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Genetic diversity analysis in underutilized medicinal climber *Mucuna pruriens* (L.) DC. germplasm revealed by inter simple sequence repeats markers

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ABSTRACT

Velvet bean is an important medicinal legume, its seeds are prominent source of L-Dopa. The present investigation on genetic diversity assessment of 58 germplasm of velvet bean by using 11 ISSR markers. Out of 63 amplified products 59 were showed polymorphism and 4 were monomorphic with an average of 5.7 bands amplified per primer. According to band statistics and efficiency parameters showed the primers UBC 827, UBC 834 and UBC 836 were more efficient. The highest genetic similarity values (0.90) were observed between IIHR MP 102 and IIHR MP 74-3. In dendrogram germplasm grouped into two major clusters at 63 per cent similarity. Among the germplasm, IIHR Selection 4, IIHR Selection 10, IIHR MP 9, IC 33243 and IIHR MP 7 were found to be distinctly divergent, can be used in the further breeding programme.

Key words: Genetic diversity, Germplasm, ISSR markers, L-Dopa, *Mucuna pruriens*, Polymorphism.

INTRODUCTION

Mucuna pruriens (L.) DC. is an important underutilized medicinal plant is indigenous to India. It is commonly called as velvet bean and it is an annual herbaceous climber belonging to the family *Fabaceae*. The species nomenclature '*pruriens*' in Latin refers to itching sensation due to pod hairs contact with human skin. It contains L-3, 4-Dihydroxyphenylalanine (L-Dopa) which is a non protein amino acid. *Mucuna* is a prominent source of L-Dopa and it act as a precursor of the neurotransmitter dopamine used in the treatment of Parkinson's disease (Manyam, 1995).

It has gained global attention in recent years as a non conventional source of protein diet due to presence of 20-30 per cent protein in the seeds. It is a self pollinated climbing shrub with long vines found in wild throughout plains of India. It tolerates a wide range of soils and suitable to grow in rain fed farming, high nitrogen fixing capability, aggressive growth habit and high productivity of biomass make it an excellent soil enriching crop, green manure cover crop, weed controller and source of food. Velvet bean is having good agronomical potential to meet demand of protein requirement of growing population. Rich genetic diversity coupled with wide ranging traditional knowledge of *Mucuna* has great scope as both medicinal plant and as food. The increased demand by the industry call for developing varieties with high L- Dopa, higher seed yield, higher protein and also with non itching type. However, very little effort has been done in its improvement through conventional or advanced biotechnological tools. Lack of basic information about diversity and variability, relationship among chemotypes and breeding behaviour etc. have seriously constrained the effective utilization of *Mucuna* genetic resources in India. Considering above information the present study undertaken to investigates the molecular diversity in 58 germplasm consisting three *Mucuna* species using eleven ISSR markers and also study on suitability of ISSR marker for assessment of genetic diversity of *Mucuna* germplasm.

MATERIALS AND METHODS

Plant materials: The study was conducted in the year of 2014-15 at Laboratory of the Section of Medicinal Crops, ICAR-Indian Institute of Horticulture Research, Bangaluru, India. The 58 germplasm of velvet bean were selected from the different collections of various parts of the country are being maintained at the institute. Collected young and fresh leaf samples of 58 germplasm (Table 1) belonging to *Mucuna pruriens* var. *utilis* (43 germplasm non itching type) and var. *pruriens* (14 germplasm itching type) and also var. *prurita* (1 germplasm itching type).

