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Research Article

OPTIMIZATION OF AMPLIFICATION CONDITIONS FOR DNA BARCODING MARKERS IN MEDICINALLY IMPORTANT GENUS MUCUNA

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ABSTRACT

Genus *Mucuna* belongs to the family Fabaceae is one of the potential underutilized legumes which include 150 species of annual and perennial species of pantropical distribution. Members of this genus possess several promising nutritional and agronomic attributes it has received wide-ranging attention from pharmaceutical industries, nutritional chemists and agronomists alike in recent years. The evolution and relationship among different *Mucuna* taxa (both at species and sub-species level) of India have remained unknown except *M. pruriens*. Because of this, it is necessary to conduct research at the species level as well as to assess phenetic relationships among different taxa in India to place the genus in right taxonomic and phylogenetic perspective. The Consortium for the Barcode of Life (CBOL) – Plant working group revealed the utility of nuclear Internal Transcribed Spacer (nrITS) region of the nuclear ribosomal cistron (18S-5.8S-26S) and chloroplast regions like *trnH-psbA, matK* and *rbcL* towards developing the plant DNA barcodes. Though these regions are considered as universal barcode regions, it observed from literature that to get successful amplification of these regions many PCR parameters needs to be optimized. Here we report different amplification parameters optimized for the amplification of nrITS2, *trnH-psbA, matK* and *rbcL* regions in *Mucuna* species of India collected from different geographical locations. The amplified regions from the optimized protocols were sequenced and obtained sequences after editing were queried in NCBI BLAST to confirm that they distinctly match the respective barcode regions of the *Mucuna* species. The results of these analysis revealed that obtained sequences were authentic and could be used for phylogenetic studies and DNA barcoding of *Mucuna* species of India.

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INTRODUCTION

Mucuna Adans a promising underutilized legume (Rai *et al.,* 2007) belongs to the family Fabaceae, which includes around 150 species of both annual and perennial varieties, distributed pantropically (Buckles, 1995). Members of this genus are capturing commercial and scientific importance as there are many reports on their medicinal, nutritional and agronomical properties (Bhat and Kharim, 2009; Siddhuraju *et al.,* 2000; Bressani, 2002). *M. pruriens*, an annual of this genus exhibits lot promise towards sustainable agricultural practices as it produces very high green biomass, high nitrogen fixing ability and an average seed yield of 2.4t/ha/yr, which are very rich in protein content (Buckles, 1995; Carsky and Ndikawa, 1998). Genus *Mucuna* is also known for its significant levels (1-5%) of L-DOPA (L-3, 4-dihydroxyphenylalanine), a non-protein amino acid which is the precursor for the synthesis of neurotransmitter Dopamine and it is used in the treatment of Parkinson's disease (Katzenschlager *et al.,* 2004; Lieu *et al.,* 2010). Besides this, different parts of this plant are supplemented as active ingredient of many ayurvedic formulations administered against several medical ailments (Amin *et al.,* 1996; Longhi *et al.,* 2011; Obogwu *et al.,* 2014).

Taxonomic revision on genus *Mucuna* in Indian subcontinent has reported the existence of ten species - of which *M. atropurpurea* (Roxb.) DC.ex Wight and Arn is endemic to peninsular India; *M. imbricata* DC.ex Bak., *M. Bracteata* DC.ex Kurz, *M. macrocarpa* Wall., *M. sempervirens* Hemsl. and *M. nigricans* (Lour) Steud. are largely distributed in the eastern Himalayas and *M. pruriens*, *M. monosperma* DC. ex Wight and *M. gigantea* (Wild.) DC. are widely dispersed

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(Wilmot-Dear, 1987). The recently reported *M.sanjappe* was identified from the Western Ghats of India (Aitawade *et al.,* 2012). Except *M. pruriens* all other species are woody perennials.

