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RESEARCH ARTICLE

AFLP assessment of genetic diversity among Indian *Mucuna* accessions

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Abstract Amplified fragment length polymorphism (AFLP) marker was used to assess diversity in germplasm collection of *Mucuna* species which has gained tremendous attention in the recent past due to its promising nutritional, agronomic and medicinal attributes. Twenty five accessions comprising five species, collected from seven states of India were evaluated with twelve AFLP primer combinations that generated a total of 1,612 fragments with an average of 134 fragments per primer combination. The values of polymorphic information content (PIC), marker index (MI) and the resolving power (Rp) demonstrated the utility of the primer combinations used in the present study for discriminating the *Mucuna* accessions. UPGMA and Principal coordinate analysis (PCoA) of the genotypic data revealed clustering of accessions as per phenetic and genetic relationships. The Jaccard's similarity coefficient values suggested good variability among the *M. pruriens* accessions indicating their utility in breeding programs. Molecular diversity presented in this study combined with the datasets on other morphological/agronomic traits will be highly useful for selecting appropriate accessions for plant improvement through conventional as well as molecular breeding approaches and for evolving suitable conservation strategies.

Keywords AFLP · *Mucuna* species · Velvetbean · Molecular characterization · Genetic diversity

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Introduction

Genus *Mucuna* belongs to the family Fabaceae and includes 150 species of annual and perennial legumes of pantropical distribution (Buckles 1995). In India, it is represented by nine species (Wilmot Dear 1987), of which *M. atropurpurea* and *M. pruriens* (var. *hirsuta*) are endemic to Peninsular India; *M. imbricata, M. bracteata, M. macrocarpa, M. sempervirens* and *M. nigricans* are restricted to the North-Eastern parts and *M. pruriens* (excluding var. *hirsuta*), *M. monosperma* and *M. gigantea* are widely distributed all over. All the species exhibit morphological features such as: climbing habit, trifoliolate leaves, hairy body parts and clustered or long inflorescence of white or purple flowers. The pods, mostly green or brown in color are thickly covered with soft or stiff bristles which, in many cases, cause intense irritation if they come in contact with the human skin. The seeds, ranging 1–6 in number contain, as a genus attribute, non protein amino acid L-3,4 dihydroxy phenylalanine (L-Dopa) that confers an important medicinal property to the plant. L-Dopa is a precursor of dopamine and is widely used in the symptomatic management of Parkinson's disease (Haq 1983).

Mucuna pruriens (velvetbean) is the key member of the genus and shows highly promising agronomic potentials. Reported to be native of China and Eastern India (Wilmot Dear 1987)—the plant is now distributed in several parts of the World with newer production niches. In addition to 20–30 % protein content (Bressani et al. 2003), it produces seed yield of 2,000 kg/hectare (Buckles 1995); performs well under dry farming and low soil fertility conditions (Siddhuraju et al. 2000); shows resistance against wide ranging diseases (Eilitta et al. 2002); exhibits allelopathic properties (Fujii et al. 1991) and is effective in lowering nematode population (Carsky and Ndikawa 1998; Queneherve and Martiny 1998).

Due to rich biomass and N_2 fixing ability, it is often described as "featured example of green manures contribution to sustainable agricultural system" (Buckles 1995). Its impact on main crop yield is documented in number of earlier studies (Tarawali et al. 1999; Jorge et al. 2007). Besides, many Indian and African tribes consume it as minor food with processing methods unique to their cultural habits (Eilitta et al. 2002).

Considering the potential attributes of velvetbean for inclusion in human food chain, there is a wide scope for its improvement to bridge the gap between increasing demand for protein diet and its limiting sources. However, toxic properties of L-Dopa has rendered it inedible as consumption without proper processing induce severe side effects such as nausea, anorexia and vomiting in human beings and intestinal ailments in ruminant animals (Szabo and Tebbett 2002). Past experiences have shown this as major bottleneck in its popularization and thus efforts are needed to breed improved varieties with safe levels of L-Dopa to make its cultivation broad based and acceptable.

Rich gene pool, owing to wide ranging geographical and climatic distribution, offers immense scope for velvetbean improvement in India. However, lack of clear-cut characterization and understanding on phenetic relationships has caused significant taxonomic problems, both at the species and subspecies levels rendering authentic identification of the taxa difficult. The problem is particularly high among *M. pruriens* varieties where, in addition to other factors, extensive exchange of the seed materials over the years has further aggravated this confusion. Consequently even the floristic literatures are ambiguous on descriptions of species and their sub groups. Studies by Capo-Chichi et al. (2004) and Padmesh et al. (2006), while acknowledging these problems have viewed that: "It is now imperative to conduct detailed research on the taxonomic characterization and phenetic relationship among different species and subspecies of *Mucuna"* before any serious attempts towards its improvement is initiated. On the other hand, even the information on the pattern of diversity and genetic relationships among the germplasm collection has remained unclear with only works of preliminary nature available in literatures (Capo-chichi et al. 2001; Capo-chichi et al. 2004; Padmesh et al. 2006; Sathyanarayana et al. 2010; Leelambika et al. 2010).

