Indian J. Genet., 72(4): 445-453 (2012)

Analysis of phylogenetic relationships in *Abelmoschus* species (Malvaceae) using ribosomal and chloroplast intergenic spacers

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(Received : June 2012; Revised : September 2012; Accepted : October 2012)

Abstract

India is an important centre of diversity for genus *Abelmoschus* belonging to family Malvaceae and about eight *Abelmoschus* species occur here. In the present study the phylogenetic relationships among *A. caillei*, *A. crinitus*, *A. esculentus*, *A. ficulneus*, *A. moschatus*, *A. tuberculatus*, *A. tetraphyllus* and *A. pungens* were analysed using modern phylogenetic tools such as nuclear ribosomal spacers (*ITS-1*, *ITS-2*) and chloroplast intergenic spacers (*trnC-trnD, trnE-trnF, trnH-psbA*)to estimate species divergence based on sequence similarity. The study exposed the lack of power of ribosomal spacers *ITS-1*and *ITS-2* in resolving the species relationships in complex polyploid genera like *Abelmoschus* while the analysis of cpDNA intronic spacers revealed that *A. ficulneus* and *A. moschatus* are the closest wild relatives of *A. esculentus*. The study was helpful in redefining the *Abelmoschus* gene pool and since these species are exclusively found in the Indian sub continent, the centre of origin of okra should be re-addressed properly.

Key words: *Abelmoschus*, chloroplast DNA, internal transcribed spacer, phylogenetic analysis, ribosomal DNA, *trnC-trnD*, *trnE-trnF*, *trnH-psbA*

Introduction

Okra, *Abelmoschus esculentus* L. (Moench) is an important vegetable crop grown throughout the world mainly for its tender fruits. It belongs to the family Malvaceae. The genus *Abelmoschus* contains 8 different species described in detail by [1, 2]. The genus *Abelmoschus* is believed to have been domesticated in the Ethiopian region [3] or in western Africa [4, 5]. But there is an alternate view regarding the Indian origin of *Abelmoschus* due to the presence of several wild relatives in the region. In addition to the cultivated okra (*A. esculentus)*, other species belonging to the same genera found in the sub-continent are, *A. pungens*, *A. crinitus*, *A. caillei*, *A. manihot*, *A. tuberculatus*, *A. tetraphyllus*, *A. moschatus*, *A. angulosus* and *A. ficulneus.*

On the basis of the cytogenetic studies, affinities between cultivated okra, *Abelmoschus esculentus* and related wild taxa have been determined. Joshi and Hardas [6, 7] studied meiosis in hybrids obtained by crossing *A. esculentus* (n=65) and *A. tuberculatus* (n=29). They observed that 29 of the 65 chromosomes of *A. esculentus* had complete homology with 29 chromosomes of *A. tuberculatus.* The remaining set of 36 chromosomes (genome Y) of *Abelmoschus esculentus* showed greater homology with 36 chromosomes of *A. ficulneus* as compared to that of *A. moschatus.* It was concluded that one of the parents of *A. esculentus* (n=65) should have been *A. tuberculatus* (n=29). However, the source of other genome of 36 chromosomes remained doubtful with regard to the two species of Indian origin, *A. ficulneus* and *A. moschatus.* The present study was undertaken to determine the phylogenetic relationships among the different taxa under the genus *Abelmoschus* using modern phylogenetic tools such as Internal Transcribed Spacers (*ITS-1* and *ITS-2*) of nuclear ribosomal DNA and selected intergenic spacers of cpDNA (chloroplast DNA).

Advanced phylogenetic tools like noncoding chloroplast DNA spacers and *ITS* sequences from nuclear ribosomal DNA (nrDNA) make phylogenetic analysis more precise and less cumbersome. These

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Published by Indian Society of Genetics & Plant Breeding, F2, First Floor, NASC Complex, PB#11312, IARI, New Delhi 110 012
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are considered as the ideal tools for phylogenetic studies [8]. The spacer regions of nrDNA are characterized by higher sequence divergence and slow rates of evolution and are used for systematics across the whole tree of life including algae [9] , angiosperms [10-12], fungi [13, 14], polyps [15], bryophytes and lichens [16]. The other advantages of this region are biparental inheritance, easy PCR amplification using universal primers, moderate size permissible for sequencing and multicopy structure [17]. Compared to the ribosomal DNA, chloroplast DNA is slower in evolution and is maternally inherited. Moreover it is haploid and lacks recombination [18]. Chloroplast genome is completely sequenced, and different genes as well as intronic sequences are extensively used for phylogenetic analysis in plant kingdom [19-21]. In the present study three chloroplast spacer regions *viz*., *trnC-trnD, trnE-trnF* and *trnH-psbA* are employed for phylogenetic reconstruction of genus *Abelmoschus*.

