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RESEARCH ARTICLE

Assessment of genetic diversity in *Mucuna* species of India using randomly amplified polymorphic DNA and inter simple sequence repeat markers

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Abstract Genus *Mucuna* which is native to China and Eastern India comprises of perennial climbing legume with long slender branches, trifoliate leaves and bear green or brown pod covered with soft or rigid hairs that cause intense irritation. The plants of this genus are agronomically and economically important and commercially cultivated in India, China and other regions of the world. The high degrees of taxonomical confusions exist in *Mucuna* species that make authentic identification and classification difficult. In the present study, the genetic diversity among the 59 accessions of six species and three varieties of *M. pruriens* has been assessed using DNA fingerprinting based molecular markers techniques namely randomly amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR) and combined dataset of RAPD and ISSR. Also, genetic relationship among two endemic species of *Mucuna* namely *M. imbricata* and *M. macrocarpa* and two varieties namely IIHR hybrid (MHR) and Dhanwantari (MD) with other species under study was investigated by using cluster analysis and principal coordinate analysis. The cluster analysis of RAPD, ISSR and combined dataset of RAPD and ISSR clearly demonstrated the existence of high interspecific variation than intra-specific variation in genus *Mucuna*. The utility and efficacy of RAPD and ISSR for the study of intra species and interspecies genetic diversity was evident from AMOVA and PCoA analysis. This study demonstrates the genetic diversity in *Mucuna* species and indicates that these markers could be successfully used to assess genetic variation among the accessions of *Mucuna* species.

Keywords DNA fingerprinting · Genetic diversity · ISSR · *Mucuna* · RAPD

Introduction

Mucuna is an important plant genus of family *Leguminosae* that contains 100 species distributed throughout tropical and subtropical regions of the world (Lackey 1981; Mabberley 2005). This genus is native to China and Eastern India from where its cultivation spreads to other regions of the world creating newer populations (Wilmot-Dear 1987). The plants of *Mucuna* are perennial climbing legume with long slender branches, trifoliate leaves and produce either clustered or long inflorescence. The flowers are white, pale yellow or purple in color. They bear green, brown pod which are covered with soft or rigid hairs causing intense irritation (Leelambika and Sathyanarayan 2011). The plants of *Mucuna* thrive well in dry farming and low soil fertility conditions and exhibit resistance against wide ranging diseases (Buckles 1995; Jorge et al. 2007; Siddhuraju et al. 2000). Various species of *Mucuna* are reported with many versatile medicinal and agronomic applications to humankind (Patil et al. 2015). *Mucuna pruriens* (Velvet bean), one

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of the most studied members of *Mucuna,* exhibits good agronomic potentials. The yield of velvet bean seed may go up to 2.4 t/ha/year; biomass and dry mass accumulation of 20–30 t and 7–9 t/ha/year (Buckles 1995; Jorge et al. 2007). Its seeds may contain as high as 20–30 % proteins (Siddhuraju et al. 2000). The study of proteins profile of *Mucuna* showed that it mainly contains albumins, glutelins and globulins with prolamins in trace concentration (Adebowale et al. 2007). Extensive studies on its nutritional and anti-nutritional properties with different processing effects also have been accomplished (Bhat et al. 2007). Seeds of *M. pruriens* contain precursor of neurotransmitter dopamine, L-DOPA (L-3, 4-dihydroxyphenylalanine) which is extensively used in management of Parkinson's disease. These seeds are also widely used against snakebite, uterine stimulant and as an aphrodisiac in traditional system of medicine predominantly in India and West Africa (Siddhuraju et al. 1996; Aguiyi et al. 1999; Amin et al. 1996; Lorenzetti et al. 1998). The alkaloids like prurienine, prurieninine, prurienidine and 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acids (Tic) have been reported from *Mucuna pruriens*. Tic shows selectivity towards μ-opioid receptors for peptide hormones and neurotransmitters (Misra and Wagner 2004). In vitro antioxidant (Tripathi and Upadhyay 2001) and anti-fungal activity have also been described (Adebowale et al. 2007, 2011).

From Indian subcontinent, nine species and three varieties of *Mucuna* were reported by Wilmot-Dear (1984, 1991). Further, a new variety of *M. pruriens; M. pruriens* (L.) DC. var. *thekkadiensis* from Kerala state of India (Thothathri and Ravikumar 1997) and one new species *M. sanjappae* have been documented (Aitawade and Yadav 2012). Consequently, at present, the genus is represented by ten species in which one species *M. pruriens* has four different varieties in India. For effective economic utilization of plants of important genus like *Mucuna*, availability of information regarding intra and interspecies diversity and variability, phylogenetic relationship between and among the species, availability of germplasm and breeding behaviour are prerequesites. Lack of such primary information has seriously constrained the effective utilization of *Mucuna* genetic resources in India. In the view of lack of this information, intra and interspecies diversity and variability among six species and three varieties of *Mucuna* was investigated. The objectives of present study was to evaluate the genetic diversity among the different accessions of six species and three varieties of *Mucuna* by using molecular markers viz. randomly amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR). In addition, how two rare and endemic species of *Mucuna* namely *M. imbricata* and *M. macrocarpa* and two varieties namely IIHR hybrid (MHR) and Dhanwantari (MD) are genetically related to other investigated species is also studied.

Materials and methods

Collection of plant material and DNA extraction

Young and healthy leaves of 59 *Mucuna* accessions from different locations were collected for the study. Most of the plant material was collected from south western parts of India and along the Western Ghats of India, whereas *M. macrocarpa* and *M. imbricata* were collected from the North Eastern India (Table 1; Fig. 1).