Members of this genus are climbers, with long slender branches. Leaves are trifoliar with lanceolate lamina and the petioles are hairy. Inflorescence contains bunch of flowers ranging from 15 to sixty large flowers with dark purple or creamy white corolla. Pods exhibit characteristic thick layer of pod-hairs on them which causes itching and irritation when they come in contact with human skin, due to the presence of purities (Wilmot-Dear, 1987). Pod shape and number of seeds is again different across the species (Jaheer and Sathyanarayana, 2010).

Though there are lot of reports and revisions on the taxonomical classification of species and subspecies of this genus, a collective review of these reports ends with considerable taxonomic ambiguities (Dassanayake and Fosberg, 1980; Sasidharan, 2004; Ellis, 1990; Saldanha, 1996). A number of taxa namely, *Mucuna aterrima*, *M. cochinchinensis*, *M. hassjoo*, *M. nivea* and *M. utilis* which were formerly considered separate species are now included as varieties of *M. pruriens*, (Wilmot-Dear, 1987). In India, two main varieties of *M. pruriens viz.,* var. *utilis* and var. *pruriens* are widely reported (Wilmot-Dear, 1987; Sur, 2002; Sasidharan, 2004) , along with a third group *viz.,* var. *hirsuta* . The latter was earlier classified as an independent species (Ellis, 1990; Saldanha, 1996); but subsequent revisions; especially the one by Wilmot Dear (1987) categorically suggested its inclusion, along with few others, under the botanical varieties of *M. pruriens.* Distribution of this genus across the wide geographical and climatic conditions of India has reflected tremendous genetic diversity among *Mucuna*. In order to understand the evolution and relationship among different *Mucuna* taxa (both at species and sub-species level), it is necessary to conduct research towards this way.

Studies by Kress & Erickson and The Consortium for the Barcode of Life (CBOL) – Plant working group revealed the utility of nuclear and chloroplast genes towards developing the plant DNA barcodes (Kress and Erickson, 2007; C.P.Group,2009). Observations made by these studies specifically suggest that nuclear Internal Transcribed Spacer (nrITS) region of the nuclear ribosomal cistron (18S-5.8S-26S) and chloroplast regions like *trnH-psbA, matK* and *rbcL* are the most commonly sequenced loci for molecular systemic investigations and species. In genus *Mucuna* there are very few reports on molecular phyologentic studies using the above said markers. Study made by Stefanovic *et al* on a large group of Phaseoloid legumes using eight chloroplast regions including *matK* and *rbcL* reported genus *Mucuna* is sister to genus *Desmodieae* (Stefanovic *et al.,* 2009). However, such reports for species in the Indian subcontinent are lacking. The present research group made the first attempt on establishing the phylogenetic inference in *Mucuna* species of India using nrITS and *trnH-psbA* sequences for only seven species (Jaheer *et al.,* 2015), BUT it is found in the later stages of the research, to amplify these regions across large number of species and subspecies few modifications are needed for the earlier reported protocols.

Though the barcode regions nrITS, *trnH-psbA, matK* and *rbcL* are considered as the universal markers, the review made by Vijayan and Tsou clearly lists out the different sets of suitable primers to amplify these candidate regions (Vijayan and Tsou, 2010). The protocols followed to amplify these regions vary from report to report in parameters like primer concentrations, annealing temperatures and utility of PCR additives etc. (Tallei & Kolondam, 2015; Lagoudakis *et al*., 2011; Phong *et al*., 2014). For example to amplify nrITS2 region the reported annealing temperatures ranges between 36°C to 60°C (Jaheer *et al*., 2015; Selvaraj *et al*., 2012; Ihrmark *et al*., 2012), to retrieve *matK* sequences always it needs extra efforts with multiple primer combinations (Parmentier *et al*., 2013; Fazekas *et al*., 2008). Thus it is very important to screen the relevant primer combination and optimize the amplification conditions for the DNA barcoding markers for the taxa under study before taking a large scale phylogenetic analysis and DNA barcoding. In this view, the present study reports the suitable primer combinations and optimized the PCR amplification conditions for regions of *nrITS2* and chloroplast *trnH-psbA, matK* and *rbcL* in *Mucuna* species of India.

