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Genetic diversity analysis in wheat (*Triticum aestivum* L.) using simple sequence repeat markers

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Abstract
To ascertain genetic diversity, total 35 wheat genotypes were analyzed by 19 SSR primers. Out of 19, nine primers showed polymorphism and revealed considerable molecular diversity among wheat genotypes. The genotypes comprising different geo-graphic regions of India. A total 33 alleles were detected, with two to six alleles per locus and mean allelic richness of 3.66. The size of amplification product ranged from 61 bp to 724 bp. Polymorphic information content (PIC) value ranged from 0.14 (Xgwm-609-2D) to 0.72 (Xgdm-109-5A) with an average of 0.50, indicating diverse nature of genotypes. The correlation coefficient between number of alleles and PIC value was r = 0.87. The heterozygosity value ranged from 0.29 to 0.97 with an average of 0.68. The major allele frequency ranged from 0.375 to 0.920 with an average of 0.610. Number of effective alleles ranged from 1.41 to 40.83 with a mean value of 10.28. The D genome contain highest mean number of alleles (4.00) followed by the A and B genome (3.66 and 3.33, respectively). The mean PIC value for three genomes *viz*., A, B and D was 0.53, 0.48 and 0.49, respectively. Dissimilarity indices between genotypes ranged from 2.013 to 4.721. Maximum dissimilarity index was found between the genotype IC0598590 and IC0595254 whereas lowest genetic dissimilarity was observed between GW-496 and EC0635787 and also between IC059923 and GW-366. The dendrogram prepared on the basis of dissimilarity matrix using the Euclidean method, delineated the 35 genotypes into two major clusters (I and II). Cluster I and II further divided in to two sub-clusters (IA, IB and IIA, IIB), while one genotype, IC0595254 remained separate which predicts that it was unique and diverse with respect to all other genotypes under study. Results indicated that wheat cultivars had high genetic diversity and these nine SSR markers could be successfully used in molecular characterization in wheat cultivars that may be useful as parental material for the wheat breeding programs.

Keywords: Genetic diversity, simple sequence repeat, polymorphic information content, genetic dissimilarity

Introduction
Wheat (*Triticum* spp.) is highly self-pollinated annual plant, belonging to family poaceae. Wheat is one of the world most important cereal grain crops, serving as a staple food for human population and widely cultivated across the globe. Wheat genome (2n = 6x = 42) is huge (~17 GB), complex (AABBDD, 3 homoeologous subgenomes), five times larger than human genome, with high repeat content (85%) and having more than 1,07,800 high confidence genes (Apples *et al*., 2018) [2]. wheat is the staple food of more than a third of the global human population and accounts for almost 20% of the total calories and protein consumed by humans worldwide, more than any other single food source and it serves as an important source of vitamins and minerals.

Genetic diversity is the underlying cause of many important agriculturally important phenomena like heterosis and transgressive segregation (Bhandari *et al.*, 2017) [3]. Crop improvement programme solely depends upon nature and magnitude of genetic variability and the extent to which the desirable characters are heritable. Species with wide range of variability provide ample scope for selection of desirable and superior genotypes for further improvement. Hence for optimal utilization and conservation of germplasm for plant breeding and other activities, studies on genetic diversity is of great importance (Uddin & Boerner, 2008) [22]. Wheat breeding through hybridization requires selection of diverse genotypes, irrespective of whether the end product is a pure line or a hybrid variety (Prasad *et al.*, 2000) [16]. Polyploidy and genome evolution of wheat are responsible for maintaining its genetic diversity (Khan *et al*., 2015) [10]. Genetic diversity can be estimated using pedigree analysis, physiological and biochemical markers and also through morphological characters and molecular markers. A large number of highly informative DNA markers such as RAPD,

RFLP, SSR, ISSR, CAPS, SCAR, SRAP and SNP have been developed for identification of genetic polymorphism, marker assisted selection and marker validation in various breeding studies.

SSR is widely used as markers for genotyping plant because they are highly informative, codominant inheritance, multi-allele genetic markers, reproducible and transferable among related species (Mason, 2015) [12]. They are widely distributed throughout the genome, especially in euchromatin of eukaryotes and, coding and non- coding region of nuclear and organellar DNA (Vieira *et al*., 2016) [23]. SSR or microsatellite markers are tandem repeats motif may composed of one to six nucleotide. SSR markers are ubiquitous, developmental stage independent, locus specific and shown high degree of polymorphism. Markers can reveal new alleles, as well as original alleles that were reduced in the wheat gene pool during the process of evolution, thereby offering a deeper understanding of wheat during domestication and selection. Nowadays more than 4000 SSR markers have been developed for wheat (Han *et al*., 2015). These markers are extensively used for QTL analysis for identification of marker-trait associations. The entire genome of wheat is estimated to contain 476,169 SSRs, which occur in the genome at a frequency of 29.73 per Mb. In recent years, these markers are extensively utilized in genomic mapping, marker-assisted breeding, evolutionary processes, genetic diversity analyses and genotype identification in self-pollinated species such as wheat (Donini *et al*., 2000) [6].