For extraction of total genomic DNA, Cetyl Trimethyl Ammonium Bromide (CTAB) method (Doyle and Doyle 1987) was used. Finally, DNA was solvated in 100 μl of sterile double distilled water (SDDW), quantified spectrophotometrically and stored at cold condition till further use. The quality of DNA was also checked by agarose gel electrophoresis.

PCR amplification of RAPD

Initially, 50 decamer oligonucleotide primers from Bangalore Genei Kit (Merck Bioscience Pvt. Ltd, Bangalore, India) were screened. The 25 μl reaction contained 40 ng of template DNA, 2.5 μl of 10× buffer A (Merck Bioscience Pvt. Ltd, Bangalore, India), 100 μM of each of dNTP's (Merck Bioscience Pvt. Ltd, Bangalore, India), 1 mM $MgCl_2$ (Merck Bioscience Pvt. Ltd, Bangalore, India), 1.2 U of *Taq* polymerase (Merck Bioscience Pvt. Ltd, Bangalore, India), 100 pmol of primer and 12.5 μl SDDW. Amplification reactions were carried out on C1000 Touch Thermal Cycler (Biorad, India).

PCR amplification of ISSR

At first, 50 ISSR primers (UBC 801-850) from UBC primer set 9 (University of British Columbia, Canada) were screened. ISSR-PCR amplification was achieved in 25 μl reactions containing 40 ng of template DNA, 1× PCR Buffer A (Merck Bioscience Pvt. Ltd, Bangalore, India), 2 mM $MgCl_2$ (Merck Bioscience Pvt. Ltd, Bangalore, India), 100 μM of each dNTP (Merck Bioscience Pvt. Ltd, Bangalore, India), 10 pmol primers and 2 U of *Taq* DNA polymerase (Merck Bioscience Pvt. Ltd, Bangalore, India). Amplification reactions were conducted on C1000 Touch Thermal Cycler (Biorad, India).

Agarose gel electrophoresis and data analysis

Amplified RAPD and ISSR fragments were separated using agarose gel (1.8 %) electrophoresis. Electrophoresis was done at 80 V for 4 h in 1xTAE, stained with dye ethidium bromide. The gel images were viewed and documented on G: Box gel imaging system (Syngene

Table 1 List of species, varieties and hybrids of *Mucuna*, their accession codes and number of accessions investigated in this study

Sr. no.	Name of species	Accession code	Location	Number of accessions
1	*Mucuna pruriens* var. *Pruriens (L.) DC*	MPP	Karnataka (sutagati Ghat, Jog fall); Maharashtra (Amboli, Ajara, Durgawadi, Gaganbavada, Junnar; Kinwat, Kolhapur, Palasambe, Tillari)	18
2	*Mucuna monosperma* Wight	MM	Karnataka (Jog fall); Maharashtra (Amboli, Gaganbavada, Palasambe)	16
3	*Mucuna sanjappae* Aitawade and S. R. Yadav	MS	Maharashtra (Junnar, Durgawadi)	9
4	*Mucuna pruriens* var. *utilis* (Wall.ex Wight) L. H. Bailey	MPU	Assam, West Bengal (Darjeeling); Karnataka (Tumkur); Maharashtra (Nanded, Kolhapur)	4
5	*Mucuna gigantea* (Willd.) DC	MG	Andaman, Maharashtra (Kolhapur)	3
6	*Mucuna atropurpurea* (Roxb.) Wight and Arn.	MA	Karnataka (Tumkur), Tamilnadu (Naraikada)	3
7	*Mucuna pruriens* var. *hirsuta (Wight and Arn.)* Wilmot-Dear	MPH	Karnataka (Jog fall)	2
8	*Mucuna imbricata Baker*	MI	West Bengal (Buxa tiger reserve forest)	1
9	*Mucuna macrocarpa Wall.*	MK	West Bengal-Darjeeling	1
10	IIHR Hybrid	MHR	Indian Institute of Horticultural Research (IIHR) Bangalore	1
11	Dhanwantari	MD	Indian Institute of Horticultural Research (IIHR) Bangalore	1

Bioimaging Pvt. Ltd, India). The band size was calculated using 100 bp DNA ladder. The polymorphic bands in primer profile were scored using GeneTool image analysis software (Syngene Bioimaging Pvt. Ltd, India). To ensure reproducibility, DNA amplified profiles with ISSR and RAPD markers were tested for repeatability at least thrice. This was done by repeating the PCR reactions on two different thermal cyclers. Clear and intense bands were considered for scoring. The presence or absence of band for specific primer was recorded as 1 or 0 respectively to generate binary data matrix. This data was used to generate similarity coefficients (SE) which were further used to build a dendrogram by UPGMA (Unweighted Pair Group Method of Arithmetic average) and cluster analysis using the NTSYS pc 2.02e (Numerical System, Applied Biostatistics, Inc., New York, USA) computer programme according to the method proposed by Nei and Li (1979). Polymorphism percentage (P%), polymorphism information content (PIC), major band frequency and gene diversity were estimated using Power Marker (Liu and Muse 2005). For estimating the diversity among and within the *Mucuna* species, a nonparametric analysis of molecular variance (AMOVA) (Excoffier et al. 1992) was performed using GenAlEx (Peakall and Smouse 2006; Table 3). Assessment of the genetic relatedness among the tested species, varieties and hybrids, PCoA was also performed using GenAlEx. For PCoA, covariance matrix of pairwise species PhiPT values with data standardization was used as input to generate two-dimensional PCoA plot.

Results

Genetic diversity in the 59 accessions of six *Mucuna* species and three varieties collected from different locations in India was assessed by using RAPD, ISSR and combined dataset of RAPD and ISSR.

