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SSR-Based Genetic Diversity Assessment Among Hexaploid Wheat (*Triticum aestivum* L.) Cultivars

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Genetic diversity of the eighteen wheat (*Triticum aestivum* L.) cultivars were evaluated using 21 SSR markers. A total of 273 alleles were detected and the mean of alleles in each genome was 7.3 (A genome), 8.6 (B genome) and 9.3 (D genome). The polymorphism information content (PIC) value were ranged from 0.630 (*Xgdm132*) to 0.928 (*Xgwm190* and *Xgwm538*) with an average of 0.848. The Pair-wise genetic similarity matrix was calculated based on all possible pairs of wheat cultivars, which varied from 0.56 (VL 914 vs Mons ald's) to 0.97 (Halna vs Iepaca rabe and Kauz/AA/Kauz vs Halna). In UPGMA based cluster analysis using Dice coefficients, 18 cultivars were grouped into 4 major groups and clearly reflected the presence of very high degree of genetic diversity. Therefore, the molecular assessment of the genetic diversity between cultivars should be a good critrion for selecting these cultivars in future breeding programs.

Key Words: Genetic diversity, Microsatellite, Molecular markers, Wheat

Introduction

Wheat (*Triticum aestivum* L.) is one of most important, widely-cultivated cereal crops on a global scale in terms of total harvested weight and amount used as nutrition for human and animal (FAO, 2015). It is one of the main cereal crops in India. It is grown in about 30.60 million ha area with a production of 88.94 million tonnes and productivity of 3.0 tonnes/ha (Anonymous, 2015). Currently, India is second largest producer in the world after China with total 12% share in world wheat production. The world's major wheat producing countries are China, India, USA, France, the Russian Federation, Turkey, UK, Pakistan, Argentina, Canada, Australia, Iran and Italy. These countries together contribute about 76% of the global wheat production (FAO, 2015). In wheat the availability of variability at DNA level is a pre-requisite for any breeding improvement program targeted towards the improvement of its production and productivity. To estimate genetic diversity in wheat germplasm, various kinds of biomolecules based markers can be used. A number of methods are currently used for analysis of genetic diversity in germplasm accessions, recombinant inbreed lines (RILs) and segregating populations. These methods are based on pedigree records, morphological traits, biochemical and molecular markers (Kotzamanidis *et al.,* 2011).

Morphological traits can be used to evaluate genetic diversity but are often influenced by environmental factors and are unreliable, while the expression of molecular markers is the direct product of genes. In wheat, many breeding programs all over the world are aimed to improve for high grain yield with better quality, disease-resistance and agricultural performance with the use of molecular markers. Large number of germplasm lines can be characterized in a short period of time with more accurate assessment of genetic variability than the any other methods using DNA based markers. DNA based markers are more useful due to highly abundant, widely distributed, hypervariability, multiallelic and co-dominant in nature which facilitates the study of genetic divergence (Gorji and Zolnoori, 2011; Valentina *et al.*, 2012), gene mapping (Feuillet and Eversole, 2007) and testing of authenticity of genetic stocks in wheat (Pestsova *et al.,* 2000).

Simple sequence repeats (SSRs) or microsatellites are short tandemly repeated sequences of 2-6 deoxy-nucleotides (Mclauchlan *et al.,* 2001). These are ubiquitous in eukaryotic genomes and the loci are highly polymorphic than the other markers due to the change in the number of repeating units among the individuals of population and each microsatellite locus can easily be amplified using polymerase chain reaction knowing the

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DNA sequence flanking the specifically tandem repeat region. Limitation in the application of SSR markers is the requirement of prior sequence information for the development of primers for locus-specific amplification (Dourar and Akkaya, 2001). The microsatellite markers are extremely useful for many different applications in wheat breeding programmes due to their high level of polymorphism and easy handling (Zeb *et al.,* 2009; Sehgal *et al.,* 2012). Therefore, present investigation was conducted to assess genetic diversity using SSR markers in 18 different wheat cultivars. Thus, the information generated could be useful to improve genetic diversity assessment and their use in plant breeding programs (Bertini *et al.,* 2006).

