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Microsatellite markers based characterization in advance breeding lines and cultivars of bread wheat

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Abstract

Aim : Studying genetic diversity is useful for wheat breeding and production of more efficient wheat cultivars under changing climatic conditions. Forty-four diverse bread wheat genotypes were assessed for genetic diversity study using 70 microsatellite (SSRs) markers covering all three genomes.

Methodology : Genomic DNA of 44 genotypes was extracted using CTAB method and quality was checked by UV spectrophotometer and agarose gel electrophoresis. PCR amplification reaction was carried out and amplified products were resolved by horizontal electrophoresis. Bands were scored and matrix was used to calculate the similarity genetic distance. Phenotypic data was subjected to Euclidean cluster analysis for estimation of genetic divergence and grouping of genotypes into clusters.

Results : A total of 181 alleles were detected and the number of alleles per locus ranged from 1-5 with an average of 2.6 alleles per locus. The overall size of PCR products amplified ranged from 100-475 bp. Significant differences in allelic diversity among various microsatellite loci were seen. The SSR primers, xgwm 428, xbarc 1165, wmc 477, psp 3071, xcfa 2129, xgwm 18, xgwm 234 and xbarc 359 amplified single allele, while the primers xgwm 46, xgwm 334 and wmc 517 amplified five alleles. The similarity coefficients among all the genotypes ranged from 0.62 to 0.81. The cluster tree analysis based on UPGMA following the software NTSYS PC led to grouping of 44 genotypes in different clusters in such a way that the genotypes within each cluster had higher similarity than between clusters.

Advance breeding lines and cultivars of bread wheat were characterized both at phenotypic and molecular level

Phenotypic data was recorded for eight metric traits. Genomic DNA of 44 genotypes was isolated using modifed CTAB method.

PCR Amplification and AGE. Scoring was done based on presence (1) or absence of band (0)

0/1 matrix was used to calculate the similarity genetic distance using 'SIMQUAL' sub-programme of NTSYS-PC software 2.02e

Phenotypic data was subjected to Euclidean Cluster Analysis for estimation of genetic divergence

Microsatellite markers can be exploited as a new generation tool in studying the polymorphism and genetic diversity among cultivars

A total of 181 alleles were detected. PCR products amplified ranged from 100-475 bp

Similarity coefficients among all the genotypes ranged from 0.62 to 0.81

Interpretation : The study ascertained that microsatellite markers can be exploited as a new generation tool in studying the polymorphism and genetic diversity among cultivars and further, selection of parental combinations for initiating breeding programs.

Introduction

The knowledge of genetic diversity can be used as an important tool for improving quantitative traits (Huang *et al.,* 2002). This knowledge can in turn become a valuable source for further improvement of modern cultivars. The differences that distinguish one cultivar from another are encoded in the crop's genetic material and is passed to each generation. Bread wheat (*Triticum aestivum*) is a hexaploid species with three sets of basic chromosomes number (x=7 and 6x= 2n = 42), and the three genomes originating from natural hybridization of related species.

Globally, India is the second largest wheat producer after China and recorded a production of 93.50 million ton from an area of 30.23 million ha with a productivity of 30.93 q ha^{-1} (Anonymous, 2016). World population is expected to increase from around 6 billion now to around 8 billion by 2020, and more than 95% of the additional population will be in the developing countries. The agricultural sector in developing countries like India and Pakistan needs increased crop production to fulfill the demand and to occupy a pivotal position in agricultural crop production and hence, contributes a lot to the gross domestic product.

Since a narrow genetic base of germplasm is highly vulnerable to biotic and abiotic stresses, diversity is important for crop breeding and its assessment provide keys to disclose ways to combat the threats of environmental fluctuations (Khan *et al.,* 2015). Molecular markers are environment insensitive, they provide most suitable tool for evaluation of genetic material. DNA has no effect of environmental conditions (Cifci and Yagdi, 2012; Abouzied *et al.,* 2013; Malik *et al*., 2013). Therefore, assessment of genetic diversity at the molecular level is more consistent than at the phenotypic level. Presently, several kinds of DNA based molecular markers are available. Use of genetic markers not only provides information about genetic diversity but also helps in understanding the genetic control of quantitative characters. They can provide detailed characterization of genetic resources (Zhang *et al.,* 2009; Mir *et al.,* 2012).

