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Full Length Research Paper

Investigation of simple sequence repeats (SSR) marker-assisted genetic diversity among upland Bt- and non Bt-cotton varieties

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Genetically modified with insect resistance gene (Cry1AC) and hybrid Bt-cotton genotypes were developed to redress cotton leaf curl virus epidemic. In this study, genetic diversity of 6 Bt- and 14 non-Bt upland cotton genotypes was detected using simple sequence repeats (SSR) or micro-satellite markers. Out of 31 primer pairs, only 7 (22.6%) yielded polymorphic amplicons of 80 to 340 bp. The average loci per primer were 3.16 and 78.6% of them were informative. The unweighed pair-group method with arithmetic averages (UPGMA) dendrogram placed Bt- and non-Bt cotton varieties into four major groups. With the exception of 4 accessions (being similar), genetic dissimilarity coefficient among all other genotypes ranged from 0.50 to 0.98 suggesting a wide genetic heterogeneity among the selected collection. The lowest similarity was found between BH-160 and CIM-482, CIM-496 and MNH-554, BH-160 and CIM-473, SARMAST and BH-160 (non-Bt cultivars), respectively as were some Bt-cultivars. In contrast to previous reports, these results show that some Bt- and non-Bt-cotton cultivars form a genetically diverse population. They could be used in future breeding programmes to develop new elite cotton cultivars.

Key words: Bt-cotton, dissimilarity matrix, DNA polymorphism, genetic diversity, SSR marker.

INTRODUCTION

Cotton is the world's most imperative natural textile fiber and a valuable oil seed crop. Cotton is the main cash crop of Pakistan and provides cotton fiber to the national textile industry. It contributes nearly 65% of our foreign exchange earnings (Asif et al., 2009). Cotton yield in Pakistan experienced unexpected fluctuations and imparted significant economic losses to our country due to large scale pest infestation since the early 1990s. Pakistan introduced a wide range of pesticides to control pests but their application increased the cost of cotton production. The pests developed resistance to these pesticides which turned out to be non-effective in controlling them. Cotton breeders isolated a gene called Cry1AC from *Bacillus thuringiensis* (Bt) and incorporated into traditional (non-Bt) upland cotton through genetic engineering. Cotton harboring Bt gene is popularly called Bt cotton and is expected to reduce input cost as well as drop in number of sprays of harmful and toxic pesticides.

There are total 50 species of cotton with 5 allo-tetraploid and 45 diploid species and they are grouped into nine genomic types (Wendel and Cronn, 2003; Cantrell, 2004). The two major cultivated species are *Gossypium herbaceum* and *Gossypium arboreum* followed *by Gossypium hirsutum*. They normally occupy the largest area in South Asia (Cantrell, 2004).

For genetic improvements, plant breeders adopt a constricted range of adopted lines in their programme. During this process, breeder relies on accurate screening methods and availability of lines with clear-cut phenotypic traits. This approach is time consuming and difficult to produce results with classical methods (Asif et al., 2009).

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Use of molecular markers accelerates these breeding processes. These markers facilitate the generation of new varieties and allow association of phenotypic traits with genomic loci (Jiang et al., 2000; Asif et al., 2009). Genetic diversity is a fundamental source of crop existence and its improvement (Xiao et al., 2009). Information of genetic diversity and relationships among breeding genome, their polymorphic nature, and co-dominance has a significant impact on crop improvement. Recently molecular markers have come up as a quite fascinating technology for identifying molecular phylogenetics, genetic linkage mapping, conservation of gene order and synteny, genetic fidelity and genetic diversity (Liu et al., 2000; Belaj et al., 2006; Joshi and Dhawan, 2007; Preetha and Raveendren, 2008). Since our upland cotton (*G. hirsutum*) possesses a large and complex genome, it requires a large collection of DNA markers to get maximum genome coverage (Qureshi et al., 2004; Xiao et al., 2009) for its genome characterization. Molecular markers not only allow the simple and consistent identification of breeding lines, hybrids and GM cultivars (Bastia et al., 2001; Rahman et al., 2002; Reddy et al., 2001; Asif et al., 2009) but also facilitate the monitoring of introgression, mapping of QTLs (Jiang et al., 2000), marker assisted selection (MAS) (Zhang et al., 2003) and evaluation of genetic diversity (Mukhtar et al., 2002; Tabbasam et al., 2006).

