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Cytogenetic and ITS-*psbA-trnH* Sequence Analysis for Phylogenetic Inference in *Mucuna* sp. of India

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Abstract This paper reports new chromosomal information and earliest proof on efficacy of nrITS and cp-*psbA-trnH* gene sequences for barcoding and phylogenetic studies in *Mucuna* sp. First cytological evidence on *M. sempervirens* and *M. bracteata* revealed symmetrical karyotype in both the species. Meiotic anomalies evident from the formation of chromosomal laggards and bridges in *M. gigantea* and *M. atropurpurea* and unreduced pollens in *M. bracteata* suggest evolutionary events that might have shaped annual species in this genus. Molecular investigations using nrITS and cp-*psbA-trnH* sequences revealed them to be phylogenetically informative. Vis-à-vis individual genes, combined sequences of ITS and *psbA-trnH* sequences offered reliable data for species delineation. The results summarized here are expected to galvanize molecular taxonomic studies and open-up newer means for answering phylogenetic questions in this genus.

Keywords *Mucuna* sp · Karyomorphological analysis · Taxonomy · Phylogeny · nrITS · cp-*psbA-trnH*

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KKR completed this research when she was student of MVIT

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Introduction

Mucuna Adans. belongs to Fabaceae family and includes 100 species of annual and perennial legumes of pantropical distribution (Buckles 1995). In India, it is represented by ten species (Wilmot-Dear 1987; Aitawade et al. 2012) - 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. Excluding *M. pruriens*, and perhaps recently reported *M. sanjappae* (Aitawade et al. 2012), all others display perennial growth habit.

Mucuna's are climbing plants with long, slender branches and lanceolate leaves on hairy petioles with large flowers. They grow in clusters of two or three, with a bluish purple or white, butterfly-shaped corolla. The pods or legumes are variously shaped and contain four to six seeds. They are of rich dark brown or green in colour, thickly covered with stiff hairs and cause intense itching (pruritis) if they come in contact with human skin (Wilmot-Dear 1987). The members of this genus particularly annual *M. pruriens* L. (DC) offers distinct agronomic and medicinal benefits. It is characterized by high nitrogen fixing ability, aggressive growth habit and high productivity of vegetative matter (Bressani 2002). Besides, the plant exhibits promising agronomic potentials in terms of protein content (20–30 %; (Buckles 1995)); seed yield (Carsky and Ndikawa 1998); and disease resistance (Eilitta et al. 2002). It is also a source of L-Dopa (L 3, 4 dihydroxy phenylalanine) -a non-protein amino acid that acts as a precursor of the neurotransmitter drug dopamine used in treatment of Parkinson's disease (Farooqi et al. 1999). Due to rich biomass and N2 fixing ability, it is often described as "featured example of green manures

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

Chromosomal studies suggest x=11 as the most frequent basic number in this genus even though x=14 is reported in *M. gigantea* and *M. benettii* (Jaheer and Sathyanarayana 2010; Sastrapradja et al. 1974). Among the Indian species, karyotype description is available for *M. atropurpurea, M. monosperma, M. nigricans* and *M. pruriens* (Agostini et al. 2009; Jaheer and Sathyanarayana 2010). Other species, including *M. sempervirens*, *M. bracteata* investigated in the present work lacked this information.

On the other side, considerable taxonomic ambiguities exist within this genus with several synonyms reported both at the species and sub-species level. Quite a few taxa that were earlier recognized as a separate species are now shown to be merely varieties of *M. pruriens* (Capo-chichi et al. 2001). Even within this species, in addition to two widely known botanical varieties: var. *pruriens* and var. *utilis* (Dassanayake and Fosberg 1980; Sasidharan 2004), presence of third group: var. *hirsuta* has been suggested (Wilmot-Dear 1987). Botanical type *hirsuta* was earlier classified as an independent species (Cecil Saldanha 1996; (Ellis 1990)), and subsequent revisions, especially by Wilmot-Dear (Wilmot-Dear 1987), has categorically suggested inclusion of the same under the botanical varieties of *M. pruriens*. However, literatures continue to refer *M. pruriens* var. *hirsuta* as an independent species (Rajaram and Janardhanan 1991) and such anomalies, although predominant in *M. pruriens* are not uncommon in other taxa of this genus. Because of this and other confusions surrounding the taxonomy, it is necessary to conduct research both at the species and sub-species level to assess the phenetic relationships among different taxa to place them in a right taxonomic and phylogenetic perspective. Moreover, the ongoing efforts under legume diversity assessment project by Asia-Pacific Biodiversity Observation Network (AP-BON) has emphasized on the genus specific phylogentic diversity (PD) assessment using DNA sequence information to gain a holistic representation of global legume diversity. The consortium has identified, among others, *Mucuna* as representative genera for the case study.