MATERIALS AND METHODS

Plant Material and DNA Isolation: Four different species of *Mucuna* namely *M.atropurpurea, M. monosperma, M.bracteata, M.nigricans* and two botanical varieties of *M. pruriens* var. *utilis* and *M. pruriens* var. *pruriens* were collected from the natural growing areas of India. DNA was isolated from the tender leaflets of the 5-7 day old plantlets using modified Doyle and Doyle method (Doyle & Doyle, 1990). 0.5 to 1 g of fresh leaf sample was ground in liquid nitrogen and homogenized in 10ml of extraction buffer containing 2% cetyltrimethylammoniumbromide (CTAB), 1% Polyvinylpyrolidone (PVP), 0.5% Charcoal, 1.4 M NaCl, 0.1M Tris-HCl, 20mM EDTA and 0.2% β mercaptoethanol. This suspension was incubated at 60^0C for 1 hour and extraction was done with two times chloroform: isoamylalcohol (24:1) and one time phenol: chloroform: isoamylalcohol (1:1) treatments. Finally the DNA was precipitated using 0.67 volumes of isopropanol. The concentrated DNA pellet was washed with 70% ethanol to remove the traces of phenol or chloroform. This pellet was air dried and dissolved in 1X Tris-EDTA buffer (pH 8). Isolated DNA was assessed for its quality and quantity using ethidium bromide staining on 0.8% agarose gel.

Internal Transcribed Spacer (nrITS) amplification

For amplification of ITS region ITS3 and ITS4 (Table 1) primers were used. Sequences of the primers used are given in Table 1. Amplification reaction was carried out in 0.025cm^3 reaction mixture containing 0.2mM dNTP's, 10mM Tris-HCl, 1.5mM $MgCl_2$, 50mM KCl, 0.1% Triton X-100, 1.0 U *Taq DNA polymerase*, 30-50ng of genomic DNA and different concentrations (0.05µM, 0.1µM and 0.2µM) of ITS3 and ITS4 primers (Eurofins Genomics India Pvt. Ltd. Bangalore). Amplification protocol was tested with the following program with different annealing temperatures: 2 min at 94^0C, 35 cycles of 30 s at 94^0C, 30 s at 54^0C /56^0C/58^0C and 45 s at 72^0C, final extension of 5 min at 72^0C were performed.

***trnH-psbA* amplification**

Amplification of *trnH-psbA* region was carried out in 0.025cm^3 reaction mixture containing 0.3mM dNTP's, 10mM Tris-HCl, 3mM $MgCl_2$, 50mM KCl, 0.1% Triton X-100, 1.0 U *Taq* DNA polymerase, two different individual concentrations of primers i.e., 0.1µM and 0.2µM forward and reverse primers (Eurofins Genomics India Pvt. Ltd. Bangalore) and 50ng of genomic DNA. Primers were used in the study are *psbA*F and *trnH*R (Table 1). Amplification program included the following steps: 1 min at 94 ^{0}C, followed by 40 cycles of 30 s at 94 ^{0}C, 40 s annealing at 53 ^{0}C and 40 s extension at 72 ^{0}C and a final extension cycle of 5 min at 72 ^{0}C.

***matK* amplification**

For amplifying the *matK* region in *Mucuna* species the three different combinations (Table 1) of *matK* primers (mat K 2.1F/5 R, matK-Mu1F/Mu2 R & matK-xf/MALP) were tested. Amplification reaction was carried out in a total of 0.025cm^3 reaction mixture containing 10mM Tris-HCl,50mM KCl, 0.1% Triton X-100, 1.5mM $MgCl_2$, 0.2mM dNTP's, 1.0 U *Taq* DNA polymerase, with different concentrations (1 µM, 0.5 µM and 0.3µM) of forward and reverse primers (Eurofins Genomics India Pvt. Ltd. Bangalore) and 50ng of genomic DNA. The thermal cycling program was tested with two different annealing temperatures and the programs is as follows; 94°C for 1 min; 35 cycles of 94°C for 30 s, 49°C /55°C for 30 s, 72°C for 50 s; final extension 72°C for 5 min.

