The comparison of inter simple sequence repeat and randomly amplified polymorphic DNA markers for genetic assessment of intra-specific cotton hybrid genotypes

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Abstract

Hybridization is one of the main strategies in cotton breeding producing new genotypes and increasing the genetic diversity in the cotton germplasm available. Thirty RAPD and ten ISSR markers were use to assess the genetic diversity in F2 progenies of tetraploid cotton hybrid genotypes (*Gossypium hirsutum*). Fifteen of 30 RAPD primers produced 126 reproducible bands with 66.44% polymorphism. Among the primers used, OPA09 primer showed highest value of number of effective allele (Ne), Shannon index (I), Nei's genetic diversity (H) (1.814, 0.638 and 0.447 respectively). The value of genetic identity varies from 0.65 between Siokra x No.200 and Tabladila x Siokra to 0.93 between Tabladila x Sindose and Sindose x No.200. Eight of 10 ISSR primers provided 87 reproducible bands with 67.82 % of polymorphism. UBC834 primer with AG repeats as a core sequence showed the highest value of Ne (1.570), I (0.477) and H (0.323) while combined primer UBC807+(GA)9T showed the highest PIC value (0.500). Based on ISSR data obtained, the hybrid cultivars Mehr x Tabladila and Tabladila x Siokra showed the lowest Nei's genetic identity (0.59), while, the hybrid cultivars No.200 x Belilzovar and Tabladila x Sindose showed the highest value. UPGMA Dendrograms obtained in RAPD and ISSR as well as combined RAPD and ISSR markers grouped together the hybrid genotypes which have Mehr cultivar as one of their parents. In conclusion, this study showed that using combined RAPD and ISSR data provides higher resolution of molecular profiles in hybrid genotypes.

Key words: cotton, hybridization, genetic diversity, ISSR, RAPD

Introduction

Cotton (*Gossypium* L.) is one of the most important commercial crops in the world. Among fifty species of this genus only five species, including two diploid (2n=2x=26) and three tetraploid (2n=4x=52) species with A and D genomes are cultivated. Continuous use of *G. hirsutum* L. (AD1AD1) as the primary genetic stock for cotton breeding could be one of the limitations in breeding programs as it may result in genetic erosion and uniformity of the genetic background (Kantartzi et al., 2009). Therefore, making new hybrid combinations for providing secondary germplasm pool in modern cotton production and studying the amount of genetic diversity obtained is important (Sheidai et al., 2008; Kantartzi et al., 2009). Hybridization is one of the important researchers' strategies with the aims to increase the yield in cotton breeding. Inter- and intra-specific hybridization is used to provide more diversity in the cotton germplasm and also to obtain elite cotton cultivars with improved agronomy traits (Esmail et al., 2008).

Cotton cultivars (diploid and tetraploid cultivars) are cultivated in 17 out of 30 provinces in Iran. Based on latest report issued by Agriculture ministry (Iran cotton report to 68th plenary meeting of ICAO, Cape Town, South Africa, 2009), cotton is cultivated in 130000 hectares with a production of 85000 tones during 2008/09 season. With compare to season 2007/08 these show an increase of 7.7% for area. Therefore, in cotton breeding program of country, hybridization is one of the important researchers' aims to increase production. Besides, Inter- and intra-specific hybridization in cotton has been mainly considered to provide more diversity due to loss of

genetic diversity which brings about the genetic erosion as well as introduce cotton cultivars with improved agronomy traits.