Molecular studies involving nuclear and chloroplast genes have resolved monophyletic grouping among several legume taxa including Phaseoloid members. Internal transcribed spacer (ITS), present between the 18S and 26S rRNA genes is well recognized for such studies (A'lvarez and Wendel 2003) and has been used earlier for resolving relationship among the members of *Phaseolus–Vigna* complex (Delgado-Salinas et al. 2006; Goel et al. 2002). On the other hand, the *trnH-psbA* intergenic spacer region lies in the inverted repeat region of the chloroplast genome near the boundary with the large single-copy region adjacent to the *trnK* gene (Suguira 1992). It is one of the most variable regions of the plastid genome in terms of having highest percentages of variable sites (Shaw et al. 2007) and can offer high levels of species discrimination (Kress et al. 2005; Shaw et al. 2007). In *Mucuna,* however, such efforts for taxonomy and phylogenetic studies are lacking. So far, it has been only shown to be member of Erythrininae - a sister tribe to Desmodieae under larger phaseologid group (Stefanovic et al. 2009) having plastid genome inversion of 78-kb with a loss of one copy of large inverted repeat (Lavin et al. 1990; Palmer et al. 1987).

In view of this, the present study aimed to ascertain chromosome number and karyo-morphological characteristics in three Indian taxa viz., *M. sempervirens*, *M. bracteata* and *M. gigantea* in addition to examining the potential of ITS and *trnH-psbA* regions as diagnostic markers for species identification and phylogenetic studies.

Materials and Methods

Plant Material

Plants representing seven *Mucuna* species viz. *M. atropurpurea* (3)*, M. monosperma* (1)*, M. nigricans* (1), *M. sempervirens* (1)*, M. gigantea* (1), *M. bracteata* (2) and botanical varieties of *M. pruriens* viz. var. *utilis* (1), var. *pruriens* (2) collected from natural growing areas in India were used for the study (Table 1 and Fig. 1).

Mitotic and Meiotic Slide Preparation

For cytological preparations young tissues collected from 10 randomly selected plants of each species/accessions, preserved in a fixative were used. Anthers and root tips were squashed in a drop of acetocarmine on a glass slide by immobilizing the root tip with cover slip. Followed by gentle tapping, strong squashing was applied to spread the cells uniformly. The chromosomes were visualized, photographed and archived using Olympus digital camera (Olympus, Japan). Analysis of the chromosome complement was done using microphotographs.

Karyotype and Idiogram Preparation

Ten metaphases stage cells chosen from each accession were used for karyotyping and idiogram construction. The length of short (s) or long arm (l) of each chromosome was obtained using published standard methods and these values were used to calculate: total length of chromosome (CL=s+l), haploid set length (HSL=ΣCL), arm ratio (AR=l/s) and relative length of chromosome (RL=CL/HSL). Chromosomes were

Table 1 Representative samples of different *Mucuna* spp. used in the study

Sl. No.	Name of the species	Accession numbers	Place of Collection	Geographical co-ordinates	For mitotic	For meiotic	For ITS and *trn*H- *psb*A
1	*M. atropurpurea*	500114TN	Madurai, Tamil Nadu	09°91′ N 78°12′ E	-	-	√
2	*M. atropurpurea*	500140TN	Teni, Tamil Nadu	10°04′ N 77°45′ E	-	√	-
3	*M. atropurpurea*	500141TN	Tirunelveli, Tamil Nadu	08°58′ N 77°21′ E	-	√	-
4	*M. monosperma*	500107KL	MSSRF, Waynad Kerala	11°60′ N 76°08′ E	-	√	√
5	*M. gigantea*	500104KL	MSSRF, Waynad Kerala	11°60′ N 76°08′ E	√	√	√
6	*M. bracteata*	500124KL	Seed company, Kerala	-	√	√	√
7	*M. bracteata*	500201ML	Shillong, Meghalaya	25°57′ N 91°88′ E	-	√	-
8	*M. nigricans*	500198WB	Gorumera, N. Park	26°70′ N 88°80′ E	-	-	√
9	*M. sempervirens*	500200WB	Darjeeling, W. Bengal	27°03′ N 88°16′ E	√	-	√
10	*M. pruriens* var. *pruriens*	500113MH	Triambakeshwar, MH	20°00′ N 73°77′ E	-	√	√
11	*M. pruriens* var. *utilis*	500101KA	Bangalore, Karnataka	13°14′ N 77°62′ E	-	√	√
12	*M. pruriens* var. *pruriens*	500123KL	Seed company, Kerala	-	-	√	√
13	*Butea* sp.	-	Bangalore, Karnataka	13°14′ N 77°62′ E	-	-	√