Simple sequence repeats (SSRs) have been extensively used in wheat due to their high level of polymorphism, co-dominant inheritance and abundant distribution in the wheat genome (Roder *et al.,* 1995; Parker *et al.,* 2002). It has also been found that they show a much higher level of polymorphism and information than any other marker system in wheat crop (Ma *et al*., 1996; Bryan *et al*., 1997). Till date, more than 4000 SSR markers have been developed and used in genetic mapping studies of wheat (Han *et al*., 2015). SSR markers also play an important role in cultivar identification and genetic diversity studies (Hao *et al.,* 2011). In the present study, both phenotypic as well as molecular data were used to assess inherent genetic diversity of the selected bread wheat advance breeding lines and cultivars.

Materials and Methods

The present study was carried out at Research Farm of Department of Genetics and Plant Breeding, Chaudhary Charan Singh Haryana Agricultural University, Hisar, a semi-tropical region of North Western Plain Zone of India. Plant material consisted of 44 bread wheat genotypes (38 cultivars and 6 advance breeding lines) (Table 1), grown during 2014-15 *rabi crop season* in a randomized block design with three replications. Each genotype was sown in 2.5 m long paired row plots with row-to- row distance of 20 cm and plant-to-plant distance of 10 cm.

Phenotypic analysis of genetic diversity : Observations were recorded on five randomly chosen plants of each genotypes in each replication for eight metric traits, namely, days to heading, plant height (cm), tiller no per plant, grains per ear, 1000-grain weight (g), biological yield per plant (g), harvest index (%) and grain yield per plant (g). Statistical analysis of data was carried out by the method of Panse and Sukhatme (1978). Genetic divergence was estimated by Euclidean cluster analysis and grouping of the genotypes into clusters was done using Ward (1963) method.

Genomic DNA isolation : Genomic DNA was isolated from each genotype using CTAB method of Saghai-Maroof *et al.* (1984). To remove RNA contamination, DNA samples were treated with 1 µl of RNase A solution (10 mg ml^{-1}) per 50 µl of DNA sample. Quality of DNA samples was checked both by UV-spectrophotometer and on agarose gel electrophoresis. Using spectrophotometer, the ratio of the absorbance at 260 nm and 280 nm was noted. PCR amplified DNA fragments were resolved by submerged horizontal electrophoresis in 2.5% (w/v) agarose gels. PCR amplified products were viewed under UV light (350 nm) fluorescence and photographs were taken by gel documentation system.

PCR amplification conditions : PCR amplification conditions were optimized with respect to concentration of template DNA, primers, $MgCl_2$, *Taq* DNA polymerase and annealing temperature. Genomic DNA concentration 50 ng $µl^{-1}$ was used for PCR amplification, which was carried out in *Benchtop thermocycler* (programmable thermal cycler from BIORAD™ INTERNATIONAL).

Allele scoring : SSR amplification profiles were scored visually, based on the presence (taken as 1) or absence (taken as 0) of bands for each wheat genotype. Only clear and unambiguous bands were scored. The size (in nucleotide base pairs) of amplified bands was determined based on its migration relative to standard molecular size marker (100 kb).

Molecular data analysis : The 0/1 matrix was used to calculate the similarity genetic distance using 'SIMQUAL' sub-programme of NTSYS-PC software 2.02e (numerical taxonomy and

Table 1 : Pedigree of bread wheat advance breeding lines and cultivars used to study genetic diversity

