Indian J. Agric. Res., 53(3) 2019: 284-290
Print ISSN:0367-8245 / Online ISSN:0976-058X

AGRICULTURAL RESEARCH COMMUNICATION CENTRE
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Variations in the *trnHpsbA* region of *Mucuna pruriens* L. (DC.) varieties of India: an insight on intraspecific diversity

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Received: 01-12-2018 Accepted: 25-03-2019 DOI: 10.18805/IJARe.A-5182

ABSTRACT

Mucuna pruriens is a nutritionally and pharmaceutically important underutilized legume belonging to genus *Mucuna* of Leguminosae family. Extensive intraspecific diversity and adverse morphological features among the members have led to taxonomical confusions in the species. A total of 12 *Mucuna pruriens* accessions including its two botanical varieties, var. *pruriens* and var. *utilis* with six accessions each were analyzed to characterize the intraspecific diversity among them using the three barcode markers *viz. ITS2, MatK* and *trnHpsbA*. Extensive intraspecific sequence diversity was observed in the *trnHpsbA* region, where as *ITS2* and *MatK* exhibited significant conserved regions. Such intraspecific sequence diversity is a limitation in adapting *trnHpsbA* as barcode as it may lead to misinterpretations, but in the present study an attempt has been made to extrapolate this diversity to explain within the species variations among *Mucuna pruriens* genotypes. Intraspecific sequence variations exhibited by *trnHpsbA* sequences were 83% in correlation with morphological features. The between groups mean distance of var. *pruriens* and var. *utilis* was found to be 0.346. Results of this study will complement with the existing molecular, morphological, ytological and biochemical markers towards Velvet bean improvement programs.

Key words: Intraspecific diversity, *ITS2, MatK, Mucuna pruriens, trnHpsbA*, Velvet bean.

INTRODUCTION

Bringing underutilized crops into mainstream agriculture is very important presently to achieve sustainable food systems and also to combat protein energy malnutrition which are the key components of SDG 2 and 12 of UN's Sustainable Development Goals (UNDP, 2015). Genetic improvement of such underutilized crops require an extensive understanding of the genotypic and phenotypic properties. *Mucuna pruriens* L. (DC.) is one among the most promising underutilized legumes which belongs to genus *Mucuna* (Family: Leguminosae) and commonly known as 'Velvet bean'. It is economically popular because of its agronomical, medicinal and nutritional applications (Patil *et al*. 2018; Jorge *et al*. 2007; Sathiyanarayanan and Arulmozhi 2007). The characteristic feature of *Mucuna pruriens* seeds is presence of L-Dopa (L-3,4 dihydroxy phenylalanine), a non-protein amino acid which is used in the treatment of Parkinson's disease (Lieu *et al*. 2010). *Mucuna pruriens* is an annual species of the genus *Mucuna* with climbing habit, trifoliate leaves, clustered or long showy inflorescence with purple or creamy white corolla. Members of *Mucuna pruriens* species exhibits significant intraspecific diversity at morphological, biochemical and molecular levels (Leelambika and Sathyanarayana 2011). *Mucuna pruriens* includes two botanical varieties var. *utilis* with all non-itching cultivated members and var. *pruriens* with wild itching members. Extensive morphological diversity has been observed among the members of both the varieties of *M. pruriens* (Fig 1). Seeds of most of the var. *uitilis* are white with few exceptions which exhibit brownish or black seed coat as in accessions IC392850 and 500101KA employed in the present study. Pods from *M.pruriens* var. *pruriens* are covered with a thick layer of golden brown itchy trichomes. The seed coat color is also highly diverse among the var. *pruriens* genotypes ranging from yellowish brown to black (Leelambika *et al*. 2010). Taxonomical ambiguities are very common among the members of *M. pruriens* posing greater challenges in their genetic improvement programs. Taxonomical revision in genus *Mucuna* in Indian subcontinent and Burma was undertaken three decades ago and this revision considered many species like *M. aterrima, M. cochinchinensis, M. hassjoo, M. nivea* and *M. utilis* as varieties of *M. pruriens* (Wilmot-Dear 1987). Within the botanical varieties of *M. pruriens* confusions and contradictions are still persistent between var. *pruriens*, var. *utilis* and var. *hirsuta* (Dassanayake and Fosberg 1980; Sasidharan 2004; Leelambika and Sathyanarayana 2011). A well characterized understanding on these intraspecific diversities is still lacking among the *Mucuna pruriens* members. Intraspecific variations are of widespread focus as they are the basis of