DNA based markers have served invaluable tool in addressing the above issues with their superiority well established over other marker systems (Belaj et al. 2003; Genet et al. 2005; Yasmin et al. 2010). Among the various molecular markers employed, PCR-based markers such as RAPD (Random amplified polymorphic DNA; Williams et al. 1990), ISSR (Inter simple sequence repeat; Zietkiewiez et al. 1994) and AFLP (amplified fragment length polymorphism; Vos et al. 1995) have become popular, as their application does not need any prior sequence information. On the other hand, microsatellite or simple sequence repeat (SSR) are the markers of choice for breeding applications, but their development is an expensive process (Gupta and Varshney 2000). Among different marker systems available at present, AFLP represents dominant marker system and has proven to be extremely proficient in revealing diversity at the species levels and provide an effective means of covering a wide area of the genome in a single assay (Karp and Edwards 1997). These features make AFLP technology suitable for molecular characterization and DNA fingerprinting studies (Azhaguvel et al. 2006). Consequently, it has found wide application in phylogeny and genetic diversity analysis in number of plant species such as *Heritiera littoralis* (Malvaceae) (Jianab and Shib 2009); banana (El-Khishin et al. 2009); mustard (Shyama Weerakoon et al. 2010) as well as in legume crops such as soybean (Singh et al. 2010); *Dolichos* bean (Venkatesha et al. 2010) and chickpea (Sudupak et al. 2004). In the direction of analyzing the diversity of *Mucuna* species, a few studies have been carried out earlier using AFLP and RAPD markers (Capo-Chichi et al. 2001; Padmesh et al. 2006).

In this background, the present study investigates the molecular diversity in 25 accessions comprising of five *Mucuna* species using twelve AFLP primer combinations thus evaluating the suitability of AFLP marker system for characterization of *Mucuna* germplasm. The genotyping data thus obtained has also been examined to strengthen the understandings on taxonomic as well as genetic relationships among the germplasm lines to reliably use them in breeding programs.

Materials and methods

Plant material

Twenty five *Mucuna* accessions belonging to five species viz., *Mucuna monosperma*, *Mucuna atropurpurea*, *Mucuna gigantea, Mucuna bracteata* and *Mucuna pruriens* (represented by its all three sub-species) were used in the present study (Table 1). Of these, 12 accessions were obtained from the National Bureau of Plant Genetic Resources (NBPGR), New Delhi and the remaining 13 are collected from diverse geographical locations across India including Andaman & Nicobar Islands in Bay of Bengal.

DNA isolation

One gram of fresh leaf material was harvested from 2 to 3 weeks old seedlings from ten individual plants and bulked for each *Mucuna* accession. DNA isolation was carried out using modified Doyle and Doyle (1990) method. The leaf material was ground in liquid nitrogen and then homogenized