The success of a cross can be determined using morphological and agronomy characters. However, these markers may not be significantly distinct and not to be proper marker for evaluation of genetic diversity in the hybrids. Molecular analysis could be an efficient alternative to this approach for estimating genetic relationships on the basis of genotype and not the phenotype. Due to its simplicity and speed, RAPD analysis is one of the most commonly used techniques because it produces numerous molecular markers with providing high genetic variations in cotton genotypes (Demeke et al., 1996; Castagna et al., 1997, Sheidai et al., 2010). Inter-simple sequence repeat (ISSR) is the efficient and reproducible marker using to evaluate genetic diversity in plants (Reddy et al., 2002; Mondel, 2002; Noormohammadi et al., 2011a; Mehrabian et al., 2011). RAPD and ISSR markers have been also used to evaluate genetic variation, hybridization as well as the occurrence of somaclonal variations in different plant crops including cotton (Kumar et al., 2003; Vafaie-Tabar et al., 2003; Dongre et al., 2004; Mehetre et al., 2004; Rana et al., 2007; Preetha and Raveendren 2008; Sheidai et al., 2008, Wei et al., 2008; Sheidai et al., 2010; Noormohammadi et al., 2011b).

In the present study, we aimed to evaluate genetic variation of F2 progenies of intra-specific cotton hybrids by using RAPD and ISSR markers as well as comparison banding patterns provided by two molecular markers.

Materials and Methods

F2 progenies of nine intra-specific hybrid genotypes of tetraploid cotton *Gossypium hirsutum* L. including Mehr x Sindose, Mehr x Siokra, Mehr x Tabladila, Siokra x No.200, Mehr x

Belilzovar, No.200 x Belilzovar, Tabladila x Sindose, Sindose x No.200, Tabladila x Siokra were cultivated in three rows of 10 m length with 20 cm interplant distance, in the experimental field of Gorgan Cotton Research Center (Iran), according to a completely randomized design (CRD) with 3 replications.

Ten randomly fresh leaves of three to five plants in each genotype were pooled and used for DNA extraction. The total genomic DNA was extracted using the CTAB method by DNeasy® Plant Mini Kit (Qiagen Inc., Valencia, CA). All procedures for DNA isolation were according to the manufacturer's protocol for the kit. Quality of extracted DNA was examined by running on 0.8% agarose gel.

Thirty decamer RAPD primers of Operon Technology (Alameda, Canada) belonging to OPA, OPB, OPC, OPH, OPI, OPM and OPR sets as well as hetero-RAPD primers were used in this study. The PCR reaction mixture consisted of 20 ng template DNA, 1 x PCR buffer (10 mM Tris-HCL pH 8.8, 250 mM KCL), 200 µM dNTPs (Bioron, Germany), 0.80 µM 10-base random primers and 1 unit of *Taq* polymerase (Bioron, Germany), in a total volume of 25 µl. DNA amplification was performed in Techne thermocycler (Germany). Template DNA was initially denatured at 94°C for 3 min, followed by 35 cycles of PCR amplification under the following parameters: denaturation for 1 min at 94°C, primer annealing for 1 min at 36°C and primer extension for 2 min at 72°C. A final incubation for 10 min at 72°C was performed to ensure that the primer extension reaction proceeded to completion.

Ten ISSR primers used were UBC810, UBC811, UBC823, UBC834, UBC849, UBC807, (CA)7GT, $(GA)_9T$, $(GA)_9A$ and $(GA)_9C$ commercialized by UBC (the University of British Columbia). Hetero-ISSR primers were also used in this study. PCR reactions were performed in a 25 µL volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM MgCl2; 0.2

mM of each dNTP (Bioron, germany); 0.2 µM of a single primer; 20 ng genomic DNA and 3 unit of *Taq* DNA polymerase (Bioron, Germany). Amplifications reactions were performed in Techne thermocycler (Germany) with the following program: 5 min initial denaturation step 94° C, 30 s at 94° C; 1 min at 50° C, 1 min at 72° C. The reaction was completed by final extension step of 7 min at 72° C.

The RAPD and ISSR PCR amplified products were separated by electrophoresis on a 2% agarose gels using 0.5 X TBE buffer (44.5 Mm Tris/Borate, 0.5 Mm EDTA, pH 8.0). The gels were stained with ethidium bromide and visualized under UV light (Sambrook et al., 2001). A 100 bp DNA ladder (GeneRuler, Fermentas) was used as the molecular standard in order to confirm the appropriate RAPD markers. These markers were named by primer origin, followed with the primer number and the size of amplified products in base pairs.