Materials and methods

The experimental material consisted of different accessions collected from various locations in India as well as some exotic specimens (Table 1). The voucher specimens of all the species used for the study are preserved in the National Herbarium for Cultivated Plants, NBPGR, New Delhi. For *ITS* and cpDNA analyses DNA from a single plant or usually from a single leaf was used. DNA extraction was done from the young leaves of okra and related species using DNeasy Plant Mini Kit from Qiagen, as per the procedure. A part of the DNA sample was diluted with appropriate amount of sterilized water to yield a working solution with a concentration of 20 ng/µl and stored at –20^{o}C until use for PCR amplification. PCR amplification was carried out with 40 ng of genomic DNA, 2.5 mM $MgCl_2$, 1U Taq DNA polymerase, 1x PCR buffer without $MgCl_2$ and 200 µM of dNTPs and the amplification products were resolved in 1.8% agarose gel. The phylogenetic relationship between different species was analyzed through amplification of *ITS-1*, *ITS-2* and selected chloroplast regions including *trnC-trnD*, *trnE-trnF* as well as *trnH-psbA* intergenic spacers using specific primers (Table 2) [22] and the primer concentration varied from 1.0 µM to 1.2 µM for ITS and cpDNA analysis respectively. Amplification products were purified using Genei Quick PCR purification kit (Bangalore Genei), and sequenced by dideoxy chain termination method using fluorescent end labeled primers. The DNA sequences were resolved by capillary electrophoresis in a single-capillary Automatic DNA Sequencer; QE Automated DNA Sequencer 310. The raw sequences were aligned

Table 1. List of *Abelmoschus* species used for ITS and cpDNA analysis. The species names are given based on the classification adopted by the International Okra Workshop (1990)

S.No.	Species	Accession number
1	*A. esculentus* (L) Moench.	EC 316073 Coll No: 28 Parbhani Kranti x 15 IC 117081
2	*A. tetraphyllus* (Roxb.ex. Hornem) R. Graham var. *tetraphyllus*	NIC 6846 IC 140980
3	*A. moschatus* Medikus	IC 141055 NIC 10442 NIC 13301
4	*A. ficulneus* (L). W & A.ex Wight	Coll No: 83 (152) IC 140961 Coll No: 85 (176) IC 141042
5	*A. tuberculatus* Pal & Singh	NIC 9286 IC 90402
6	*A. crinitus* Wall	N/SS 2759
7	*A. caillei* (A. Chev.) Stevels	EC 305672-2
8	*A.tetraphyllus* var. *pungens* (Roxb.) Hochr.	IC 253305

Table 2. Particulars of primers used for amplification of *ITS* and cpDNA regions in different *Abelmoschus* species

Region	Primer code	Sequence 5'-3'	Annealing Temp. (^{o}C)
psbA-trnH	*psbA*	GTTATGCATGAACG TAATGCTC	55^{o}C
	trnH	CGCGCATGGTGGA TTCACAATC	55^{o}C
trnC-trnD	*trn*L-F	CGAAATTGGTAGA CGCTGCG	55^{o}C
	Intron R	GGGGGTAGAGGGA CTTGAAC	55^{o}C
trnE- trnF	*trn*L$^{(UAA)}$	GGTTCAAGTCCCTC TATCCC	55^{o}C
	trnF $^{(GAA)}$	ATTTGAACTGGTGA CACGAG	55^{o}C
ITS-1	ITS-L	TCGTAACAAGGTTT CCGTAGGTG	50^{o}C
	ITS-4	TCCTCCGCTTATTG ATATGC	50^{o}C
ITS-2	ITS1	GAACCTGCGGAAGG AAGGATCATTG	60^{o}C
	ITS25S-5.8S	ACGAATTCCCTCCGC TTATTGATATGCTTA	60^{o}C

and curated using software CLUSTAL W [23] and the Neighbor Joining and Minimum Evolution trees were constructed using software MEGA [24]. The trees were rooted using sequences from *Gossypium barbadense* and *G. hirsutum* as outgroups since the genus is distantly related to *Abelmoschus* and belongs to the same family, Malvaceae.

Results and discussion

Analysis of ITS-1 region

Gossypium barbadense and *G. hirsutum* were included as out groups for analyzing the sequences of nrDNA comprising of ITS-1 and ITS-2 regions. The *ITS-1* region between 18s and 5.8s nuclear ribosomal DNA was amplified using *ITS L* and *ITS 4* primer pairs. The length of aligned sequences came to 783 bp. Moreover the sequenced region contained 26 indels including 11 insertions and 15 deletions. Most of the insertions were 1-2 bp in length. The longest deletion observed was of 31bp and was common to *A. esculentus* and *A. caillei*. Deletions at 5 sites were found unique to *A. tetraphyllus* var. *pungens* with lengths differing from 1-17 bp. The Minimum Evolution tree (Fig. 1) placed *A. tetraphyllus* var. *tetraphyllus* and *A. ficulneus* together with bootstrap value of 92%. This cluster was joined to *A. tuberculatus* with 94% bootstrap value while *A. caillei* and *A. esculentus* formed a separate group with a higher value of 99%.

The closely placed *A. tetraphyllus var tetraphyllus* and *A. ficulneus* differed from each other for deletions at 4 sites involving single bases. *A. caillei* and *A. esculentus* had 4 deletions common to each other including the longest deletion of 31 bp and the second longest one of 30 bp. The pyrimidine base'C' was most common in all the species having an average value of 31.8% followed by 'G' with a value of 28.8%. 'T' was the least common base at 18.5% average value. The total G/C content in *Abelmoschus* species for *ITS-1* region was 60.6%. The closely placed species *A. caillei* and *A. esculentus* had an almost identical share of all the four nucleotides. But differed for 'A' and 'T' at position-1.