RAPD banding pattern

At first, a preliminary screening of 24 individual specimens representing all six species and three varieties was done with 30 RAPD primers to obtain reproducible polymorphic and distinguishable banding patterns. Of these 30 RAPD primers screened, 16 primers amplified clear and reproducible profiles with highly informative polymorphic banding pattern (Table 2). In total, RAPD fingerprinting produced 266 clear, reproducible fragments and all were polymorphic. Mean of 16.62 fragments per primer was formed. The size range of the amplified fragments was 0.3–3.6 kb. Total number of bands, polymorphic bands, percentage of polymorphism (P%), polymorphism information content (PIC), major band frequency, gene diversity is shown in Table 2. The number of polymorphic bands obtained ranged from 11 (RPI 1) to 25 (RPI 18). The percentage of polymorphism for each marker was 100 %. In the RAPD analysis, PIC which is a measure of marker for detecting polymorphism within species, ranged from 0.63 (RPI 9) to 0.86 (RPI 6) indicating primer RPI 9 as a least and most polymorphic respectively. The gene diversity ranged from 0.66 (RPI 9) to 0.87 (RPI 6) whereas

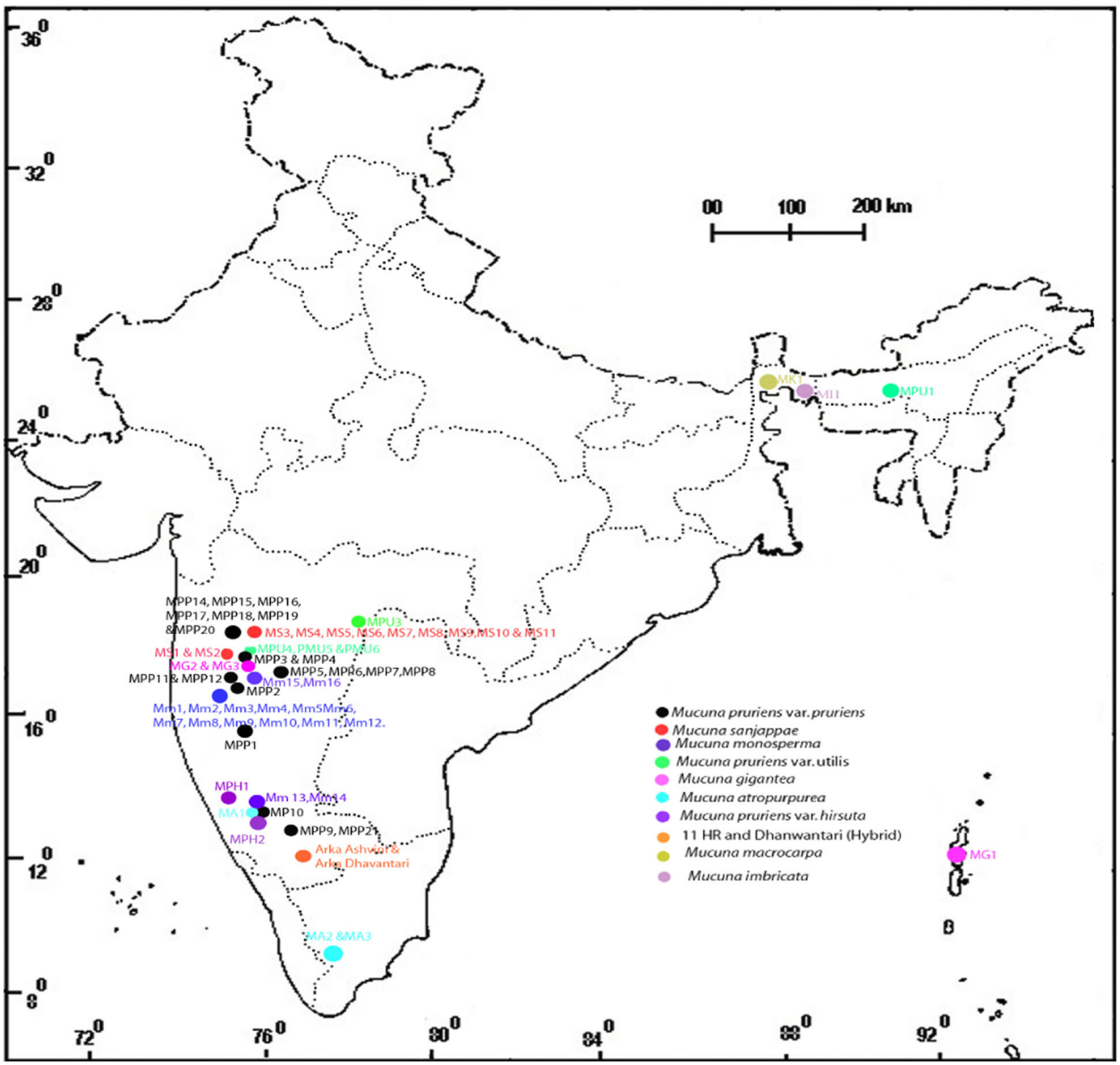

Fig. 1 Geographical locations in India from where the accessions of different *Mucuna* species were collected (the accessions codes indicate name of the species as represented in Table 1)

major band frequency ranged from 0.16 (RPI 6) to 0.25 (RPI 9). The mean polymorphism information, mean gene diversity and mean major band frequency were 0.81, 0.83, and 0.24 respectively (Table 2).

