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Assessment of agro-morphological and molecular diversity among fertility restorer lines in wheat (*Triticum aestivum* L.)

S. M. S. Tomar, Pallavi Sinha, Asad Ali, Vinod, Bhanwar Singh and H. S. Balyan[1]

Division of Genetics, Indian Agricultural Research Institute, New Delhi 110 012
[1]Department of Genetics & Plant Breeding, C.C.S. University, Meerut

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Abstract

Genetic diversity among eight diverse fertility restorer lines of exotic and indigenous origin was studied using agronomic characters and molecular markers. Thirty eight of the 79 SSR markers used were found polymorphic yielding a total of 113 alleles with mean polymorphism information content (PIC) of 0.37 per marker. Dendrograms resulting from an UPGMA cluster analysis classified the fertility restorer lines into two main clusters with distinct sub-grouping. On the whole a very low range of genetic similarity (GS) was observed, which indicated that the fertility restorer lines used in the study are genetically diverse. There was low correspondence between the similarity matrices as obtained with morphological and molecular data. The information deduced from the study can be utilized in hybrid breeding programme as well as in producing combination of different fertility restorer genes in a single genetic background.

Key words: Wheat, fertility restorer, genetic similarity, molecular diversity and SSR markers

Phenomenal increase in crop yields, particularly in cereals witnessed in mid of last century is mainly due to the application of principles of genetics in crop improvement. Exploitation of hybrid vigour in maize, pearl millet [1], sorghum [2] and now in rice registered a considerable increase in grain yields [3] and continues to be the most appropriate means for enhancing production [4]**.** Apprehensions about heterosis breeding in a major cereal crop like rice were constantly made till hybrid rice became the reality. With similar floral biology as of rice, wheat also has a chasmogamous flowers, which do not promote out crossing naturally. Efforts to change the pollination system of wheat were made as early as 1951 [5] to facilitate hybrid breeding programme. Later, cytoplasmic male sterility (CMS)-fertility-restoration system was developed by Wilson and Ross [6]. Since then, many studies have been carried out on various aspects of cytoplasmic male sterility-fertility-restoration and the effect of nuclear x cytoplasm interaction on different yield components [7-10]. Commercial hybrids yielding10-15 per cent higher under normal row spacing and low seed rates have been successfully developed. In the pursuit of novel system for hybrid seed production, chemical hybridizing agents (gametocides) inducing male sterility were also developed [11] and wheat hybrids based on CHA (Genesis and Croisor) were recommended for commercial cultivation in many countries. Recently, China has taken a great leap forward in releasing high heterotic hybrids. For successful exploitation of heterosis, both CMS and fertility restorers have to be genetically divergent. Unavailability of diverse fertility restorers is considered to be a major lacuna in hybrid breeding programme in wheat. Release of commercial hybrids is hampered by poor fertility restoration of the *T. timopheevi* cytoplasm based male sterile lines. Therefore, future progress in heterosis breeding depends on identification of diverse fertility restorers. The analysis of agronomic traits is also an important pre-requisite for efficient use of fertility restorer lines. Several molecular markers including the SSRs have been utilized for characterizing genetic diversity in wheat [12, 13, 14]. SSR markers have the advantages of co-dominance and offer uniform genome coverage. A limited information on genetic diversity at agro-morphological and molecular levels is available among fertility restorers. The objective of the present study was, to estimate genetic diversity among eight fertility restorers based on agronomical traits and SSR markers

Corresponding author's e-mail: smstisgpb@gmail.com

with a view to understanding their utility in attaining adequate levels of heterosis in hybrid breeding programme in wheat.

Materials and methods

Plant material

Material comprised of eight fertility restorer lines of wheat (*Triticum aestivum* L.) of which four were original lines identified from a set of 700 genotypes tested for fertility restoration, while remaining four were derived from the crosses involving the original lines (Table 1). These fertility restorers are being maintained in the Division of Genetics at Indian Agricultural Research Institute (IARI), New Delhi.