classified based on arm ratio according to Levan et al. (Levan et al. 1964). Karyotype symmetry and asymmetry was measured by the criteria proposed by Stebbins (Stebbins 1958).

Meiotic Analysis

Meiotic studies were performed in four species which included two accessions of *M. atropurpurea* (500140TN and 500141TN), two accessions of *M. monosperma* (500107KL and 500167KA), two accessions of *M. bracteata* (500124KL and 500201ML) and one accession of *M. gigantea* (500104KL). Meiotic abnormalities such as, chromosome laggards, bridges and unreduced meiocytes were analyzed in 50 to 300 meiocytes from five slides per species/accessions under light microscope and percentage of abnormalities were estimated.

Fig. 1 Collection locations of accessions sampled for the study. Courtesy: (Maps of India)

Internal Transcribed Spacer (ITS) Amplification

Amplification of ITS region was carried out in 0.025 cm^3 reaction mixture containing 0.2 mM dNTP's, 10 mM Tris–HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 % Triton X-100, 1.0 U *Taq DNA polymerase*, 0.2 μM forward and reverse primers (Eurofin Genomics India Pvt. Ltd, Bangalore) and 50 ng of genomic DNA. Two primer combinations- ITS-3 and ITS-4 (White et al. 1990) were used (Table 2). Amplification was performed in Peltier thermal cycler (MJ Research, USA) using the following protocol: After the initial cycle of 2 min at 94 °C, 2 min at 36 °C and 2 min at 72 °C, 38 cycles of 1 min at 94 °C, 1 min at 36 °C and 2 min at 72 °C were performed. The last cycle was followed by 7 min extension at 72 °C. Reaction mixture in which template DNA was replaced by distilled water, was used as a negative control. Amplification product were resolved on 1.5 % agarose gel (1× TAE) followed by ethidium bromide staining.

trnH-psbA Amplification

Amplification of *trnH-psbA* region was carried out in 0.025 cm^3 reaction mixture containing 0.3 mM dNTP's, 10 mM Tris–HCl, 3 mM $MgCl_2$, 50 mM KCl, 0.1 % Triton X-100, 1.0 U Taq DNA polymerase, 0.2 μM forward and

Table 2 Nucleotide sequences of the two genes used in the study

SL. NO.	PRIMER	PRIMER SEQUENCE (5′→3′)
ITS primers[a]		
1.	ITS 3	GCATCGATGAAGAACGCAGC
2.	ITS 4	TCCTCCGCTTATTGATATGC
trnH-psbA primers[b]		
1.	*psbA*F	GTTATGCATGAACGTAATGCC
2.	*trnH*R	CGCGCATGGTGATTCACAAATC

[a] White et al. 1990

[b] Sang et al. 1997

reverse primers (Sigma Aldrich Chemicals Pvt.Ltd) and 50 ng of genomic DNA. Primers were used in the study: *psbAF* and *trnHR*. The sequences of these primers are provided in Table 2. Amplification was performed in Peltier thermal cycler (MJ Research, USA) using the following protocol: 1 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 40 s annealing at 53 °C and 40 s extension at 72 °C and a final extension cycle of 5 min at 72 °C. Reaction mixtures in template DNA replaced by distilled water was used as negative control. Amplification product were resolved on 1.5 % agarose gel (1× TAE) followed by ethidium bromide staining.

Sequencing, Alignment and Phylogenetic Analysis

Sequencing works were carried out at Bangalore Genei on an ABI 3100 sequencer following the manufacturer's protocols. The Sequences so obtained were exported to Molecular Evolutionary Genetics Analysis software version 4.0 (MEGA-4) for editing, alignment and converting into nexus format (Tamura et al. 2007) and were subjected to analyses such as estimating percentage of G and C content, variable sites, and parsimony informative sites. Alignment gaps were coded as missing characters.