The profiles produced by RAPD and ISSR markers were scored manually: each allele was scored as present (1) or absent (0) for each of loci studied. The level of intra-population genetic diversity of cotton genotypes was determined by Nei's gene diversity (He), the Shannon information index (I, Nei, 1972), number of effective alleles (Ne) as well as Polymorphic Information Content (PIC) (Anderson et al., 1993) with POPGENE version 1.31 (1999) based on allelic frequencies among nine hybrid genotypes analyzed.

Jaccard similarity as well as Nei's genetic distance (Nei, 1973) were determined among the cultivars studied and used for grouping of the genotypes by UPGMA (Unweighted Paired Group with Arithmetic Average) and NJ (Neighbor Joining) clustering methods and ordination based on principal components analysis (PCoA) (Podani, 2000; Weising et al., 2005). Cophenetic correlation was performed to check the fit of dendrograms obtained. NTSYS Ver. 2.02 (1998) and GenAlex Ver. 6.4 (2006) were used for clustering and PCoA analyses.

Results

RAPD analysis

Fifteen of 30 RAPD primers produced 126 reproducible bands with 66.44% polymorphism. Among the primers used, OPA09 primer showed highest value of number of effective allele (Ne), Shannon index (I), Nei's genetic diversity (H) (1.814, 0.638 and 0.447 respectively) and OPC12 primer revealed the highest value of Polymorphic Information content (0.500) (Table 1). OPI12 primer produced the lowest value of I (0.113), H (0.07) and PIC (0.25) while OPC06 showed the lowest value of Ne (1.07) (Table 1). Only one specific band was observed in Mehr X Belilzovar for band No.4 (6200bp) of the primer.

Some bands were present only in two genotypes, for example band No.8 (1000bp) of OPA02, No. 3 (6100 bp) of OPC06 and No.7 (2500bp) of OPA3+OPA9 were specific for Siokra x No.200 and Mehr x Belilzovar. Band No.2 (500bp) of OPC09 was specific for Mehr x Belilzovar and Tabladila x Sindose.

Some bands were present in all the genotypes except one, for example bands N0. 5 (2500 bp), No.7 (3200bp), No.8 (3400bp) and No.9 (3600) of the primer OPA10 as well as No.4 (3000bp) of OPM10 and No.3 (1000bp) of OPA03+OPA09 were only absent in Tabladila x Siokra. Bands No.1 (1000bp) and No. 10 (4000bp) of OPA05 as well as No.8 (3000bp), No.9 (4000bp) and No. 10 (5000bp) of OPC12 were only absent in Mehr x Sindose.

Nei's genetic identity for RAPD data determined among the cotton genotypes are given in Table 2. The value of genetic identity varies from 0.65 between Siokra x No.200 and Tabladila x Siokra to 0.93 between Tabladila x Sindose and Sindose x No.200.

UPGMA and NJ clustering of RAPD data produced similar results (Fig. 1). Based on Jaccard's similarity matrix, Tabladila x Sindose and Sindose x No.200 cultivars showed the highest similarity (0.92) while Siokra x No.200 and Siokra x Tabladila cultivars showed the lowest similarity (0.63). The Cophenetic correlation of UPGMA tree was higher (r = 0.80) than NJ and is discussed bellow.

In general two major clusters were obtained. The cultivars Mehr x Sindose and Tabladila x Siokra formed the first major cluster standing far from the other genotypes. In the second major cluster Tabladila x Sindose and Sindose x No.200 showed high similarity. Mehr x Siokra and Mehr x Tabledila also showed affinity while, No.200 x Belilzovar joined them with some distance.

ISSR analysis

Eight of 10 ISSR primers provided 87 reproducible bands with 67.82 % of polymorphism. UBC834 primer with AG repeats as core sequence showed the highest values of Ne (1.570), I (0.477) and H (0.323) while, the combined primer UBC807+(GA)9T showed the highest PIC value (0.500) (Table 1).