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species co-existence and community composition. As reported in many reviews, ecological interactions and adaptive phenotypic variations are highly influenced by intraspecific genetic variations (Hughes *et al*. 2008; Ehlers *et al*. 2016). Among the plant communities, intraspecific variations are mostly due to interbreeding within varieties resulting in natural hybrids with intermixed or transitional morphological characters leading to taxonomic ambiguities (Padmesh *et al*. 2006). Morphological variability within the species is very common in Leguminosae members and such variations pose challenges in correct identification of species. Genetic diversity analysis using morphometric features or nuclear markers are the common methods to characterize intraspecific diversities (Razvi *et al*. 2018; Thakur *et al*; Vir and Singh 2015).

Enormous information on the plant DNA barcodes like *nrITS*, *matK*, *rbcL*, *trnHpsbA*, and their utility in species identification and phylogeny analyses have been generated in the past two to three decades (Kress and Erickson 2008; Bieniek *et al*. 2015; Singh *et al*. 2017; CBOL Plant Working Group 2009). As defined by Kress and Erickson (2008), the key criteria of a DNA barcode marker is significant species level genetic variability and divergence. In other words, gene segments used as barcodes must have high level of variation between the species but should be conserved within the species. But if there are plenty of variations within the species, can it be used for characterization of intraspecific diversities based on some pattern of variations? In the present study we tried to find the answer for this question and explored the possibilities of characterizing the intraspecific variations *Mucuna pruriens* members.

MATERIALS AND METHODS

Plant materials: A total of 12 *Mucuna pruriens* accessions including its two botanical varieties *viz* var. *pruriens* and var. *utilis* with six accessions each were selected for the present study. These plant samples were collected from different geographical locations of India (Table 1). Voucher specimens of the plant samples are deposited at the Herbarium and Raw Drug Repository (National Herbarium) of Foundation of Revitalization of Local Health Traditions (FRLHT), Bengaluru.

Fig 1: Morphological diversity among the *M.pruriens* accessions used in the present study. a. 500101KA, b. 500102KA, c. IC392850, d. 500155AP, e. 500170UP, f. 500179MH, g. 500112KA, h. 500109KA, i. IC391885, j. IC391941, k. IC265577, l. 500199WB.

Table 1: Details of multiple accessions of *Mucuna pruriens* used in the present study.

Accession Name	Place of Collection	Plant Accession Number	Genbank Accession Numbers			Voucher specimen id
			ITS2	*matK*	*trnh_psbA*	
M. pruriens var. *utilis*	Bengaluru, Karnataka	500102KA	HM355852	KX606946	KX606891	FRLH119723
M. pruriens var. *utilis*	Bengaluru, Karnataka	500101KA	HM355851	KX606956	KX606909	FRLH 119727
M. pruriens var. *utilis*	NBPGR, Moudi, Orissa	IC392850	KX499624	KX606951	KX606890	NBPGR
M. pruriens var. *utilis*	Ayurvedic vendor, Tandur, Andhra Pradesh	500155AP	HM355839	KX606958	KX606898	FRLH 119724
M. pruriens var. *utilis*	AyurvedicVendor, Lucknow, Uttar Pradesh	500170UP	KX499621	KX606953	KX606893	FRLH 119750
M. pruriens var. *utilis*	Andhanair, Aurangabad, Maharashtra	500179MH	KX499623	KX606954	KX606894	FRLH 119719
M. pruriens var. *pruriens*	Mysore, Karnataka	500112KA	HM355846	KX606949	KX606873	FRLH 119742
M. pruriens var. *pruriens*	NBPGR, Njeezhoor, Kerala	IC265577	HM355848	KX606950	KX606888	NBPGR
M. pruriens var. *pruriens*	NBPGR, Gaatogaon, Orissa	IC391941	HM355834	KX606955	KX606881	NBPGR
M. pruriens var. *pruriens*	NBPGR, Anandpur, Orissa	IC391885	HM355835	KX606957	KX606889	NBPGR
M. pruriens var. *pruriens*	Shimoga, Karnataka	500109KA	HM355847	KX606947	KX606882	FRLH 119745
M. pruriens var. *pruriens*	Gorumera, N. Park, West Bengal	500199WB	KX499614	KX721058	KX606878	FRLH 119711

