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Genetic analysis of wild species in support of evolutionary changes of the genus *Gossypium* through ISSR markers

A. B. Dongre, M. R. Bhandarkar and V. T. Parkhi

Central Institute for Cotton Research, Post Box No. 2, Shankar Nagar P.O., Nagpur 440 001

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Abstract

Twenty-two wild species of *Gossypium* belonging to seven genomic groups were studied using Inter Simple Sequence Repeat (ISSR) markers to establish phylogenetic relationship within the genus. Among 25 ISSR primers used, 19 were found to be scorable on agarose gel of which 88% were polymorphic. Genome specific and unique markers were observed in this study. In support of the evolutionary study, 3 major clusters were found in the dendrogram, one composed of A and B genome species and the other composed of D, AD and C genome species. E, F and G genome species were placed out side the major cluster at the end of dendrogram. These results suggested that ISSR-PCR markers are potentially useful in establishing genetic relationship, genome specificity and genetic basis of evolution among the wild species of genus *Gossypium*.

Key words: Cotton, genetic relationship, ISSR markers

Introduction

The genus *Gossypium*, belonging to family *Malvaceae* includes about 50 species, out of which only four are cultivated, while 46 species are wild. These 50 species including 43 diploid (2n = 26 chromosomes) classified into 7 genomes from A to G, and 7 tetraploid species (2n = 52 chromosomes) with genome designation AD [1] are summarized in Table 1.

The genus *Gossypium* evolved 5-15 million years ago [2, 3]. This genus is distributed in four centers of diversity - Africa, Arabia, Australia and Mesoamerica. Two old world cotton species, *G. arboreum* and *G. hirsutum* arose from African-Arabian gene pool while two are New World cotton [4].

The wild species are important sources of useful traits such as special and superior fiber properties, cytoplasmic male sterility, resistance to biotic and abiotic stresses etc. that can be introgressed into the cultivated species for crop improvement. Genome and breeding values relationship does not exist except in D-genome, which was found to contain cytoplasmic male sterility, fusarium wilt and verticillium wilt resistance traits [5]. Since the variability available in the cultivated germplasm is limited and has been exhaustively utilized in breeding programs, it has become a necessity to develop basic germplasm materials enriched with rare/useful genes from wild species through introgression.

PCR based DNA markers have been used in the identification, evolutionary, genetic diversity studies, and plant genome analysis [6]. Among them Inter Simple Sequence Repeat (ISSR) is a reliable, fast and reproducible marker [7]. ISSR analysis involves PCR amplification of genomic DNA using a single primer that targets the repeat *per se*, with 1-3 bases that anchor the primer at the 3' or 5' end [8]. ISSR is a technique that overcomes most of limitations of other PCR based DNA marker systems such as low reproducibility of RAPD, high cost of AFLP & the need to know the flanking sequences to develop species-specific primers for SSR polymorphism [9].

Keeping these facts in view and the importance of wild species in cotton improvement program, the present investigation was undertaken to see the genetic relationship among available wild species of cotton at molecular level using ISSR, so as to utilize these species for cotton improvement programme.

Materials and methods

Fresh young leaves of twenty-two wild species (three plants of each species) of *Gossypium* representative of A to G genomic groups (diploid 2n = 2x = 26) and AD genome (tetraploid 2n = 4x = 52) were collected from wild species garden of Central Institute for Cotton Research, Nagpur, India as summarized in Table 1. Modified protocol of Edward (1991) [10] was used for DNA isolation. 2% poly vinyl pyrrolidone (PVP) was added in DNA extraction buffer to avoid co-isolation of phenolics and polysaccharides. Quality and quantity of DNA were estimated spectrophotometrically and electrophoretically. The DNA was finally diluted to 40ng/ul and used for the PCR amplification using the ISSR primers.