DNA extraction, PCR amplification and electrophoresis

Plant DNA was isolated from fresh leaves as per the protocol of Dellaport *et al.* [15]. The quality and quantity of DNA was determined using a UV spectrophotometer (Beckman, USA). The DNA samples were diluted to 30 ng/µl. A set of 79 SSR primer pairs were used in the present study. These were selected based on their high polymorphism information content (PIC) values and uniform distribution across the wheat genome (Table 2) based on information available in http://www.graingene.com. DNA samples (50 ng) were amplified in 25-µL reaction volume containing 1x PCR buffer [10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.01% (v/v) gelatin] (Bangalore Genei, India), 0.2 m*M* of each dNTPs (Bangalore Genei, India), 10 pmol of each primer and 1 U of Taq polymerase (Bangalore Genei, India). PCR was carried out in a Thermal cycler (Perkin-Elmer-Gene Amp PCR System 9700, USA). The basic PCR profile was 5 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C and 7 min at 72°C for final extension. PCR amplified products were resolved on 3% metaphor gels, stained with ethidium bromide and visualized under UV in a gel documentation system (Alpha Innotech, USA). The sizes of the amplified fragments were estimated with the help of Alphaease software utility of the gel documentation system using 50 bp and 100 bp DNA ladder (MBI Fermentas, Lithuania) as the size standard.

Observations recorded

Observations on agronomical (morphological) traits, namely, plant height (cm), spike length (cm), number of spikelets/spike, number of tillers/plant, number of grains/spike and 1000 grain weight were recorded. The material was sown in three replications in RBD. Ten randomly chosen plants from each replication were taken for recording observations and the data were subjected to statistical analysis. The features of fertility restorer lines are given in Table1.

Statistical analysis

All agronomical (morphological) traits were standardized by subtracting the mean value and dividing by the standard deviation. This allowed scaling effect before calculating Euclidian distances. Based on the value of standardized traits, Euclidian distance (md_{ij}) between the lines was calculated and matrix of these values denoted as MD. Morphological similarities (ms_{ij}) were also calculated as 1 - md_{ij} and matrix of these values was denoted as MS. Using the matrix MS (1 – MD), UPGMA cluster analysis was performed using the statistical package NTSYS-pc 2.0, producing a dendrogram and PCA map depicting the relationship among the lines relative to the morphological characteristics.

The polymorphism information content (PIC) value for each SSR marker was calculated using the formula:

Table 1. Characteristic features of fertility restorer lines

S.No.	Fertility restorer lines	Pedigree	Features
1.	PWR4101R	CBHW-R CHN 89R 4294 OCHN S-2 BV97 = EC414148	Exotic, spring medium tall
2.	PWR4099R	CBHW-R CHN QI RR925 OCHN S-4 BV97 = EC414149	Exotic, spring
3.	T2003 R	HD69/NP839//S310//NP830	Indigenous, spring
4.	EC368169R	Not known	Exotic, winter
5.	T280 R	KMS-8/HD2329*5/C80-1//PWR4101	Derived line
6.	T2995 R	KMS-8/HD2329*5/C80-1//PWR4099	Derived line
7.	T1771 R	EC368167A/ Ures//Jaun-Kauz/ EC368169	Derived line
8.	T1752 R	Not known	Derived line

Table 2. Number of alleles and PIC values of 38 SSR markers among eight fertility restorer lines

S.No.	Marker name	Size range of alleles(bp)	Chro-mosome number	No. of alleles	PIC value
1.	Xgwm174	148-166	5D	4	0.34
2.	Xgwm341	222-238	3D	4	0.35
3.	Xgwm297	80-150	7B	4	0.32
4.	Xgwm265	165-199	2A	4	0.35
5.	Xbarc77	239-290	3B	3	0.38
6.	Xgwm540	138-410	5B	4	0.32
7.	Xgwm335	96-116	5B	2	0.21
8.	Xgwm339	166-172	2A	2	0.46
9.	Xwmc273	209-206	7B	2	0.46
10.	Xwmc432	192-195	1D	3	0.32
11.	Xgwm397	104-155	4A	2	0.50
12.	Xgwm334	246	6A	3	0.35
13.	Xbarc170	165-183	4A	2	0.46
14.	Xbarc171	164-194	6A	3	0.43
15.	Xgwm427	189-204	6A	3	0.39
16.	Xgwm610	124	4A	2	0.46
17.	Xwmc44	210-400	1B	5	0.31
18.	Xgwm484	118-126	2D	4	0.32
19.	Xbarc71	98-104	3D	4	0.34
20.	Xwmc532	104-124	3A	4	0.35
21.	Xwmc169	106-194	3A	3	0.39
22.	Xgwm630	85-153	2B	4	0.28
23.	Xbarc57	134-215	3A	3	0.41
24	Xbarc397	148-193	4A	2	0.46
25.	Xbarc140	253-304	1B	3	0.35
26	Xgwm459	243-303	6A	5	0.31
27.	Xbarc497	86-130	5B	3	0.41
28.	Xgwm273	165-177	7B	3	0.40
29.	Xgwm337	214-255	1D	2	0.46
30.	Xgwm257	98-118	2B	2	0.21
31.	Xgdm67	92-94	7D	2	0.46
32.	Xwmc283	151	4A	2	0.21
33.	Xwmc134	137	1B	2	0.21
34.	Xwmc332	169	2B	3	0.39
35.	Xwmc11	177	3A	3	0.35
36.	Xgwm376	143-147	3B	3	0.43
37.	Xgwm513	146-152	4B	2	0.21
38.	Xgwm495	160-178	4B	2	0.37