Mehr x Siokra genotype showed three specific bands obtained by (GA)9T (500, 600 and 750 bps) while, Three genotypes including Tabladila x Sindose, No.200 x belilzovar and Mehr x Tabladila showed one specific bands (UBC834-1200, UBC834-500 and UBC807+(GA)9T-500 respectively).

Some bands were present only in two genotypes, for example band UBC811 (1300bp) was specific for Mehr x Siokra and Mehr x Belilzovar. Band UBC807 (1200bp) was specific for Mehr x Sindose and No.200 x Belilzovar.

Some bands were present in all the genotypes except one. For example, bands 1600 and 2000 bps of UBC811 were absent only in Tabladila x Siokra. Similarly, band 1100bp of UBC807 and band 600bp of UBC810 were absent in Tabladila x Sindose.

Nei's genetic identity and genetic distance for ISSR data determined among the cotton genotypes are given in Table 3. Based on Nei's genetic identity, Mehr x Tabladila and Tabladila x Siokra showed the lowest identity value (0.59) while, No.200 x Belilzovar and Tabladila x Sindose showed the highest value (0.91).

Nei's genetic identity for ISSR data determined among the cotton genotypes are given in Table 2. The value of genetic identity varies from 0.62 between Siokra x No.200 and No.200 x Belilzovar to 0.91 between Tabladila x Sindose and No.200 x Belilzovar.

UPGMA and NJ dendrograms of ISSR data produced similar results (Fig. 2). Based on Jaccard's similarity matrix, Tabladila x Sindose and No.200 x Belilzovar genotypes showed the highest similarity (0.88) while, Mehr x Tabladila and Siokra x Tabladila cultivars showed the lowest similarity (0.53). The Cophenetic correlation of UPGMA tree was higher (r = 0.98) than other method, therefore it is discussed bellow.

In general two major clusters were obtained. Mehr x Tabladila, Siokra x No.200 and Mehr x Belilzovar are grouped closely in the first cluster while, Mehr x Siokra and Mehr x Sindose are placed far from them. In the second cluster, No.200 x Belilzovar and Tabladila x Sindose showed the highest similarity forming a cluster to which, Sindose x No.200 shows affinity, partly supporting RAPD tree discussed before.

Combined RAPD and ISSR data

In regards to comparison RAPD and ISSR data, mean of genetic diversity indices (Table 4) showed that RAPD data provided higher Shannon index (0.393) while high value of polymorphism (67.82%) obtained by ISSR data. Both ISSR and RAPD+ISSR data showed the highest PIC value (0.474). RAPD+ISSR data also showed the highest value of genetic diversity (0.281).

UPGMA dendrogram carried out on pooled RAPD and ISSR data (Fig. 3) grouped the genotypes in 3 major clusters. The first cluster consists of Mehr x Sindose genotype while the second cluster is comprised of the rest of genotypes except Tabladila x Siokra which forms the third cluster.

In general view, major cluster of the RAPD and ISSR analysis as well as combined data were found to be similar and arrangement of genotypes are agree in most parts of these trees. According to RAPD+ISSR analysis, Mehr x Sindose and Tabladila x Siokra showed less similarity to the rest of the genotypes while the other genotypes are grouped closely with more than 75% similarity. The same is true for RAPD analysis alone but in ISSR data, they show more affinity to the other genotypes (Fig 1, 2 and 3).

Analysis of dendrograms in detail revealed that Sindose x No.200, Tabladila x Sindose and No.200 x Belilzovar are grouped together in all three dendrograms. The hybrid genotypes in those which one of the parents was Mehr cultivar including: Mehr x Siokra, Mehr x Tabladila and Mehr x Belilzovar were placed closely in RAPD+ISSR as well as ISSR trees while in

RAPD tree only Mehr x Siokra and Mehr x Tabladila showed high similarity. PCoA ordination based on pooled molecular data studied could support UPGMA analysis (Fig. 4).