Analysis of ITS-2 region

The *ITS-2* region between 5.8s and 28s nuclear ribosomal DNA was amplified using primer pairs *ITS 1* and *ITS 25S-5.8S*. The average size of the region amplified in all the eight species was same as *ITS-1* about 700 bp. In addition to the major band observed in gel of size ˜700bp a second band of ˜600bp was also found in some species which was eluted from gel and sequenced; but due to poor quality these sequences were not included in the final analysis. The length of the aligned region was 788 bp in all the species. The

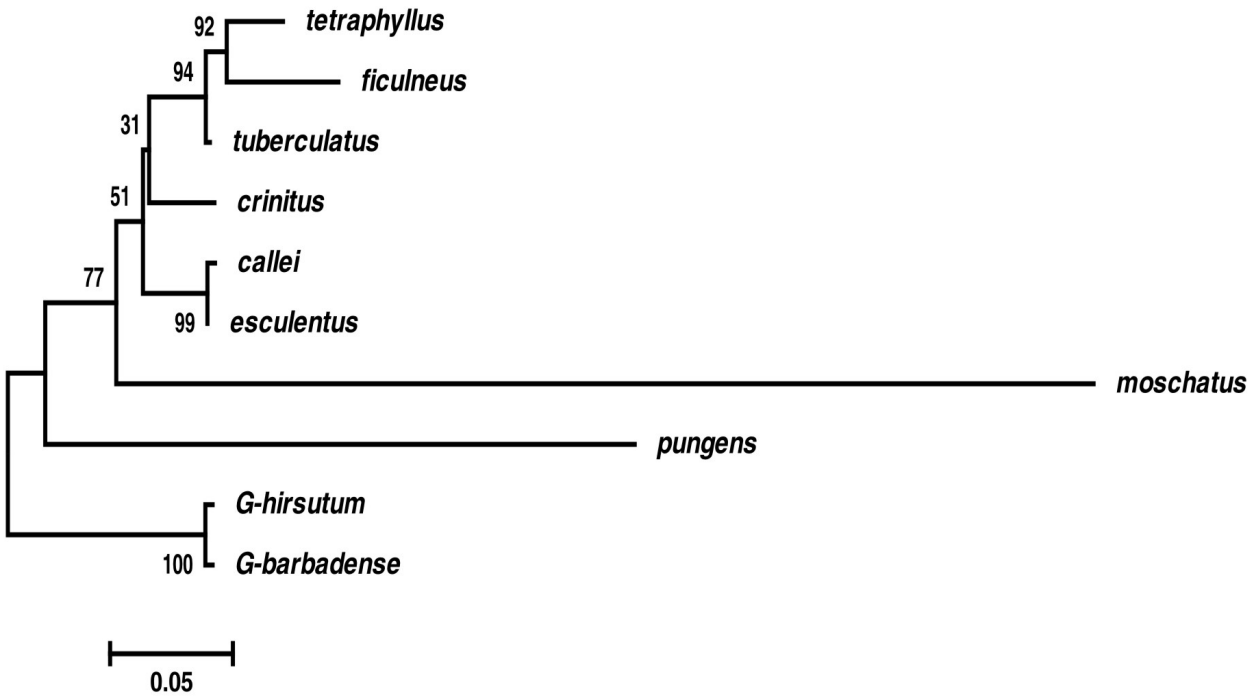

Fig. 1. Minimum Evolution tree for ITS-1 region indicating greater affinity between *A. esculentus* and *A. caillei*

region amplified had 29 variable sites including 11 insertions. Most of the insertions were of 1-3 bp in length. The longest deletion was of 32 bp found in *A. caillei*. The longest insertion was of 6 bp found in *A. esculentus* and *A. tetraphyllus* var. *pungens.* The Minimum Evolution tree (Fig. 2) placed *A. esculentus* in between the outgroups and *A. crinitus*. The bootstrap value between *A. esculentus* and *A. crinitus* was 99% indicating closer affinity of *A. esculentus* with *A. crinitus*. These two species were connected to *A. tetraphyllus var tetraphyllus* with bootstrap value of 98%.

Study of the sequence alignments indicated that *A. esculentus* and *A. crinitus* vary with respect to two deletions at two sites for single nucleotides C and G. *A. esculentus* and *A. tetraphyllus var tetraphyllus* vary with each other for 6 deletions of length varying from 1-6 bp. These two species also differ with respect to two insertions of 3 bp and 6 bp observed only in *A. esculentus* including the longest insertion of 6bp found in the amplified region and also for one insertion of three bases found in *A. tetraphyllus var tetraphyllus.* The species under study differed significantly for observed nucleotide frequencies. 'G' was the most common nucleotide averaging at 31.5% followed by 'T' at 29.7%. Between species the frequency of 'T' differed significantly with *A. tetraphyllus var pungens* having the lowest value of 15.7% and *A. crinitus* having the highest value of 22.2%. *A. crinitus* and *A. esculentus* vary greatly with respect to the amount of total 'T' i.e., 22.2% in *A. crinitus* against 17.6% in *A. esculentus.* The nucleotides, 'C' and 'G' at position-1 and 'T' at position-3 also differed significantly with *A. crinitus* having a greater percentage than others. The total G/C content in *Abelmoschus* species for *ITS-2* region was 61.2%.