$$PIC = 1 - \sum_{i=n} P_i^2$$

Where, P = Frequency of an allele

n = Number of alleles

For each SSR marker-genotype combination, the presence or absence of band was given a score of 1 or 0. Pairwise GS was estimated using SIMQUAL of the software package NTSYS-pc version 2.0. The similarity matrices were used to construct a dendrogram for all the genotypes using SAHN of NYSYS-pc based on UPGMA. The correlation between similarity matrices based on morphological and molecular data was determined by MXCOMP module of the NTSYS.PC software.

Results and discussion

Informativeness of SSR markers

Out of 79 SSR markers used in the study, 38 were found to be polymorphic. Which amplified a total of 113 alleles. The number of alleles per marker ranged from 2 for 14 SSRs to 5 in Xwmc44 and Xgwm459 with an average of 2.9 (Table 2). The markers, namely, Xgwm174, Xgwm341, Xgwm297, Xgwm265, Xgwm540, Xgwm484, Xbarc71, Xwmc532 and Xgwm630 generated four polymorphic alleles each, while 3 polymorphic alleles each were produced by Xbarc77, Xwmc432, Xgwm334, Xbarc171, Xgwm427, Xwmc169, Xbarc57, Xbarc140, Xbarc497, Xgwm273, Xwmc332, Xwmc11 and Xgwm376.

The polymorphic information content (PIC) for these 38 SSR primer pairs ranged from 0.21 to 0.50. Detailed information of 38 polymorphic SSR primer pairs along with PIC values are given in Table 2. The range of PIC value indicated that the locus specific PCR-based microsattelite markers were quite informative. However, this observed PIC range is lower than the range reported in barley [16, 17] and wheat [18] using 26 and 71 accessions respectively. The average PIC value (0.37) obtained for eight fertility restorers confirmed that SSR markers are highly informative and would be useful in hybrid breeding.

Genetic similarity among the restorers

The average genetic similarity among the fertility restorer lines at molecular level was 0.32 with a range from 0.10 between PWR4099 and T1752R to 0.45 between T1752R and T1771R (Table 3a). At morphological level the mean genetic similarity was 0.43 ranging from 0.09

to 0.67 the minimum similarity being between an indigenous line T2003R and T1771R, a derivative of EC368169 as well as between PWR 4099 and EC368169 (Table 3b). The average genetic similarity within the original (PWR4101, PWR4099, T2003R and EC368169) and derived restorer lines (T280R, T2995R, T1771R and T1752R) was 0.22 and 0.28 respectively indicating a high degree of genetic diversity. The microsatellite markers used were also able to discriminate between fertility restorer lines even though some of them had original parents in their pedigree (Fig. 1). The average genetic similarity value of 0.32 among fertility restorers reflected some common SSR alleles between them that was supported by the mean genetic similarity value observed at morphological level. Out of 113 alleles detected by using 38 microsatellite markers, 20 were common between PWR4099 and its derivative T2995R indicating their close genetic relationship. However, T1771R, a derivative of EC368169 had only seven alleles in common indicating that they are genetically distinct. The detection of more number of alleles would be possible if a large number of SSR markers are used in the study. Although, information on morphological and molecular diversity in emmer and common wheat has been reported [13, 19], reports on diversity of fertility restorers having different origin are not available and thus the present study provides new information that would be of use in wheat hybrid breeding.