Phylogenetic Analysis

Phylogenetic analyses using maximum parsimony (MP) was performed with PAUP ver. 4.0b10 (Swofford 2011) where *Butea* - which also belongs to Erythrininae, was used as an out-group. Heuristic searches with 100 addition replicates were conducted using tree bisection reconnection (TBR) branch swapping and MULPARS in effect. Support for individual clades was tested using bootstrap values (Felsenstein 1985) using 200 replicates. Different measures of homoplasy such as consistency index (Kluge and Farris 1969), retention index (Farris 1989a, 1989b) and rescaled consistency index (Farris 1989a, 1989b), were also estimated in MP analysis.

Results

Karyotype Analysis

Chromosomes of different *Mucuna* species observed in the present study fit into *'very small to small'* category as per the classification given by Lima De Faria (1980). The karyotypic details of the three studied species are described below:

M. gigantea The mitotic chromosome length (Fig. 2a) ranged from 1.35 to 0.7 μm and the average Haploid Set Length (HSL) recorded was 10.51 μm with a standard error of 0.35. The idiogram is given in the Fig. 2d. The taxon revealed karyotypic formula: 2n=28, *n*=14, 3 M+8 m+2sm+1st and the karyotype is of symmetrical type (Table 3).

M. sempervirens The mitotic chromosome length (Fig. 2b) ranged from 1.63 to 0.5 μm and the average Haploid Set Length (HSL) was 10.38 μm. with a standard error of 0.16 The idiogram is given in the Fig. 2e. The taxon produced a karyotypic formula: 2n=22, *n*=11, 3 M+6 m+2sm. The karyotype is of symmetrical type (Table 3).

M. bracteata The mitotic chromosome lengths (Fig. 2c)) ranged from 1.1 to 0.7 μm and the average Haploid Set Length (HSL) was 10.23 μm with a standard error of 0.28. The idiogram is given in the Fig. 2f. The taxon display a karyotypic formula: 2n=22, *n*=11, 10 m+1sm. The karyotype is of symmetrical type (Table 3).

Meiotic Analysis

M. monosperma and *M. pruriens* did not show any meiotic anomalies. However, chromosome laggards and bridges were observed in *M. gigantea* (Fig. 3a and b) and *M. atropurpurea* (Fig. 3c–e; f–h). Both the samples of *M. bracteata* showed unreduced meiocytes (Fig. 3i–j). The percentage of these anomalies was measured and the data is presented in Table 3.

Characterization of ITS and *trnH-psbA* Region

All the species, after PCR amplification, yielded single ITS fragment without any spurious bands. The size of the PCR products, after sequencing, ranged from ~303 to ~382 bp among different *Mucuna* species. The *Butea* sp. selected as an out-group produced sequence length of ~250 bp. The highest sequence length (382 bp) was observed in *M. pruriens* var. *utilis* and lowest (303 bp) in *M. nigricans*. The G+C content among different sequences varied from 54 to 63 % indicating well conserved regions. Likewise, for *trnH-psbA*, the amplicon size