Materials and Methods

Plant Materials and DNA Extraction

Eighteen wheat cultivars used for the evaluation of genetic diversity were chosen to represent material from diverse sources (Table 1). Leaves from 7-10 days old wheat seedlings were used for genomic DNA extraction. DNA was isolated using CTAB method (Doyle and Doyle, 1987). The quantity of DNA was measured with a UV spectrophotometer at 260 nm and adjusted to a concentration of 50 ng/µl and stored in -20 ^{o}C.

SSR Analyses

The 21 SSR (*Xgwm, Xgdm, Xpsp, Barc* and *Wmc*) markers specific for wheat chromosomes were used for diversity analysis (Table 2). The details of SSR primers were gathered from Grain genes: a database for *Triticeae* and *Avene* (http://graingenes.org) and PCR procedure was used as described by Roder *et al.* (1998). The polymerase chain reaction (PCR) was performed in a volume of 15 µl, containing, 0.5U of Taq DNA polymerase, 1.7 mM dNTPs, 1.5 mM $MgCl_2$, 1X PCR buffer, 3 µM of forward and reverse primer, 50 ng of template DNA and remaining nuclease free water. The amplification was carried out in thermocycler from Biometra, Germany with initial denaturation at 94°C for 7 min, denaturation at 94°C for 30 sec, annealing at 51-61°C for 30 sec, extension at 72°C for 30 sec and final extension at 72°C for 10 min followed by 36 cycles. After PCR amplification, the 15 µl samples were loaded in amount of with 2 µl of 6X loading dye (0.03% bromophenol blue and 0.03% xylene cyanol FF) in 1.5% agarose gel and run at 95 V and 400 mA for about one hour. A DNA standard (Fermentas, Lithuania) marker of 50 bp loaded along with samples for determination of amplification product sizes and documented using gel documentation system from Bio-Rad (Berkeley, U.S.A.).

Data Collection and Diversity Analysis

Marker Polymorphism

The informativeness of the SSR markers was determined using polymorphism information content (PIC). The PIC content was calculated as described by Anderson *et al.* (1993).

Table 1. Wheat cultivars and their sources used in present investigation

Cultivars	Source	Pedigree	Country of origin
Mons Ald's	IARI, New Delhi	-	-
HD 2285	IARI, Pusa	249/HD2150 //HD 2186	India
Sonalika	IARI, New Delhi	II54-388 /AN/3/YT54/N10B/LR 64	India
Iepaca rabe	IARI, New Delhi	-	-
AKAW 4008	IARI, New Delhi	-	-
Halna	IARI, New Delhi	HD 1982/K816	India
Pusa gold	RAU, Pusa	KALYANSONA/HD 1999//HD 2204/DW 38	India
HD 2733	IARI, Pusa	ATTILA /3/ TUI /CARC // CHEN / CHTO /4/ ATTILA	India
PBW 343	RAU, Pusa	ND/VG9144//KAL/BB/3/YCO"S' /4/VEE#S "S"	India
C 306	RAU, Pusa	RGN/CSK3//2*C591/3/C217/N14 //C281	India
Kauz/AA/Kauz	IARI, New Delhi	JUP/BJY//URES	Mexico
Raj 3765	RAU, Pusa	HD 2402/VL639	India
HD 2888	RAU, Pusa	C 306/*T.sphaerococcum*//HW 2004	India
VL 914	IARI, New Delhi	-	India
F5-995	NBPGR, New Delhi	-	-
K0583	RAU, Pusa	-	India
SAWSN 3010	NBPGR, New Delhi	-	-
Cuo/79/Prulla	IARI, New Delhi	-	-

Table 2. SSR locus, their sequences, chromosomal location (Cl), repeat motif and annealing temperature (AT)