A variety of DNA markers, namely, restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter simple sequence repeats (ISSR), sequence related amplified polymorphism (SRAP) and simple sequence repeats (SSR) in combination with the analysis of morpho-physiological traits (Gutiérrez et al., 2002; Preetha and Raveendren, 2007; Sharma et al., 2008; Xiao et al., 2009) has been reported to investigate genetic variability among cotton genotypes. The application of a molecular marker approach is dependent on its simplicity, reproducibility, cost-effectiveness and reliability.

The SSR primers consist of repeating sequences of 1 to 6 base pairs of DNA (Preetha and Raveenden, 2008) and have been successfully employed in many genetic diversity studies (Liu et al., 2000; Semagan et al., 2006). The present investigation was conducted to check the genetic polymorphism among 20 cotton genotypes (6 Bt- and 14 non-Bt) using 31 PCR-based SSR markers and to assess the genetic relationships among these genotypes not previously reported in Pakistani approved varieties. We found that GM varieties of Bt-cotton resembled their parental lines but were different from Bt-hybrids and other non-Bt genotypes. These varieties can be adopted for incorporating yields related QTLs and other genes for development of commercial GM varieties. Thus, SSR markers are excellent genomic methods and can be successfully applied for deciphering genetic background of unknown genotypes for their genetic diversity and and heredity.

MATERIALS AND METHODS

20 cotton genotypes of upland cotton (Table 1) from Cotton Research Institute Faisalabad (FH accessions), Central Cotton Research Institute Multan (CIM accessions), Cotton Research Station Multan (MNH accessions), Cotton Research Station Bahawalpur (BH accessions), National Institute for Biotechnology and Genetic Engineering, Faisalabad (IR-3701 = Bt.09-V3, IR-1524 = Bt.09-V6: GM varieties), Guard Agricultural Research Services Lahore (GM-31 = Bt.09-V1, GM-2085 = Bt.09-V2, hybrid accessions), Center of Excellence in Molecular Biology Punjab University Lahore (CEMB accession = Bt.09-V5, GM variety), Ali Akbar Seeds Multan (Ali Akbar 802 accessions = Bt.09-V4, hybrid), Nuclear Institute of Agriculture, Tandojam (SARMAST) and Nuclear Institute for Agriculture and Biology (NIAB-111 accession), Faisalabad Pakistan, were selected for this study.

DNA extraction

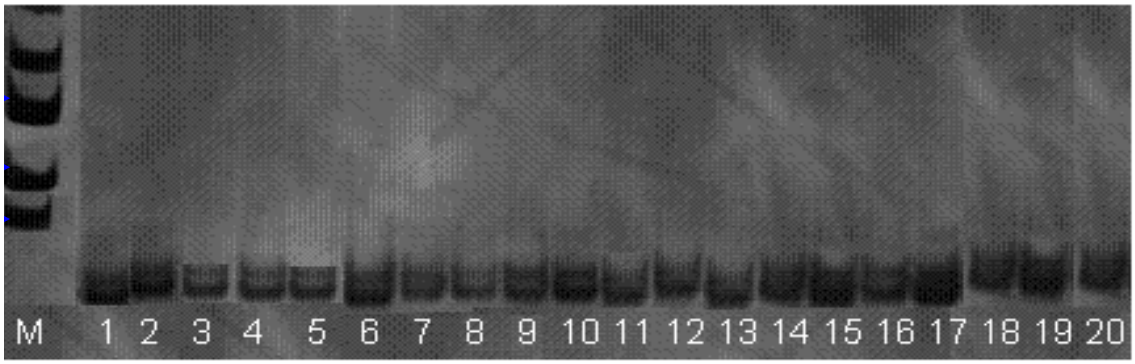
The plants were grown under controlled environmental conditions and young leaves from each genotype were harvested. Total genomic DNA was extracted by using a CTAB-based procedure reported previously, with 3% (v/v) b-mercaptoethanol in a 3% (w/v) CTAB buffer (Powell et al., 1996). DNA concentrations were quantified using Nano drop method and by inspection of electrophoregrams after electrophoresis in 1% agarose gels stained with ethidium bromide. The concentration of the DNA samples was adjusted to 30 ng/µl and the DNA aliquots were stored at -20°C. After electrophoresis, the amplicons were visualized using ultraviolet trans-illuminator and photographed using Genesnap software of Syngene Gel Documentation System.