Table 1 List of *Mucuna* accessions used for the study

Sl. No.	Name of the Accession	Accession Number	Place of collection	Latitude and longitude
1.	*M. pruriens* var. *utilis*	500102KA	Karnataka	–
2.	*M. pruriens* var. *utilis*	IC385928	NBPGR	–
3.	*M. pruriens* var. *utilis*	IC392850	NBPGR, Moudi, Mayurbhanj, Orissa	22° 06′ N, 86° 40′ E
4.	*M. pruriens* var. *utilis*	IC185926	NBPGR	–
5.	*M. pruriens* var. *utilis*	IC395793	NBPGR, Narmada, Gujarat	21° 89′ N, 73° 48′ E
6.	*M. pruriens* var. *utilis*	IC392241	NBPGR, Dhanbad, Jharkand	23° 78′ N, 86° 43′ E
7.	*M. pruriens* var. *utilis*	IC326953	NBPGR, Jaunaji, Solan, HP	30° 90′ N, 77° 09′ E
8.	*M. pruriens* var. *utilis*	IC471870	NBPGR, Delhi	28° 62′ N, 77° 23′ E
9.	*M. pruriens* var. *utilis*	IC385841	NBPGR, Pakud, Jharkand	24° 62′ N, 87° 84′ E
10.	*M. pruriens* var. *utilis*	IC385842	NBPGR, Mohanpur, Jharkand	24° 48′ N, 86°.69′ E
11.	*M. pruriens* var. *pruriens*	IC265577	NBPGR, Kottayam, Kerala	9° 58′ N, 76° 52′ E
12.	*M. pruriens* var. *pruriens*	IC391941	NBPGR, Keonjhar, Orissa	21° 65′ N, 85° 63′ E
13.	*M. pruriens* var. *pruriens*	IC391885	NBPGR, Anandpur, Keonjhar, Orissa	21° 65′ N,85° 63′ E
14.	*M. pruriens* var. *pruriens*	500113MH	Triambakeshwar, Maharastra	20° 00′ N, 73° 77′ E
15.	*M. pruriens* var. *hirsuta*	500144AP	Pathur, Andhra Pradesh	18° 03′ N, 78° 18′ E
16.	*M. pruriens* var. *hirsuta*	500146AP	Ghanapur, Andhra Pradesh	18° 39′ N, 78° 10′ E
17.	*M. pruriens* var. *hirsuta*	500147AP	Durzgaon, Andhra Pradesh	18° 39′ N, 78° 10′ E
18.	*M. pruriens* var. *hirsuta*	500148AP	Mancheppa, Andhra Pradesh	18° 40′ N, 78° 10′ E
19.	*M. pruriens* var. *hirsuta*	500149AP	Pochampad dam, Andhra Pradesh	18° 89′ N, 78° 10′ E
20.	*M. pruriens* var. *hirsuta*	500150AP	Kanakapur, Andhra Pradesh	19° 05′ N, 78° 45′ E
21.	*M. monosperma*	500107KL	Wynad, Kerala	11° 60′ N, 76° 08′ E
22.	*M. gigantea*	500104KL	Waynad, Kerala	11° 60′ N, 76° 08′ E
23.	*M. monosperma*	500103AN	Andaman Islands, Bay of Bengal	11° 61′ N, 92° 72′ E
24.	*M. atropurpurea*	500114TN	Azhagarkoil, Madurai, Tamil Nadu	9° 91′ N, 78° 12′ E
25.	*M. bracteata*	500124KL	Seed company, Kerala	–

in extraction buffer containing cetyltrimethylammonium bromide (CTAB), 0.5 % charcoal along with 0.2 % β-mercaptoethanol and incubated at 60 °C for 1 h. Purification steps were carried out twice with chloroform: isoamylalcohol (24:1) and once with phenol: chloroform: isoamylalcohol (1:1). Finally the DNA was pelleted using 0.67 volumes of propanol. Followed by ethanol (70 %) wash, the pellets were air dried and re-suspended in 0.5 ml of 1X Tris-EDTA buffer (pH 8.0). Recovered DNA was quantified fluorometrically on 0.8 % agarose gel using ethidium bromide staining.

AFLP method

AFLP fingerprinting was carried out as described by Capo-chichi et al. (2001) with some modifications as follows: Genomic DNA (500 ng) was digested with 10 U of *Eco* RI and 4 U of *Mse* I (New England Biolabs, USA) at 37 °C for 3 h. Without inactivating the restriction enzymes, adapters [*Eco* RI (5 pmol) and *Mse* I (50 pmol)] were ligated to the restricted DNA fragments in ligation buffer [1x T4 DNA ligase buffer, 1 μl of T4 DNA ligase (New England Biolabs, USA)] and incubated at 37 °C for 12 h. Preamplification of the diluted (2-fold), ligated DNA was carried out with primers complimentary to the *Eco* RI and *Mse* I adapters, with two sets of selective nucleotides, one with cytosine and guanine and the other with adenine and cytosine respectively in PTC-200™ (MJ Research Inc., USA) thermocycler using the following cycling parameters: 20 cycles of 94 °C (denaturation) for 30 s, 56 °C (annealing) for 60 s, 72 °C (extension) for 60 s. The diluted (4-fold), amplified products were then used as the template for selective amplification. The second amplification was carried out with twelve selective primer combinations of *Eco* RI and *Mse* I each with three selective nucleotides (Table 2) in a total volume of 10 μl. The PCR program consisted of two segments: The first segment comprised of 12 cycles with one cycle at 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 60 s. The annealing temperature was then lowered by 0.7 °C per cycle during the first 12 cycles to reach an optimum temperature of 56 °C. The second segment comprised of 23 cycles at 94 °C for 30 s, 56 °C for 60 s and 72 °C at 60 s.