Locus	Sequence (5′-3′)	Cl	Repeat motif	AT (^{o}c)
Xgwm44	(L) GTTGAGCTTTTCAGTTCGGC (R) ACTGGCATCCACTGAGCTG	7D	$(GA)_{28}$	60
Xgwm160	(L)TTCAATTCAGTCTTGGCTTGG (R) CTGCAGGAAAAAAAGTACACCC	4A	$(GA)_{21}$	60
Xgwm190	(L) GTGCTTGCTGAGCTATGAGTC (R) GTGCCACGTGGTACCTTTG	5D	$(CT)_{22}$	60
Xgwm261	(L) CTCCCTGTACGCCTAAGGC (R) CTCGCGCTACTAGCCATTG	2D	$(CT)_{21}$	55
Xgwm265	(L) TGTTGCGGATGGTCACTATT (R) GAGTACACATTTGGCCTCTGC	2A	$(GT)_{23}$	55
Xgwm272	(L) TGCTCTTTGGCGAATATATGG (R) GTTCAAAACAAATTAAAAGGCCC	5D	$(CA)_{17}$	50
Xgwm408	(L) TCGATTTATTTGGGCCACTG (R) GTATAATTCGTTCACAGCACGC	5B	$(CA)_{22}(TA)(CA)_{7}(TA)_{9}$	55
Xgwm413	(L) TGCTTGTCTAGATTGCTTGGG (R) GATCGTCTCGTCCTTGGCA	1B	$(GA)_{18}$	60
Xgwm437	(L) GATCAAGACTTTTGTATCTCTC (R) GATGTCCAACAGTTAGCTTA	7D	$(CT)_{24}$	50
Xgwm 499	(L) ACTTGTATGCTCCATTGATTGG (R) GGGGAGTGGAAACTGCATAA	5B	$(GA)_{32}$	60
Xgwm513	(L) ATCCGTAGCACCTACTGGTCA (R) GGTCTGTTCATGCCACATTG	4B	$(CA)_{12}$	60
Xgwm538	(L) GCATTTCGGGTGAACCC (R) GTTGCATGTATACGTTAAGCGG	4B	$(GT)_{6}(T)(GT)_{10}$	60
Xgwm570	(L) TCGCCTTTTACAGTCGGC (R) ATGGGTAGCTGAGAGCCAAA	6A	$(CT)_{14}(GT)_{18}$	60
Barc55	(L) GCGGTCAACACACTCCACTCCTCTCTC (R) CGCTGCTCCCATTGCTCGCCGTTA	1B, 2B, 5B	$(ATCT)_{10}$	55
Barc75	(L) AGGGTTACAGTTTGCTCTTTTAC (R) CCCGACGACCTATCTATACTTCTCTA	3B	$(TAG)_{2}(TAGA)_{5}$	52
Wmc341	(L) ACATGGGCTACATGAGAGAAGA (R) AGAGTGGCTCCCTTTTCACCTA	6B	$(CA)_{24\ 55\ to\ 102}$	61
Wmc397	(L) AGTCGTGCACCTCCATTTTG (R) CATTGGACATCGGAGACCTG	6B	$(CA)_{9\ 69\ to\ 86,}\ (GA)_{14\ 93\ to\ 120}$	61
Xbarc71	(L) GCGCTTGTTCCTCACCTGCTCATA (R) GCGTATATTCTCTCGTCTTCTTGTTGGTT	3D	$(TAGA)_{7}(TA)_{2}$	55
Xbarc198	(L) CGCTGAAAAGAAGTGCCGCATTATGA (R) CGCTGCCTTTTCTGGATTGCTTGTCA	6B	$(ATT)_{19}$	50
Xgdm132	(L) ACCGCTCGGAGAAAATCC (R) AGGGGGGCAGAGGTAGG	6D	$(CT)_{24}$	60
Xpsp3071	(L) CGTGCCCTACACCTCCTTTTCTCTC (R) TCCGTACATACTCCGGGAGACC	6A	$(TC)_{14}$	61

$$PIC = 1-\sum_{j=1}^{k} P^{2}_{ij}$$

Where k is the total number of alleles detected for a given marker locus and P_{ij} is the frequency of the j^{th} allele for i^{th} marker in the set of eighteen wheat cultivars.