DNA extraction and ISSR-PCR amplification: Total genomic DNA was extracted by modified CTAB method (Dellapotra *et al*., 1983), dissolved in TE buffer and then

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Table 1: List of velvet bean germplasm used for the study

Germplasm	State / Source of collection	Species type
IIHR MP 17	New Delhi	*M. pruriens* var.*utilis*
IIHR MP 11	Tamil Nadu	*M. pruriens* var.*utilis*
IIHR MP 5	Karnataka	*M. pruriens* var.*utilis*
Arka Dhanwantari	IIHR, Bengaluru	*M. pruriens* var.*utilis*
IIHR MP 101	Andhra Pradesh	*M. pruriens* var.*utilis*
IIHR MP 99	NBPGR, N Delhi	*M. pruriens* var.*utilis*
IIHR MP 82	Gujarat	*M. pruriens* var.*utilis*
IIHR MP 91	NBPGR, N Delhi	*M. pruriens* var.*utilis*
IIHR MP 84	NBPGR, N Delhi	*M. pruriens* var.*utilis*
IIHR MP 89	NBPGR, N Delhi	*M. pruriens* var.*utilis*
IIHR MP 89-1	IIHR, Bengaluru	*M. pruriens* var.*utilis*
IIHR MP 7	Karnataka	*M. pruriens* var. *pruriens*
IIHR MP 88	NBPGR, N Delhi	*M. pruriens* var.*utilis*
IIHR MP 90	NBPGR, N Delhi	*M. pruriens* var.*utilis*
IIHR MP 92	NBPGR, N Delhi	*M. pruriens* var.*utilis*
IIHR MP 95	NBPGR, N Delhi	*M. pruriens* var.*utilis*
IIHR MP 96	NBPGR, N Delhi	*M. pruriens* var.*utilis*
IC33243	NBPGR, N Delhi	*M. pruriens* var.*utilis*
IIHR Selection 1	IIHR, Bengaluru	*M. pruriens* var.*utilis*
IIHR Selection 9	IIHR, Bengaluru	*M. pruriens* var.*utilis*
IIHR MP 104	Bihar	*M. pruriens* var.*utilis*
IIHR Selection 2	IIHR, Bengaluru	*M. pruriens* var.*utilis*
EC 25334	NBPGR, N Delhi	*M. pruriens* var.*utilis*
IC202969	NBPGR, N Delhi	*M. pruriens* var.*utilis*
IIHR MP10	Tamil Nadu	*M. pruriens* var.*utilis*
IIHR Selection 8	IIHR, Bengaluru	*M. pruriens* var.*utilis*
IIHR Selection 3	IIHR, Bengaluru	*M. pruriens* var.*utilis*
Arka Aswini	IIHR, Bengaluru	*M. pruriens* var.*utilis*
IIHR Selection LP	IIHR, Bengaluru	*M. pruriens* var.*utilis*
IC 2199	NBPGR, N Delhi	*M. pruriens* var.*utilis*
EC17827	NBPGR, N Delhi	*M. pruriens* var.*utilis*
IC2534	NBPGR, N Delhi	*M. pruriens* var.*utilis*
IC 332432	NBPGR, N Delhi	*M. pruriens* var.*utilis*
IIHR MP 44	Tamil Nadu	*M. pruriens* var. *pruriens*
IIHR MP 47	Karnataka	*M. pruriens* var. *pruriens*
IIHR MP 63	Kerala	*M. pruriens* var. *pruriens*
EC2533A	NBPGR, N Delhi	*M. pruriens* var.*utilis*
IIHR MP 90-1	IIHR, Bengaluru	*M. pruriens* var.*utilis*
IC 21998	NBPGR, N Delhi	*M. pruriens* var.*utilis*
IIHR MP 87	NBPGR, N Delhi	*M. pruriens* var.*utilis*
IC 83195	NBPGR, N Delhi	*M. pruriens* var. *prurita*
IIHR MP 85	NBPGR, N Delhi	*M. pruriens* var.*utilis*
IIHR MP 98	NBPGR, N Delhi	*M. pruriens* var.*utilis*
IIHR MP 93	NBPGR, N Delhi	*M. pruriens* var.*utilis*
IIHR MP 22	Gujarat	*M. pruriens* var.*utilis*
IIHR Selection 4	IIHR, Bengaluru	*M. pruriens* var.*utilis*
IIHR Selection 10	IIHR, Bengaluru	*M. pruriens* var.*utilis*
IIHR MP 9	Karnataka	*M. pruriens* var.*utilis*
IIHR MP 62-1	Kerala	*M. pruriens* var. *pruriens*
IIHR MP 62-2	Kerala	*M. pruriens* var. *pruriens*
IIHR MP 62-3	Kerala	*M. pruriens* var. *pruriens*
IIHR MP 63 -1	Kerala	*M. pruriens* var. *pruriens*
IIHR MP 45	Karnataka	*M. pruriens* var. *pruriens*
IIHR MP 74-3	Karnataka	*M. pruriens* var. *pruriens*
IIHR MP 74	Karnataka	*M. pruriens* var. *pruriens*
IIHR MP 105	Bihar	*M. pruriens* var. *pruriens*
IIHR MP 21	Himachal Pradesh	*M. pruriens* var. *pruriens*
IIHR MP 102	Andhra Pradesh	*M. pruriens* var. *pruriens*