Table 2 Sequences of oligonucleotide adapters and primers used in AFLP

Name	Code	Sequence
Eco RI adapter	E-0	5′-AAT TGG TAC GCA GTC TAC-3′
		3′-CC ATG CGT CAG ATG CTC-5′
Mse I adapter	M-0	5′-TAC TCA GGA CTC AT-3′
		3′-G AGT CCT GAG TAG CAG-5′
Eco RI primer	E-A00	5′-GAC TGC GTA CCA ATT C A-3′
Mse I primer	M-C00	5′-GAT GAG TCC TGA GTA A C-3′
Eco RI primer	E-C00	5′-GAC TGC GTA CCA ATT C C-3′
Mse I primer	M-G00	5′-GAT GAG TCC TGA GTA A G-3′
Eco RI + 3-CAC	E-CAC	5′- GAC TGC GTA CCA ATT C CAC-3′
Eco RI + 3-CAA	E-CAA	5′-GAC TGC GTA CCA ATT C CAA-3′
Eco RI + 3- ACT	E-ACT	5′-GAC TGC GTA CCA ATT C ACT-3′
Eco RI + 3- AAC	E-ACC	5′-GAC TGC GTA CCA ATT C AAC-3′
Mse I + 3-GCT	M-GCT	5′-GAT GAG TCC TGA GTA A GCT-3′
Mse I + 3-GCA	M-GCA	5′-GAT GAG TCC TGA GTA A GCA-3′
Mse I + 3-CAT	M-CAT	5′-GAT GAG TCC TGA GTA A CAT-3′
Mse I + 3-CAG	M-CAG	5′-GAT GAG TCC TGA GTA A CAG-3′
Mse I + 3-CTA	M-CTA	5′-GAT GAG TCC TGA GTA A CTA-3′
Mse I + 3-CTC	M-CTC	5′-GAT GAG TCC TGA GTA A CTC-3′

Gel electrophoresis was carried out using Sequegen DNA sequencer (BIORAD™, USA). Following the amplification reaction, the PCR products were mixed with 8 μl of formamide stop/loading buffer and denatured at 94 °C for 5 min and then chilled immediately to 4 °C. Eight microliters of each reaction was loaded onto a 6 % denaturing PAGE. The gel was pre-run in 1X TBE, freshly prepared from a 10X TBE stock solution. The key electrophoresis parameters include voltage set at 1,200 V and temperature at 45 °C. The DNA bands were visualized using silver staining (Sigma Aldrich India Pvt. Ltd).

Statistical analysis

Genotyping data obtained for AFLP primer combinations was used for assessing the discriminatory power of AFLP primer combinations by evaluating: (i) Polymorphism information content (PIC), (ii) Gene diversity, (iii) Marker index (MI) and (iv) Resolving power (Rp). The PIC value and mean gene diversity for each AFLP primer combination was calculated as part of the summary statistics using software: Power Marker version 3.25 (Liu and Muse 2005) and was averaged over the fragments for each primer combination. The marker index was calculated as given in Varshney et al. (2007) as MI = PIC × EMR where, EMR is "the effective multiplex ratio (E) and is defined as the product of the total number of loci/fragments per primer (n) and the fraction of polymorphic loci/fragments (β) (E=n. β)". Resolving power of each primer was calculated according to Prevost and Wilkinson (1999) which is given as $Rp = \sum I_b$ where I_b represents the band informativeness. The I_b is represented into a 0–1 scale by following the formula: $I_b = 1 - [2^*|0.5 - P|]$ where, p is the proportion of the accessions containing the band/fragment.

Construction of phenogram

Each AFLP marker was treated as unit character and scored as a binary code (1/0). The results were analyzed using NTSYS-pc version 2.21c (Rohlf 2009) with SIMQUAL option on the basis of Jaccard's coefficient to generate similarity coefficients among all the possible pairs and ordered in similarity matrix (Jaccard 1908). The resulting matrices were subjected to clustering method by UPGMA (Sokal and Michener 1958). To find the robustness of the phenogram, bootstrapping was carried out (1,000 replicates) with Winboot software (Yap and Nelson 1996). The goodness of fit of the clustering to the data matrix was calculated by the COPH and MXCOMP programs. The data was also analyzed using multivariate method such as principal coordinate analysis (PCoA) to visualize the dispersion of individuals in relation to the first two principal co-ordinates.

AMOVA analysis

Percentage of molecular variance among 25 accessions based on geographical regions and different population groups was assessed using GENALEX 6 (Peakall and Smouse 2006). Statistical significance of variances was tested by random permutations with number of permutations set at 9,999.

Results

Marker attributes

A total of 1,612 fragments were generated using 12 primer combinations with an average 134 fragments/primer combinations. The details of markers attributes for different AFLP primers combinations are given in Table 3. The Polymorphic information content (PIC) values ranged from 0.138 to 0.209 with an average of 0.166 per fragment. In order to distinguish different primer combinations, the PIC values for all the polymorphic fragments generated by a primer combination were averaged to obtain an average PIC value for the corresponding primer combination. As a result, the highest PIC value (0.209) was observed for the primer combination E-ACT/M-CAT and the lowest (0.138) was recorded for the primer combination E-CAA/M-GCT. Mean gene diversity index for different primer combination varied from 0.160 to 0.253 with an average of 0.194 indicating good diversity among the presently studied accessions. Marker index (MI) as a feature of marker diversity was also calculated for all the primer combinations. The MI values ranged from 12.64 to 33.23 with an average of 22.43 per primer combination. Highest value (33.23) was scored with the primer pair for E-ACT/ M-CAT and the lowest values (12.64) for the primer pair E-CAC/M-GCT. A positive correlation was observed between MI and PIC values (r^2=0.69, p<0.005). The Resolving power, Rp is a feature that indicates the discriminatory potential of the marker. Rp ranged from 18.88 to 55.44 with an average of 33.53 per primer combination. Highest value (55.44) was scored with the primer combination E-ACT/ M-CAT and the lowest value (18.88) for E-CAC/ M-GCT. The Rp values were also positively correlated with MI (r^2=0.98, p<0.005).