Genetic Similarity Estimation and Cluster Analysis

The amplified DNA bands were scored as 1 for presence and 0 for absence for each SSR marker (Fig.1) and entered into binary matrix as discrete variables. The average SSR difference between cultivars were calculated using Dice genetic similarity (GS) coefficient (Dice, 1945) using the NTSYS-pc software ver. 2.1 from Exeter Software, East Setauket, NY. The GS between two cultivars i and j is equivalent to the formula:

$$GS\ [Dice] = \frac{2a}{(2a + b + c)}$$

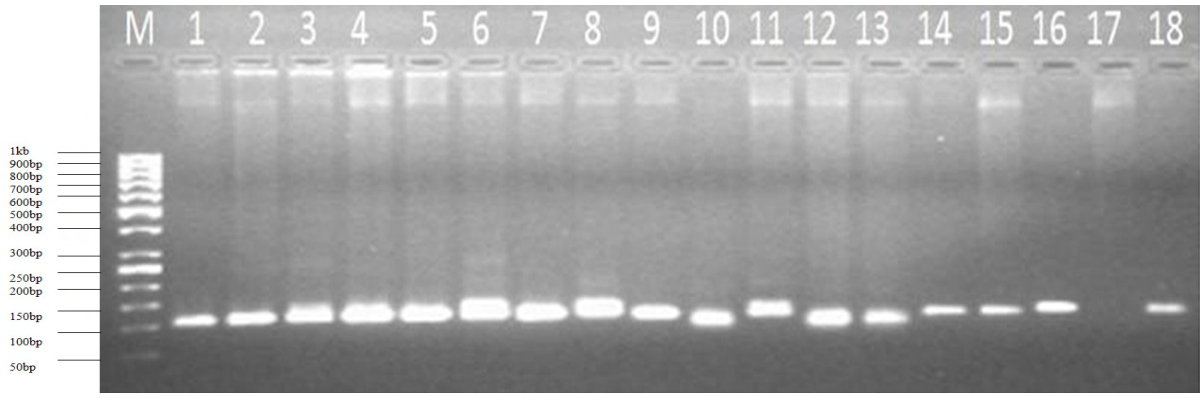

Fig. 1. Amplification of *Xbarc71* microsatellite marker in eighteen wheat cultivars (Lane-M= 50 bp molecular weight ladder; 1: Mons Ald's, 2: HD 2285, 3: Sonalika, 4: Iepaca rabe, 5: AKAW 4008, 6: Halna, 7: Pusa gold, 8: HD 2733, 9: PBW 343, 10: C 306, 11: Kauz/AA/Kauz, 12: Raj 3765, 13: HD 2888, 14: VL 914, 15: F5-995, 16: K0583, 17: SAWSN 3010, 18: Cuo/79/Prulla)

Where, a is number of bands present in cultivars i and j, b is the number of bands present in cultivar i and c is the number of bands present only in cultivar j but absent in cultivar i. The dendrogram was constructed for clustering of wheat cultivars based on matrix of genetic similarities by the unweighted pair group arithmetic mean average (UPGMA) method using the SAHN subprogram of NTSYS-pc 2.1 (Rolf, 1997). The two-dimension principle component analysis (PCA) was also performed for further validation of results of dendrogram. The nature and extent of diversity between cultivars were assessed by identifying the clusters at appropriate similarity coefficient.