NGPBR: National Bureau of Plant Genetic Resources (accessions collected from NBPGR).

Molecular methods (DNA Extraction, PCR, Sequencing): DNA was extracted from the young leaflets of *Mucuna* using the modified Doyle and Doyle (1990) method and quantified by using 0.8% agarose gel electrophoresis with ethidium bromide staining. Two chloroplast (*matK* and *trnHpsbA*) and one nuclear (*ITS2*) markers were used in the present study (Table S1). Amplification was done in Peltier thermal cycler (MJ Research, USA) in a total reaction mixture of 25µl containing 0.2mM dNTP's, 10mM Tris-HCl, 1.5mM $MgCl_2$, 50mM KCl, 0.1% Triton X-100, 1U *Taq DNA polymerase* and 30-50ng of DNA. PCR program included Initial denaturation at 94°C for 1 min; Denaturation at 94°C for 30 s; Annealing temperature was 56°C, 49°C and 53°C for *ITS*2, *matK* and *trnH-psbA* regions respectively for a duration of 30 s; Extension at 72°C for 50 s; Total number of cycles were 35 followed by the final extension 72°C for 5 min. Negative controls were kept for all the primers which included all the components of the reaction mixture except template DNA and it was replaced with nuclease free water. Amplified products were observed on 1.5% agarose gel (1XTAE) with ethidium bromide staining. The PCR products were sequenced for both forward and reverse directions at Eurofins Genomics India Pvt. Ltd., Bangalore, using ABI 3730XL (Applied BioSystems) sequencer following the manufacturer's protocols. Obtained sequences were edited manually using Chromas lite version 2.01 (Technelysium 2015). All the sequences generated in this work were submitted to NCBI Genbank and the accession numbers are given in Table 1.

Sequence alignment, estimation of intraspecific genetic diversity and phylogenetic analysis: Multiple sequence alignment was done by using Clustal W (Larkin *et al*. 2007) and MUSCLE (Edgar 2004) in software MEGA version 6.06 (Tamura *et al*. 2013). Estimation of genetic distance over sequence pairs within the groups and between the groups were calculated in MEGA version 6.06 taking p-distance model. Neighbor Joining (NJ) and UPGMA of the aligned matrices was done as implemented in MEGA version 6.06 with 1000 bootstrap replications.

RESULTS AND DISCUSSION

The results of the present study indicate the molecular distinction between the botanical varieties of *Mucuna pruriens* at the *trnHpsbA* region whereas the other two barcode markers *ITS2* and *matK* considered all the members as almost similar. *Mucuna pruriens* is originated from South Asia possibly from China or Eastern India (Burkill 1966; Wilmot Dear 1984) and India has the largest collections of varieties of the same. Varieties of *Mucuna pruriens* are economically popular due to their pharmaceutical, nutritional and agronomical properties but

Table 2: Sequence diversity information among the *M.pruriens* accessions

		ITS2	*matK*	*trnH_psbA*
Amplified product length (bp)		~300	~850	~350
Aligned matrix		223	557	277
G+C content		60%	32%	22%
Conserved sites		211	404	102
Variable sites		7	148	167
Parsimony informative		4	19	121
Singleton variable sites		3	129	45
Within the group	var. *pruriens*	0.006	0.035	0.239
mean distance	var. *utilis*	0.011	0.071	0.154
Between the groups mean distance		0.008	0.053	0.346
Mean diversity in entire population		0.008	0.053	0.278

the taxonomical confusions among them are the challenges in their genetic improvement programs (Jaheer *et al*. 2015).