ISSR banding pattern

For the amplification of ISSR markers from tested accessions of *Mucuna* species, initial screening was performed with first 50 ISSR primers (UBC 801-850) from UBC primer set ≠ 9. Only 11 primers resulted amplification and remaining could not amplify consistently and reproducibly. Of these 11 primers, five were used further because of their ability to amplify clear, reproducible and polymorphic ISSR profile. The ISSR profile was greatly depended on annealing temperature and also affects the efficiency of amplification. The difference between the optimum annealing temperature and the theoretical melting temperature (Tm) ranged from 18 to 4 °C. Five ISSR primers (Table 2) that generated polymorphic banding patterns were subsequently used to amplify ISSR fragments from all accessions of *Mucuna* species and generated 104 bands with 20.8 bands per primer. The amplified fragments ranged from 0.25 to 3.5 kb. The number of polymorphic bands generated ranged from 18 (UBC 807) to 23 (UBC 826).

Table 2 List of RAPD and ISSR primers, their annealing temperatures and polymorphism obtained (no. of total bands, no. of polymorphic bands, major band frequency, gene diversity and PIC)

Marker	Optimum temperature (°C)	No. of total/polymorphic bands	Major band frequency	Gene diversity	PIC
RPI-1	36	11	0.237	0.837	0.816
RPI-2	38	18	0.254	0.842	0.824
RPI-5	36	15	0.305	0.807	0.783
RPI-6	38	16	0.169	0.878	0.865
RPI-7	38	21	0.203	0.879	0.868
RPI-8	37	16	0.237	0.830	0.809
RPI-9	38	13	0.525	0.667	0.634
RPI-10	38	19	0.203	0.854	0.837
RPI-11	37	13	0.186	0.845	0.825
RPI-12	38	19	0.254	0.852	0.836
RPI-13	38	17	0.288	0.796	0.767
RPI-14	37	14	0.254	0.808	0.780
RPI-15	38	17	0.237	0.824	0.801
RPI-18	38	25	0.186	0.877	0.865
RPI-19	37	16	0.203	0.860	0.844
RPI-22	38	16	0.220	0.873	0.860
ISSR-807	52.7	18	0.152	0.878	0.865
ISSR-826	46	23	0.288	0.800	0.773
ISSR-847	46	19	0.322	0.799	0.772
ISSR-848	56	22	0.252	0.802	0.772
ISSR-849	52	22	0.266	0.812	0.782
Mean		17.61	0.249	0.829	0.808

Similar to RAPD profile, the percentage of polymorphism for each ISSR marker was 100 %. The analysis of ISSR profile indicated that PIC ranged from 0.77 (UBC 847 and 848) to 0.86 (UBC 807) indicating primer UBC 847 and 848 as the least polymorphic and UBC 807 as the most polymorphic. Gene diversity varied in the range from 0.79 (UBC 847) to 0.87 (UBC 807), and major band frequency ranged from 0.15 (UBC 807) to 0.32 (UBC 847). The mean PIC, mean gene diversity and mean major band frequency were 0.79, 0.81, and 0.25 respectively (Table 2).

Clustering based on RAPD

The dendrogram based on UPGMA analysis of the RAPD data of 59 accessions of *Mucuna* species is given in Fig. 2. The 0.17–0.96 range of similarity coefficients indicates a good level of genetic diversity in the accession of *Mucuna* species sampled. The 59 accessions were grouped into five clusters. The first major Cluster I comprised of 15 of 16 accessions of *M. monosperma* and one accession of *M. pruriens* var. *pruriens* (MPP-13). The second major cluster grouped together 15 of 18 accessions of *M. pruriens* var. *pruriens* whereas cluster III emerged as single species cluster that grouped together all 9 accessions of *M. sanjappae*. Two accessions of *M. pruriens* var. *pruriens* (MPP-9 and MPP-21) were not clustered with any of the cluster formed. These two accessions were collected from Tumkur district of Karnataka which shows some distinct morphological differences from remaining accessions of *Mucuna pruriens* var. pruriens (MPP). For example, the shape of pod which is not exactly S-shaped (as found in other MPP accessions), only tip is curved, covered with black colored hairs (rather than brown color hairs). The cluster IV was also multispecies clustered that grouped together four *M. pruriens* var. *utilis* accessions, two *M. pruriens* var. *hirsuta* accessions and single accession each of *M. imbricata*, IIHR hybrid and Dhanwantari. The cluster V was also multispecies and grouped together three accessions of each of *M. atropurpurea*, *M. gigantea* and single accession of *M. macrocarpa*. Based on RAPD, it was observed that sampled single acceesions of Dhanwantari, *M. imbricata* and IIHR hybrid were genetically closely related to *M. pruriens* var. *utilis* and *M. pruriens* var. *hirsuta* since they were clustered together with the accession of these two sampled species. Likewise, *M. macrocarpa* was found genetically close to *M. atropurpurea* and *M. gigantea* (Fig. 2).