Analysis of trnC-trnD spacer from chloroplast genome

The intergenic spacer region *trnC-trnD* spacer between *trn*L(UAA) gene and the intron R in the chloroplast genome was amplified using specific primer pairs (Table 2). The aligned sequences contained 750 bp in all the species. The aligned sequences from *trnC –trnD* region was highly informative with a total of 32 indels including 10 insertions and 22 deletions. The longest insertion of 6 bp was found in *A. esculentus.* Of the total insertions found in the region about 5 were having length greater than 3 bp. *A. esculentus* had 7 unique deletions when compared to other species. The longest deletion observed was for 10 bp in *A. ficulneus*. The phylogenetic analysis (Fig. 3a) resolved the eight species into two

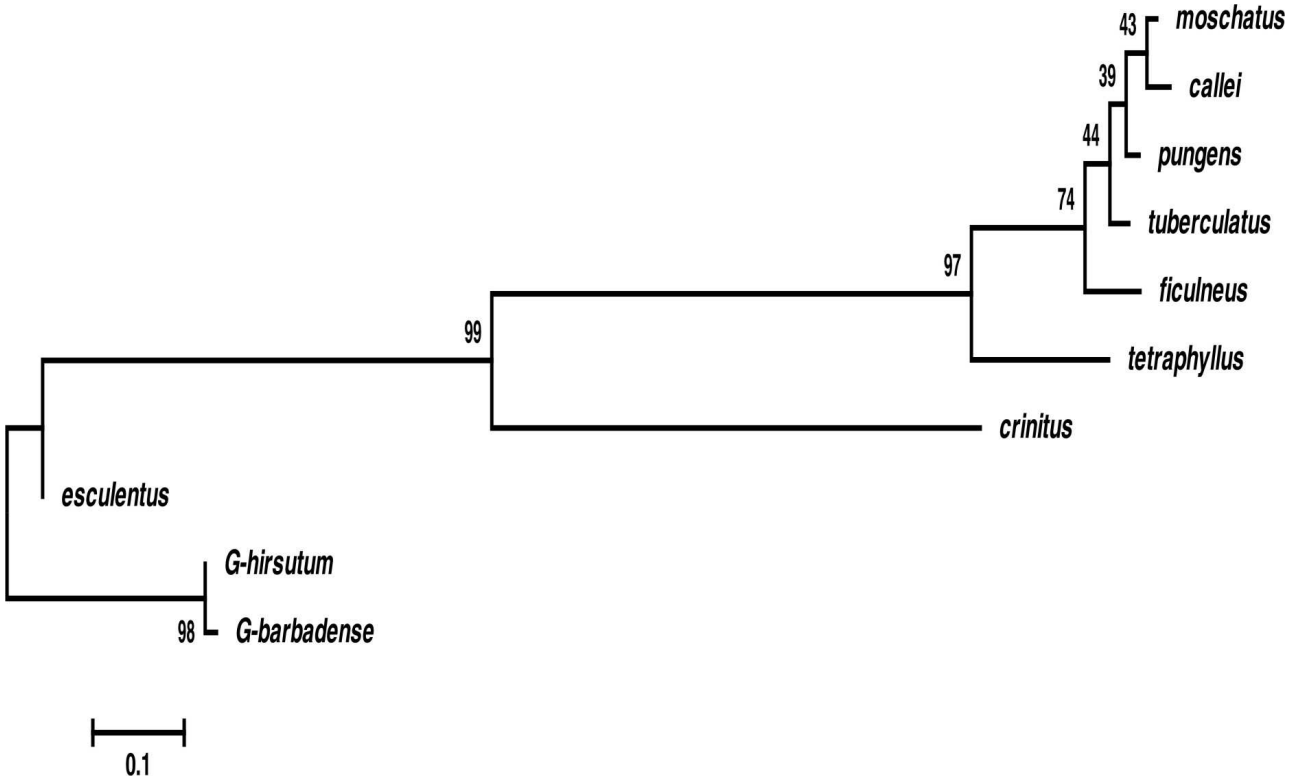

Fig. 2. Minimum Evolution tree for ITS-2 region

clusters with *A. tetraphyllus var pungens*, *A. crinitus, A. caillei, A. tuberculatus* and *A. tetraphyllus var tetraphyllus* falling into one group and the rest three *viz*., *A. moschatus, A. esculentus* and *A. ficulneus* forming a separate one. *A. esculentus* and *A. ficulneus* were grouped together but with a low bootstrap value of 42% to which *A. moschatus* was joined with a bootstrap value of 100% in both ME and NJ trees. *A. moschatus* and *A.esculentus* were found to be related, *A. ficulneus* was also part of the cluster. In the second cluster *A. tetraphyllus var tetraphyllus* and *A. crinitus* formed a separate group with a high bootstrap value of 99%. All the three species shared 3 deletions of nucleotides C and T stretching to 4-5 bp. *A. esculentus* had 4 deletions unique to itself whereas in *A. ficulneus;* the numbers of unique deletions were 3. *A. crinitus* contained a deletion of 5 bp when compared to *A. tetraphyllus var tetraphyllus.*