The aligned sequence matrix of *ITS2, matK* and *trnHpsbA* had 223, 557 and 277 characters respectively (Table 2). The aligned matrix for all the three regions included 12 *M. pruriens* accessions with six individuals from both botanical varieties. Highest G+C percentage among the sequences was observed with *ITS2* region (60%) and the least was with *trnHpsbA* region (22%). More conserved regions were found in the *ITS2* and *matK* regions. The *trnHpsbA* region showed highest variable (167) and parsimony informative (121) sites. The *ITS2* and *matK* regions did not show much difference between var. *pruriens* and var. *utilis* and the mean diversity in entire population was 0.008 and 0.053 respectively but in contrast, the *trnHpsbA* region exhibited a greater diversity within the group and between the groups (Table 2). The mean diversity in entire population by *trnHpsbA* region was found to be 0.278. Extensive intraspecific sequence diversity was observed in the *trnHpsbA* region, where as *ITS2* and *matK* exhibited significant conserved regions (Table 2). Thus *trnHpsbA* sequences were further analyzed to characterize the intraspecific diversities among *M. pruriens* varieties. This region exhibited many indels and parsimony informative regions (Fig 2). The overall sequence analysis indicated the distinguishing differences between most of the var. *pruriens* and var. *utilis* at sites 69, 70, 95 and between 166 to 174. Except for 500155AP and 500199WB sites 69, 70 and 95 exhibited unique pattern of indels for var. *pruriens* and var. *utilis*. A unique insertion was observed at region 166 to 170 in *M.pruriens* var. *pruriens* accession 500112. Intraspecific sequence variations exhibited by *trnHpsbA* sequences were 83% in correlation with morphological features. We cannot rule out the point that, barcode region *trnHpsbA* exhibits intraspecific inversions which leads to separation of closely related lineages and uniting of more distantly related taxa if they share similar inversions (Whitlock *et al*. 2010). However, it is important to examine and characterize if there are any unique patterns in such intraspecific inversions in the plant group under study. Our experimentation with *trnHpsbA* dataset from 12 accessions of *Mucuna pruriens* revealed unique sequence variations between var. *pruriens* and var. *utilis*.

NJ and UPGMA trees exhibited same topologies (Fig 3). Cluster I included five *M. pruriens* var. *pruriens* with one var. *uitilis* (500155AP) with 100% bootstrap support. Cluster II included five *M.pruriens* var. *uitilis* and one var. *pruriens* (500199WB) with 100% bootstrap support. In both the trees *M.pruriens* var. *pruriens* accession 500112 showed out clustered to Cluster I members and this is probably due to its variation at region 166 to 170 (Fig 2). This exceptional clustering of 500155AP and 500199WB is probably indicative of interbreeding within the varieties. These results open up the hopes for a scaled up analysis to reveal more on these two botanical varieties. The biochemical profiling information for the 12 individuals under study were retrieved from a previous report (Leelambika and Sathyanarayana 2011). There was not much variation between the L-Dopa percentage and protein content among the 12 accessions studied but carbohydrate content was varying from 6-28g/100g seed powder. The highest carbohydrate containing accession 500112KA showed an independent clustering in Cluster I of both NJ and UPGMA trees. Interestingly this accession 500112KA also showed an insertion at region 166 to 170 in the *trnHpsbA* marker. The least carbohydrate content was observed in genotype 500199WB which showed an association with var. *utilis* members. The biochemical parameters did not show significant direct correlations with the morphological and molecular inferences. A comparative evaluation of genetic diversity among the Indian *Mucuna* species based on the morphometric, biochemical and RAPD markers also reported the difficulties in resolving the botanical varieties of *Mucuna pruriens* (Leelambika *et al*. 2010). Genetic diversity studies in *Mucuna pruriens* varieties from RAPD and AFLP markers also concluded the limitations of these nuclear markers in infra-species studies (Sathyanarayana *et al*. 2011). Apart from diversities at genetic marker level, these *Mucuna pruriens* varieties also exhibited highest variability in response to abiotic stress like salinity (Mahesh and