Results and Discussion

SSR Polymorphism

To assess genetic diversity among 18 wheat cultivars, 21 microsatellite markers located on 13 different chromosomes with 32 loci were used (Table 3). A total of 273 allelic variants were detected. The number of alleles per locus varied from 5.0 (*Xpsp3071*) to 11.5 (*Xgwm570*) with an average of 8.5 alleles per locus. The ten primer pairs generated amplified products from single locus while remaining 11 primer pairs generated amplified products from two loci.

The genetic diversity at DNA level was estimated using PIC values of microsatellite markers. The highest number of alleles per locus was detected in genome D with 9.3 alleles, compared to 7.3 and 8.6 alleles for genomes A and B (Table 4), respectively. Similar observation of higher polymorphism level in genome D was also reported earlier (Naghavi *et al.,* 2007). This finding was accordance with (Huang *et al.,* 2002), the number of alleles can be used to evaluate genetic diversity because of the significant correlation between gene diversity and the number of alleles. The D genome of *Aegilops squarrosa*, which is the donor of the D genome of common wheat, exhibited more variation than that of common hexaploid wheat (Pestsova *et al.,* 2000; Dudnikov, 2000; Gianibelli *et al.,* 2001). The gene diversity of the B genome was found to be considerably lower than the gene diversity of the genomes A and D. This is consistent with the fact that the B genome is quite different from A and D genomes whereas the A and D genomes are more similar (Bonjean and Angus, 2001). The PIC varied from 0.630 to 0.928 with an average of 0.848 indicating that the markers were highly informative (PIC>0.5). The PIC value in 11 wheat cultivars was reported (Akkaya and Buyukunal-Bal, 2004) in the range of 0.36 to 0.87 with mean of 0.68 using 19 SSR loci. Among the A, B and D genome the mean PIC value were 0.734, 0.888 and 0.857, respectively. The PIC value was higher than the earlier observations (Prasad *et al.*, 2000; Schuster *et al.,* 2009) who reported an average 0.510, 0.710 and 0.490, respectively. The PIC values in Bulgarian winter wheat ranged between 0.100-0.810 (Landjeva *et al.,* 2006).

Cluster Analysis

The genetic relationships among eighteen wheat cultivars were investigated using cluster analysis. Genetic similarity varied from 0.56 (VL 914 Vs. Mons ald's) to 0.97 (Halna Vs. Iepaca rabe and Kauz/AA/Kauz Vs. Halna) indicating a higher level of variation among the cultivars. UPGMA dendrogram divided all wheat cultivars into four main groups (Fig. 2) at 0.81 genetic similarity coefficients. Within the each clusters several sub-cluster were observed and cultivars with more

Table 3. Fragment size, number of loci, number of alleles and polymorphism information content (PIC) in 18 wheat cultivars based on 21 SSR markers

Marker	Fragment size (bp)	No. of loci	No. of alleles	PIC
Xgwm44	140-238	2	22	0.862
Xgwm160	192-252	2	13	0.772
Xgwm190	222-269	1	12	0.928
Xgwm261	178-256	2	14	0.898
Xgwm265	117-194	2	12	0.632
Xgwm272	147-168	1	8	0.841
Xgwm408	161-266	2	12	0.874
Xgwm413	107-161	1	8	0.886
Xgwm437	66-104	1	12	0.922
Xgwm 499	91-151	2	11	0.843
Xgwm513	158-203	1	13	0.916
Xgwm538	167-246	2	18	0.928
Xgwm570	104-206	2	23	0.832
Barc55	138-198	1	15	0.883
Barc75	128-158	1	14	0.922
Wmc341	142-174	1	10	0.910
Wmc397	163-198	1	8	0.910
Xbarc71	125-156	1	11	0.922
Xbarc198	65-174	2	12	0.812
Xgdm132	180-447	2	14	0.630
Xpsp3071	74-208	2	10	0.700
Total		32	273	–
Mean			8.5	0.848