***rbcL* amplification**

rbcL region amplification in *Mucuna* species was tried with three different *rbcL* primer combinations (Table 1). Amplification reaction was carried out in a total of 0.025cm^3 reaction mixture containing 10mM Tris-HCl,50mM KCl, 0.1% Triton X-100, 2.5mM $MgCl_2$, 0.4mM dNTP's, 1.0 U *Taq* DNA polymerase, 0.3µM of forward and reverse primers (Eurofins Genomics India Pvt. Ltd. Bangalore) and 50ng of genomic DNA. The same reaction was also tested with 5% DMSO The thermal cycling program was as follows; 94°C for 1 min; 35 cycles of 94°C for 30 s, 49°C /55°C for 30 s, 72°C for 50 s; final extension 72°C for 5 min.

Amplification of all the above marker regions was performed in Peltier thermal cycler (MJ Research, USA). In all the reactions a negative control was kept, which contained all PCR reactants except the template DNA, which was replaced with nuclease free water.

Amplification products were resolved on 1.5% agarose gel (1X TAE) followed by ethidium bromide staining.

Sequencing of the amplicons, sequence alignment and analysis of the data

The nrITS2, *trn*H-*psb*A*, mat*K and *rbc*L amplification products were sequenced in both forward and reverse directions at Eurofins Genomics India Pvt. Ltd. using ABI 3730XL (Applied BioSystems) sequencer following the manufacturer's protocols. The sequence data obtained was manually assessed and trimmed based on the quality parameters like background noise, peak intensity in respective chromatograms using Chromas lite version 2.01 (Technelysium, 2015). Successful sequencing was considered only when bidirectional sequences obtained in different sequencing runs could be assembled into a reliable contig a BLAST (http://blast.ncbi.nlm.nih.gov/) run was done to confirm their identity. Obtained sequences were submitted to NCBI Genbank. Genbank accession numbers are given in table 2.

RESULTS

ITS2 region of Mucuna species showed a single non-spurious amplification product of size ~300-400bp at the concentration of 0.1µM of ITS3 and ITS4 primers with an annealing temperature of 56^{0}C (Figure 1). 0.05µM & 0.2µM concentrations yielded less intense bands and primer dimers respectively. Amplification at 58^{0}C annealing temperature yielded poor amplification products, where as with 54^{0}C the product intensity was almost comparable with 56^{0}C with minor reduction in the intensity. The average G+C content among different sequences of ITS2 region varied from 54 to 63 % indicating well conserved regions.

The chloroplast *trn*H-*psb*A spacer region gave an intense sharp amplification product of size ranging between 350 to 450bp with 0.1µM concentration of *psbA*F and *trnH*R primers (Figure 1). The G+C content varied from 27 to 31 % indicating less conserved regions compared to ITS.

To amplify *mat*K region of *Mucuna* species three different combinations were tried, out of which only matK-xf/MALP primer combination showed the amplification at a concentration of 0.3µM of forward and reverse primers. This amplification was seen at an annealing temperature of 49°C and the product size was between 900 to 1100bp (Figure 1).