Most frequently occurring base is 'A' with an average value of 38.3% across the species. The highest % for 'A' (43.5) was found in *A. tuberculatus.* Second most common base is 'T' with a value of 25.4% and the least common one is 'C'. *A. moschatus* had a comparatively higher % of 'T' and lower % of 'C' when compared to *A. esculentus* and *A. ficulneus*. Between them *A. esculentus* and *A. ficulneus* differed greatly for the total % of 'A' (Table 3 and Fig. 3b). The total G/C content in *Abelmoschus* species for *trnC- trnD* region was 36.3% which was comparatively very low when compared to *ITS-1* and *ITS-2* regions.

Analysis of trnE - trnF intergenic spacer

The aligned sequences from *trnE- trnF* intergenic spacer were more uniform and phylogenetically less informative. All the species were limited to a single cluster and none of the relationships could be proven conclusively since the bootstrap values were less than 50 (Fig. 4). The total length of aligned sequences was 490 bp. A total of 6 variable sites were found including 4 insertions. The largest insertion observed was of 6 bp in *A. crinitus.* Here also, the phylogenetic analysis revealed a close association between *A. moschatus, A. esculentus* and *A. ficulneus*. Most frequently found base is T (U) with an average value of 38.7% among the species. This was followed by 'A' with 27.3% and the least common one is 'G'. *A. moschatus* had the highest % of 'T' 'C' and 'G'. *A. esculentus* differed from *A. ficulneus* and *A. moschatus* for having lower percentage of 'T' and 'G' at position -3 and a comparatively higher amount of other two nucleotides. The total G/C content of the intergenic spacer region *trnE-trnF* was 34%.

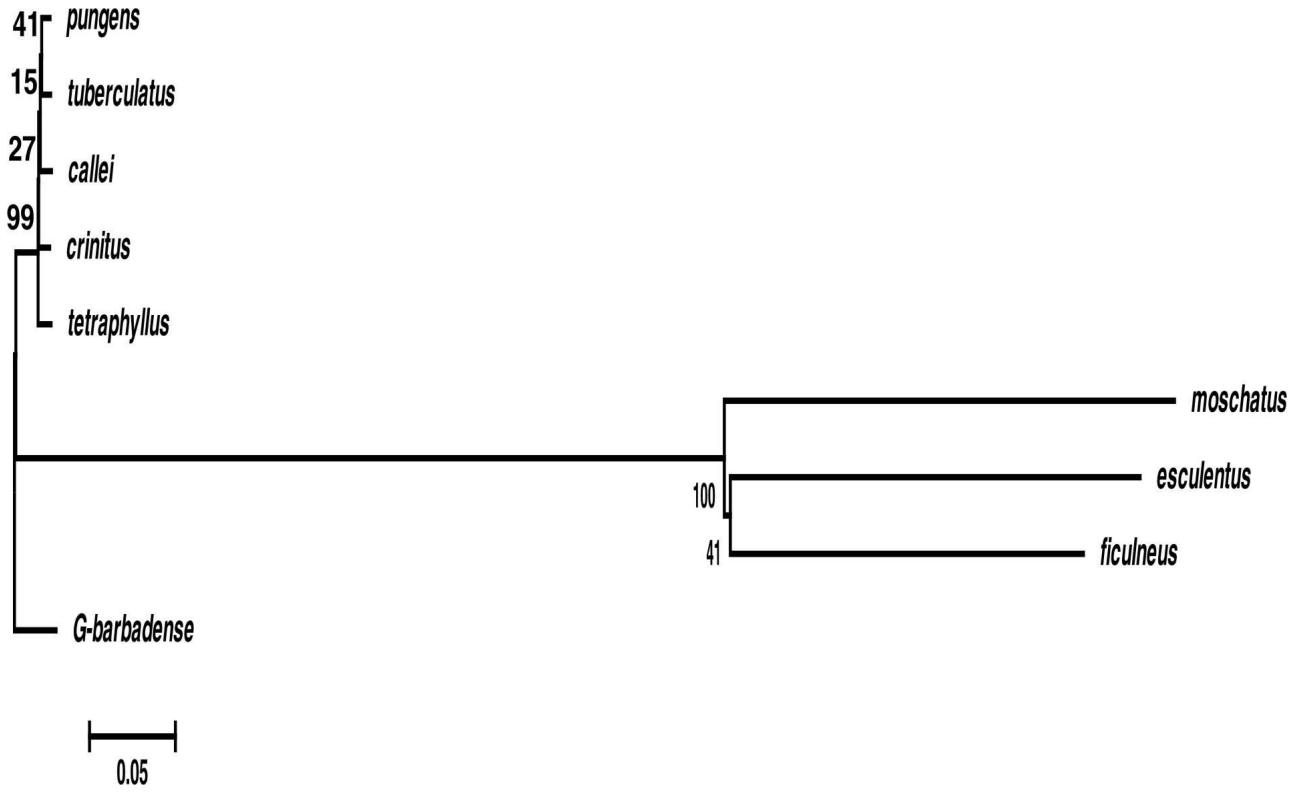

Fig. 3a. Minimum Evolution Tree for trnC-trnD region

Table 3. Pair wise distance based on nucleotide sequences from *trnC-trnD* region. *Abelmoschus esculentus*, *A. ficulneus* and *A. moschatus* form a cluster with least possible distance