Taxonomy and genetic diversity analysis

Genotyping data obtained for 12 primer combinations were used to estimate pairwise similarity among 25 accessions and genetic similarity matrix was calculated using Jaccard's similarity coefficient. Subsequently, this matrix was used to construct phenogram using unweighted pair group method with arithmetic average (UPGMA) algorithm with the help of NTSYS-pc software. The similarity coefficient value of the phenogram ranged from 0.087 to 0.332 suggesting broad genetic base among the studied accessions. The extent of variability was highest among wild accessions (var. *pruriens* + var. *hirsuta*) as similarity index is from 0.10 to 0.25 with mean value at 0.20, whereas for cultivated accessions (var. *utilis*) it ranged from 0.13 to 0.33 with mean value at 0.31.

The UPGMA dendrogram (Fig. 1) grouped 25 *Mucuna* accessions into two major clusters (Cluster I and Cluster II) based on their phenetic and genetic relationships. Cluster I consisted of all annual plants belonging to *M. pruriens* while cluster II distinctly grouped perennial accessions such as: *M. monosperma*, *M. atropurpurea*, *M. gigantea* and *M. bracteata*. Within cluster I, two sub-clusters IA and IB were formed of which cluster IA included all cultivated members belonging to *M. pruriens* var. *utilis* with a lone exception of 500113MH (var. *pruriens*), while cluster IB discretely separated other wild accessions of *M. pruriens* belonging to var. *pruriens* and var. *hirsuta*. The correlation of tree data with similarity coefficient data was tested through Mantel's test using MXCOMP subroutine of

Table 3 Marker attributes for different AFLP primer combinations

Primer combination	Gene diversity	PIC[a]	EMR[b]	MI[c]	Rp[d]
E-CAC/M-GCT	0.178	0.156	81.02	12.64	18.88
E-CAC/M-GCA	0.198	0.170	101	17.17	25.36
E-CAA/M-GCT	0.160	0.138	113	15.59	22.96
E-CAA/M-GCA	0.210	0.179	120	21.48	32.96
E-ACT/M-CAT	0.253	0.209	159	33.23	55.44
E-ACT/M-CAG	0.209	0.181	116	21.0	29.92
E-ACT/M-CTA	0.176	0.152	146	22.19	32.40
E-ACT/M-CTC	0.224	0.191	172	32.85	49.20
E-AAC/M-CAT	0.186	0.160	156	24.96	35.60
E-AAC/M-CAG	0.179	0.152	140	21.28	32.88
E-AAC/M-CTA	0.176	0.153	149	22.80	31.92
E-AAC/M-CTC	0.176	0.152	158	24.02	34.88
Maximum	**0.253**	**0.209**	**172**	**33.23**	**55.44**
Minimum	**0.160**	**0.138**	**81.02**	**12.64**	**18.88**
Average	**0.194**	**0.166**	**134.25**	**22.43**	**33.53**

[a] Polymorphic Information content (PIC)

[b] Effective Multiplex Ratio (EMR)

[c] Marker Index (MI)

[d] Resolving power (Rp)