SSR amplification and SSR analysis

All PCR reactions were performed in 20 µl volumes using GeneAmp PCR system. For 1 reaction, PCR mixture contained 2 µl volume of 10 × buffer ($MgCl_2$ + $(NH_4)_2SO_4$), 3 µl volume 0.2 mM dNTPs, 2.5 µl volume of 50 mM $MgCl_2$, 0.4 µl volume of 5 unit/µl Taq polymerase, 1.5 + 1.5 µl volume of 30 ng/µl primer pairs (forward and reverse of BNL series), 1 µl volume of 30 ng/µl DNA, and 8.3 µl of double distilled water (d_2H_2O). Temperature for PCR programmes consisted of first denaturation step for 5 min (94°C), followed by 35 cycles of denaturation for 30 s (94°C), annealing for 30 s at (55°C) and final extension for 10 min at (72°C). PCR products were incubated at 72°C for another 10 min to ensure complete extension. The amplicons were resolved on 3.0% agarose (Genechoice, Inc USA) or PAGE gels and stained with ethidium bromide or silver nitrate respectively followed by data recording using photo-documentation system.

31 primers of the BNL series with known sequences (Liu et al., 2000) and available in cotton genomic library of Brookhaven National Laboratory (BNL), namely, BNL-113, BNL-116, BNL-118, BNL-119, BNL-137, BNL-150, BNL-193, BNL-226, BNL-252, BNL-256, BNL-285, BNL-390, BNL-530, BNL-542, BNL-580, BNL-597, BNL-598, BNL-632, BNL-834, BNL-852, BNL-946, BNL-673, BNL-827, BNL-840, BNL-1047, BNL-1053, BNL-1061, and BNL-1064 were used for genotyping assays. All BNL clone sequences available at CottonDB (http://ars-genome.cornell.edu) for forward and backward sequences were downloaded and sent to Research Genetics, Inc., Hunstville, AL-35801, USA for synthesis. The size of the marker was determined by comparison with a DNA marker (1 kb). They were used to fingerprint the 20 cotton genotypes (Figure

A



B

Figure 1. (A) DNA finger prints of 20 cotton genotypes using SSR primer pair BNL-827. Number stands for codes of genotypes shown in Table 1. This SSR primer displayed genetic polymorphism in two non-Bt (8 and 13) and two Bt-cotton (17 and 20) genotypes. M stands for 1 kb marker; (B) DNA finger prints of 20 cotton genotypes (Table 1) using SSR primer, BNL-1061. Genotype 3,7,13 and 17 showed genetic diversity.

1a and b). The SSR reaction products were evaluated for polymorphisms on 3.0% agarose (Genechoice, Inc USA) or PAGE gels and stained with ethidium bromide or silver nitrate respectively followed by data recording using photo-documentation system as described earlier.

Data analysis

SSR markers give bistate (present-absent) type of scoring. Each DNA fragment amplified by a given primer pair was treated as a unit character and the amplicons were scored as present (1) or absent (0) for each of the primer-accession combinations. The fragments with the identical mobility were scored as identical fragments. Only major bands were scored and faint bands were ignored. The molecular size of the amplicons was calculated from a standard curve based on the known size of DNA fragments of a 1 kb DNA marker. The presence and absence of the bands was scored in a binary data matrix. Pair-wise comparison of the accessions based on the presence or absence of unique and shared amplificons was used to generate similarity coefficients. DNA bands shared by all the accessions were excluded from the data analysis as described earlier (Powell et al., 1997). The resulting similarity coefficients were used to evaluate the relationship among the accessions with a cluster analysis using UPGMA and then plotting in the form of a dendrogram using computer software NTSyspc version 2.20. Genetic similarities were estimated using Nei and Li's standard method (1979) for genetic distances using computer software NTSyspc version 2.20.

RESULTS AND DISCUSSION

A total of 31 SSR primers were used to find out genetic heterogeneity among 20 cotton genotypes of *G. hirsutum* L (Table 1). Only seven primer pairs (BNL-150, BNL-193, BNL-226, BNL-BNL-827, BNL-834, BNL-1064 and BNL BNL-1061) detected 98 reproducible and scorable amplification products across all the accessions, out of which 77 (78.6%) fragments were polymorphic in one or more of the 20 accessions. They also generated 1 to 6 polymorphic fragments. BNL-193 amplified 6 polymorphic loci among the all accession. Four primer pairs generated indecipherable products while 20 detected monomorphic amplicons in each sample. Similar results were reported by Liu et al. (2000, 2006). For example, Liu et al. (2006) used 358 SSR primer pairs of BNL series, 200 detected monomorphic amplicons in each sample. Their 84 primer pairs produced indecipherable bands and only 74 primer pairs yielded 165 polymorphic amplicons only. Accession-specific SSR markers were identified based on SSR band formation patterns. BNL-827 and BNL-1061

Table 1. Names, ID and accessions of cotton genotypes.