	A. pungens	*A. crinitus*	*A. tuberculatus*	*A. tetraphyllus*	*A. caillei*	*A. esculentus*	*A. ficulneus*	*A. moschatus*
A. pungens								
A. crinitus	0.0							
A. tuberculatus	0.0	0.0						
A. tetraphyllus	0.0	0.0	0.0					
A. caillei	0.0	0.0	0.0	0.0				
A. esculentus	0.7	0.7	0.7	0.7	0.7			
A. ficulneus	0.6	0.6	0.6	0.6	0.6	0.4		
A. moschatus	0.7	0.7	0.7	0.7	0.7	0.5	0.5	

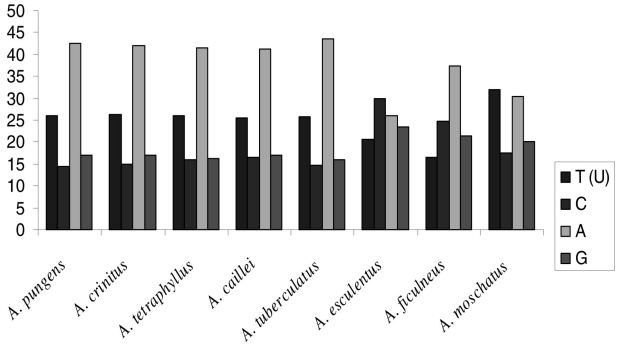

Fig. 3b. Nucleotide frequencies calculated as percentage from *trnC-trnD* region

Analysis of trnH –psbA intergenic spacer

The intergenic spacer between the chloroplast genes *psbA* and *trnH* was found to contain the greatest number of variable sites. The aligned sequences covered a length of 760 bp. The region of amplification included 38 indels with 13 insertions and 25 deletions. The longest insertion of 12 bp was found in *A. crinitus* while *A. ficulneus* carried the largest deletion of size 10 bp. Around 50% of the insertions were having length greater than 3 bp. The phylogenetic analysis resolved the eight species into two clusters (Fig. 5a) in a similar manner as *trnC- trnD* intergenic spacer with. *A. moschatus,*

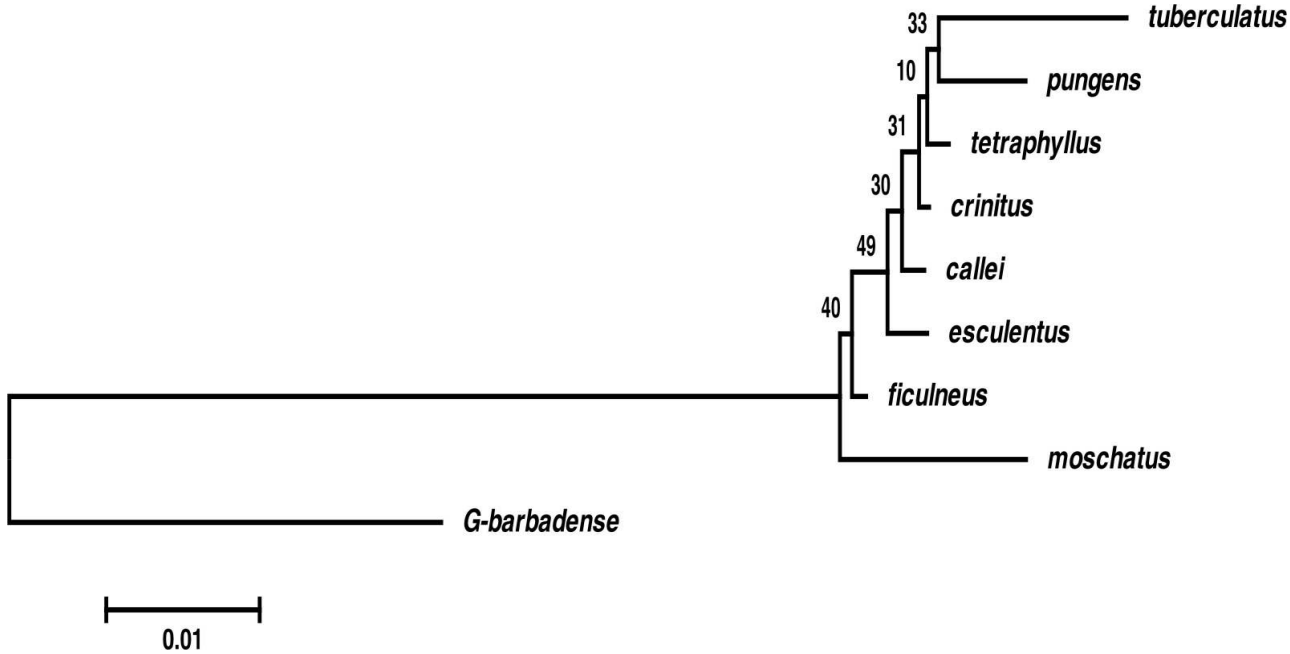

Fig. 4. Minimum Evolution Tree for trnE-trnF region. All the branches are showing low boot strap value indicating that the region remained uniform throughout the genera