Table 1. Wild species of *Gossypium* used for ISSR marker analysis

S. No.	Genomes	No. of species	Geographical location	Samples taken for ISSR studies	Lane 1-22	Name of species taken for ISSR studies
1.	A	3	Africa/Asia	3	1.	*G. africanum* (A)
					2.	*G. herbaceum* (A_1)
					3.	*G. sinence* (A_2)
2.	B	4	Africa	4	4.	*G. anamolum* (B_1)
					5.	*G. triphylum* (B_2)
					6.	*G. barbosanum* (B_3)
					7.	*G. capitis viridis* (B_4)
3.	C	18	Australia	1	8.	*G. sturtianum* (C_1)
4.	D	12	America	9	9.	*G. thurberi* (D_1)
					10.	*G. harknessii* ($D_{2.2}$)
					11.	*G. davidsonii* ($D_{3.d}$)
					12.	*G. klotzsianum* ($D_{3\text{-}k}$)
					13.	*G. aridum* (D_4)
					14.	*G. raimonondii* (D_5)
					15.	G. gossypoides (D_6)
					16.	*G. lobetum* (D_7)
					17.	*G. trilobum* (D_8)
5.	E	4	Arabia	2	18.	*G. stockssii* (E_1)
					19.	*G. somalence* (E_2)
6.	F	1	Africa	1	20.	*G. longicalyx* (F_1)
7.	G	1	Australia	1	21.	*G. bickii* (G_1)
8.	AD	7	America	1	22.	*G. mexicanum* (AD_1)
	Total	50		22		

Total 19 ISSR primers synthesized from Bangalore Genei Pvt. Ltd., India were used to amplify the DNA isolated from 22 wild species. Among the nineteen primers, 4 primers were with 3 bp repeats anchored at 3' end, 12 primers were 2 bp repeats anchored at 3'end and 3 primers were 2 bp repeats anchored at 5'end as summarized in Table 2.

Table 2. ISSR sequences used for genetic analysis

S.No.	Name	Primer sequence
1.	IS-1	5'(AGC) 5 GA 3'
2.	IS-2	25'(AGC) 5 GG 3'
3.	IS-3	5'(AGC) 5 GT 3'
4.	IS-4	5'(AGC) 5 GC 3'
5.	IS-5	5'(CA) 7 AT 3'
6.	IS-6	5'(CA) 7 AC 3'
7.	IS-7	5'(CA) 7 GT 3'
8.	IS-8	5'(CA) 7GC 3'
9.	IS-9	5'(CA) 7GA 3'
10.	IS-10	5'(CA) 7AA 3'
11.	IS-11	5'(GT) 7 TA 3'
12.	IS-12	5'(GT) 7 TG 3'
13.	IS-13	5'(GT) 7 CA 3'
14.	IS-14	5'(GT) 7CT 3'
15.	IS-15	5'(GT) 7 AT3'
16.	IS-16	5'(GT) 7 AC3'
17.	IS-17	5'CAG (GA) 7 3'
18.	IS-18	5'GCT (GA) 7 3'
19.	IS-19	5'GCA (GA) 7 3'

PCR reaction was carried out in a 25 µl reaction volume containing 40 ng of genomic DNA, 2.5 µl of 10X *Taq* polymerase buffer with 15 mM MgI_2, 200 µM of each dNTPs (Q-Biogene), 15 ng of ISSR primers and 1 unit of *Taq* polymerase (Q-Biogene, USA). A negative control, without template DNA was included in each round of reactions. DNA amplification was performed in a Biometra UNO-Thermoblock, DNA thermocycler and was programmed for initial denaturing step of 5 min at 94°C, 45 cycles of 1 min denaturation at 94°C, 45 sec annealing at 49°C and 2 min primer elongation at 72°C. Final step of 5 min at 72°C was given for polishing the ends (making smooth) of PCR products. Amplification products were analyzed by electrophoresis on 1.6 % agarose gel containing 0.1 µg/µl of ethidium bromide and was photographed with Tracktel GDS-2 gel documentation system. Reproducibility of ISSR was tested by performing duplicate reactions at different times using identical genotypes and primer combinations under stringent experimental conditions and only reproducible bands were scored. Amplicons from nineteen primers were scorable.

Comparison of genotypes was carried out based on the presence or absence of fragment produced by ISSR amplification. '1' was designated for presence of fragments and '0' for the absence of fragments. The cluster analysis was performed for the molecular data using UPGMA method [11]. The dendrogram (Fig. 3) was generated with the SAHN subroutine of NTSYS-PC to show the of similarity coefficient between the genotypes [12].

Results and discussion

Amplicons from nineteen primers as summarized in Table 2 were selected to determine the genetic relationships of the 22 wild species as given in Table 1. The amplification profile generated by the ISSR primers is summarized in Table 3. Unique markers were generated in primers IS3, IS8, IS1, and IS15. Many primers generated species-specific bands. Primer IS4 (Fig. 1) and IS7 (Fig. 2) generated 'B' genome specific marker of about 700 bp and 800bp respectively, which is only present in that B genome samples. Also marker generated by primer IS4 and IS7 for A genome species was found in and B genome species. Primer IS15 generated a marker of about 200bp size in D genome species and AD genome species.

