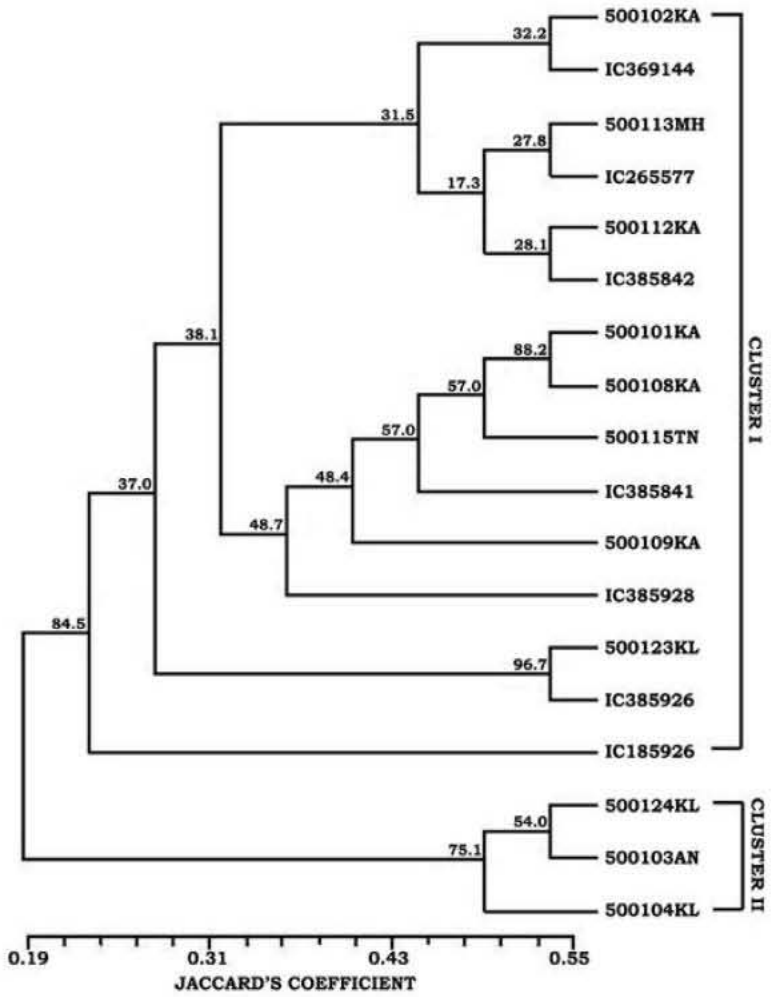

Fig. 3: UPGMA dendrogram based on Isozyme data

Table 4: Diversity for L-Dopa content in different *Mucuna* accessions

Sl. No.	Accession Number	Percentage L-Dopa[Y]
1.	500104KL	2.85±0.001[A]
2.	500113MH	2.76±0.005[B]
3.	IC265577	2.51±0.002[C]
4.	500123KL	2.37±0.003[D]
5.	500109KA	2.32±0.001[E]
6.	500112KA	2.17±0.003[F]
7.	500124KL	2.15±0.002[F]
8.	500108KA	2.10±0.002[G]
9.	IC385926	1.94±0.012[H]
10.	500102KA	1.94±0.008[H]
11.	500101KA	1.67±0.002[I]
12.	IC385928	1.64±0.010[I]
13.	IC385842	1.63±0.011[I]
14.	500115TN	1.57±0.015[J]
15.	IC385841	1.55±0.002[J]
16.	IC369144	1.48±0.011[K]
17.	500103AN	1.48±0.008[K]
18.	IC185926	1.09±0.024[L]

[Y] Values are mean ± standard deviation of 4 independent experiments. Means followed by same letter are not significantly different at 1% significance level as determined by Tukey's HSD test.

diversity indices *viz*. 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 based on genotype and allelic frequency of isozymes are shown in Table 3. Cluster analysis based on isozyme data grouped 18 *Mucuna* accessions into two major clusters (Fig. 3) with broad delineation achieved only between annual (cluster-I) and perennial (cluster-II) species. AMOVA based on isozyme loci resulted in very less molecular variance (12%) among the populations of *M. pruriens*.

The content of L-Dopa among 18 *Mucuna* accessions ranged from 1.09 to 2.85% with large number of accessions (66%) falling in the frequency range between 1.4-2.2%. Only 16% were found in the extreme ends (Table 4). The lowest and highest contents of L-Dopa were found in accessions IC185926 and 500104KL respectively.