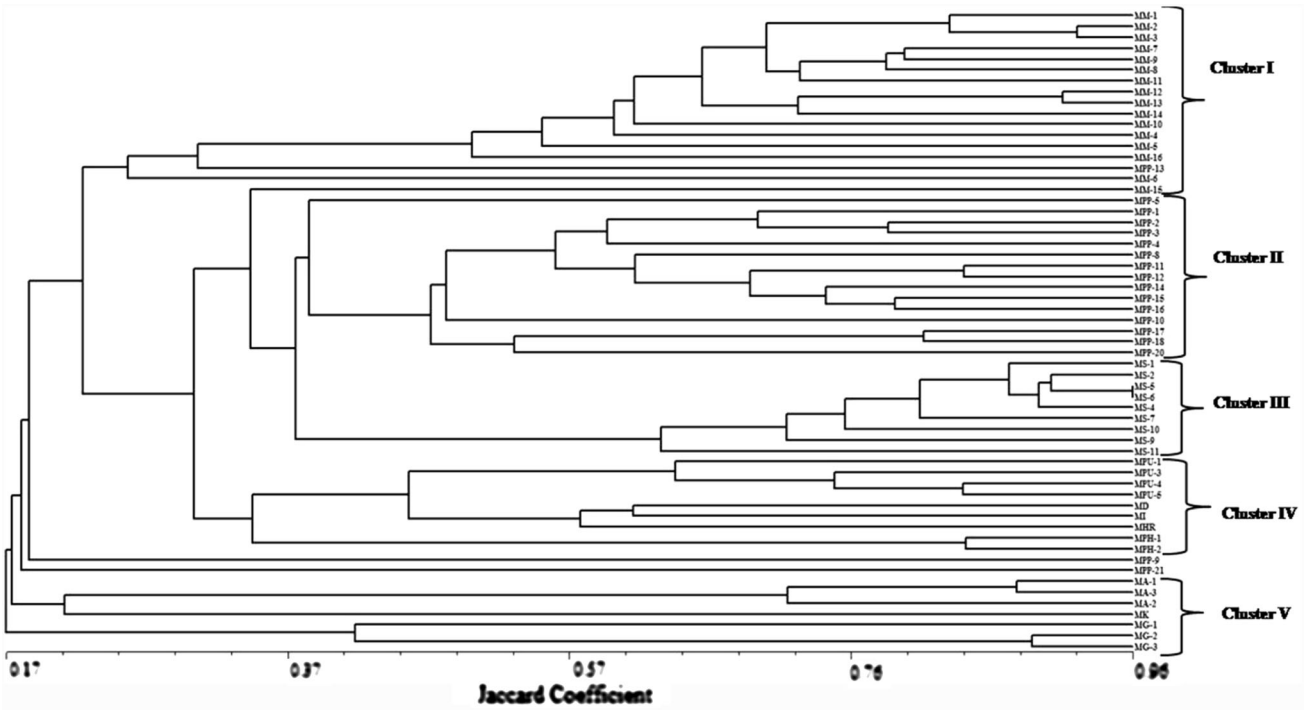

Fig. 2 RAPD based Unweighted Pair Group Method with Arithmetic average (UPGMA) dendrogram of *Mucuna* species accessions. The codes in the figure correspond to the codes listed in Table 1 and Fig. 1

Clustering based on ISSR

The grouping of 59 accessions of *Mucuna* species in the dendrogram based on ISSR was similar to dendrogram based on RAPD and clustered tested accessions into five clusters (Fig. 3). Major cluster I grouped together all the 16 accessions of *M. monosperma* whereas, cluster II comprised of all 9 tested accessions of *M. sanjappae*. Both of these clusters, I and II emerged as comprised of all tested accessions of single species *M. monosperma* and *M. sanjappae* respectively. Similarly, cluster III also emerged as single species cluster and comprised of most (15) of the tested accessions of the *M. pruriens* var. *pruriens*. Similar to the clustering based on RAPD, two accessions of *M. pruriens* var. *pruriens* (MPP-9 and MPP-21) were not clustered with any of the cluster formed. Small cluster IV comprised the four *M. pruriens* var. *utilis* accessions, two *M. pruriens* var. *hirsuta* accessions and single accession of *M. macrocarpa*. The grouping in cluster V based on ISSR was similar to grouping in cluster IV based on RAPD. Tested single acceesion of *M. imbricata* and two hybrids Dhanwantari, and IIHR hybrid were clustered together with *M. gigantea* and *M. atropurpurea* indicating their genetically close relationship (Fig. 3).

Clustering based on RAPD and ISSR

To make more robust results, the combined dataset of RAPD and ISSR markers was also used to construct dendrogram and analyzed (Fig. 4). Unlike dendrogram based on individual dataset of RAPD and ISSR, dendrogram based on combined dataset formed six clusters. Additional one cluster was formed because accession of *M. pruriens* var. *utilis* and *M. pruriens* var. *hirsuta* which were grouped together in cluster IV of both, RAPD and ISSR, here formed separate clusters. Four accessions of *M. pruriens* var. *utilis* were observed to cluster together in a small cluster III whereas, two accession of *M. pruriens* var. *hirsuta* formed separate cluster V together with single accessions of Dhanwantari, *M. imbricata* and IIHR hybrid. Clustering of accessions of remaining species was in congruence with clustering in dendrograms based on individual dataset of RAPD or ISSR. 16 of 18 accessions of *M. monosperma* were grouped together in major cluster I, 15 of 16 accession of *M. pruriens* var. *pruriens* were grouped together in cluster II and all 9 accessions of *M. sanjappae* were grouped together in cluster IV. Similar to cluster V in dendrogram based on RAPD, cluster VI in dendrogram based on combined dataset of RAPD and ISSR comprised of three accessions of *M. atropurpurea,* three accessions of