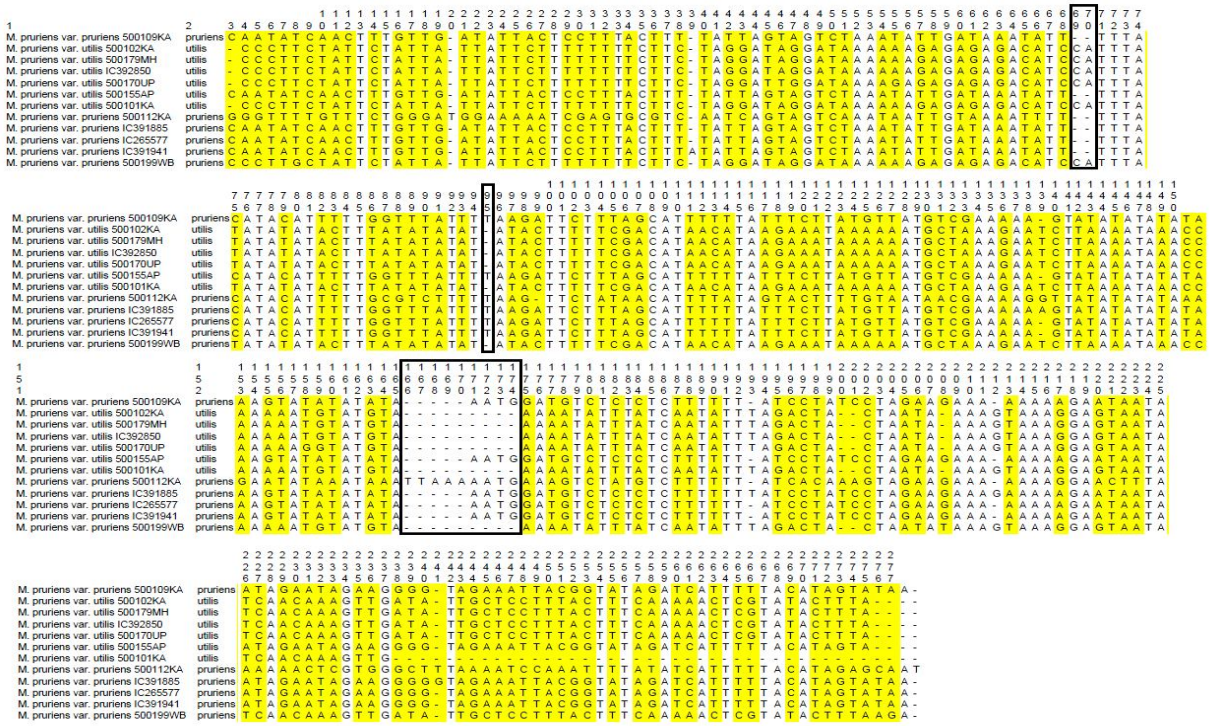

Fig 2: Portion of *trnHpsbA* aligned matrix from *M.pruriens* accessions indicating the variability. Highlighted regions are variable sites. Marked regions are indels.

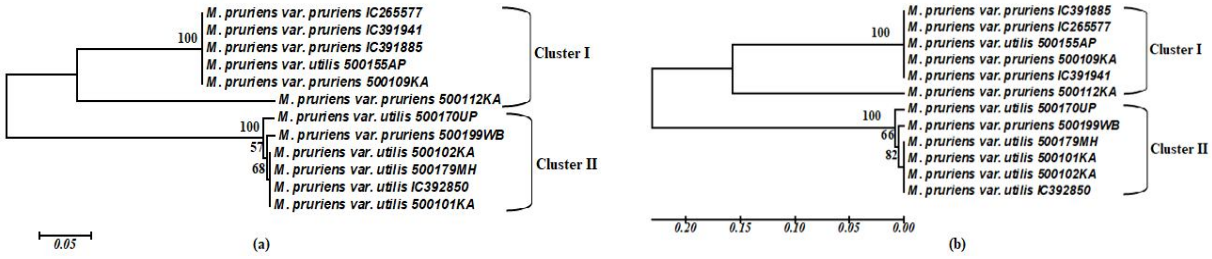

Fig 3: Neighbor Joining (a) and UPGMA (b) trees based on *trnHpsbA* data from *M.pruriens* accessions. Values on the branches are bootstrap support percentages.