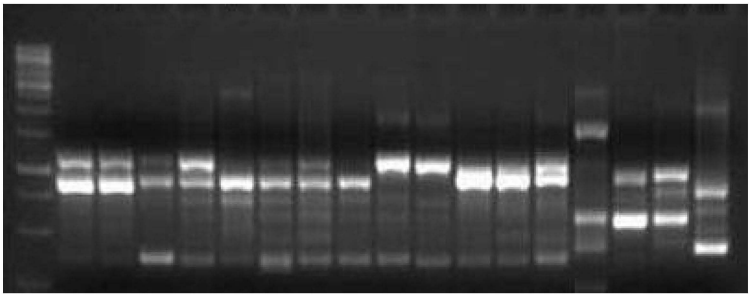

Fig. 4: RAPD profile obtained for the primer OPA-08

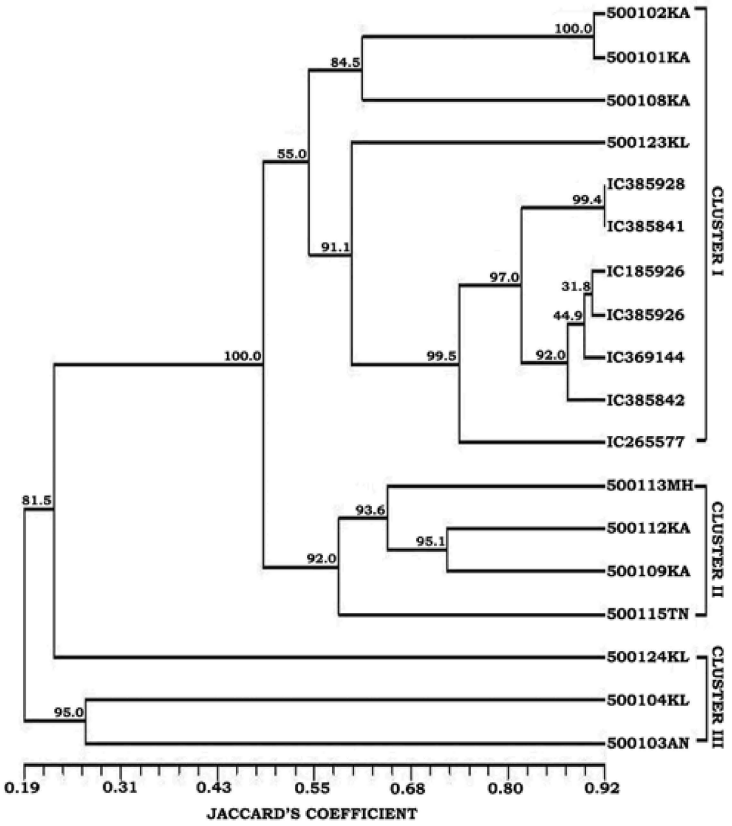

Fig. 5: UPGMA dendrogram based on RAPD data

RAPD Analysis: Of the 50 decamer primers used, 44 generated a total of 412 reproducible polymorphic markers with 98.32% polymorphism. The number of amplification products ranged from 1 (OPA-04) to 19 (OPAK-17). On an average, single primer generated 10.48 products of which nearly 10.3 were polymorphic. As an illustration, the profiling pattern obtained for RAPD primer OPA-08 is shown in Fig. 4.

Jaccard's pair-wise similarity estimates between genotypes were calculated after grouping the accessions on the basis of their growth habits (annual/perennial) and taxonomic assemblage. It is evident from the Table 5 that there is good overall diversity within annual *M. pruriens* accessions, with similarity value ranging from 36% to 89% with mean at 63%. Among its subgroups, var. *pruriens* was found to be more diverse with similarity index value ranging from 37% to 63% with mean at 50% compared to var. *utilis* where it ranged from 36% to 89% with mean at 63%. The results are in conformity with the results of morphometric analysis. The diversity within perennial accessions was found to be 79%.