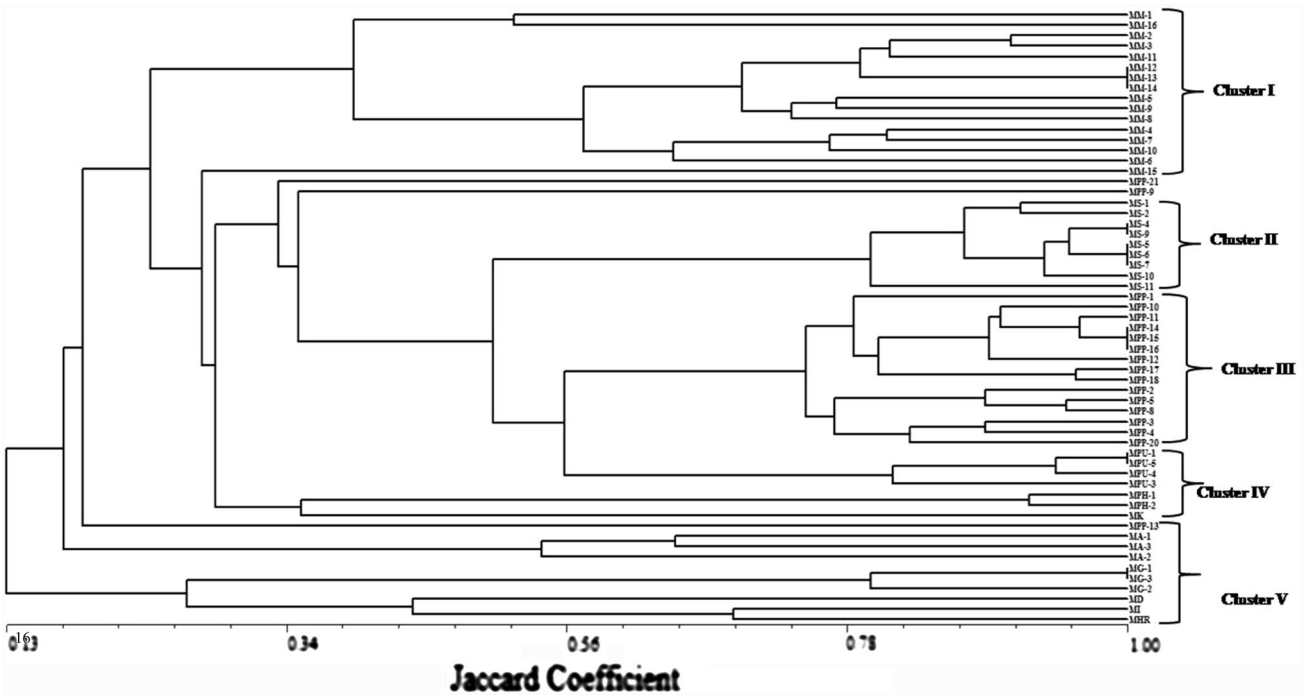

Fig. 3 ISSR based Unweighted Pair Group Method with Arithmetic average (UPGMA) dendrogram of *Mucuna* species accessions. The codes in the figure correspond to the codes listed in Table 1 and Fig. 1

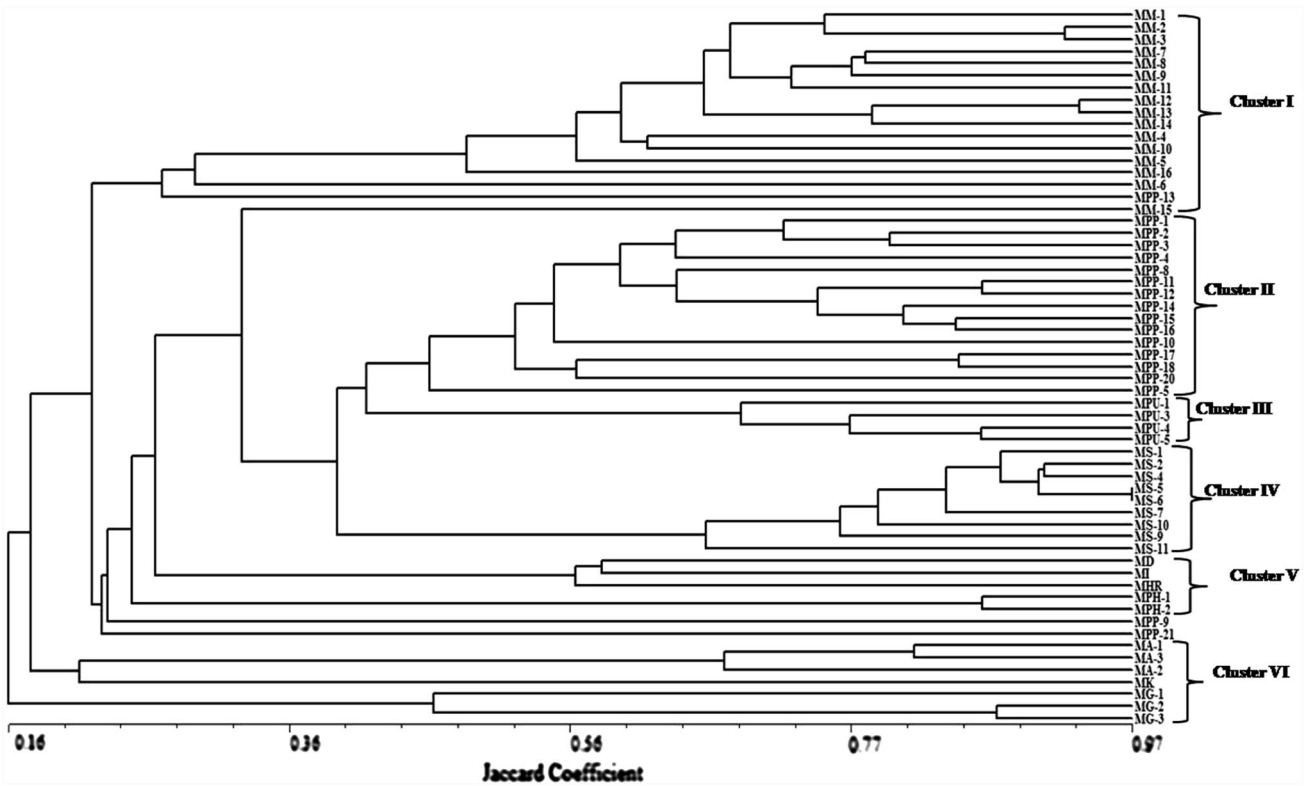

Fig. 4 Combined data of RAPD and ISSR based Unweighted Pair Group Method with Arithmetic average (UPGMA) dendrogram of *Mucuna* species accessions based on combination of RAPD and ISSR data. The codes in the figure correspond to the codes listed in Table 1 and Fig. 1