checked the quality of DNA on 0.8 % agarose gel and quantified with Hoefers DyNA Quant (Pharmacia Biotech, USA) and diluted in double distilled water to 25 ng/µl for further PCR reaction.

Initially 20 ISSR primers screened, and 11 of them (Table 2), which yielded bright, reproducible and discernible bands, were used for the analysis of all 58 germplasms diversity and PCR were carried out in 25 µl reaction mixer containing 2 µl of DNA (25ng/µl), 2.5 µl primer, 1 µl 10 mM dNTPs, 2.5 µl 10 X Taq buffer, 1 µl $MgCl_2$, 0.33 µl Taq DNA polymerase (1U/µl) and 15.67 µl of sterile distilled water. Amplification were performed using thermal cycler (Eppendorf, Germany) with following program: Initial denaturation at 94°C for 4 min, 35 cycles of 94°C for 1 min, appropriate annealing temperature (Table 2) for 45 s, 72°C for 1 min with a final extension at 72°C for 10 min. Amplified products were separated on 1.4% agarose gel in 1X TAE buffer at 75 V for 1hour and stained with ethidium bromide (0.5µg/ml) and photographed under UV light using Gel Documentation System (Syngene, G Box, UK).

Data analysis: The ISSR markers are dominantly inherited, each band assumed to represent the phenotype at a single biallelic locus. ISSR bands were scored as presence (1) and absence (0) characters, to construct binary data matrix. The binary data were used to generate genetic similarity coefficient matrices following unweighted pair group method with arithmetic average (UPGMA) using SAHN function of NTSYS pc, ver. 2.02 program (Rohlf, 1998).

Polymorphic information content (PIC) was calculated as per Roldan-Riuz *et al.*, (2000). Effective multiplex ratio (EMR) was estimated according to Weir (1996). Observed heterozygosity (H_o) was calculated by average of the sum of bands (S) present for each allele divided by number of genotypes (NC) under study, (H_o=ΣS/NC). Marker index (MI) is the product of PIC and EMR Powell *et al*., (1996). Resolving power was calculated as, R_p = ΣIb (Prevost and Wilkinson, 1999). Where, Band informativeness (Ib) = 1 – [2(0.5 – p)] Where, 'p' is the proportion of accession containing the band. Diversity index is the expected heterozygosity and was calculated as DI= 1-1/L $\Sigma_l \Sigma_l P_i^2$ by Weir (1996) Where, p_i is the frequency of i^{th} allele at the 1 locus and L is the number of loci.