Discussion

The number of RAPD bands and degree of polymorphism obtained in the cotton cultivars studied is mostly in agreement with the other studies performed in cotton. Dongre et al. (2004), Tafvizi et al. (2010), Sheidai et al. (2010) and Noormohammadi et al. (2011) studied the genetic diversity of intra-specific hybrid genotypes (*G. hirsutum* L.) and their parents by RAPD markers and obtained 50%, 47%, 73% and 80% polymorphism respectively. They also could differentiate parental and hybrid genotypes studied. In another study, Vafaie-Tabar et al. (2003) reported 79% average genetic similarity among Indian tetraploid cotton cultivars while Rana and Bhat (2005) reported 74% average genetic similarity. Rana and Bhat (2005) studied the genetic variation in tetraploid cotton cultivars outside India and report the similar ranges of average genetic similarity in them. Mehetre et al. (2004) showed 50% genetic polymorphism in RAPD primers among cotton genotypes which were produced by inter-specific crossing between *G. hirsutum* and *G. raimondii* cultivars. Using RAPD data also could produce polymorphic bands in regenerated tetraploid cultivars (Sheidai et al., 2008).

In the present study, ISSR primers with dinucleotide GA and AG repeats as core sequences showed a higher degree of polymorphism (67%) compared to the report of Dongre et al. (2004) in *G. hirsutum* cultivars studied (54%). This difference may be due to the difference in the cotton samples as well as ISSR loci used.

In this study, primers with GA and AG as core sequences and different terminal single nucleotide were used for study of genetic variation of intra-specific hybrid genotypes while,

Dongre et al. (2004) mostly used dinuclotide CA and GT as core sequences. Genomic study showed that dinucleotide GA has relative abundance in cotton genome (Liu and Wendel 2001) with one or two anchored nucleotides producing the polymorphic bands. On the other hand, using the hetero-ISSRs (those flanked on each end by different primer site) lead to enhanced applicability of ISSR analysis to eukaryotic genomes. Mixed primer UBC807+(GA)9T used in this study could produce high polymorphism although other combination of different ISSR primers did not provide reproducible bands. Using other mixture of dinucleotide GA repeats primers may bring more polymorphic loci.

The genetic diversity indices based on both RAPD and ISSR markers revealed the highest values of genetic diversity in intra-specific hybrid genotypes studied. One of the useful parameter is PIC value which has been suggested that markers with PIC values more than 0.5 are informative (Bandelj et al., 2004). Therefore in the present study seven RAPD and ISSR loci may be considered informative to characterization and evaluation of genetic diversity in cotton genotypes.

Although Mantel test did not show significant correlation between RAPD and ISSR data, the grouping of the genotypes obtained by RAPD and ISSR markers are partly in agreement. This may be due to the fact that different markers detected different components of DNA variation within the genome. However, molecular data indicated the genetic distinctness of the cotton genotypes.

Dendrograms obtained in RAPD and ISSR as well as combined RAPD and ISSR markers show similarity between hybrid crosses which one of their parents was Mehr cultivar. Noormohammadi et al. (2011) also reported more affinity of F1 Mehr crossing genotypes. It is suggested that Mehr cultivars could influence on different hybrid combinations and make them

have more affinity to each other. The exception is Mehr x Sindose genotype which is separated from the other genotypes in both RAPD and ISSR data. Furthermore, the hybrids obtained from different crosses showed different molecular affinities not necessary having similar parents, possibly due to further molecular changes occurring in them. For example, Siokra x No.200 and Mehr x Belilzovar hybrids with different parents show highest genetic identity in both RAPD and ISSR analysis.

In conclusion, RAPD and ISSR marker systems could discriminate hybrid genotype and evaluate genetic variations of F2 crossing progenies of tetraploid cotton cultivars. Genetic diversity indices showed that using combined RAPD and ISSR data provides higher resolution of molecular profiles in hybrid genotypes. Using hetero-ISSR and hetero-RAPD primers are suggested for future study to evaluate genetic diversity of hybrid progenies.