Table 1 List of Primers and their sequences used in the present study

Region	Primer Name	Sequence (5'-3')	Direction	References
ITS2	ITS3	GCATCGATGAAGAACGCAGC	F	White *et al.* 1990
	ITS4	TCCTCCGCTTATTGATATGC	R	White *et al.* 1990
***trn*H-*psb*A**	psbA3_f	GTTATGCATGAACGTAATGCT C	F	Sang *et al*. 1997
	trnHf_05	CGCGCATGGTGGATTCACAATCC	R	Tate & Simpson, 2003
***mat*K**	matK-xf	TAATTTACGATCAATTCATTC	F	Ford *et al*. 2009
	matK-MALP	ACAAGAAAGTCGAAGTAT	R	Dunning & Savolainen, 2010
	mat K 2.1F	CCTATCCATCTGGAAATCTTA	F	Ford *et al*. 2009
	mat K 5 R	GTTCTAGCACAAGAAAGTCG	R	Ford *et al*. 2009
	matK-Mu1	GTCCGT TGA TGR DTT TTA CTT G	F	Wiriyakarun *et al.* 2013
	matK-Mu2	TTAATG AAT CCC GAA TCC TG	R	Wiriyakarun *et al.* 2013
***rbc*L**	F	ATGTCACCACAAACAGAAACTAAAGCAAGT	F	Parfitt & Badenes,1997
	R	ACTACAGATCTCATACTACCCC	R	Parfitt & Badenes,1997
	RbcLa-F	ATGTCACCACAAACAGAGACTAAAGC	F	Levin *et al*. 2003
	RbcLa-R	GTAAAATCAAGTCCACCRCG	R	Kress & Erickson , 2007
	rbcLajf634R	GAAACGGTCTCTCCAACGCAT	R	Fazekas *et al*.2008

Table 2 Genbank accession numbers of the four barcode regions from *Mucuna* species

	nrITS2	*matK*	*rbcl*	*trnH_psbA*
M.atropurpurea	KX499608	KX606940	KX606913	KX606865
M.bracteata	KX499611	KX606943	KX606937	KX606870
M.monosperma	KX499613	KX606942	KX606930	KX606872
M.nigricans	JQ340034	KX721059	KX721060	KX606907
M. pruriens var. utilis	KX499624	KX606951	KX606921	KX606890
M. pruriens var. pruriens	KX499617	KX606952	KX606928	KX606877

The average The G+C content was comparable with *trn*H-*psb*A spacer and it varied from 27 to 31 % indicating less conserved regions.

The last chloroplast region under study was *rbc*L and the amplification was obtained with both RbcLa-F/R and RbcLa-F/jf634R combinations with 5% DMSO in the reaction mixture. DMSO was found critical for the amplification of *rbc*L region, as it was observed in the absence of it this region never amplified. The optimum annealing temperature for the above primer combinations was 55^{0}C and the product size was ranging between 550-650bp (Figure 1). The G+C content varied from 40 to 43% indicating better conserved regions than *matK* and *trn*H-*psb*A.

Amplification profile of DNA barcode regions in Mucuna species

DISCUSSION

The concept of DNA barcoding highlights the utility of conserved regions to identify species more accurately using the standardized DNA sequences (Hebert *et al.,* 2003). Although the regions ITS, *trnH-psbA, matK* and *rbcL* are considered as the universal markers, literature reveals different optimum conditions for amplifying these regions. Nuclear Internal Transcribed Spacer region (nrITS2) amplification conditions in the present study are in consensus with the earlier reports (Fazekas *et al.,* 2008; Madesis *et al.,* 2012). However, it is observed attempts to amplify nrITS2 at 36^{0}C and 48^{0}C were also successful (Jaheer *et al.,* 2015; Baldwin, 1998). This suggests probably the combination of primers ITS 3 and ITS4 used to amplify nrITS2 region of nrITS which is located between 5.8Sr DNA and 26Sr DNA has a wide annealing temperature range (White *et al.* 1990). Being shorter than full length ITS region, ITS2 also exhibited efficient marker attributes in DNA barcoding and phylogenetic analysis (Han *et al.,* 2013). CBOL recommended the inclusion of chloroplast *trn*H-*psb*A spacer region along with the *matK* and *rbcL* regions to develop plant DNA barcodes because of its molecular resolving power at species level and ease in amplification (Shaw *et al.,* 2007). Several studies reported the combined analysis of non-coding regions of *ITS* + *trnH-psbA* resulted a better discrimination between the closely related species (Bolson *et al*., 2015; Pang *et al*., 2012).