RESULTS AND DISCUSSION

ISSR polymorphism: In the initial screening of 20 ISSR markers, of which 11 primers were exhibited good amplification with clear, sharp, reproducible bands. Eleven ISSR primers produced total 63 amplified products were shown polymorphism ranged from 67 to 100 % among the germplasm which may be due to the difference in sequence of the primers. They produced a total of 63 bands, out of which 59 were polymorphic and 4 bands were monomorphic across the germplasm are shown in the Table 2. The number of bands produced by each primer varied from 3 to 9 with

Table 2: Per cent polymorphism in ISSR primers used in velvet bean germplasm

Primer	Sequence (5'-3')	Size (bp)	Tm (°C)	Annealing temp. (°C)	Total no. of bands	Polymorp -hic bands	Monomorph -ic bands	Percent of polymorph-ism
UBC 817	CACACACACACACACAA	17	45	52.8	6	5	1	83.33
UBC 820	GTGTGTGTGTGTGTGTC	17	47	48.0	6	6	0	100
UBC 827	ACACACACACACACACG	17	47	46.4	7	7	0	100
UBC 830	TGTGTGTGTGTGTGTGG	17	47	48.0	3	3	0	100
UBC 834	AGAGAGAGAGAGAGAGYT	18	46	48.4	9	9	0	100
UBC 836	AGAGAGAGAGAGAGAGYA	18	46	41.8	6	6	0	100
UBC 843	CTCTCTCTCTCTCTCTRA	18	46	46.4	5	5	0	100
UBC 850	GTGTGTGTGTGTGTGTYC	18	48	44.6	3	2	1	67
UBC 853	TCTCTCTCTCTCTCTCRT	18	46	48.4	5	4	1	80
UBC 855	ACACACACACACACYT	16	48	53.0	5	5	0	100
UBC 880	GGAGAGGAGAGGAGA	15	45	48.0	8	7	1	87.5
	Total	**-**	**-**	**-**	**63**	**59**	**4**	**-**
	Mean	**-**	**-**	**-**	**5.72**	**5.36**	**0.36**	**92.53**

Table 3: Efficiency parameters of ISSR primers utilised in germplasm of velvet bean

Primer	Polymorphic Information Content (PIC)	Resolving Power (Rp)	Effective Multiplex Ratio (EMR)	Diversity Index (DI)	Marker Index (MI)	Observed heterozygosity(Ho)
UBC 817	0.12	1.48	4.17	0.71	2.96	0.29
UBC820	0.33	3.83	6.00	0.59	3.57	0.41
UBC 827	0.26	2.10	7.00	0.47	3.29	0.53
UBC830	0.25	0.48	3.00	0.30	0.9	0.70
UBC 834	0.25	4.59	9.00	0.70	6.26	0.30
UBC836	0.46	4.62	6.00	0.39	2.34	0.61
UBC843	0.42	3.59	5.00	0.43	2.14	0.57
UBC 850	0.11	0.69	1.33	0.55	0.74	0.45
UBC 853	0.22	1.72	3.20	0.48	1.54	0.52
UBC855	0.43	2.03	5.00	0.28	1.40	0.72
UBC880	0.29	4.24	6.13	0.43	2.63	0.57
Mean	**0.28**	**2.67**	**5.07**	**0.58**	**2.52**	**0.51**

an average of 5.7 bands per primer in 58 germplasm of velvet bean. Similar banding pattern was reported by Vyas *et al*., (2018) in the study of balck gram using 20 ISSR markers. Out of 11 primers, UBC 834 gave maximum number (9) of total bands showing 100 per cent polymorphism. The primers UBC 820, UBC 827, UBC 830 and UBC 836 produced 3 to 7 bands with 100 per cent polymorphism. In similar study Kalidass and Mohan (2010) reported that 65.12 % polymorphism using 5 RAPD primers in wild population of velvet bean. These results are in conformity with Padmesh *et al*., (2006) who reported 90.1 per cent polymorphism using 15 RAPD primers in 11 accessions of *Mucuna*. Similarly Leelambika *et al*., (2010) reported 98.32 per cent polymorphism by using 50 RAPD primers among the 18 accession of *Mucuna*. The polymorphism delineated through ISSR markers in the present study is very high. Even 11 ISSR primers could delineate the diversity in a large 58 germplasm population of velvet bean. ISSR markers were also reported to be more efficient compared to other primers in diversity studies of crops like *Solanum trilobatum* (Shilpha *et al*., 2013) and *Abrus precatorius* (Mathur *et al*., 2012). The size and range of amplicons also differed with selected primers as well as the genetic material used.