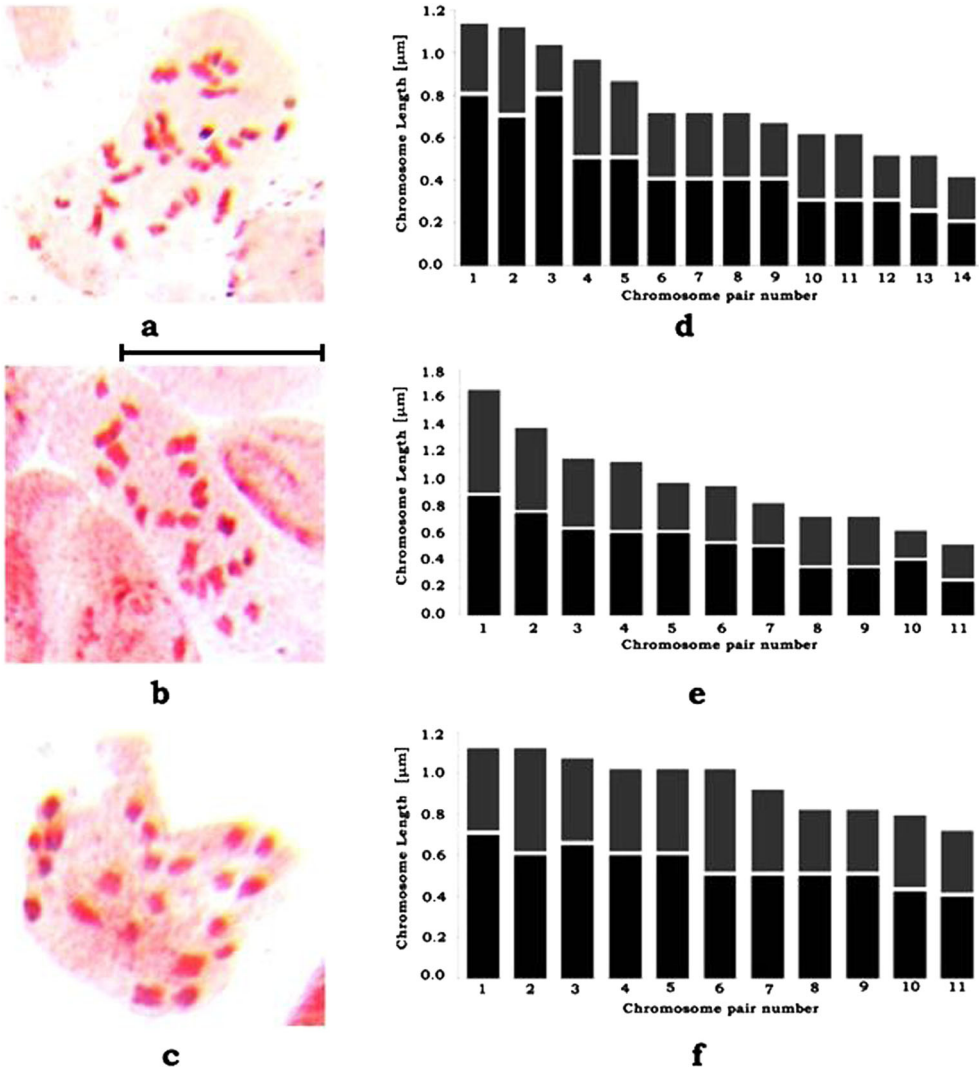


Fig. 2 Mitotic plate and idiogram of *M. gigantea* (**a** and **d**); *M. sempervirens* (**b** and **e**) and *M. bracteata* (**c** and **f**). *Bar*= 10 µm. The gray and black shaded bars indicate the short and long arms of the chromosomes respectively

varied from ~367 to ~420 bp between different *Mucuna* sp. and 403 bp in case of *Butea* sp. The highest sequence length (420 bp) was observed in *M. monosperma* and lowest (367 bp) in *M. bracteata.* The G+C content varied from 27 to 31 % indicating less conserved regions compared to ITS.

Phylogenetic Analysis

Grouping based on ITS sequences produced two distinct clusters (Fig. 4). The most parsimonious tree obtained by an out group root method with TBR as branch-swapping algorithm was monophyletic. The heuristic search indicated that all 463

Table 3 Chromosomal data from mitotic and meioticstudies in three *Mucuna* spp

Sl. No.	Name of the Species	Diploid Number (2n)	Chromosome Length (µm)	Average Haploid set Length±SE	Karyotype Formula (*)	Chromosome laggards (%)	Chromosome Bridges (%)	Unreduced Pollens (%)
1	*M. gigantea*	28	1.12 to 0.4	10.51±0.35	3 M+8 m+2sm+1st	2.1	3.7	–
2	*M. bracteata*	22	1.1 to 0.7	10.23±0.16	10 m+1sm	–	–	6.5
3	*M. bracteata*	–	–	–	–	–	–	6.9
4	*M.atropurpurea*	–	–	–	–	2.6	1.9	–
5	*M.atropurpurea*	–	–	–	–	3	4	–
6	*M. sempervirens*	22	1.65 to 0.5	10.38±0.28	3 M+6 m+2sm	–	–	–