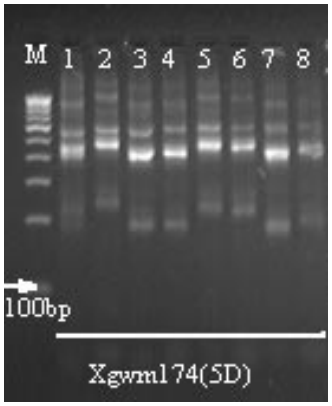


Fig. 1. Molecular profile of eight fertility restorers using SSR Xgwm174 (5D)

Variation in morphological traits

The data on six morphological traits, namely, plant height, spike length, number of tillers/plant, number of spikelets/spike, number of grains/spike and 1000 grain

Table 3a. Genetic similarity among eight fertility restorer lines on the basis of molecular data

	T2003	4099R	4101R	EC69R	1752R	1771R	T2995R	T280R
T2003	1.00							
4099R	0.16	1.00						
4101R	0.40	0.22	1.00					
EC69R	0.18	0.16	0.43	1.00				
1752R	0.15	**0.10**	0.24	0.43	1.00			
1771R	0.18	0.22	0.32	0.42	**0.45**	1.00		
T2995R	0.24	0.15	0.24	0.18	0.28	0.22	1.00	
T280R	0.24	0.15	0.24	0.18	0.28	0.22	0.13	1.00

Table 3b. Genetic similarity among eight fertility restorer lines on the basis of morphological data

	T2003	4099R	4101R	EC69R	1752R	1771R	T2995R	T280R
T2003	1.00							
4099R	0.20	1.00						
4101R	0.38	0.10	1.00					
EC69R	0.33	0.09	0.22	1.00				
1752R	**0.09**	0.20	0.38	0.33	1.00			
1771R	0.20	0.33	0.10	0.33	0.33	1.00		
T2995R	0.22	0.22	0.25	0.22	0.38	0.22	1.00	
T280R	0.57	0.22	**0.67**	0.22	0.22	0.10	0.43	1.00

weight (TGW) were analysed for computing the genetic diversity (Table 4). ANOVA revealed the significant difference in mean values among the restorer lines with respect to all the traits. PWR4099 had more number of spikelets and high grain number per spike with moderate thousand grain weight, whereas T2003R showed highest TGW. However high tiller number in EC368169 was observed, obviously because it had winter growth habit.

PWR4099 was distinct with respect to number of grains and spikelet/spike, whereas T2003 was identified with high 1000 grain weight with moderate no. of grains per spike. PWR4099 is an exotic line while T2003 is an indigenous line. The seed weight is an important character indicating seed dimension. Thousand grain weight was increased in some of the derived lines due to an increased selection pressure. Although PWR4099 is involved as one of the parent in T2995R but morphologically these two fertility restorers were different. The similarity index based on molecular analysis also did not indicate closeness. It could be deduced that genetic factors with respect to fertility restoration was only retained in T2995, which was a recombinant quite diverse from PWR4099.

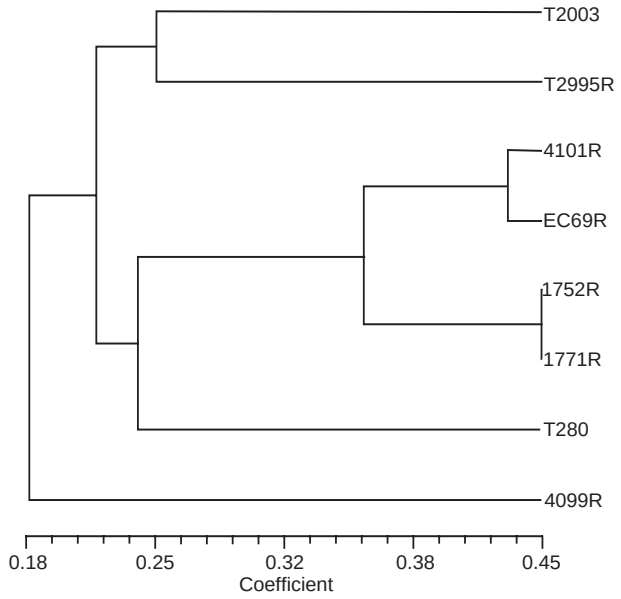
Significant differences in respect of morphological traits of all the fertility restorers were observed (Table 4). The spikelet number per spike and the number of grains per spike were significantly higher in PWR4099 but it produced grains with low 1000 gr. wt. On an average T2003R indicated a balance between all the yield components. It is expected that such genotypes would be useful in generating a heterotic combination. A winter wheat genotype EC368169 produced significantly higher number of tillers and moderate number of grains/spike with low thousand grain weight. Sufficient degree of morphological diversity among all the fertility restorers was displayed.