The dendrogram based on UPGMA analysis (Fig. 5) separated 18 accessions into three distinct groups as follows: All the *M. pruriens* accessions grouped in

Table 5: Jaccard's similarity estimates of different groups

Groups	No. of genotypes	Mean (±SD)	Range
M. pruriens var. *utilis*	9	0.63±0.22	0.42-0.88
M. pruriens var. *pruriens*	3	0.51±0.12	0.43-0.97
M. pruriens var. *hirsuta*	3	0.47±0.08	0.37-0.56
Mucuna perennials	3	0.21±0.05	0.15-0.26

the first two clusters, I and II, with bootstrap support of 100%. In this majority of the *M. pruriens* var. *utilis* accessions grouped in cluster I along with two out grouped members namely 500123KL and IC265577. Within this, accessions possessing green pods (IC369144, IC385926, IC185926, IC85842) and black velvety pods (IC385841, IC385928) formed separate subgroups, except 500101KA (black pod) and 500102KA (green pod) which despite difference in pod color remained in the same group. However, accession 500108KA is unique as it bears creamy-white flowers as against dark purple flowers in all other accessions. In cluster II, all the accessions of var. *pruriens* and var. *hirsuta* grouped together without any clear-cut distinction.

All the perennial accessions formed distinct third cluster with boot strap support of 81.5%. *M. monosperma* and *M. gigantea* formed cluster III, while *M. bracteata* positioned as an out-group indicating its transitional status between annual and perennial species [13]. The groupings, either within annual or perennial accessions did not reveal any clear-cut pattern of geographical affiliation except few instances where accessions of var. *utilis* from one region grouped together.

A cophenetic-value (ultrametric) matrix was generated from the coefficients of SAHN's cluster analysis of the similarity matrix. The cophenetic correlation coefficient between the cophenetic matrix and the data matrix of RAPD data was 0.983. Such a high value is considered very good fit [30] and shows that the original matrix was well represented by cluster analysis.

The mean heterozygosity based on RAPD data was recorded to be 0.24 on assuming the populations to be in Hardy- Weinberg equilibrium. The analysis also established high genetic similarities between var. *pruriens* and var. *hirsuta* with Nei's genetic identity [29] value of 0.94. This is further supported by other genetic diversity indices *i.e.* observed number of alleles (A), expected number of alleles (Ne), Shannon index of gene diversity (I) and Nei's gene diversity (h) (Table 3). The results of AMOVA revealed 67% variance among the studied samples of which nearly 16% is derived from the *M. pruriens* accessions. Zero percent variance was recorded between var. *pruriens* and var. *hirsuta* accessions in this study.

DISCUSSION

Morphometric Analysis: The analysis of 26 morphometric characters from 15 velvetbean accessions revealed significant variability among the studied collection. It also produced vital clues on taxonomic grouping with *M.pruriens*. Such a comprehensive study was lacking in Indian *Mucuna* species despite numerous reports available on their biochemical and nutritional characterization [3, 11 and 31]. The dendrogram based on taxonomic distances placed these accessions in two distinct clusters of which greater diversity was observed among var. *pruriens*/var. *hirsuta* accessions (Cluster-I) compared to var. *utilis* (cluster-II). This is plausible as the former represents collectively the wild gene pool of velvetbean as against the domesticated genotypes in the latter group. Pugalenthi and Vadivel [32] have recently surveyed agro-botanical diversity of 11 var. *utilis* accessions and reported some interesting findings from breeding perspective. However, less variability observed for this group in the present study, despite examining collections from diverse geographical location point towards narrow genetic base among the cultivated genotypes and need for diversification through either wide-hybridization or other genetic and/or biotechnological methods.

Interestingly, the analysis did not divulge any difference between var. *pruriens* from var. *hirsuta* - the two botanical varieties of *M. pruriens*. The taxonomic status of var. *hirsuta* is a matter of much debate. Many earlier studies [33-37] have categorized it as a separate species as it differs from typical variety in many characters such as: shape and size of the leaflets, bracts and pods, thickness as well as color and density of the indumentum on vegetative and floral parts, color of the hilum on the seeds etc. But according to Wilmot Dear [13], the only character providing absolute distinction between the two is long, noticeable, dense, crisp indumentum in var. *hirsuta* as against fine indumentum in var. *pruriens* due to which he opines that var. *hirsuta* cannot be maintained as distinct species. Present study confirmed further the latter views of Wilmot Dear [13].

Isozyme Analysis: The diversity estimates based on Isozyme analysis were in broad consensus with RAPD results except for low levels of differentiation obtained for sub-groupings within *M. pruriens*. This might be due limitations of the isozyme markers for resolving infra-species differences. The latter has been confirmed in earlier studies also [38, 39]. Nonetheless, many

diagnostic bands revealed for resolving inter-species differences could play an important role in related applications as has been successfully employed in *Vicia* [40] and Pines [41].