Genotypes	Pedigree	Genotypes	Pedigree
DPW 621-50	KAUZ//ALTAR84/AOS/3/ MILAN/KAUZ/4/HUITES	WH 1138	PBW65*2/PASTOR
HD 2967	ALD/CUC//URES/HD2160/HD2278	WH 1129	CS/TH.CS//3*PVN/3/MIRLO/B UC/4/MILAN/5/TILHI
Tobari	TZZP/AN64A	DBW 88	KAUZ/ALTAR84//ADS/3/MIL AN/KAUZ/4/HUITES
WH 1080	PRL/2*PASTOR	HD 3086	DBW14/HD2733//HUW468
DBW 17	CMH79A.95/3*CN079//RA J3777	WH 1179	OASIS/SKAUZ//4*BCN/3/3* PASTOR
PBW 550	WH594/RAJ3856//W485	WH 1124	MUNIA/CHTO//AMSEL
Aus 15854	-	DBW 71	PRINIA/UP2425
WH 1021	ALD/CUC//URES/HD2160/ Hd2278	DBW 90	HUW468/WH730
WH 542	JUPATECO/BLUEJAY//URES	PBW 343	ND/VG1944//KAL//BB/3/YAC O's'/4/VEE5's'
Veery's	KAVKAZ/BUHO'S'//KAL YANSONA/BLUEBIRD	WH 157	NP876/S308//CNO/8156
WH 730	CPAN2092/IMPROVED LOK 1	KRL 210	PBW65/2*PASTOR
Raj 3765	HD2402/VL639	WH 1164	RL6043/4*NAC//2*PASTOR
Raj MR-1	J24/Aus15854	HD 2329	HD1962/E4870/3/K65/5/SKA/6/ Up262
WH 595	PRL6045/NAC76	WH 1098	TILHI/PASTOR
WH 147	PJ SIB/P14//KT54B/3/C286/ C273/4/S339/PV-18	WH 1182	KLDR/PEWIT1//MILAN/ DUCULA
WH 711	ALD'S'/HUAC//HD2285/3/HFW-17	WH 416	WH147/UP368
WH 1105	MILAN/S87230//BABAX	DBW 16	Raj3765/WR484//HUW468
WH 283	HD1981/RAJ821	WH 1185	SONALIKA/RAJ3777
WH 1081	PBW65/2*PASTOR	P-5-3	Registered genetic stock with NBPGR (Ic296709)
WH 1142	CHEN/Ae.Sq.(TAUS)//FCT/3/2*WEAVER	Naphal Selection	Indian Landrace
WH 1025	C591/PBW231	Atlas 66	FRONDOSO/REDHART3// NOLL28
C 306	REGENT1974/3*CHZ//*2C599/3/119/C281	WH 712	TRAP#1/BBW//PFAU

multivariate analysis system programme) (Rohlf, 2000). Dendrogram was constructed by using distance matrix by the unweighted pair-group method with arithmetic average (UPGMA) sub-programme of NTSYS-PC. 2D and 3D Principal component analyses were performed.

Results and Discussion

Assessment of genetic diversity at molecular level is more useful than at phenotypic level; but, together, they provide an additional source of information.

A set of 100 microsatellite primer pairs covering all three wheat genomes and almost all the chromosomes were used, out of which 70 microsatellites were found polymorphic (Table 2). These primers were located almost equally on whole wheat genome *i.e.,* on genome A (chromosome 1, 2, 3, 4, 5, 6 and 7), B (chromosome 1, 2, 3, 4, 5 and 7) and D genome (chromosome 2, 3, 4, 5, 6 and 7).

A total of 181 alleles were detected. The number of alleles per locus ranged from 1-5 with an average of 2.585 alleles per locus. This level of polymorphism was lower than the average of 3.2, 5.7, 8.44, 10 and 11.84 alleles per locus reported by Schuster *et al*., (2009); Sharma *et al*., (2010); Spanic *et al*., (2012); Nasab *et al*. (2013) and Zhang *et al*., (2006) respectively in their genetic diversity studies on bread wheat using microsatellite markers. The overall size of amplified PCR products ranged from 100-475 bp (Table 2). Mir *et al.* (2011) used 90 SSRs markers to examine genetic diversity in a collection of 263 Indian bread wheat cultivars and found that the SSR loci were equally distributed on all the three sub-genomes of wheat, although average number of alleles per locus differed (8 alleles/locus in A sub-genome; 7.15 alleles/locus in B sub-genome and 5.92 alleles/locus in D sub-genome; ESM 2). Arora *et al*. (2014) assessed the status of genetic diversity among 319 Indian wheat varieties distributed across different agro-climatic regions of India using 30 primers,

Table 2 : DNA amplification profile generated in advance breeding lines and cultivars of wheat using 70 polymorphic SSR primers