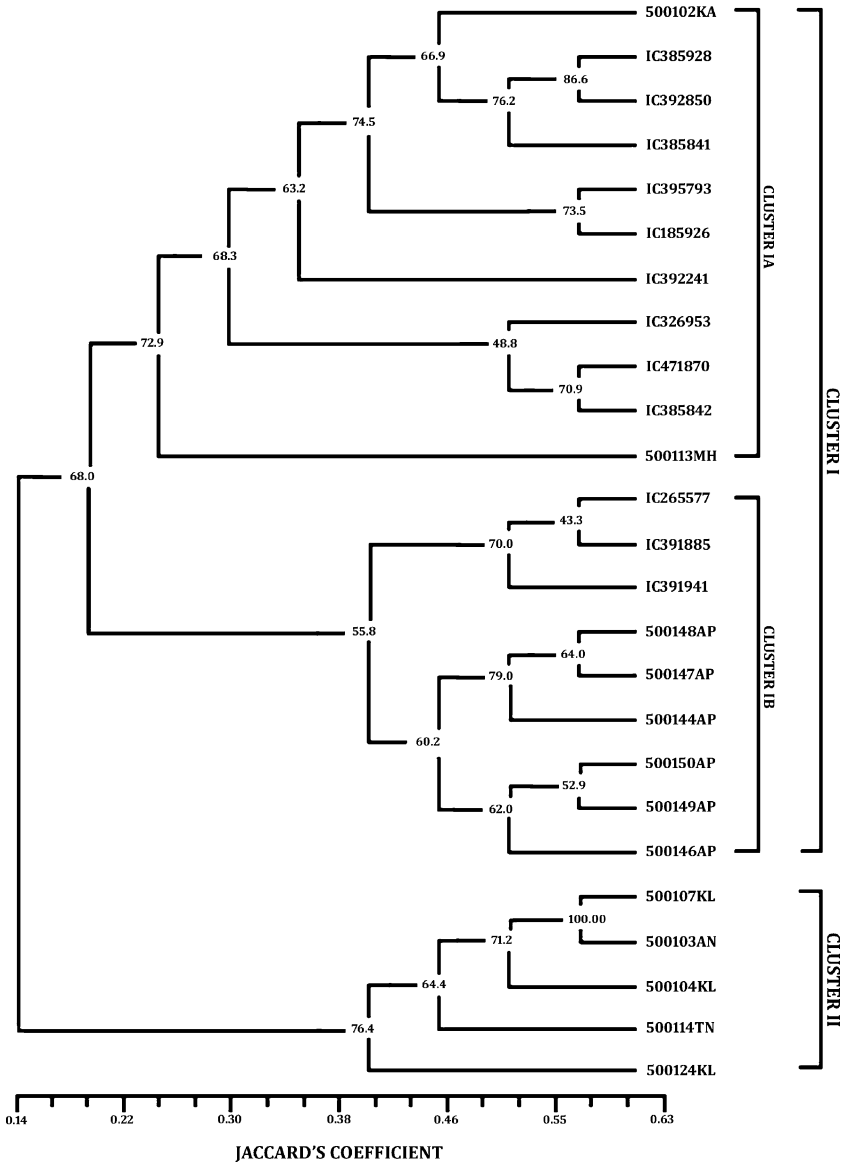

Fig. 1 UPGMA phenogram based on AFLP data from 12 primer combinations. The values on the nodes indicate bootstrap support values and the scale represents Jaccard's similarity coefficient values

NTSYS-pc program. High *r*-value of 0.92 suggested that the similarity data obtained for AFLP fragments is well represented by the cophenetic matrix generated from the tree data.

Genetic similarity matrix obtained on Jaccard's coefficient was also subjected to principal coordinate analysis. The distribution of 25 accessions among the four co-ordinates represented in a two-dimensional scatter plot (Fig. 2) was in consensus with the UPGMA tree. PCoA formed six groups of which four comprised annual plants of *M. pruriens* and other two contained perennial accessions.

AMOVA analysis

Analysis of molecular variation was carried out both on geographical regions as well as known population groups and the results are summarized in Table 4. Two population groups (cultivated and wild) were considered among annual members, while four population groups each representing one species (*M. atropurpurea*, *M. monosperma*, *M. gigantea* and *M. bracteata*) were deduced under perennial group. The percentage of variance was 26.5 % among different populations and 73.5 % within populations. High molecular variance of 91.33 % was observed for within populations and trivial differences were observed between populations of *M. pruriens* indicated by low molecular variance of 8.67 %. The AMOVA based on geographical locations was restricted only to *M. pruriens* accessions due to limited perennial accessions sampled. The results showed significant variation among the individuals of both between and within the regions. The molecular variance was found to be about 45.17 % for the individuals between different geographical