**M* Median point, *m* Median region, *sm* Submedian region, *st* Subterminal region; *SE* standard error

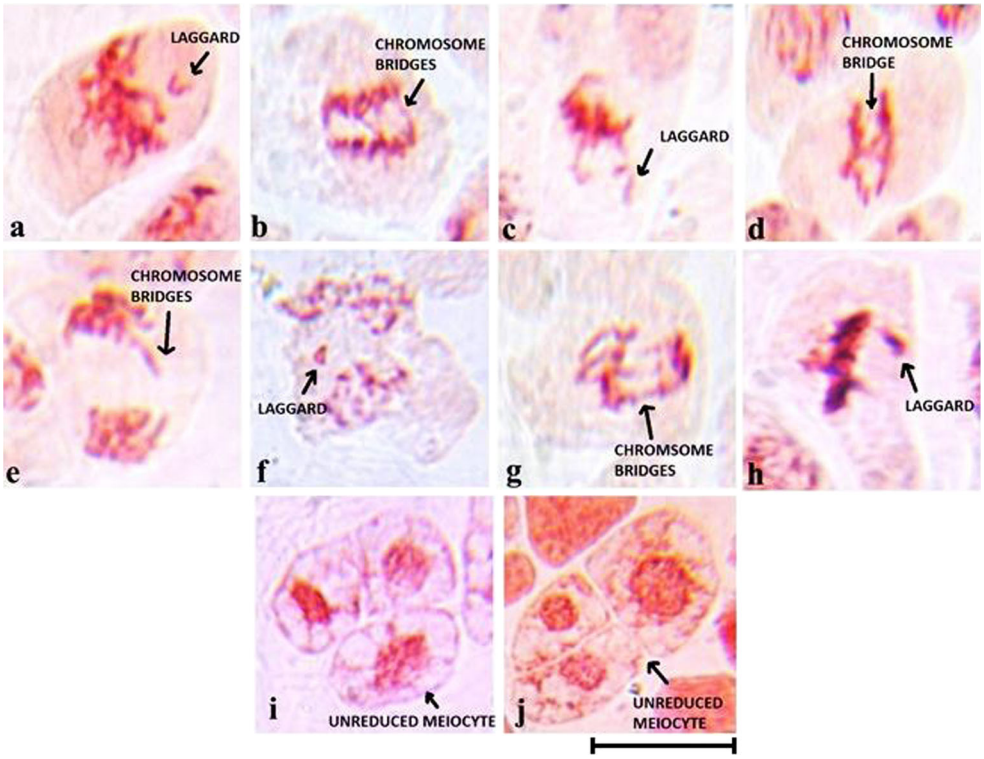


Fig. 3 Meiotic chromosome abnormalities (indicated with pointed arrow) in different species: Laggards in *M. gigantea* (**a**) *M.atropurpurea* (**c**, **f** and **h**). Chromosome bridges in *M. gigantea* (**b**) and *M. atropurpurea* (**d**, **e** and **g**); unreducedmeiocytes in*M. bracteata* (**i** and **j**). *Bar*= 10 μm

characters were of type 'unord' and have equal weight. Of these, 158 characters were constant, 89 variable characters were parsimony-uninformative and only 194 were parsimony-informative. The consensus tree generated from 423 most parsimonious trees showed a high value of consistency index (0.87), retention index (0.75) and rescaled consistency index (0.71) indicative of relatively low homoplasy (homoplasy index- 0.1277). In cladogram, majority of the accessions was found to be concentrated around cluster I, which was further divided into 2 sub-clusters of which the first one (1A) resolved monophyletic grouping. This contained perennial species of non-eastern origin such as: *M. monosperma, M. gigantea,* with *M. atropurpurea* as an ancestor. The second sub-cluster (1B) comprised varieties of *M. pruriens* with *M. bracteata* forming an out-group. The cluster II encompassed two perennial species of eastern Indian origin viz. *M. sempervirens* and *M. nigricans*.

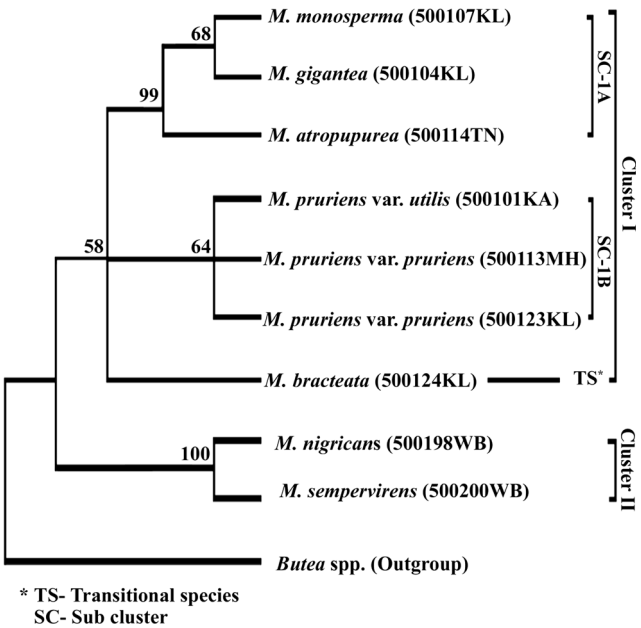


Fig. 4 Parsimonious tree obtained from the ITS nrDNA dataset. Numbers above the branches are bootstrap percentages (1000 replicates)