RAPD Analysis: RAPD analysis in the present study revealed very high level of polymorphism (98.32%) for different *Mucuna* accessions. Padmesh *et al.* [42] observed that 11 velvetbean accessions produced 90.1% polymorphism. The enhanced polymorphism seen in this case can be attributed to diverse materials which belonged to four different species sampled for the analysis. Moreover, the various genotypes within a species, particularly *M. pruriens*, represented different agro-climatic regions of growth.

Using RAPD markers greater diversity was observed for var. *pruriens*/var. *hirsuta* (SI 52%) accessions compared to var. *utilis* (SI 63%). This observation (for var. *puriens*) has been documented earlier [42] and is also supported by current morphometric analysis. Prevalence of greater diversity within var. *pruriens* necessitates further collections and conservation of these genetic resources in the country. The average genetic similarity reported by Padmesh *et al.* [42] for *M. pruriens* and its sub groups, var. *pruriens* and var. *utilis* was 68%, 70% and 82% respectively; while in our study we have found this to be 63%, 50% and 63% respectively. The results clearly indicate that the genetic stock available in the present germplasm collection is more diverse than the above. Collection from diverse geographical regions might have contributed to this diversity.

The UPGMA dendrogram (Fig. 5) grouped 18 accessions broadly into three clusters with bootstrap support of 81.5. All the annual accessions belonging to *M. pruriens* grouped in Cluster-I, II while perennial accessions such as *M. monosperma* and *M. gigantea* grouped in Cluster-III. The two out-grouped members of cluster-I, *viz.* 500123KL and IC265577 probably represent inter-varietal hybridization as the two, in opposition to their dendrogram position closer to var. *utilis*, resemble var. *hirusta* and var. *pruriens* in their morphometric characters. 500123KL is similar to var. *hirsuta* in all other respects except the size and shape of the leaf, while IC265577 has unusually longer pods like that of var. *utilis* and otherwise is similar to var. *pruriens*. In this background, it can be predicted that these might be naturalized hybrids of *utilis* X *hirsuta* (500102KA) and *utilis* X *pruriens* (IC265577) sharing greater commonalities with the wild parents.

The analyses based on genetic distances and AMOVA has clearly established high similarities between var. *pruriens* and var. *hirsuta*. The same is supported by morphometric analysis also. Even going by the taxonomic descriptors, the differences between the two are so narrow that often relative judgments are necessary to distinguish them. This has resulted in a situation that many literatures use the names var. *hirsuta* and var. *pruriens* interchangeably for either one of them. In an attempt to resolve this, Wilmot Dear [13] described the key characters for these three varieties, including the basis for not raising the var. *hirsuta* to a level of new species. But, in doing so, the author has missed an important consideration that, the natural hybridization between these two has allowed several intermediate phenotypic forms of these in the wild determining whose taxonomic status will remain a real challenge. Considering these complexities in the background of close genetic similarities between the two, it might be prudent to combine all the wild forms of *M. pruriens*: var. *pruriens*, var. *hirsuta* and their intermediate types in one subgroup under the name var. *pruriens* thus allowing only two groups *viz.* var. *utilis* (cultivated + non-itching trichomes) and var. *pruriens* (wild + itching trichomes) to be recognized under *M.pruriens*. This would not only reduce nomenclatural redundancies and resulting ambiguities, but also help assign specific systematic name for the genotype under consideration in breeding programs.

L-Dopa Analysis: The results on variability for L-Dopa content among different accessions is by and large in consensus with the earlier report of Padmesh *et al.* [42] on south Indian accessions; and in lower range than reported by Pugalenthi *et al.* [11]. The latter might be due to difference in the analytical methods used by these authors for isolation and estimation of L-Dopa. Presence of good variability for this trait in the studied accession corroborates their utility for future L-Dopa breeding programs.

CONCLUSION

The information on the genetic diversity of the 18 Indian *Mucuna* accessions evaluated with three diverse systems were consensus with each other evolving better clarity on the genetic relationship among the studied *Mucuna* accessions in hierarchical order of their resolving abilities (Morphometry<Isozyme<RAPD). The results obtained provides a short and snappy view on the total

genetic variability for *Mucuna* species in India and thus help in evolving pragmatic strategies for breeding improved varieties of *Mucuna* for food, feed and pharmaceutical applications. The taxonomic discussions throws important hints on placing potential breeding lines in right taxonomic perspective especially in the context of emerging concerns on plant breeder's right.

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