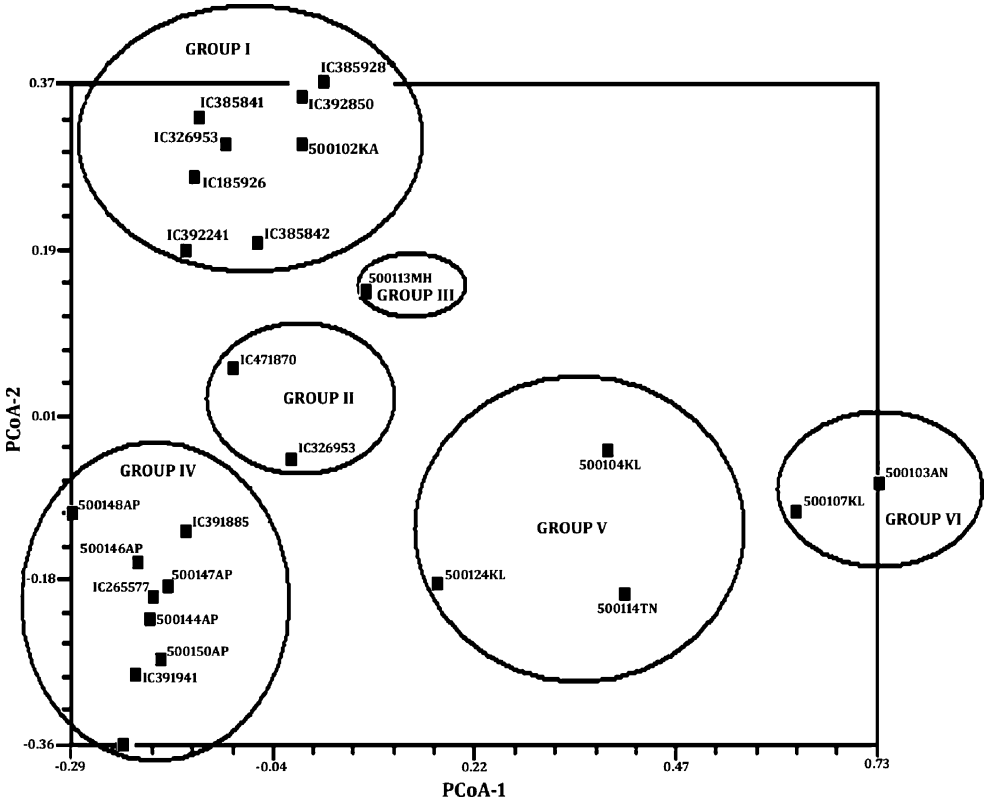

Fig. 2 PCoA plot of 25 *Mucuna* accessions based on AFLP data

regions and about 54.83 % for the individuals within different regions.

Discussion

Discriminating power of AFLP based on marker attributes

Marker attributes like PIC, MI and Rp have been used in several studies to assess the discriminatory power of AFLP markers for genetic diversity analysis (Powell et al. 1996; Bohn et al. 1999; Muminovic et al. 2004; Leela Tatikonda et al. 2009). In this first comprehensive study, we have analyzed these attributes for AFLP studies in *Mucuna* species. The PIC provides the estimate by taking into account not only the number of alleles, but also their relative frequencies. In the present study, PIC values ranged between 0.138 and 0.209, with mean at 0.166 and the distribution of PIC scores was nearly uniform (random) for all the polymorphic AFLP markers. This shows that most of these markers have high discrimination power. Another parameter, MI values ranged from 12.64 to 33.23 with mean at 22.43 and exhibited a positive correlation with PIC (r^2=0.69, p<0.005). The Resolving power, Rp which is an indicator of the discriminatory potential of the primer combination, ranged from 18.88 to 55.44 with an average of 33.53 per primer combination. Highest value (55.44) was scored with the primer combination E-ACT/ M-CAT and the lowest value (18.88) for E-CAC/ M-GCT. The Rp values were also found positively correlated with MI (r^2=0.98, p<0.005). Positive correlations observed between PIC/MI and MI/Rp suggests the usefulness of these parameters to select

Table 4 Summary of AMOVA results *Mucuna* accessions based on known population groups and geographical locations

Source of Variation	Df	SS	MS	Est. Var.	% Var.	PhiPT*
Geographical locations						
Individuals between regions	9	340.224	37.803	10.157	45.17 %	0.451
Individuals within regions	16	532.558	12.020	12.020	54.83 %	
Total	25			22.177	**100 %**	
Population groups						
Among populations	5	170.203	41.897	6.167	26.5 %	0.264
Within populations	22	371.300	16.877	16.844	73.5 %	
Total	27	541.503		23.011	**100 %**	

*PhiPT is based on 9,999 permutations across full data set

informative primer combination in future studies. Prevost and Wilkinson (1999) and Fernandez et al. (2002) observed a strong and linear relationship between the ability of a primer combination to distinguish accessions and Rp; considering this, the primer combination E-ACT/M-CAT from the present study should be the most informative primer combination for distinguishing the *Mucuna* accessions. Large number of polymorphic bands and the superior marker attributes observed in the present study suggest that AFLPs are highly discriminatory and powerful markers for classification, fingerprinting and diversity analysis in *Mucuna* species and can offer potential tool for genotyping large number of accessions in gene banks and mapping population.

Taxonomy and genetic diversity analysis

To understand the genetic diversity in the germplasm collection, Jaccard's similarity coefficients were calculated using genotyping data from all the twelve primer combinations. The similarity coefficients ranged from 0.087 to 0.332 with mean at 0.19 indicating good variability among the studied collection. Significant gene diversity was also revealed with mean diversity index varying from 0.160 to 0.253 for different primer combination. Within *M. pruriens*, wild accessions (comprising var. *pruriens* + var. *hirsuta*) were more diverse (SI-20 %) than the cultivated ones (var. *utilis*: SI-31 %). Padmesh et al. (2006) have reported similar findings for accessions collected from Western Ghats of Kerala. Narrow genetic base among the cultivated accessions revealed from both these studies emphasizes the need to broaden the genetic base of cultivated velvetbean accessions in India.