In case of *trnH-psbA,* the heuristic search indicated that out of 467 total characters, all were type 'unord' and have equal weight. Of this, 289 were constant, 119 variable characters were parsimony-uninformative and 59 were parsimony-informative. The consensus tree from the 232 most parsimonious trees showed high value of consistency index (0.88), retention index (0.79) and rescaled consistency index (0.70) indicative of low homoplasy (homoplasy index- 0.1164). The tree showed two major clusters (Fig. 5) of which cluster I comprised only annual *M. pruriens* varieties with *M. bracteata* as ancestor and cluster II grouped all the perennials species together.

Phylogenetic tree obtained by combining both ITS and *trnH-psbA* sequence was more informative. The parsimonious tree generated by the out group root method with TBR as branch-swapping algorithm was monophyletic (Fig. 6). The heuristic search indicated that out of 862 total characters all are of type 'unord' with equal weight. Of these, 374 constant and 248 variable characters were parsimony-uninformative

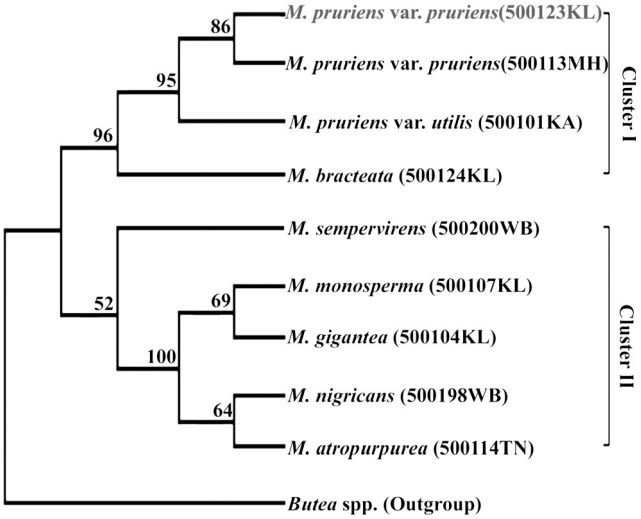
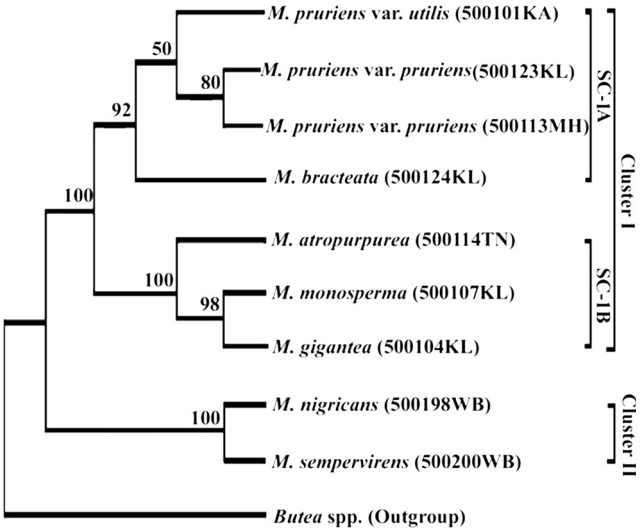


Fig. 5 Parsimonious tree obtained from the *trnH-psbA* dataset. Numbers above the branches are bootstrap percentages (1000 replicates)

and only 240 were parsimony-informative. The consensus tree from the 734 most parsimonious trees showed a high value of consistency index (0.86), retention index (0.75) and rescaled consistency index (0.65) indicative of relatively low homoplasy (homoplasy index- 0.1376). The tree depicted two major clusters, I and II (Fig. 5) with first one further classified into two sub-clusters 1A and 1B. The sub-cluster 1A grouped together botanical varieties of *M. pruriens* with *M. bracteata* as an ancestor which is in consistent with the results of other analyses. Sub-cluster 1B clearly separated perennial species of non-eastern origin such as: *M. monosperma, M. gigantea* with *M. atropurpurea* as an ancestor. The cluster II distinctly separated two perennial accessions *M. sempervirens* and *M. nigricans* belonging to the eastern region.