M. gigantea and single accession of *M. macrocarpa* (Fig. 4).

Analysis of molecular variance and PCoA

The data matrix generated by RAPD, ISSR and combined dataset of RAPD and ISSR was used to generate non-parametric analysis of molecular variance (AMOVA) (Table 3). The variation percentage among the species was 56, 68 and 62 % and within the species was 44, 32 and 38 % based on RAPD, ISSR and combined data of RAPD and ISSR, respectively. Our results demonstrate that the inter species variation is higher than intra species. Such variance among species was more in ISSR (Table 3), signifying that ISSR is a superior marker for studying diversity among *Mucuna* species than RAPD markers or combined data of RAPD and ISSR.

In PCoA, the grouping of species closely corresponded to the clustering obtained in UPGMA clustering. It was observed that *M. macrocarpa*, *M. imbricata*, *M. gigantea* and *M. monosperma* were genetically closely related. Likewise, two varieties IIHR hybrid and Dhanwantari were genetically more close to *M. pruriens* var. *pruriens* than other species under study. *M. pruriens* var. *hirsuta*, *M. pruriens* var. *utilis*, *M. atropurpurea* and *M. sanjappae* did not group together and were found genetically far from each other (Fig. 5).

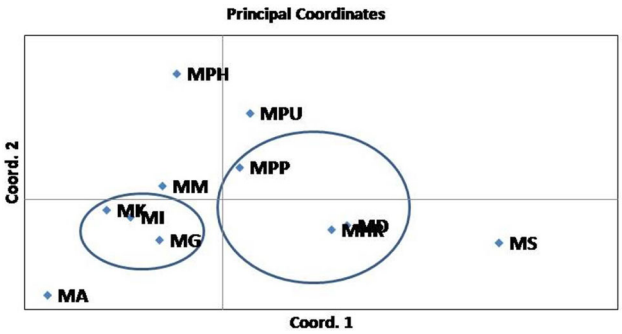

Fig. 5 Two-dimensional plot of principal coordinate analysis (PCoA) based on combined dataset of RAPD and ISSR generated from 59 accessions of *Mucuna* species. The codes in the figure correspond to the codes listed in Table 1 and Fig. 1

Discussion

The economic utilization of plants of genus like *Mucuna* is greatly hampered owing to unavailability of information on intra and interspecies diversity, variability and phylogeny. High degree of taxonomical confusion in *Mucuna* species further adds the difficulty in authentic identification and classification. Based on morphometric characteristics, a lot of ambiguities arise for proper identification of *Mucuna* species or varieties. In many literatures, name var. *hirsuta* and var. *pruriens* are used interchangeably (M. Leelambika et al. 2010). Moreover, few *Mucuna* species such as, *M. macrocarpa* and *M. imbricata* are endemic and restricted to certain geographical regions like North Eastern parts of India, and make it difficult to collect enough number of specimen for the assessment of genetic diversity. In the view of such confusions and ambiguities, we investigated the genetic diversity of *Mucuna* using molecular biology tools. For the present study, specimen for six *Mucuna* species, three varieties of *M. pruriens* and two hybrids cultivars (MHR and MD) were studied. Except for *M. imbricata* and *M. macrocarpa* which are endemic and restricted to specific area, we could collect specimen from minimum three accessions (Table 1; Fig. 1).

For this study, we relied on DNA-based markers, RAPD and ISSR, because they provide several advantages over traditional morpho-biochemical markers. DNA markers are not affected by the environmental factors, have low cost per assay, require lower level of skill, and the primers are readily available that allow the scanning of the entire genome and efficient genotype characterization. Thus, RAPD and ISSR have characteristics higher efficiency for detecting polymorphisms and intra or inter-specific genetic diversity. Recently, these markers were successfully used

Table 3 Analysis of molecular variance (AMOVA) for the accessions of *Mucuna* species based on RAPD, ISSR and combined dataset of RAPD and ISSR

Marker	Among Species					Within Species					PhiPT
	DF	SS	MS	Variation	% of total variation (%)	DF	SS	MS	Variation	% of total variation (%)	
RAPD	10	1309.6	130.9	19.03	56	56	1360.840	24.301	24.301	44	0.439
ISSR	10	473.8	47.3	7.80	68	56	203.944	3.642	3.642	32	0.682
RAPD + ISSR	10	1783.5	178.3	26.84	62	56	1564.785	27.943	27.943	38	0.620

DF degree of freedom (n − 1), *SS* sum of squares, *MS* mean of square, PhiPT = VAP/(VAP + VWP) (where, *VAP* variance among the species and *VWP* variance within the species)

to calculate the intra or inter-specific genetic diversity in different domestic and wild species (Escandón et al. 2005; Muthusamy et al. 2008; Li et al. 2011).