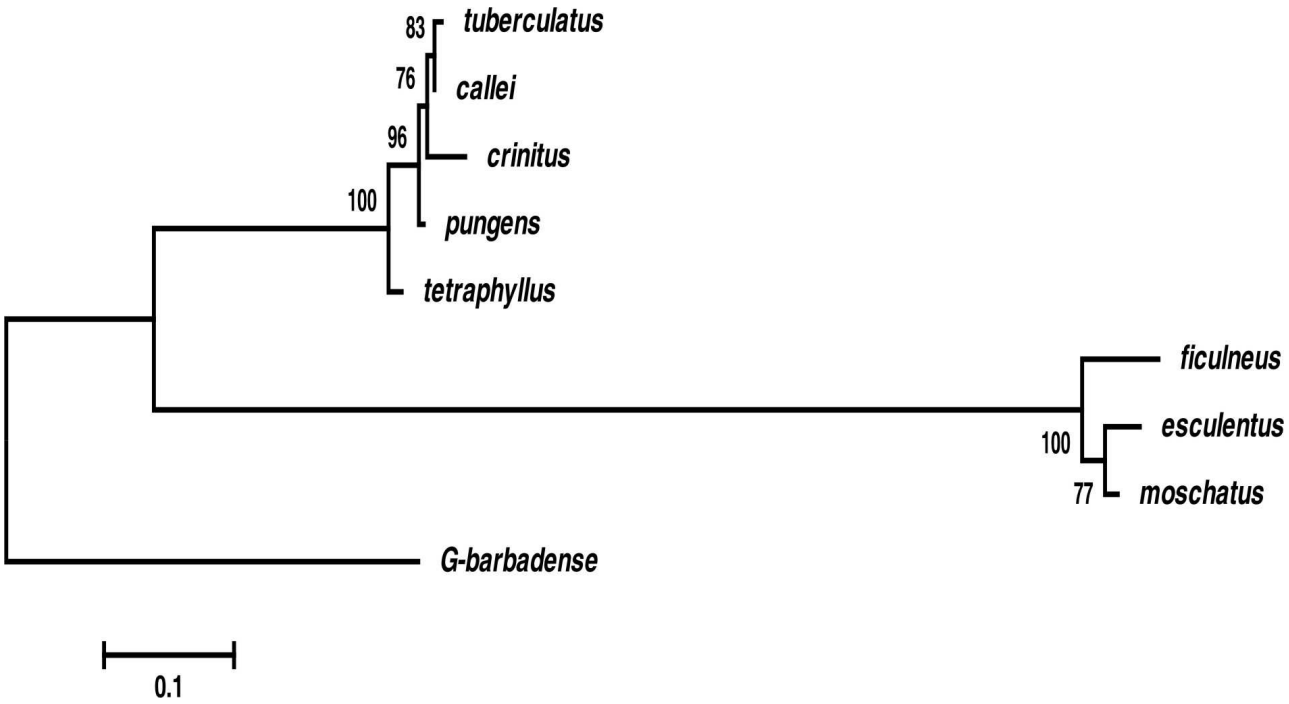

Fig. 5a. Minimum Evolution Tree for trnH-psbA region. *A. esculentus, A. ficulneus* and *A. moschatus* formed a single cluster indicating the involvement of these two species in the origin of cultivated okra; *A. esculentus*

A. esculentus and *A. ficulneus* forming a separate single cluster. *A. moschatus* and *A. esculentus* formed a group with bootstrap value of 77% to which *A. ficulneus* was joined with a bootstrap value of 100%. In the second cluster *A. tuberculatus* was joined to *A. caillei* and again to *A. crinitus* with bootstrap values 83 and 76 % respectively. This cluster was connected to *A. tetraphyllus var pungens* with bootstrap value of 96% and the large cluster was joined with *A. tetraphyllus var tetraphyllus* with a bootstrap value of 100%.

Among the three species forming one cluster 6 deletions were found unique to *A. moschatus*, 4 to *A. esculentus* and 3 found only in *A. ficulneus.* A total of 4 insertions were found unique to *A. ficulneus* with size range of 1-6 bp. All of them shared 5 insertions of 2-5 bp. *A. crinitus* had one insertion and deletion each; unique to itself. *A. tetraphyllus var tetraphyllus* had 6 deletions in the amplified region. The most frequently found base in the amplified region was T (U) with an average of 36.7 %. The % of 'A' found across different species showed greater variability; from 25.5% in *A. moschatus* to 39.5% in *A. caillei.* The least common base was 'G' with the lowest % (10.1) found in *A. tetraphyllus var pungens*. *A. esculentus* and *A. moschatus* differed from *A. ficulneus* for the total percentage of pyrimidine base 'T' (Table 4 and Fig. 5b). The total G/C content in *Abelmoschus* species for the *trnH-psbA* intergenic spacer region was 28.9%; the lowest among the three chloroplast intergenic spacer regions analysed.