Comparison of efficiency parameters : ISSR primers produced more number of banding patterns and exhibited higher polymorphism in velvet bean germplasm. The different band statistics and efficiency parameter results are as follows (Table 3). The PIC among the markers ranged from 0.11 to 0.46 with mean of 0.28 across 58 germplasm. Maximum PIC value (0.46) was recorded for UBC 836 among primers. The results of PIC values are conformity with genetic diversity of 22 black gram genotypes by using 15 RAPD primers (Vyas *et al*., 2016). Similarly the highest PIC value (0.55) Bishoy *et al*., (2014) reported UBC 887 in genetic diversity study of 17 accessions of *Clitoria ternatea* using ISSR markers. Similar results reported by earlier Tonk *et al*., (2011) in *Hypericum perforatum* L. using RAPD primers. In contrast to our results the higher PIC (0.907) reported by Sao *et al*., (2015) in molecular profiling of mungbean using ISSR and SSR markers. But lower PIC values (0.22 to 0.36) reported by Kameli *et al*., (2013) in *Satureja* species using 10 ISSR primers and also it was ranged from 0.13 to 0.20 reported by Satyanarayana *et al*., (2010) in Mucuna using AFLP primers. The PIC provides an estimate of the discriminatory power of a locus or loci by taking into account not only the number of alleles that are

expressed but also relative frequencies of those alleles. The primers UBC 836, UBC 855 and UBC 843 recorded higher PIC values and are more efficient in distinguishing among the germplasm. The resolving power of the eleven ISSR primers ranged from 0.48 to 4.62 with a mean value of 2.67. The maximum Rp value was found in primer UBC 836 (4.62), followed by UBC 834 (4.59). Tonk *et al*., (2011) reported maximum Rp value of 10.83 using RAPD primers in *Hypericum perforatum* L. The maximum Rp value exhibits the efficiency of primers according to Prevost and Wilkinson (1999). Among the 11 primers, UBC 836 (4.62) and UBC 834 (4.59) with high Rp value can be useful to differentiate the germplasm. Highest Rp value (4.62) noticed in UBC 836 whereas highest Rp value it ranges from 18.88 to 55.44 reported in Mucuna using AFLP primers (Satyanarayana *et al*., 2010). Effective multiplex Ratio ranged from 1.33 to 9.00 with an average of 5.07. The highest EMR value was observed in UBC 834 followed by UBC 827 (7.00) can be used to discriminate the germplasm in velvet bean. Higher MI highlights the distinctive nature of the markers and this is due to the simultaneous detection of several polymorphic markers per single reaction. Among the primers highest MI value recorded in 6.36 in UBC 836. MI values in the range of 0.28 to 7.32 were reported in mung bean (Saini *et al*., 2010). In previous studies it ranges from 12.64 to 33.33 reported in *Mucuna* using AFLP primers (Satyanarayana *et al*., 2010). The primers with higher MI value are more efficient. The primers which recorded maximum MI i.e. UBC 836 and UBC 820 will be more efficient primers to distinguish *Mucuna* germplasm. The diversity index values ranged from 0.28 to 0.71 and with a mean of 0.58 among primers studied in the germplasm. The high value of diversity index (DI) was noticed in primer UBC 817 followed by UBC 834 and very low value recorded in UBC 855. The high DI value is the indication of high efficiency of the primers and hence UBC 817 and UBC 834 can be selected for further studies in velvet bean. The marker index (MI) value ranged from 0.74 to 6.26 with a mean of 2.25. It was highest in UBC 836 (6.26), followed in UBC 820 (2.96). The observed heterozygosity ranged from 0.29 to 0.72 with a mean of 0.51. The maximum heterozygosity was observed in UBC 855 (0.72) followed by UBC 830 (0.70) and UBC 836 (0.61). According to Powell *et al*., (1996) estimation of marker utility and detection of polymorphism can be quantified in term of more heterozygosity and marker index. The primers with higher Ho and MI UBC 817 and UBC 855 can be utilised in molecular diversity estimation of velvet bean.