Present study demonstrates the efficacy of RAPD and ISSR markers for the analysis of genetic diversity in *Mucuna* species and indicates that these markers could be successfully used to assess genetic variation among the accessions of *Mucuna* species. In all tested accessions, both the marker systems yielded 100 % polymorphism, however, in terms of average number of bands produced per primer, RAPD was found better suited than ISSR. Likewise, PIC and gene diversity revealed by RAPD was slightly higher than ISSR. The extent of polymorphisms revealed by both markers was comparable since the similarity indices based on Jaccard's similarity coefficient matrix were almost similar. This was also evident from results obtained from the cluster analysis. The patterns of grouping of *Mucuna* species accessions in cluster analysis based on RAPD and ISSR were also comparable and in congruence with each other. Both of these markers grouped 59 accessions into five clusters, many of which grouped together majority of tested accessions of single species. RAPD clustered majority of *M. monosperma*, *M. pruriens* var. *pruriens* and *M. sanjappae* accessions into cluster I, II and III respectively, whereas ISSR also clustered majority of accessions of these species into clusters I, III and II respectively. Thus, the clustering patterns generated by both the marker systems did not vary greatly. However, resolution of accessions into clusters was further improved when cluster analysis was performed on combined dataset of RAPD and ISSR. This analysis grouped tested accessions in six clusters, majority of which also grouped all tested accessions of single species. With very few exceptions, cluster I, II, III and IV grouped together accessions of species of *M. monosperma, M. pruriens* var. *pruriens* and *M. pruriens* var. *utilis* and *M. sanjappae* respectively. Among these clusters, few were supported by morphological characters as well. Accessions of species grouped in cluster II, III and IV shared morphological characters like annual nature of habit, small sized, 1–8 seeded pods covered with highly irritating hairs (excepts for *M. pruriens* var. *utilis*), whereas, cluster I grouped accession of species that show perennial nature of habit with broad sized, 1–4 seeded pods covering less irritating hairs. Surprisingly, two of *M. pruriens* var. *pruriens* (MPP-9 and MPP-21) accessions were not clustered with any of the cluster formed in the RAPD, ISSR and combined dataset of RAPD and ISSR. These two accessions were collected from Tumkur district of Karnataka which shows some distinct morphological differences from remaining accessions of *Mucuna pruriens* var. *pruriens* (MPP). For example, the shape of pod which is not exactly S-shaped (as found in other MPP accessions), only tip is curved, covered with black colored hairs (rather than brown color hairs). This pattern of grouping indicated the high utility of RAPD and ISSR for revealing the polymorphism and genetic diversity in *Mucuna* accession. The cluster analysis clearly demonstrated the existence of high interspecific variation than intra specific variation in genus *Mucuna*. The utility and efficacy of RAPD and ISSR for the study of intra species and interspecies genetic diversity in *Mucuna* species was also evident from AMOVA and PCoA analysis. The AMOVA indicated that genetic diversity within the species was comparatively lower than among the species. Both of these markers and their combined dataset revealed higher among the species (inter species) variation than within the species (intra species) variation. However, in comparison between the two marker systems used, ISSR revealed higher among the species variation than RAPD (Table 3), whereas RAPD was found better suited for revealing genetic diversity within the species.

In the present study, we also studied the genetic relationship of *M. macrocarpa*, *M. imbricata* and two hybrids namely IIHR hybrid and Dhanwantari with other tested species. Cluster analysis based on combined dataset indicated that *M. macrocarpa* was genetically close to *M. atropurpurea* and *M. gigantea* whereas; *M. imbricata* and hybrids IIHR hybrid and Dhanwantari were genetically more close to two varieties of *M. pruriens* namely var. *pruriens* and var. *hirsuta* (Fig. 4). To confirm this observation, PCoA was performed on combined dataset of RAPD and ISSR (Fig. 5). According to our literature survey, the parentage of IIHR hybrid and Dhanwantari is *Mucuna pruriens.* The grouping in PCoA was also comparable and in congruence with this observation. In PCoA, it was found that two tested hybrids were genetically more close to *M. pruriens* var. *pruriens,* whereas, *M. macrocarpa* and *M. imbricata* were closely related to *M. gigantea*. Similar pattern of grouping was observed when PCoA was done for RAPD and ISSR separately (data not shown).

M. pruriens is by far the most investigated species for the study of intra species genetic and chemodiversity. Previously, many workers have studied and estimated the genetic diversity in both cultivated and wild *Mucuna pruriens* and its varieties using RAPD and morpho-agronomical characters, and found that *M. pruriens* has high genetic variation (Padmesh et al. 2006; Kalidas and Mohan 2010; Sathyanarayana et al. 2012). But very few studies have attempted to study the genetic diversity among the various *Mucuna* species. Most of these studies are based on either single genetic marker, mostly RAPD or morphometric and biochemical approaches. Leelambika and coworkers (2010, 2011) comparatively evaluated the genetic diversity among the accessions of four Indian *Mucuna* species using morphometric, biochemical and

molecular markers and found good variability among the studied accessions. Likewise, a study by Capo-chichi et al. (2001) used AFLP to investigate the genetic similarity in a collection of 40 velvet bean accessions of cultivated *Mucuna* species from different eco-geographic regions and found that accessions used in this study are similar. In congruence with these reports, our study also showed high genetic similarity among all tested accessions of the same species and grouped them together in the same cluster. None of these studies used more than one molecular marker system. Here we have used two marker systems (RAPD and ISSR) and included more number of accessions and *Mucuna* species. Moreover, we also investigated how *M. macrocarpa*, *M. imbricata* and two hybrids Dhanwantari and IIHR hybrid are genetically related to other species of *Mucuna*. However, to establish the molecular phylogenetic relationship of *M. macrocarpa*, *M. imbricata,* and two hybrids with other, the use of more advance and informative DNA based molecular approach such DNA barcode will be necessary.

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Compliance with ethical standards

Conflict of interest No conflict of interest.

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