In the present case, sequence analysis from the *ITS-1* region showed that *A. esculentus* and *A. caillei* are related to each other, which was not supported by the *ITS-2* analysis which supported a strong relationship between *A. esculentus* and *A. crinitus*. One of the major concerns with the use of the rDNA locus in phylogenetic analyses is the existence of polymorphisms among repeated units, which may cause extensive differentiation even within a single individual. For some species a smaller second band was observed in addition to the main band which confirmed the presence of intra-individual and intra-species heterogeneity for the ITS region [12].

From the analysis of intergenic spacers of chloroplast DNA it can be inferred that cultivated okra is more closely related to the wild species, *A. ficulneus* (2n=72) and *A. moschatus* (2n=72). The contribution of *A. tuberculatus* in the genome of *A. esculentus* remained doubtful throughout the study as opposed to the early reports [6, 7]. This may be attributed to the complexity of genome of *Abelmoschus* species and the abnormal

Table 4. Pair wise distance based on nucleotide sequences from *trnH-psbA* region *Abelmoschus esculentus*, *A. moschatus* and *A. ficulneus* form a cluster with least possible distance

	A. tuberculatus	*A. caillei*	*A. pungens*	*A. crinitus*	*A. tetraphyllus*	*A. esculentus*	*A. moschatus*	*A. ficulneus*
A. tuberculatus								
A. caillei	0.0							
A. pungens	0.0	0.0						
A. crinitus	0.0	0.0	0.0					
A. tetraphyllus	0.1	0.1	0.1	0.1				
A. esculentus	6.3	6.5	6.5	6.1	5.8			
A. moschatus	5.9	6.1	6.1	5.7	5.4	0.0		
A. ficulneus	5.0	5.2	5.3	4.8	4.5	0.3	0.2	

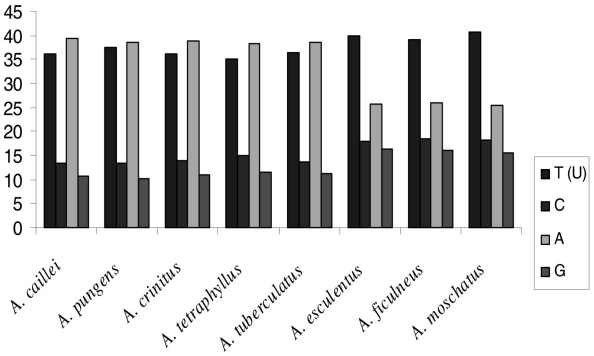

Fig. 5b. Nucleotide frequencies calculated as percentage from *trnH-psbA* region

pairing of chromosomes during meiosis.

The *ITS* and cpDNA analysis produced entirely different pattern of relationships among the eight *Abelmoschus* species analysed. The *ITS* sequences failed to capture the general evolutionary history of cultivated okra, established through the extensive cytological studies conducted earlier. Although the multiple copy number of ribosomal units is considered as an advantage in inferring phylogeny, it may become problematic in hybrids and polyploids were thousands of tandem copies are present [17]. This may lead to the prominence of paralogous sequences which on recombination lead to errors in phylogenetic trees. The result supported the view that ITS rDNA should be excluded as a species and population-level phylogenetic marker due to its complicated and undistinguishable characteristics of molecular evolution [14, 15].

The cpDNA sequence analysis were more or less in agreement with the general theory, and advocated strong relationship between cultivated okra, *A. esculentus* and two of the purported ancestors i.e., *A. ficulneus* and *A. moschatus*. Since the chloroplast DNA is nonrecombinant and maternally inherited it can be reliably stated that both of these genomes are present in the allopolyploid, *A. esculentus*. Moreover, these two wild species are present extensively in India, and enjoys specific ethanobotanical references. The center of origin of cultivated okra, *A. esculentus* needs to be precisely established, away from Vavilovian concepts [3].

The phylogeny inferred from nuclear ITS sequences showed much weaker divergence and partly conflicting patterns of separation between the different *Abelmoschus* species used in the study as well as within the same species when compared to cpDNA studies. *ITS-1* sequence analysis puts *A. esculentus* and *A. caillei* together in a cluster which is not supported by cytological or morphological studies. None of the supposed progenitors are related to *A. esculentus* as per the study; while *A. caillei* was found to be more related to *A. tuberculatus*. The similarities observed in ITS studies can be attributed as entirely due to chance. All the cpDNA studies placed *A. esculentus* in close relationship with *A. ficulneus* and *A. moschatus*, and gave rather conflicting evidence about the probable progenitor compared to previous studies. This disagreement among nuclear and cytoplasmic gene trees might be a consequence of the different transmission genetics of nuclear and organelle genes and their different rates of lineage sorting. Due to the complexity of the *Abelmoschus* genome and the high ploidy level, abnormal meiotic pairing of chromosomes can be expected, leading to greater divergences. Since the chloroplast genome is maternally inherited and nonrecombinant, the pattern obtained from the cpDNA

analysis is more reliable than ITS studies. This study also point out the failure of *ITS* sequences in extensive phylogenetic studies of polyploid species and the possibility of Indian sub-continent being the center of origin of okra.

Acknowledgements

The authors are thankful to the Indian Council of Agricultural Research (ICAR), New Delhi for funding the research.

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