Genetic similarity and cluster analysis : The pooled binary data from eleven ISSR primers was analyzed to generate pair wise band similarities for 58 velvet bean germplasm. The simple matching coefficients between each pair of germplasm were used to construct a dendrogram. The genetic similarity value in the matrix ranged from 0.26 to 0.90 (data not shown), suggesting a high to moderate diversity among the velvet bean germplasm. The least genetic similarity values (0.26) are recorded between IIHR MP 89-1, IIHR MP 7 and IIHR MP 62-1 and also between IIHR MP 62-1 and IC 21998, high genetic similarity values (0.90) observed between IIHR MP 102 and IIHR MP 74-3. The least genetic similarity values (0.26) are recorded between IIHR MP 89-1, IIHR MP 7 and IIHR MP 62-1 and also between IIHR MP 62-1 and IC 21998. These germplasm are more divergent types which is used to construct the core germplasm and useful in future hybridization programs of velvet bean. High genetic similarity values (0.90) observed between IIHR MP 102 and IIHR MP 74-3, these are similar types among the 58 germplasm. These results are consistent with earlier report by Capo- chichi *et al*., (2003) on genetic variation analysis in *Mucuna sp*, where genetic similarity index ranged from 0.68 to 1.00. Compared to earlier reports the genetic variability recorded in the present study was higher. The high level of polymorphism detected in self pollinated germplasm of *Mucuna pruriens* may be attributed to broad genetic base of the germplasm used in the study, as the number of entries used are more in number and comprises of *M. pruriens* var. *pruriens*, var. *prurita* and var. *utilis* .

The dendrogram showed two (**I**, **II**) major clusters at 63% similarity (Fig. 1). The first major cluster I consisting of 13 genotypes in which 10 itchy types and three non itchy types**.** Major cluster I consists of two sub clusters (Ia and Ib). Ia sub cluster has a single line i.e. IIHR Selection 10 which is distinctly grouped from others (Fig. 1). Ib sub cluster consists of 12 genotypes which clustered in two sub-sub cluster Ib1 and Ib2. The Ib1 sub-sub cluster formed by single genotype IIHR MP 62-1 itchy type. Sub-sub cluster of Ib2 consists of 11 genotypes in which IIHR Selection 4 distinctly separated, which is a non itchy type and remaining all 10 itchy types formed in rest of the group. Among 10 itchy types IIHR MP 102 and IIHR MP 74-3 showed 90 per cent similarity. In I major cluster the genotypes IIHR Selection 4, IIHR Selection 10 and IIHR MP 62-1 have formed single entry cluster showing their distinctness.

The major cluster II grouped into two sub cluster (IIa and IIb). IIa sub cluster grouped into two sub-sub cluster (IIa1 and IIa2). The sub-sub cluster IIa1 formed of 18 genotypes in which IIHR Selection 3 and IC 17827 showed 90 per cent similarity but these two posses contrasting white and black seed coat colour, respectively. Among 18 genotypes, 3 genotypes viz., IIHR MP 44, IIHR MP 47 and IIHR MP 63 were itchy type. Sub-sub cluster IIa2 formed 8 genotypes in which IIHR MP 104 and IIHR Selection 2 showed 88 per cent similarity. In this group IC 83195 was *Mucuna* species *prurita* grouped together with other genotypes formed *M.pruriens utilis* showing grouping of both itchy and non itchy types.