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Table 1 Genetic parameters based on RAPD and ISSR data. Na: Number of different allele, Ne: Number of effective allele, I: Shannon index, H: Nei's genetic diversity, PIC: polymorphic Information Content, Number in parenthesis: Standard Deviation

RAPD loci	**Size range**	**Total band**	**Ne**	**I**	**He**	**PIC**
OPA02	350-1800	12	1.395	0.305	0.212	0.424
OPA03	600-1600	4	1.412	0.321	0.225	0.350
OPA04	300-2400	12	1.404	0.313	0.219	0.378
OPA05	500-4000	10	1.670	0.516	0.362	0.490
OPA09	300-1700	8	1.814	0.638	0.447	0.474
OPA10	750-3600	9	1.511	0.416	0.288	0.338
OPA13	500-4000	9	1.502	0.370	0.263	0.398
OPB05	1000-3500	3	1.618	0.533	0.358	0.480
OPC06	4000-6200	4	1.077	0.126	0.065	0.498
OPC09	400-4200	14	1.587	0.516	0.347	0.498
OPC10	300-1400	8	1.430	0.366	0.249	0.416
OPC12	250-5000	10	1.613	0.526	0.358	0.500
OPI12	250-3500	10	1.126	0.113	0.076	0.252
OPM10	900-3000	4	1.617	0.481	0.337	0.456
OPA03+OPA9	400-2500	7	1.334	0.339	0.217	0.498
ISSR loci	**Size range**	**Total band**	**Ne**	**I**	**H**	**PIC**
(GA)9A	500-2100	10	1.462	0.402	0.272	0.498
(GA)9C	350-2000	8	1.407	0.317	0.222	0.430
(GA)9T	500-2500	9	1.204	0.236	0.141	0.498
UBC807-(AG)8T	300-1500	11	1.387	0.318	0.218	0.420
UBC810-(GA)8T	300-1500	14	1.544	0.432	0.299	0.478
UBC811- (GA)8C	1000-3000	10	1.354	0.326	0.217	0.472
UBC823-(TC)8C	600-1700	7	1.395	0.364	0.241	0.496

UBC834-(AG)8YT	500-2300	10	1.570	0.477	0.323	0.476
UBC807+(GA)9T	500-2000	8	1.420	0.388	0.257	0.500

Table 2. Nei's genetic identity for RAPD (above diagonal) and ISSR (below diagonal) among cotton genotypes

Genotype	Mehr x Sindose	Mehr x Siokra	Mehr x Tabladila	Siokra x No.200	Mehr x Belilzovar	No.200 x Belilzovar	Tabladila x Sindose	Tabladila xSiokra	Sindose x No.200	Tabladila x Sindose
Mehr x Sindose	---	0.722	0.714	0.666	0.698	0.793	0.714	0.793	0.730	0.801
Mehr x Siokra	o.712	---	0.912	0.801	0.801	0.865	0.833	0.690	0.849	0.777
Mehr x Tabladila	0.724	0.781	---	0.793	0.857	0.825	0.825	0.682	0.841	0.785
Siokra x No.200	0.747	0.827	0.862	---	0.857	0.825	0.777	0.650	0.730	0.722
Mehr x Belilzovar	0.724	0.804	0.839	0.862	---	0.809	0.793	0.666	0.793	0.769
No.200 x Belilzovar	0.735	0.632	0.643	0.620	0.689	---	0.825	0.761	0.825	0.817
Tabladila x Sindose	0.724	0.689	0.655	0.655	0.701	0.919	---	0.746	0.936	0.801
Tabladila xSiokra	0.712	0.632	0.597	0.620	0.620	0.839	0.827	---	0.777	0.817

Sindose x No.200	0.712	0.701	0.666	0.712	0.712	0.839	0.896	0.793	---	0.849
Tabladila x Sindose	0.632	0.689	0.678	0.747	0.655	0.666	0.724	0.666	0.804	---

Table 3 Mean genetic parameters of molecular data studied. Ne: Number of effective allele, I: Shannon index, He: Nei's genetic diversity, PIC: polymorphic Information Content, Number in parenthesis: Standard Deviation