Primer	Chromosomal location	Size of bands	Total no. of bands	Annealing temp (C°)	Primer	Chromosomal location	Size of bands	Total no. of bands	Annealing temp (C°)
Xgwm 341	3D	150-425	4	50.5	cfa2153	1A	400-410	2	60
barc 13	2B	200-325	2	52	xgwm 334	6A	200-430	5	55
xgwm 325	6D	100-200	3	60	xgwm428	7D	200	1	53
xgwm 102	2D	175-200	2	53	xbarc1165	6A	300	1	61.3
xgwm 469	6D	175-200	2	52	xgdm132	6D	150-170	2	54.6
xgwm 124	1B	200-325	3	52	xgwm397	4A	150	1	55
xgwm 666.2	5A	150-300	4	51.8	barc065	7B	100-120	2	54
xgwm 400	2A	250-375	3	52	cfd50	2D	200-270	3	60
xgwm 425	1B	150-175	2	53	xcfd7	5D	380-420	3	61
wmc 364	1B	200-225	2	55	xgwm368	4B	200-280	2	56
xgwm 6	4D	175-225	3	50	xbarc1152	1B	200-250	2	64.8
xgwm 295	4D	150-250	4	49.5	wmc477	2B	170	1	52
xgwm538	4D	100-200	2	51	psp3071	6A	200	1	52.5
xgwm 273	1B	175-425	4	50	cfd34	3D	100-400	2	59.5
barc8	2B	250-275	2	51	xcfa2129	1A	120	1	54
xgwm374	2B	100-300	5	52	Xbarc012	3A	190-300	4	57.3
xgwm 413	4B	100-125	2	52.5	psp2999	1A	130-150	2	52
xgwm 46	7B	125-250	5	49.5	xbarc228	2D	150-200	3	57
psp 3000	1B	200-300	2	51.5	Xbarc24	6B	300-475	4	60.5
barc 7	3B	300-325	2	55	xbarc359	3A	120	1	56.5
xgwm 498	1B	150-175	2	50.5	xbarc12	3B	200-240	2	55
xgwm 359	2A	175-290	4	49.5	xgwm18	1B	190	1	51
xgwm 635	7A	125-150	3	52	xbarc084	3B	170-200	2	52
xgwm 314	3D	100-175	3	52	wmc419	4B	200-220	2	51
xgwm 566	3B	150-175	2	52	xbarc196	6D	170-185	2	51
xgwm 249	2D	280-320	2	53.5	xgwm37	2D	120-150	2	50
wmc 312	2A	200-225	2	52	xgwm611	7B	140-180	3	51
wmc517	7B	100-400	5	55	xgwm102	2D	160-190	2	50
wmc 527	3B	400-425	4	57	xgwm260	7A	280-450	3	55
wmc 170	2D	200-250	3	56	xgwm234	5A	200	1	50.5
gwm133	1B	100-140	3	58	wmc1	3B	180-200	2	51
xgwm 617.2	6A	100-300	4	50.7	xbarc219	7D	200-220	2	58
xgwm 111.2	2B	135-300	4	51.5	xbarc310	3A	190-250	3	56
xgwm 33	1A	145-195	3	55	xbarc04	5B	100-120	3	56
xgwm 513	4B	135-195	3	60.2	cfa2135	1A	100-200	3	55

out of which 16 primers were found polymorphic; and could be used effectively for future breeding practices.

The present study also showed that SSR primer xgwm 428, xbarc 1165, wmc 477, psp 3071, xcfa 2129, xgwm 18, xgwm 234 and xbarc 359 generated least number of bands i.e., one while three primers namely xgwm 46, xgwm 334 and wmc 517 produced maximum number of bands i.e., five. The alleles revealed by markers showed a higher degree of polymorphism (Fig. 1). Significant differences in allelic diversity among various microsatellite loci have been found in present study which was also supported by the studies of other authors (Kumar *et al*., 2016, and Kara *et al*., 2017). Drikvand *et al*. (2015) assessed genetic diversity of some durum and bread wheat genotypes using 37 microsatellites and distinguished a total of 71 alleles (2-4 alleles per each locus with an average number of 2.36 alleles per locus). The results of the present study along with other studies discussed above clearly demonstrate the utility of microsatellite markers in fast and high throughput fingerprinting of numbers of genotypes/ or germplasm collection for detecting polymorphism and estimation of genetic diversity.