Sub cluster IIb grouped in to two sub-sub clusters IIb1 and IIb2. The IIb1 is an IIHR MP 7 distinctly formed separate sub-sub cluster, which is an itchy type with seed

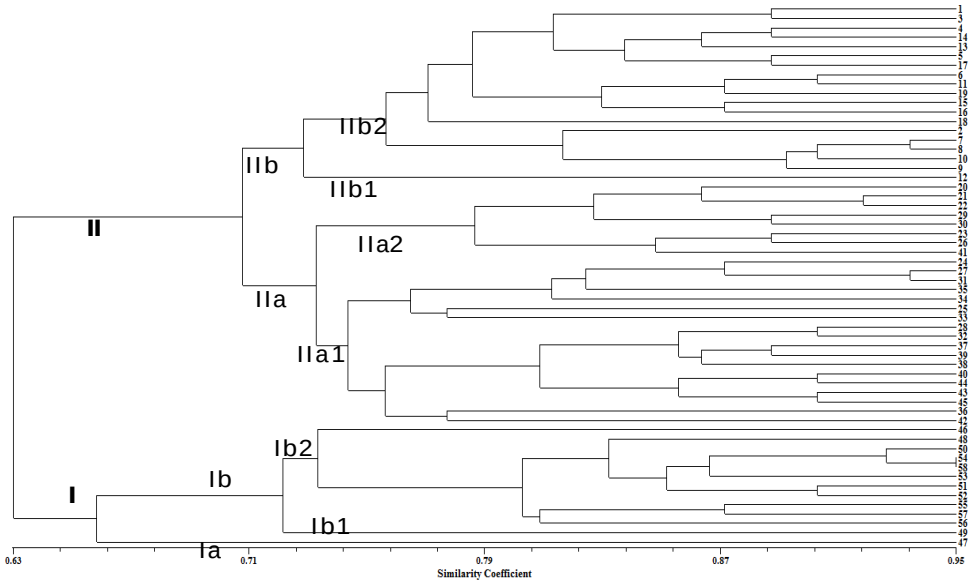

Fig 1: UPGMA dendrogram of velvet bean germplasm based on ISSR primers

coat grey in colour. Eighteen genotypes formed into a sub-sub cluster IIb2 in which IC 33243 formed as distinct group and two germplasm IIHR MP 91 and IIHR MP 82 have shown 90 per cent similarity.

The perusal of the result, it is evident from the dendrogram there is no distinct grouping according to species, geographical origin and its other morphological traits of the genotypes. The genotypes used in the study belong to *M. pruriens* var. *utilis* is non itchy types and *M. pruriens* var. *pruriens* and var. *prurita* having itchy trichomes on the pod and in major clusters combined of lines of both groups. Though the distinct grouping systems of itchy lines and non itchy lines of velvet beans observed in sub clustering of dendrogram. But in a similar study of using RFLP primers Capo-chichi *et al*., (2003) reported that UPGMA dendrogram generated two major clusters based on existing phenological difference with maturity. The clustering of *Mucuna* germplasm in two major cluster according geographical locations reported in velvet bean using RAPD primers (Padmesh *et al*., 2006) and also Bishoyi *et al*., (2014) clustering pattern was reported that all the accession of *Clitoria ternatea* grouped according to geographical origin rather than their morphological variation of flower colour.

CONCLUSION

In Mucuna, many researchers studied genetic diversity by using AFLP and RAPD makers with few collections. The assessment of genetic diversity analysis by using RAPD and ISSR (five) primers was also reported in collection of 59 accessions of six *species* and three varieties of Mucuna and reported that existence of high interspecific variation than intra specific variation of genus Mucuna. The present investigation reveals that ISSR markers UBC 827, UBC 834 and UBC 836 markers are found to be efficient markers are identified based on band statistics. The present study identified distinctly diverse germplasm IIHR Selection 4, IIHR Selection 10, IIHR MP 9, IC 33243 and IIHR MP 7 and these germplasm can be useful in the further breeding programme of the velvet bean.

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