UPGMA analysis of the ISSR markers and pairwise genetic similarities among 22 wild species estimated and ranged from 0.63 to 0.93 indicating that the genetic distance among the species tested were high and grouped into three major clusters (Fig. 3). Old World

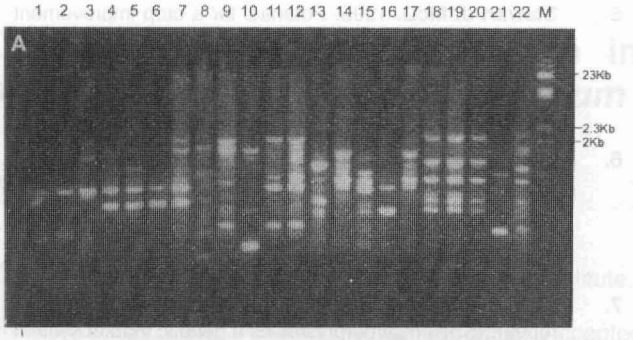

Fig. 1. ISSR profile of 22 wild cotton species obtained with primer IS 2. Lane 1-22 corresponds to 22 wild species listed in Table 2. Lane 23, M = *Lambda/Hind III/ Eco RI* double digest DNA ladder

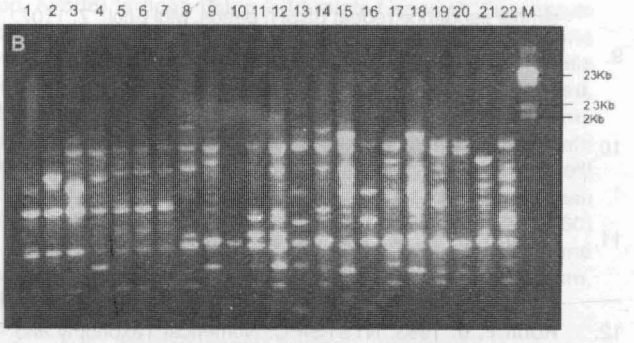

Fig. 2. ISSR profile of 22 wild cotton species obtained with primer IS 7. Lane 1-22 corresponds to 22 wild species listed in Table 1. Lane 23, M = *Lambda/Hind III/ Eco RI* double digest DNA ladd

species included in 'A' and 'B' genomes, domesticated in Asiatic region (Asia and Africa) and New World species contained in D and AD genome, domesticated in Mesoamerica. 'A' and 'B' genome were branched out from each other at the genetic distance, 0.83 on the scale. In the subcluster of A genome, A, A1 and A2 were arranged in the hierarchical fashion, while in the subcluster of B genome, B3 and B4 and B1 and B2 placed nearer to each other. The only representative of the ADI genome *G. mexicanum*, which was domesticated in Mesoamerica placed in the cluster of 'D' genome. *G. mexicanum* is placed near to *G. raimondi* in the D genome cluster in the dendrogram. *G. sturtianum* with C_1 genome found close to *G. lobetum* with D_7 genome. *G. thurberi* (D_1), *G. harknessii* ($D_{2\text{-}2}$), *G. klotzianum* ($D_{3\text{-}k}$) and *G. trilobum* (D_8) genome species found closed to each other and formed minor cluster in major cluster of D genome. While 'E' genome representatives, *G. stockssi* (E_1) and *G. somalence* (E_2) along with *G. longicalyx* (F) and *G. bickii* (G) genome representatives domesticated in Australia and Arabia found the places at the end of the dendrogram.

Gossypium genus is believed to have originated 5-15 million years ago. Genomic diversity has arisen during the global radiation of the genus leading to the evolution of eight genomic groups (A through G and AD) [13]. In the process of evolution and diversification allopolyploid cotton appears to have arisen within the last 2 million years, as consequence of transoceanic dispersal of A genome taxon to the new world followed by hybridization with indigenous D genome diploid [14]. This is very likely reason that A genome of Old World cotton and D genome of New World cotton form two major cluster in the dendrogram.