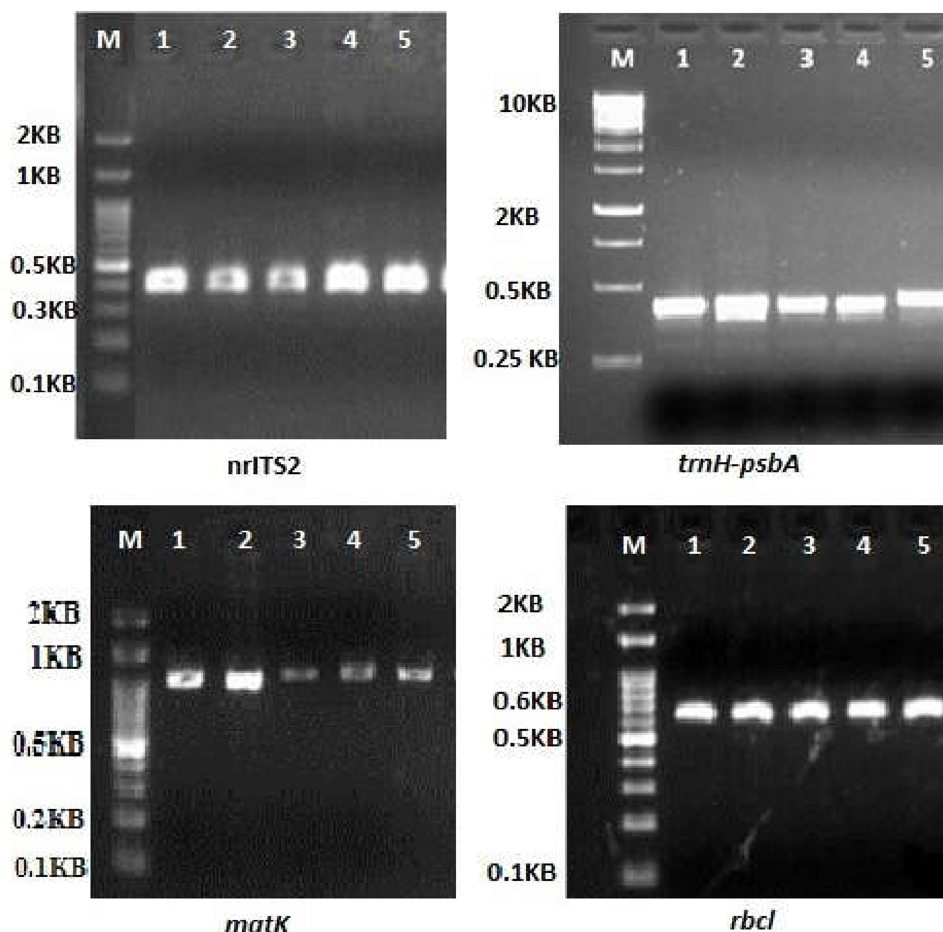

Figure 1 Amplification profile of nrITS2 , *trnH-psbA*, *matK* and *rbcL* regions in *Mucuna* species; **M**: 1KB Ladder; Lanes 1- 5: *Mucuna* species (*M.atropurpurea, M.monosperma, M.bracteata, M.pruriens* var. *pruriens* and *M.pruriens* var. *utilis*)

Though *matK* is one among the two locus universal barcode suggested by CBOL plant working group (C.P.Group, 2009), its amplification across the species using universal primers is not successful (Vijayan and Tsou, 2010). Because of this, to amplify *matK* region of the species under study, always relevant primers selection and optimization of other PCR parameters arises. In the present study out of the three different combinations of *matK* primers screened only matK-xf/MALP was able to amplify *matK* region in *Mucuna*. Where as to amplify another chloroplast barcode region *rbcl* such universality related issues were not found but it is observed for successful amplification of *rbcl* region PCR additive DMSO plays a very critical role. CBOL plant working group has recommended the two locus combination of *rbcL* + *matK* as the plant barcode based on the recoverability, sequence quality and levels of species discrimination (C.P.Group, 2009).

CONCLUSION

This study reports the amplification efficiency of the DNA barcode markers like ITS and *trnH-psbA, matK* and *rbcL* in medicinally and nutritionally important genus *Mucuna*. The results of the present study lead foundation to expand the sample size towards inferring the phylogeny of the genus to get a better understanding on the relationship among the members of the genus *Mucuna*.

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