S/N	Sample ID	Genotype	S/N	Sample ID	Genotype
1	41	CIM-482	11	51	BH-160
2	42	BH-118	12	52	CIM-496
3	43	MNH-554	13	53	MNH-786
4	44	FH-900	14	54	SARMAST
5	45	FH-901	15	55	GM-31 (B.T.09 V1)
6	46	CIM-473	16	56	GM-2085(B.T.09 V2)
7	47	FH-1000	17	57	IR-3701 NIBGE(B.T.09 V3)
8	48	NIAB-111	18	58	Ali Akbar-802(B.T.09 V4)
9	49	CIM-506	19	59	CEMB01 (B.T.09 V5)
10	50	CIM-707	20	60	RI-1524 NIBGE(B.T.09 V6)

detected 3 polymorphic loci each (280, 290 and 300 bp and 135, 140 and 150 bp respectively) in both MNH-786 and GM Bt variety IR-3701. BNL-150 resolved 4 polymorphic loci (125,135,140, and 150 bp) in GM-2085 (Bt.09-V2). BNL-827 was a unique primer which generated polymorphic amplicons of higher molecular masses (280, 290 and 300 bp) indicating its dispersive nature of annealing on genome. Its sequence was located on NIAB-111, MNH-786, IR-3701, and IR-1524. BNL-1064 (150 and 155 bp) and BNL-1061 (160 and 165 bp) generated polymorphic amplicons of different sizes among non-Bt cottons as well. These loci were observed in CIM-482, BH-118, MNH-554, FH-900, BH-160, CIM-496, and FH-1000. Thus the size distribution of these genotypes' amplicons was relatively concentrated on the genome.

The high level of polymorphism for SSR system compared to other systems was also reported in previous studies (Belaj et al., 2003; Ferreria et al., 2004). It is concluded that SSR analysis is a reliable method to study genetic diversity and varietal relationship. It has been observed that SSR markers can scan only one locus **at** a time (Liu et al., 2006) and generate low level of polymorphism in cotton genotypes (Gutierrez et al., 2002; Wang et al., 2006) but this is not always applicable. Since BNL series of primers were developed from tetraploid cotton genome, annealing between primer pairs and template may be affected if cotton genome is different. They exhibit polymorphism on the basis of differences in number of repeats in amplifiable regions of chromosomes. These variations are caused by mutations within primer binding regions on genome and may yield null alleles if annealing fails. Whereas a mutation between the primer binding regions may lead to abrupt binding of primer on mutated site and may result in new alleles (Asif et al., 2009). It was interesting to note that among 31 primer pairs used in this investigation, only 7 primers yielded unambiguous and well defined DNA fragments in our assays while 20 pairs showed monomorphic bands and 6 pairs produced undecipherable fragments on agarose gels.

Cluster analysis

Based on UPGMA analysis, dendrogram constructed for 20 genotypes with 31 microsatellite primers is presented in Figure 2. Four main clusters and some sub-clusters have been distinguished in the present material. These sub-clusters are further divided into sub clusters. In first cluster A, there are two sub clusters named as A1 and A2. In sub cluster A1, there are 3 genotypes, CIM-482, FH-900 and CIM-473. Genotypes, CIM-482 and FH-900, are closely related. In sub cluster A2, there are 5 genotypes FH-901, NIAB-111, CIM-506, Bt.09-V1 and Bt. 09-V5 and they are very similar to two Bt-cotton genotypes (Bt.09-V1 (a hybrid variety) and Bt.09-V5 (non-approved Bt-cotton landrace). They may have evolved with the same genetic inheritance. In second cluster B, there are three sub-clusters named as B1, B2 and B3. In B1, there are 3 genotypes, namely, GM-2085 (Bt.9-V2), IR-3701 (Bt.09-V3, a GM variety having Cry1Ac gene) and Ali Akbar-802, Bt.09-V4). Thus, these three Bt-cotton genotypes are closely related and may have common genetic background. In B2, there is only 1 genotype named as SARMAST (a mutant from Tandojam) and in B3, there is also 1 genotype named as MNH-786. These two are genetically diverse. SARMAST is from Tandojam and MNH-786 is from Multan; both are geographically distantly located and are expected to be genetically diverse as they may have evolved through different evolutionary processes. In third cluster C, there are 3 sub-clusters named as C1, C2 and C3. In sub cluster C1, there are 2 genotypes CIM-707 and CIM-496. Both are closely related and may have evolved from same parental material. In C2, there is only 1 genotype named as IR-1524 (Bt.09-V6) and in C3, there is also 1 genotype named as FH-1000. They appear genetically diverse from other genotypes; one of them is a Bt-cotton (IR-1524) approved variety. These both may be used in future crop improvement programme. In fourth cluster D, there are two sub-clusters named as D1 and D2. In D1, there is only 1 genotype (BH-118) and in D2, there are 2 genotypes (MNH-554 and BH-160). They are all non-Bt