Allopolylpoid species, *G. mexicanum* (ADI genome) was placed in the major cluster of D genome close to *G. raimondi* (D_5) in the dendrogram. But *G. mexicanum* showed great divergence from A genome species (*G. africanum*, *G. herbaceum* and *G. sinence*). *G. raimondi* is the closest living donor of parental 'D' genome [4]. Phylogenetic analysis place 'D' subgenome of allopolyploid sister to *G. raimondi* [15]. Multivalent frequencies and segregation ratio in synthetic allohexaploid combining D-genome species with *G. barbedense* and *G. hirsutum* suggest that *G. raimondi* genome is more similar structurally to the D subgenome of diploids [16, 17]. Similar relationship showed in present study in which *G. raimondi* (D genome) and *G. mexicanum* (AD genome) were placed closed to each other in major cluster of D genome in dendrogram. While AD genome species was found at great genetic distance from A genome.

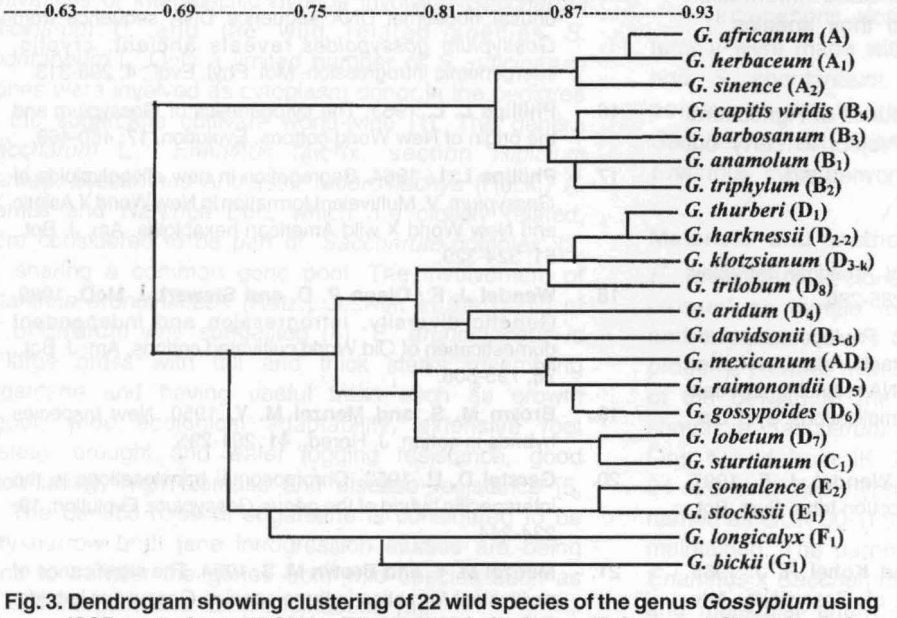

Fig. 3. Dendrogram showing clustering of 22 wild species of the genus *Gossypium* using ISSR analysis on UPGMA of Jaccard's similarity coefficients and Shan clustering

Table 3. ISSR analysis results in *Gossypium*

S.No.	ISSR analysis	Observations
1.	Total no. of primers used	25
2.	No. of polymorphic primers	19
3.	Total no. of bands amplicons	201
4.	Total no. of polymorphic amplicons	177
5.	Average number of polymorphic markers	9.3 per primer
6.	Polymorphic percentage	88%
7.	Size of amplified products	100-2500 bp

The A subgenome donor lineage is probably extinct [18]. Two existing Old World diploids, *G. herbaceum* and *G. arboreum* share a common ancestor and are equidistant from A subgenome progenitor [2]. The A subgenome progenitor of allopolyploid differs from the genomes of *G. herbaceum* and *G. arboreum* by two and three arm translocations respectively [19, 20, 21].

During the process of evolution, A genome and B genome maintained their genetic status with respect to each other and hence have found place close to each other in the dendrogram. C genome species found the place in the major cluster of D genome in dendrogram. While E genome representatives along with F and G genome representatives find the places at the end of the dendrogram. In the present study the high level of ISSR polymorphism among wild species of cotton and amplification of the unique as well as genome specific markers indicate that ISSR analysis is efficient, reliable and highly informative system in establishing genetic diversity, genome specificity and genetic basis of evolution among wild species of *Gossypium*. Cloning and sequencing of unique and genome specific amplicons and optimizing the primer design can establish highly authenticate information about specificity of the genome and their species.

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