The similarity coefficients between all genotypes ranged from 0.62 to 0.81 and averaged 0.71. It showed the most closely related wheat genotypes were WH 1182 and WH 1124 and highest similarity index was 0.81. On the contrary, most diverse genotypes were Tobari and HD 2967, with low similarity index of 0.62 (Fig. 2). While the average value of similarity coefficient was reported to be 0.48 by Drikvand *et al*. (2015), 0.54 by Yildirim *et al*. (2011) and 0.60 by Kara *et al*. (2017) in genetic diversity studies in wheat using SSR markers. Sharma *et al*. (2010) estimated similarity index of pair-wise comparisons on the basis of 10

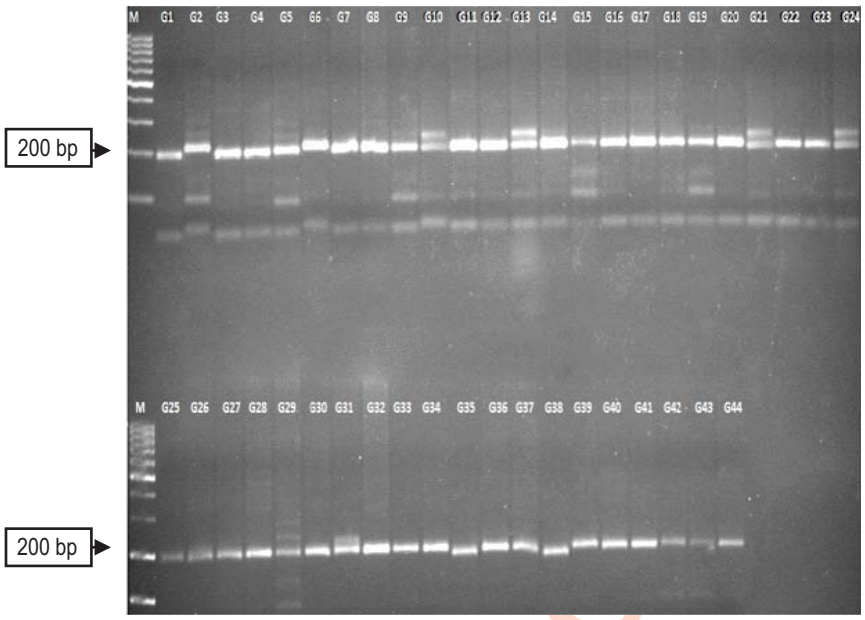

Fig. 1 : Electrophoretic pattern of advance breeding lines and cultivars in SSR analysis using primer xgwm 312, M-marker (100 kb standard ladder marker) and G1—G44 (Names are available in table 1)