Molecular markers	Ne	P%	I	He	PIC
RAPD	1.479(0.035)	66.41	0.393(0.026)	0.270(0.018)	0.430
ISSR	1.425(0.040)	67.82	0.367(0.030)	0.247(0.021)	0.474
RAPD/ISSR	1.460(0.027)	66.67	0.382(0.020)	0.281(0.014)	0.474

Fig. 1. UPGMA dendrogram based RAPD data. . Abbreviation: Mehr x Sindose (MxSIN), Mehr x Siokra (MxSIO), Mehr x Tabladila (MxT), Siokra x No.200 (SIOx200), Mehr x Belilzovar (MxB), No.200 x Belilzovar (200xB), Tabladila x Sindose (TxSIN), Sindose x No.200 (SINx200), Tabladila x Siokra (TxSIO)

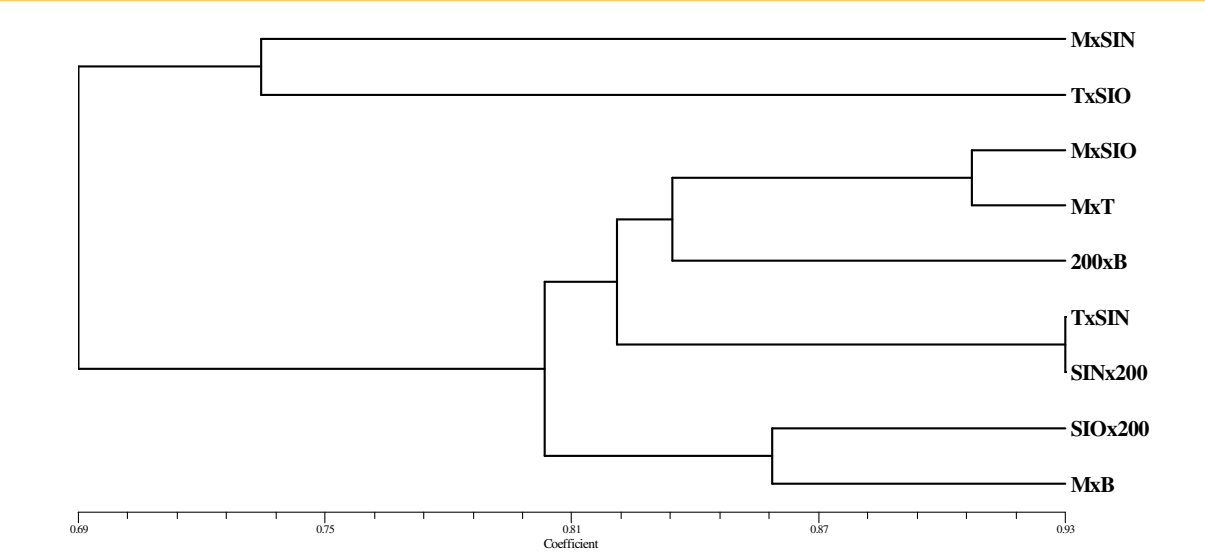

Fig. 2. UPGMA dendrogram based ISSR data. Abbreviation like those explained in figure 1