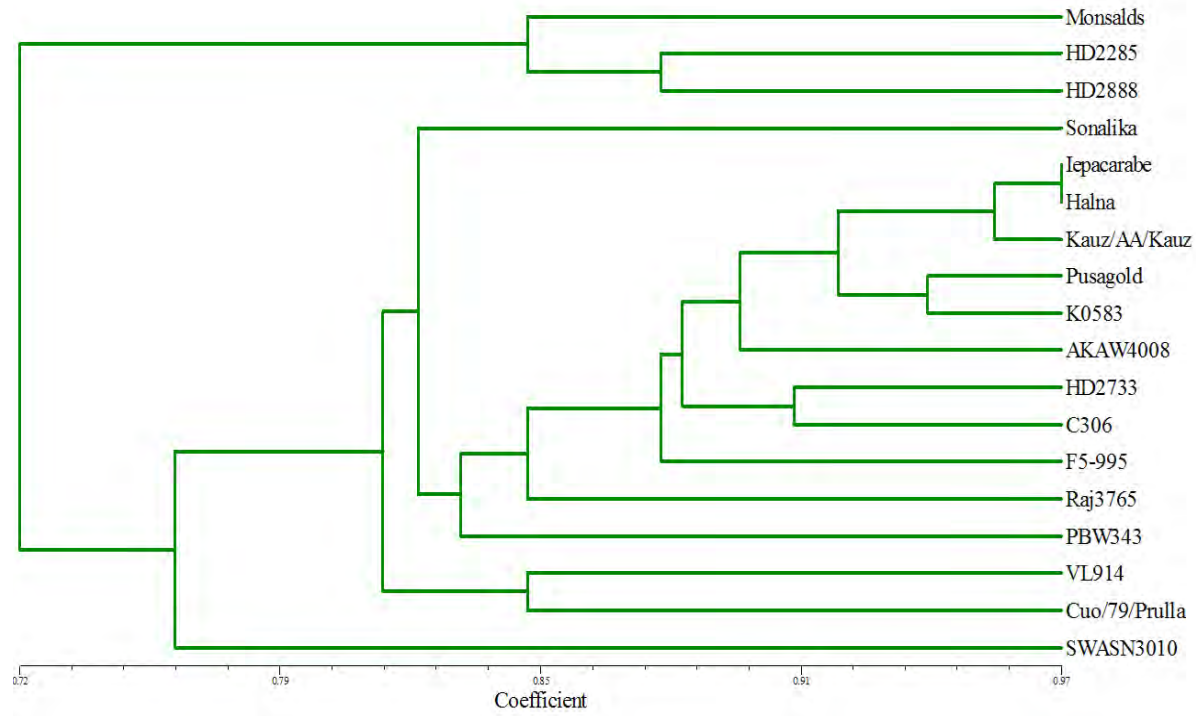

Fig. 2. UPGMA dendrogram showing genetic relationship between 18 cultivars based on Dice similarity coefficient using SSR markers

Table 4. Number of SSR alleles, number of alleles per locus and mean PIC of 32 microsatellite loci in three genomes

Item	Genome			Total
	A	B	D	
Loci	8	14	10	32
Number of alleles	59	121	93	273
Number of alleles/locus	7.3	8.6	9.3	25.2
Mean PIC value	0.734	0.888	0.857	0.826