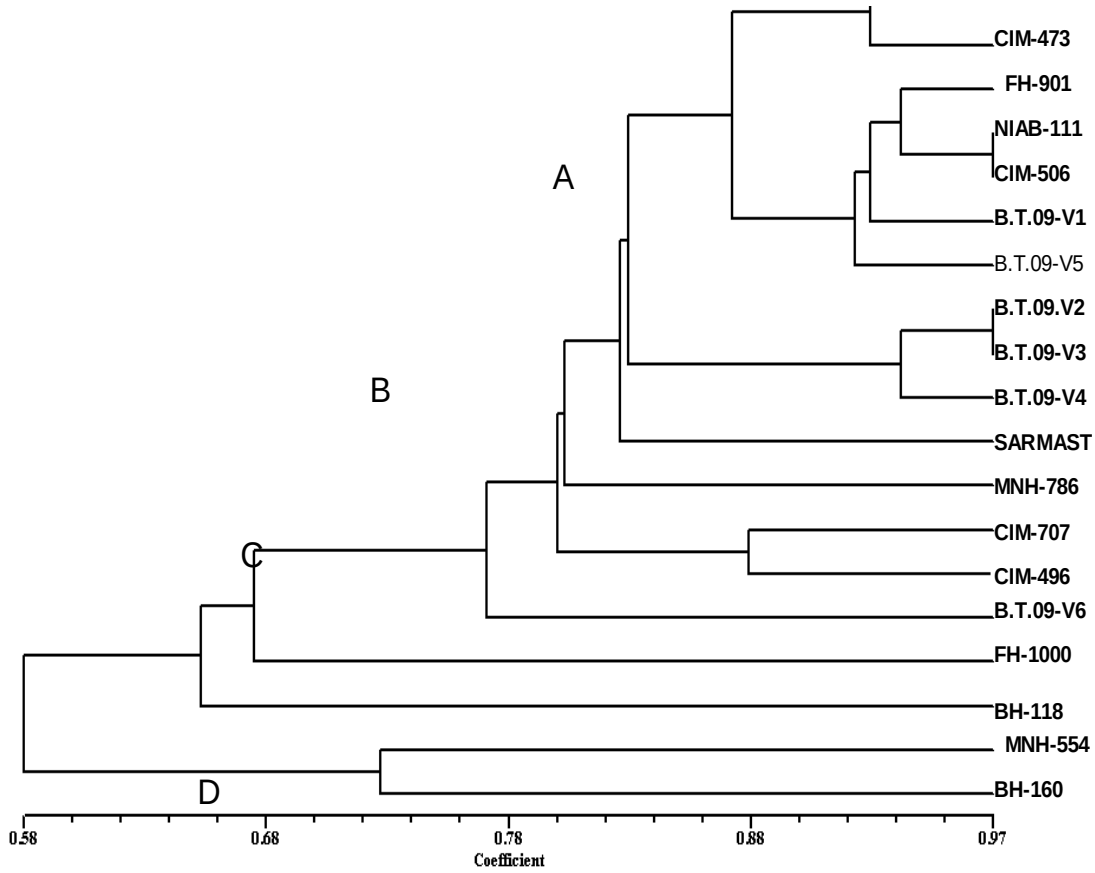


Figure 2. An UPGMA dendrogram of 6 Bt- and 14 non-Bt cotton genotypes samples constructed based on 31 SSR data. For abbreviation of germplasm sources see Table 1.

varieties and appear genetically dissimilar from other genotypes.

Similarity matrix

The highest similarity (98%) was observed between CIM-506 and NIAB-111 and 95% between Bt. 09-V3 and Bt. 09-V2. The lowest similarity was found between BH-160 and CIM-482, CIM-496 and MNH-554, BH-160 and CIM-473, SARMAST and BH-160 (Table 2) respectively. Among them, the similarity ranged from 50 to 98% with average similarity index of 0.83 among 20 cotton genotypes and exhibited genetic diversity. Thus, these markers showed a significant power for identifying genotypes and their most probable genetic background.