Cluster analysis

Unweighted Pair Group Method with Arithmetic Averages (UPGMA) cluster analysis was performed using Jaccard's similarity coefficient matrices calculated from SSR markers to generate a dendrogram of eight fertility restorer lines analyzed (Fig. 2a.). The similarity coefficient ranged from 0.18 to 0.45. All the genotypes were grouped into two major clusters at 21 percent similarity except PWR4099. The first cluster was divided in to two sub-clusters at 25% similarity having only two fertility restorers, namely, T2003R and T2995R in the group. The second cluster also consisted of two sub-groups. Among them the first sub-cluster comprised of four restorer lines viz., PWR4101, EC368169R, T1752R and T1771R. Restorer lines T1752R and T1751R grouped together sowing maximum value of similarity (36%) between them. In the second sub-cluster had only T280R. The similarity coefficient based on agro-morphological traits ranged from 0.19 to 0.67 (Fig. 2b) indicating a great degree of morphological diversity among the fertility restorers which concurred with previous results in barley [13, 20] and in wheat [14]. All the genotypes were grouped into two major clusters at 19 percent average similarity. One major cluster was sub-divided into two sub-cluster at 25 % similarity and consisting of six restorer lines. Restorer lines PWR4101 and T280R grouped together along with T2003R. At 33% similarity second sub-cluster was divided with two restorer lines 4099R and 1771R in this group.

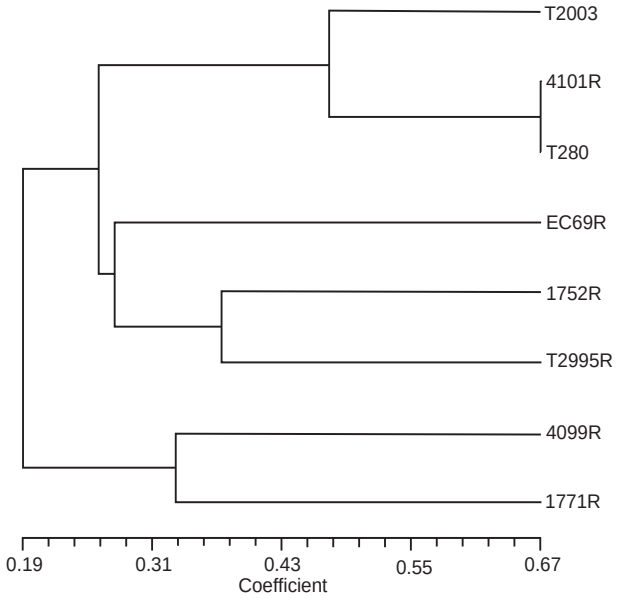
Table 4. Morphological characteristics of eight fertility restorer lines under study

TGMS lines	Plant height (cm)	Spike length (cm)	Spikelet/ear (No.)	Tillers/plant (No.)	Grains/spike (No.)	1000 grain wt. (g)
4101R	92.25	10.00	24.00	7.81	57.22	45.85
4099R	88.56	11.33	27.8	9.80	64.12	33.63
T280R	97.66	12.56	24.00	14.63	52.43	45.17
T2003R	101.46	10.00	20.6	17.76	55.86	49.00
T2995R	110.80	13.2	21.33	11.53	52.13	33.46
EC69R	82.26	12.15	22.10	28.14	57.90	34.00
1771R	101.33	11.33	23.00	9.63	53.00	39.00
1752R	104.80	11.30	23.46	8.50	54.13	40.76
SE±	3.28	0.40	0.78	2.41	1.41	2.17
CD	7.03	0.85	1.67	5.16	3.02	4.65



(a)

Fig. 2a. Dendrogram derived from UPGMA cluster analysis of eight fertility restorer lines based on 38 SSR markers



(b)

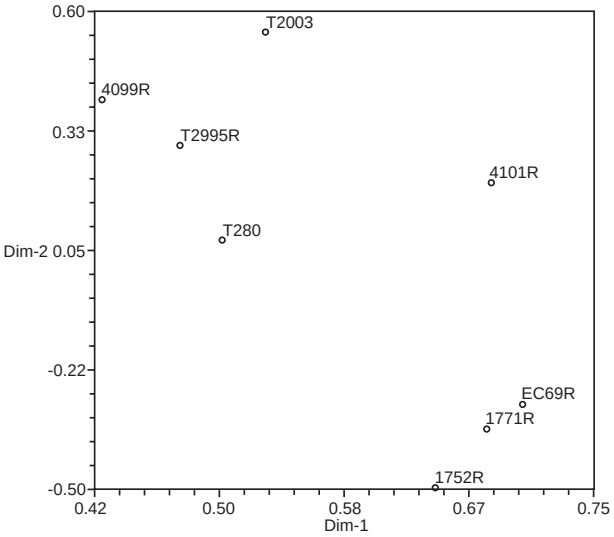
Fig. 2b. Dendrogram derived from UPGMA cluster analysis of eight fertility restorer lines based on six morphological traits