Cluster analysis based on UPGMA (Fig. 1) grouped 25 *Mucuna* accessions into three distinct groups. Cluster I separated all annual *M. pruriens* members while cluster II distinctly grouped perennial accessions. Within cluster I, accessions belonging to *M.pruriens* further sub-grouped based on their phenetic and genetic relationships. All the cultivated plants belonging to var. *utilis*—characterized by their small silky, non-itching trichomes on pods clustered in group-IA with a lone exception of 500113MH (possessing itching trichomes) with 72.9 % confidence interval limits. This accession might be naturalized hybrid of *pruriens* x *utilis* sharing more commonalities with var. *utilis* at genetic level. This is justified by the large phenotypic similarities the plant shared with var. *utilis* accessions (data not shown) except itching trichomes which was the main basis to classify it under var. *pruriens*. Presence of naturalized hybrids is not uncommon in *M. pruriens* as its varieties exhibit considerable crossability with each other. Special attention is needed while evaluating these accessions as some of them might harbor valuable recombination of interest in breeding program.

All the var. *pruriens* accessions along with those identified as var. *hirsuta* grouped in cluster IB with 68 % confidence interval limit. AFLP data did not discretely separate var. *hirsuta* from the typical variety (var. *pruriens*), probably due to very close genetic similarities between the two, although a sub-set of it collected from Andhra Pradesh grouped separately at 55.8 % confidence limits. Taxonomic status of the var. *hirsuta* is a matter of much debate. Many authors earlier have classified it as separate species as it reportedly differs from typical variety in characters such as shape & 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 (Baker 1876; Nair and Henry 1983; Ellis 1987; Saldanha 1996). However, according to widely referred review of Wilmot Dear (1987), the only character of it providing absolute distinction with *var. pruriens* is long, noticeable, dense, crisp indumentum as against fine indumentum in var. *pruriens* due to which he opines that: var. *hirsuta* can at best be maintained as botanical variety under *M. pruriens*. However, even to date many reports overlook this finer distinction and treat var. *pruriens* synonymously with var. *hirsuta*. The present study confirms very close genetic similarities between the two varieties even at genetic level as discussed elsewhere in this paper. In this background, 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 sub-groups viz., var. *utilis* (cultivated + non-itching trichomes on pod) and var. *pruriens* (wild + itching trichomes on pod) 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.

The high levels of polymorphism observed in otherwise self pollinated members of the genus *Mucuna* in the present study suggests broad genetic base among its different members, possibly due to accumulation of novel gene combinations in response to dynamic pressures of natural selection. The observation is in consistence with earlier reports (Capo-chichi et al. 2001; Padmesh et al. 2006). Attempts to resolve taxonomic status of *M. pruriens* by amplified ITS regions of nuclear DNA showed no variation between accessions (St. Laurent et al. 2002). Superiority of fingerprinting methods over ITS sequences for such a purpose, especially when the problem is pertaining to taxonomic group below the rank of species, has been well documented in number of earlier reports (Muir and Schlötterer 1999; St. Laurent et al. 2002; Padmesh et al. 2006). The present study clearly affirms the utility of AFLP technique for this purpose.

AMOVA analysis

The results of AMOVA based on known population groups showed high variation (91.33 %) among *M. pruriens* accessions but recorded low inter-varietal differences. Low variance in perennial populations is due to limited number of accessions sampled for this group. Molecular variance based on the geographical locations revealed significant variations for individuals of both within regions (54.83 %) as well as between regions (45.17 %). Within region variation may be due to a number of reasons. One of the important factors is domestication of diverse seed materials obtained through seed exchange programs resulting in a wide parental diversity within a region. Likewise between regions variation may have been caused due to differences in genetic resources exploited in breeding and varietal production programmes in different regions. However, further studies must be conducted, employing larger samplings of germplasm collections to authentically verify the divergence of *Mucuna* gene pool.

Conclusion

In conclusion, AFLP technology was used to investigate the genetic diversity and taxonomic relationships among 25 *Mucuna* accessions belonging to five species. Total genetic diversity within *M. pruriens* germplasm used in the present study was considerably high suggesting their utility in breeding programs; however narrow genetic base among the cultivated accessions emphasizes the need for broadening the same. Taxonomically, close genetic similarities between var. *pruriens* and var. *hirsuta* of *M.pruriens* demand consideration on merging these two botanical varieties under one name var. *pruriens*. Limited sample size used in this study however restricts the relevance of analysis and the reliability of results for more generalized conclusions on the diversity levels in *Mucuna*. Further studies should therefore be carried out using larger samples derived from more extended geographical regions to classify the general attitude of *Mucuna* genetic variation and define valuable germplasm for the improvement of this multi-potential crop. In general, considering the high polymorphism and superior marker attributes revealed by AFLP, the technique is recommended for *Mucuna* genetic studies and for the evaluation and identification of elite germplasm.

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