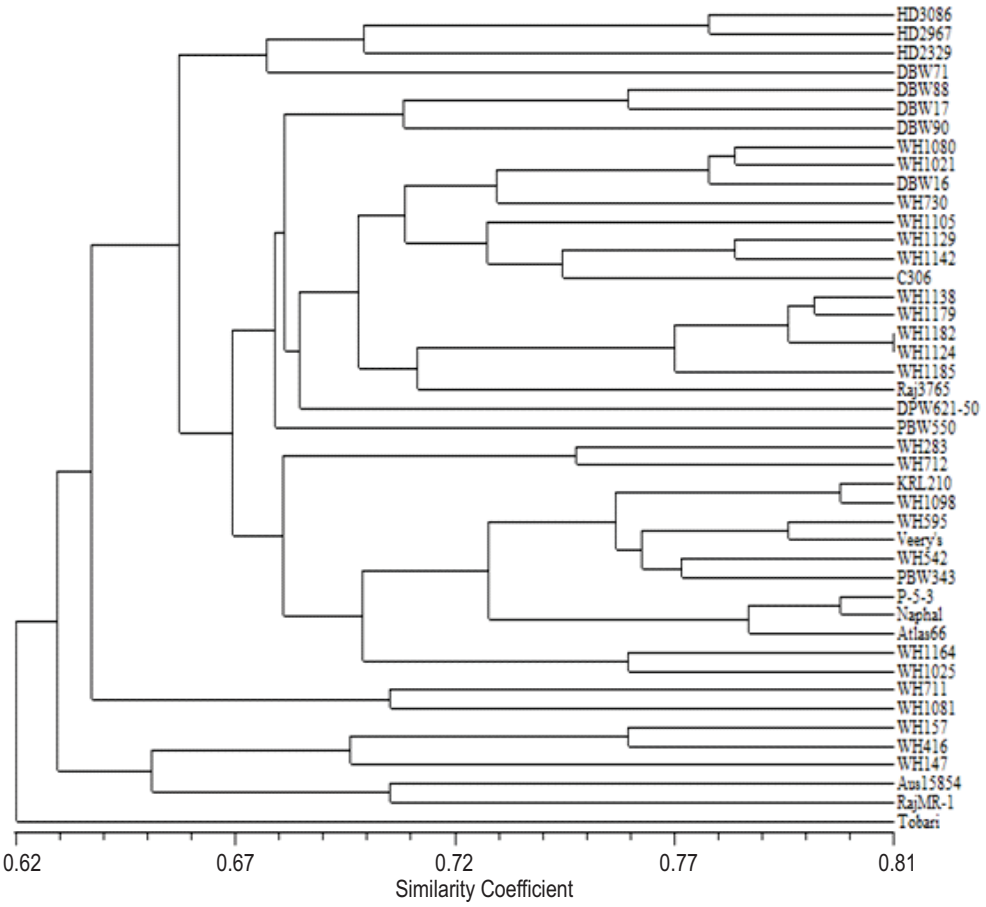

Fig. 2 : Dendrogram constructed with UPGMA clustering method among advance breeding lines and cultivars using SSR primers

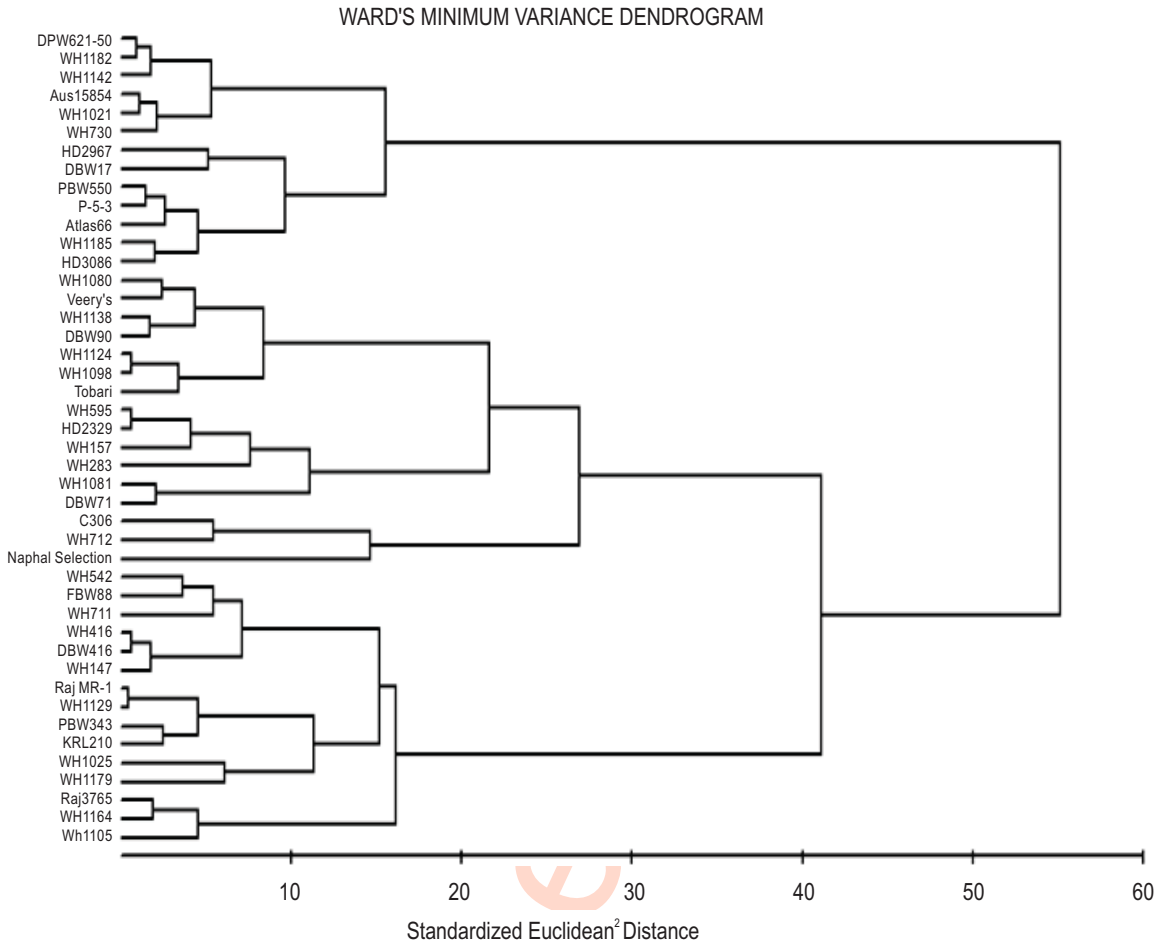

Fig. 3 : Ward's minimum variance dendrogram of advance breeding lines and cultivars based on standardized Euclidean 2 distance