SC- Sub cluster

Fig. 6 Parsimonious tree obtained from the combined ITS and *trnH-psbA* dataset. Numbers above the branches are bootstrap percentages (1000 replicates)

Discussion

Karyotypic Analysis

The diploid chromosome number of 2n=22 and 2n=28 observed in two *Mucuna* species viz. *M. sempervirens* and *M. gigantea* is in consensus with the earlier reports (Agostini et al. 2009; Sastrapradja et al. 1974). In addition, new counts reported in *M. bracteata, M. sempervirens* and *M. nigricans* (all 2n=22) corroborate with the trend of chromosome number conservation in the tribe *Phaseoleae* with basic number x=11. Even the chromosome lengths of different species which ranged from 1.63 to 0.4 μm with an average at 0.53 and 1.28 μm is in agreement with reports from the subfamily Papilionoideae (Mercado-Ruaro and Delgado-Salinas 1998; Souza and Benko-Iseppon 2004). However, presence of two basic numbers, $n=11$ and $n=14$ suggests possible occurrence of dysploidy in the genus which needs further investigations.

Meiotic Analysis

Incidence of meiotic anomalies in different species points towards chromosomal instability – the reason for which could not be ascertained. However, presence of unreduced meiocytes and uneven pollen grains in *M. bracteata* suggest possible evolutionary pressure towards annual growth habit which needs to be confirmed. This species also shows several characters that are transitional between annual and perennial species.

Phylogenetic Analysis

The efficacy of nrITS and chloroplast-*trnH-psbA* regions for taxonomic and phylogenetic studies is well established ((Kress et al. 2005); Renaud et al. 2008). Thus, variations harbored in these sequences in 7 *Mucuna* sp. were analyzed to determine their usefulness for species identification and phylogenetic studies. However, both the genes, when used individually were found less effective for this purpose. For instance, ITS analysis placed *M. atropurpurea, M. monosperma and M. gigantea* in a single cluster with *M. atropurpurea* as an ancestor. The tree also clustered together two perennial species from the eastern Himalayas viz. *M. sempervirens* and *M. nigricans* with strong bootstrap support. However, it failed to divulge relationship between *M. pruriens* and *M. bracteata* and the latter clustered as an out group in this branch. The *trnH-psbA* sequence analysis, on the other side, resolved monophyletic tree for annual species

represented by *M. pruriens* varieties with *M. bracteata* emerging as an ancestor. However, it did not clearly resolve relationship between perennial accessions and produced skewed tree comprising intermixing of species from eastern Himalayas and other places.

Combined ITS+*trnH-psbA* analyses however provided better insight into the relationship among the *Mucuna* taxa in which the tree not only separated annual and perennial species, but also separated them based on the geographical areas of their presence. Within cluster I, sub-cluster IA revealed close affinities between *M. bracteata* and *M. pruriens* varieties. *M. bracteata*, even though reported to occur in North-East India, is now commonly distributed even in peninsular India. It exhibits characteristics of both annual and perennial species and shares close morphometric affinities with *M. pruriens* - with which it quite agrees in calyx, corolla and shape and texture of leaflets (Wilmot-Dear 1987). The pods are also similar in texture and shape. The two resemble each other even in their chromosomal characteristics (Jaheer and Sathyanarayana 2010). These similarities, along with the chromosomal anomalies discussed earlier, suggests *M. bracteata* as possible immediate ancestor for *M. pruriens* and other annual species in this genus. Likewise, cluster 1B grouped perennial species of non-eastern region. Further, cluster II clearly separated two perennial species of eastern Himalayas viz. *M. sempervirens* and *M. nigricans* establishing the superiority of the combined gene sequence analysis for phylogenetic inferences in *Mucuna* species.

Conclusions

The paper reports first information on chromosome number and meiotic behavior in selected *Mucuna* species. It also provides first proof on efficacy of combined ITS+*trnH-psbA* analysis for taxonomic and phylogenetic studies in this genus. Nonetheless, limited sample size restricts relevance of the results for the larger phylogenetic inferences. Thus, further work involving large number of species is needed to get clear-cut understanding on relationship among the members of this genus and evolutionary events that shaped them.

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