In this study, we have found that SSR markers discriminated the genotypes in the competent way. The genetic divergence provided an idea about variability presented in cotton genotypes and could be used in the future breeding programmes to develop new cotton cultivars that possess high yield. Zhang et al. (2005) characterized and evaluated commercial cotton cultivars with microsatellites and found some specific SSR alleles for discriminating cotton germplasm. We also confirmed the efficacy of SSR analysis for the verification of hybridity and parentage of Bt-cotton approved varieties.

Molecular markers linked to a gene of interest possess pivotal importance and are useful tools for identification of genes of interest. Once the molecular markers closely linked to desirable traits have been detected, MAS can be performed in early segregating populations or at early stages of plant development (Zhang et al., 2005; Asif et al., 2009). The future for improvement of polygenic traits through DNA markers appears quite promising. Moreover, by adopting new and novel marker systems like the one currently developed by Monsanto (Xiao et al., 2009); they are expected to amplify several cotton marker loci. In future, *cry*2Ab under suitable plant expression promoter may be incorporated in Bt-cotton genotype to extend pesticide resistance to other pests as well. Yield enhancing gene/s may be incorporated to enhance yield of new GM cotton varieties.

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Table 2. Similarity matrix coefficients calculated for 20 cotton genotypes from 31 SSR marker loci.

Cotton genotype	CIM-482	BH-118	MNH-554	FH-900	FH-901	CIM-473	FH-1000	NIAB-111	CIM-506	CIM-707	BH-160	CIM-496	MNH-786	SAR-MAST	B.T.09-V1	B.T.09-V2	B.T.09-V3	B.T.09-V4	B.T.09-V5	B.T.09-V6
CIM-482	1																			
BH-118	0.73	1																		
MNH-554	0.68	0.65	1																	
FH-900	0.95	0.78	0.73	1																
FH-901	0.90	0.73	0.68	0.95	1															
CIM-473	0.90	0.73	0.68	0.95	0.9	1														
FH-1000	0.68	0.55	0.55	0.73	0.78	0.68	1													
NIAB-111	0.90	0.68	0.63	0.90	0.95	0.85	0.70	1												
CIM-506	0.88	0.65	0.65	0.88	0.93	0.83	0.73	0.98	1											
CIM-707	0.80	0.68	0.58	0.85	0.90	0.80	0.68	0.90	0.88	1										
BH-160	0.50	0.58	0.73	0.55	0.60	0.50	0.53	0.60	0.58	0.60	1									
CIM-496	0.73	0.60	0.50	0.73	0.78	0.68	0.60	0.78	0.75	0.88	0.53	1								
MNH-786	0.75	0.63	0.63	0.80	0.85	0.75	0.73	0.85	0.83	0.85	0.65	0.73	1							
SAR-MAST	0.85	0.68	0.58	0.85	0.80	0.90	0.58	0.80	0.78	0.80	0.50	0.78	0.75	1						
B.T.09-V1	0.90	0.68	0.58	0.85	0.90	0.80	0.68	0.95	0.93	0.85	0.55	0.83	0.80	0.85	1					
B.T.09-V2	0.73	0.55	0.55	0.78	0.83	0.83	0.60	0.83	0.80	0.83	0.58	0.70	0.78	0.83	0.78	1				
B.T.09-V3	0.75	0.58	0.53	0.80	0.85	0.85	0.63	0.85	0.83	0.85	0.55	0.73	0.80	0.85	0.80	0.98	1			
B.T.09V-4	0.80	0.63	0.58	0.85	0.90	0.80	0.68	0.90	0.88	0.90	0.60	0.78	0.85	0.80	0.85	0.93	0.95	1		
B.T.09-V5	0.85	0.63	0.58	0.85	0.90	0.80	0.68	0.95	0.93	0.85	0.55	0.73	0.80	0.75	0.90	0.83	0.85	0.90	1	
B.T.09-V6	0.75	0.63	0.53	0.80	0.85	0.75	0.68	0.80	0.78	0.75	0.50	0.73	0.70	0.70	0.80	0.73	0.75	0.80	0.85	1

B.T.09-V1 to B.T.09-V6 is GM-31, GM-2085, IR-3701 NIBGE, Ali Akbar-802, CEMB01, and RI-1524 NIBGE, respectively.

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