primers and found that genetic similarity coefficient ranged from 0.0938 to 0.7586, while Nasab *et al*. (2013) reported gene diversity statistics in the range of 0.66 to 0.94 for 37 microsatellite loci. This variation in genetic similarity coefficient values may be indicated either to the disparity in number of genotypes or SSR primers used to detect DNA variegation.

The NTSYS-PC UPGMA cluster tree analysis led to the grouping of 44 genotypes broadly into two groups at the similarity coefficient of 0.62. The group I was very large and include 43 genotypes, while group II includes only one genotype (Tobari), which indicates that this genotype was highly diverged with respect to other genotypes and its pedigree also proved that it was an exotic cultivar. Group I, which includes 43 genotypes, was further divided into 2 clusters at similarity coefficient of 0.629. Cluster I includes 38 genotypes, namely HD 3086, HD 2967, HD 2329, DBW 71, DBW 88, DBW 17, DBW 90, WH 1080, WH 1021, DBW 16, WH 730, WH 1105, WH 1129, WH 1142, C 306, WH 1138, WH 1179, WH 1182, WH 1124, WH 1185, Raj 3765, DPW 621-50, PBW 550, WH 283, WH 712, KRL 210, WH 1098, WH 595, Veery's, P-5-3, Naphal, Atlass 66, WH 1164, WH 1025, WH 711, WH 1081, PBW 343 and WH 542. These genotypes may have similar gene pool contributing to greater similar repetitive sequence in their ancestors. In cluster I, WH 1182 and WH 1124 were found similar, although, they had different pedigree, this may be due to the reason that present day wheat varieties have been derived from six broad genotypes, *viz.*, Cross II-8156, Pitic 62, Sonora-64, Lerma Rojo-64, Ciano-67 and Bluebird. Cluster II consisted of only 5 genotypes, namely, WH 157, WH 416, WH 147, Aus 158854, and Raj MR-1.

Two and three dimension principal component analysis based on SSR data, showed similar clustering of 44 wheat genotypes as evident from cluster tree analysis.

Morphological traits have been studied for the estimation of genetic diversity and as selection criteria for wheat breeding (Marti *et al.,* 2007). For quantifying the genetic divergence between a numbers of genotypes based on morphological data, Euclidean cluster analysis was employed. On the basis of relative

magnitude of distances, 44 genotypes were grouped into 5 clusters (Fig. 3). Further, cluster pattern revealed that cluster V was the largest cluster consisting of 15 genotypes which was followed by cluster I (with 13 genotypes), cluster II (with 7 genotypes), cluster III (with 6 genotypes) and cluster IV which was the smallest cluster (with 3 genotypes). Cluster I comprised of genotypes which had highest cluster mean value for grains per ear (49.49), harvest index (%) (40.81) and grain yield per plant (g) (21.14). Cluster II had low mean value for plant height (cm) (89.52) which was desirable in present semi-dwarf varieties, for tiller number per plant (19.08). The cluster III can be exploited for characters like 1000-grain weight (g) with highest cluster mean value (44.97). Cluster IV had highest mean value for biological yield per plant (g) (54.59) and Cluster V genotypes had lowest cluster mean value for plant height (cm) (89.35). Thus, incorporation of genotypes from respective cluster for contrasting traits can be beneficial. Divergent parents in hybridization programme can led to recovery of transgressive segregants with high genetic yield potential in segregating generations.

Molecular variation evaluated in this study in combination with morphological characters of wheat can be useful in traditional and molecular breeding programs. Thus, microsatellite markers can be exploited as a new generation tool in studying the polymorphism and genetic diversity among cultivars and identification of parental combinations effectively to maintain maximum genetic variability in progenies.

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