similarity were placed in the same cluster. In group I, cultivars differentiated into two subgroups comprising, one Mons ald's which is exotic cultivar with lesser similarity on one hand and two Indian cultivars HD 2285 and HD 2888 with higher similarity value (0.88) on the other hand. The larger group II comprised twelve cultivars, can be divided into further two subgroups, subgroup IIA has only one cultivar *i.e.* Sonalika, the most common and widely growing cultivar, while subgroup IIB has eleven cultivars namely, Iepaca rabe, Halna, Kauz/AA/Kauz, Pusa Gold, K0583, AKAW 4008, HD2733, C306, F5-995, Raj3765, PBW343 and most of these cultivars belong to Indian subcontinent. The maximum genetic similarity value (0.97) in this group was observed between Iepaca rabe *vs*. Halna and Kauz/AA/Kauz *vs.* Halna. Cultivars HD 2733 and Sonalika used as check cultivar in many parts of India and in other countries like Bangladesh, Nepal etc. Group III comprise of VL 914 which is an Indian origin cultivar and Cuo/79/Prulla *i.e.* exotic cultivar with 0.84 similarity value and group IV contains only one exotic cultivar, SAWSN 3010 which has different growth type than the other cultivars. Each group consist of similar responsive cultivars with respect to their growth, flowering and maturation time. Cultivars of group II and group III showed maximum similarity while group I and IV showed comparatively less similarity. Among all groups, group I cultivars had completely different growth behaviour than the remaining groups. Results showed that some of the cultivars showing higher similarity of SSR loci were placed in a group, due to similarity of their pedigree from and relative uniformity of their genetic structure. For diversity and population structure analyses different types of marker has been extensively used in wheat, among them most important are Diversity array technology (DArT) (Nielsen *et al.,* 2014) and a single t nucleotide polymorphism markers (SNP) (Ren *et al.,* 2013). In addition, Balestre *et al.* (2008) compared similarity and dissimilarity coefficients using SSR markers data in maize (*Zea mays* L.) and found that the coefficient with the smallest stress value was Dice similarity coefficients. The two-dimensional principle component analysis (PCA) plot was carried out including all cultivars and SSR loci using allele frequency to summarize relationship and validated the results of the dendrogram (Fig. 3). In this study, SSR markers were efficient in discrimination and unambiguous identification of all the eighteen wheat cultivars used in the analysis and information on genetic diversity may be a good tool of selecting genotypes in breeding programs.

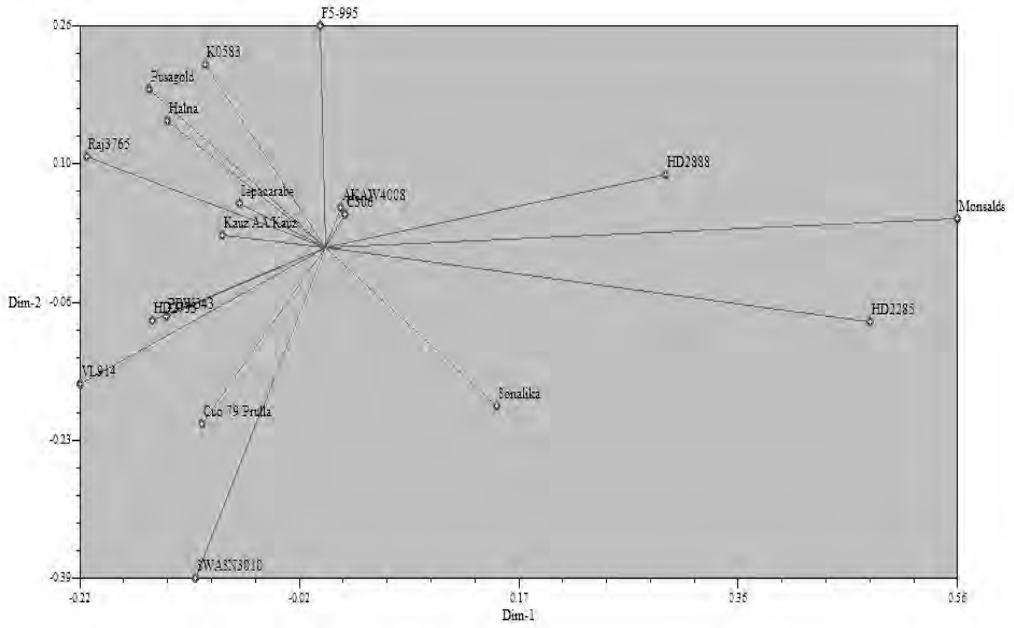

Fig. 3. Scatter plot of the 2-D principal coordinates analyses matrix of SSR marker data.

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