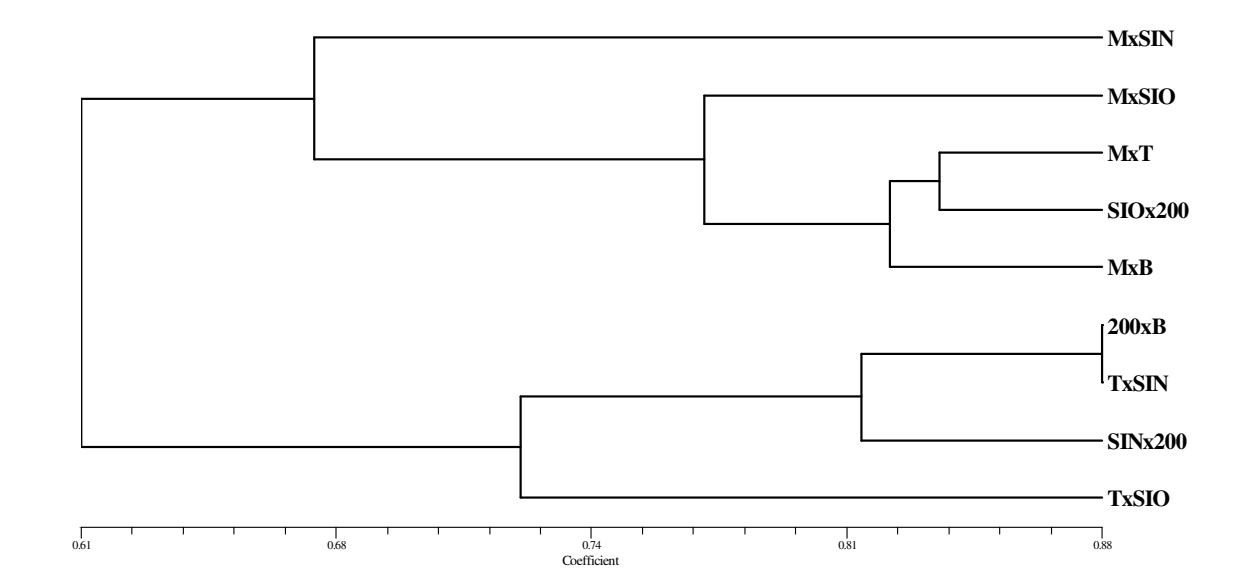

Fig. 3. UPGMA dendrogram based RAPD and ISSR data. Abbreviation like those explained in figure 1

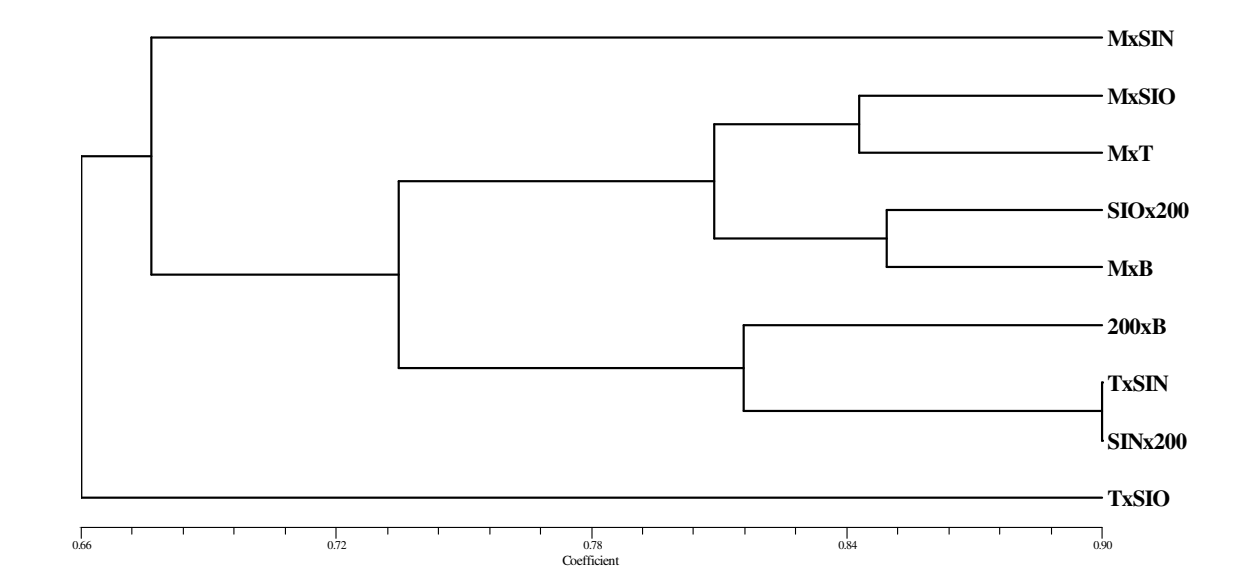

Fig. 5. PCoA ordination based on RAPD and ISSR data. Abbreviation like those explained in figure 1