Sathyanarayana 2015). Most of these studies concentrated on genetic diversities based on polymorphic markers but intraspecific diversities among *Mucuna pruriens* species based on barcode markers are least attempted. Patil and coworkers evaluated the genetic diversity of Indian *Mucuna* species using RAPD and ISSR markers and reported the highest genetic diversity among the *Mucuna pruriens* species and this study conclused that DNA barcode based approach is necessary to understand the phylogenetic relationships among the *Mucuna* species (Patil *et al*. 2016). In the recent years, DNA barcode markers like ITS, *matK* and *trnHpsbA* are popular in understanding the intraspecific diversities among the closely related plant groups. Nucleotide diversity, sequence variation and secondary structure variations from the barcode markers are carefully investigated to characterize the intra species variations (Jiang *et al*. 2016; Li *et al*. 2017; Bieniek *et al*. 2015; Singh *et al*. 2017). While discriminating very closely related taxa, it is equally important to consider the indels in the highly variable sequences. A study by Liu and group also highlighted the potential utility of coding indels and they also observed the *trnHpsbA* is very informative in differentiating closely related taxa (Liu *et al*. 2012). In congruence with this report, our analysis also exhibited highest variability among the *trnHpsbA* sequences of the *Mucuna pruriens* varieties with remarkable indels.

Mostly the members of *Mucuna pruriens* undergo self-pollination, however the tendency of few accessions grouping with morphologically dissimilar members is probably attributed to cross pollination leading to natural hybrids and the existence of such natural hybrids have been reported earlier (Padmesh *et al*. 2006). It is evident through their extensive polymorphism that the accessions of *Mucuna pruriens* have acquired novel gene pools for helping them in adapting to different geographic and environmental

conditions. With such a heterogeneous population it is very important to investigate every available difference towards understanding the diversity and also to characterize the genetic similarities or dissimilarities. Primitively the results from *trnHpsbA* region from 12 *Mucuna pruriens* accessions is showing molecular differences between the botanical varieties but it certainly require a large scale evaluation to validate the results. However, the outcomes of this study will definitely complement the existing molecular, morphological, cytological and biochemical markers towards Velvet bean improvement programs.

CONCLUSION

In conclusion, there is an extensive intraspecific diversity observed among the *Mucuna pruriens* varieties which needs to be understood and characterized with better marker systems to improve this underutilized crop. Many markers like morphological, biochemical and molecular have failed in completely characterizing the intraspecific variations of *Mucuna pruriens*. Here we attempted to observe the variations among 12 *Mucuna pruriens* accessions including 6 each botanical varieties from var. *pruriens* and var. *utilis* using commonly employed DNA barcode markers like *ITS2*, *matK* and *trnHpsbA*. The *ITS2* and *matK* regions indicated all the 12 accessions are almost same but the *trnHpsbA* region exhibited the between the groups mean distance of var. *pruriens* and var. *utilis* is 0.346. Intraspecific sequence variations exhibited by *trnHpsbA* sequences were 83% in correlation with morphological features. A large scale evaluation of this region is recommended to reveal more on these two botanical varieties in order to complement the genetic improvement programs.

ACKNOWLEDGEMENT

We acknowledge the support of Sri Krishnadevaraya Educational Trust (Sri KET) Bengaluru, Sikkim University, Gangtok, Sikkim and NMAM Institute of Technology, Nitte, Udupi dist., Karnataka for providing facilities to carry out this study.

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Compliance with Ethical Standards

Conflict of Interest No conflict of interest

Author Contributions Statement

Sathyanarayana N and Vidya SM conceptualized and designed the study. Rashmi KV conducted the experiments, analyzed the results and prepared the manuscript.

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