Principal component analysis (PCA)

Principal component analysis (PCA) revealed that more than 75% variation in the estimates was explained by the first four components (Fig. 3a). The first principal component explained approximately 36% variation among exotic PWR4099 and indigenous T2003R lines on one hand and essentially derived restorers, T280R and T2995R on the other. One of the original (exotic) restorer lines, PWR4101 remained distinct as compared to others separated by second principal component, which explained approximately 22% variation in the data. Thus, a total of 56% of variability is explained by the first two components indicating the suitability of the selected SSR markers for genetic diversity analysis. Rest of the restorer lines falls in the third component. It is significant to mention that EC368169R involved in the development of T1771R clustered together displaying close similarity. T1752R was also in the same group, however, its lineage is not known. The study indicated considerable genetic variability between the exotic and indigenous fertility restorers. More than 60% variation in the estimates was explained by the first three components. The first principal component explained approximately 37 % significant variation with three restorer lines namely T280R, 4101R and T2003R. Second principal component separated the fertility restorer line T2955R, while remaining restorer lines were grouped based on another principal component (Fig. 3b).

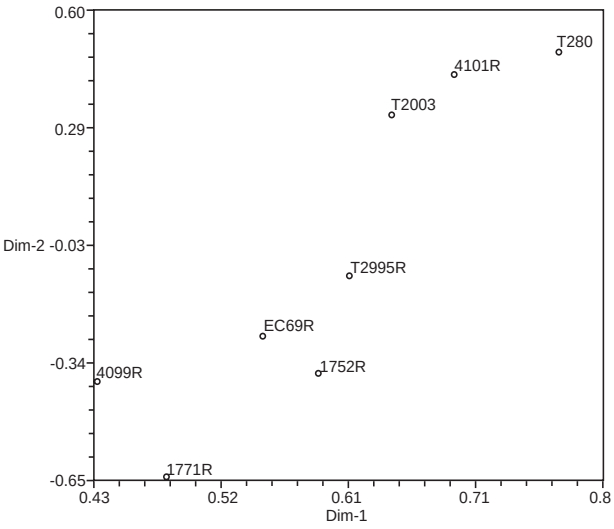
Comparison of data derived from SSR markers and morphological traits

Genetic similarity between and within the group of fertility restorer lines on the basis of morphological and SSR data is presented in Table 3. The correlation between morphological similarity matrix (MS) and molecular similarity matrix (GS) was very low (Mantel test, $r = 0.147$; $P = 0.278$). The mantel Z test statistic was also not significant between MS and GS matrices ($Z = 1.93$, $P < 0.0001$) indicating low correspondence between the two. The poor relationship reflects diverse genetic background of fertility restorers. Besides, diversity at SSR loci may not reflect diversity in quantitative traits [21]. Low correlation between morphological and molecular data has been reported in rice [22]. It was elaborated that there is a fundamental difference in the concepts underlying both the measures of genetic diversity. The morphological data is an indirect measure of genetic diversity; which qualifies the degree to which two genotypes are 'identical by morphology'. In contrast the rationale for using genetic similarity estimates based on molecular data is that the proportion of bands shared between two genotypes is an indicator of their resemblance in the DNA sequence across the entire



(a)

Fig. 3a. Principal Component Analysis (PCA) of eight fertility restorer lines based on 38 SSR markers



(b)

Fig. 3b. Principal Component Analysis (PCA) of eight fertility restorer lines based on six morphological traits

genome [23]. Consequently genetic similarity is a direct measure and should reflect the proportion of 'gene alike in state' irrespective of whether the identity is caused by alleles 'identical by morphology' or only those 'alike in state'. The low value for correlation obtained may be due to poor relationship between two measures. As pointed out by Kim and Ward [24], the alleles are not always transmitted equally from female and male parents to the progeny and therefore, each parent may not contribute equally to the cross.

The present study demonstrated that SSRs are markers useful for the assessment of genetic diversity of fertility restorers in wheat. Considerable genetic diversity was detected in exotic and indigenous fertility restorers and their derivatives. The result revealed the number of alleles per SSR marker can be a good indicator to assess diversity. The comparison of relationship between SSR based polymorphism and morphological traits showed that SSR markers are more precise to examine genetic diversity. Further, the observations suggested that the diverse fertility restorers can be utilized to produce commercial hybrids in wheat.

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