Materials and Methods

Plant materials

The experimental material was comprised of total 35 wheat genotypes procured from the Regional Research Station, Anand Agricultural University, Anand, Gujarat. The details of genotypes are given in table 1.

DNA isolation

Genomic DNA was isolated from fresh leaves of each of the 35 genotypes using CTAB method (Doyle and Doyle, 1997) [7]. The isolated DNA was checked spectrophotometrically at A_{260}/A_{280} for quantity and quality (In terms of protein and RNA contamination) using software N.D. 1000 (V.3.3.0). To check the form of DNA (Linear or sheared) and RNA contamination, gel electrophoresis was performed using 1% agarose gel and quality was checked by viewing the image of single intact DNA band.

SSR genotyping

Total 19 SSR primers prescreened, among them nine SSR primers were used for PCR amplification. The markers Xgdm were described by Pestsova *et al*., (2000) [15] and Xgwm by Röder *et al*., (1998) [18]. The details of the primers are given in table 2. Amplification reaction was performed in 15µl volume containing 2µl template DNA, 6µl PCR master mix (2X), 1µl diluted primer and 6µl nuclease free water. The cyclic amplification was carried out by using following thermal amplification conditions: Initial denaturation at 94 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 45 seconds, annealing at ΔT °C (Primer specific) for 45 seconds, extension at 72 °C for 45 seconds, and a final extension at 72 °C for 7 minutes. The amplified products of SSR was analyzed using 2.5% agarose gel.

Statistical Analysis

Each band amplified by primer was scored as presence (1) or absence (0) by using Alpha Ease FC 4.0 software (Version 4.0, Alpha Innotech, USA), and the data were entered in to binary matrix. The binary data further analyzed by R software (R core team, 2013) for phylogenetic analysis. Genetic relationships among genotypes were investigated using Euclidean distance. Summary statistics for all the markers derived using Microsoft Excel programme (Microsoft Excel, 2018).

Squared Euclidean distance $d_E(i,j)=\sqrt{\sum_{k=1}^{n}(X_{ik} - Y_{jk})}$

As per Botstein *et al*., (1980) [4], allelic polymorphic information content (PIC) was calculated using the following formula:

$$PIC = 1 - \left(\sum_{i=1} P_i^2\right)$$

Table 1: List of genotypes used in study

Sr. No	Genotype	Sr. No	Genotype	Sr. No	Genotype	Sr. No	Genotype
1	EC0595292	10	EC0635609	19	IC0594599	28	GW-366
2	EC0597833	11	EC0635735	20	IC059117	29	GW-496
3	EC0598050	12	IC0598590	21	IC059923	30	Lok 1
4	EC0520959	13	IC0591099	22	IC0598587	31	GAW-16-04
5	EC0520970	14	IC0591062	23	IC0538797	32	GAW-16-13
6	EC0635810	15	IC0595254	24	IC118745	33	GAW-16-14
7	EC0635796	16	IC0598602	25	IC443748	34	GAW-16-21
8	EC0635787	17	IC059737	26	GW-11	35	MACS-6222
9	EC0633783	18	IC0596737	27	GW-322		

Table 2: List of SSR markers

Sr. No	Microsatellite locus	F/R	Sequence (5'-3')	Annealing Temperature (^{0}C)
1	Xgdm-62-3D	F	GATCTTGAAGCACTCTTGGT	58.5
		R	GAAAGCCGTCCACTGCC	
2	Xgdm-87-2D	F	CCCAAGCCCCAATCTCTCTCT	61.3
		R	AATAATGTGGCAGACAGTCTTGG	
3	Xgdm-88-4A	F	AAGGACAAATCCCTGCATGA	55.3
		R	TCCCACCTTTTTGCTGTAGA	
4	Xgdm-93-2A	F	GGAGGCATGGCTACATCCTTC	58.5
		R	AAAAGCTGCTGGAGCATACA	
5	Xgdm-93-4B	F	GGAGCATGGCTACATCCTTC	57.3
		R	AAAAGCTGCTGGAGCATACA	
6	Xgdm-109-5A	F	AAAGCTGCTCATCGTGGTG	58.3
		R	GGTCCGCCTGACAGACC	
7	Xgwm-609-2D	F	ACATTCTGTGTGGGGCC	58.3
		R	GATCCCTCTCCGCTAGAAGC	
8	Xgwm-340-3B	F	GCAATCTTTTTTCTGACCACG	56.6
		R	ACGAGGCAAGAACACACATG	
9	Xgwm-148-2B	F	GTGAGGCAGCAAGAGAGAAA	56.6
		R	CAAAGCTTGACTCAGACCAAA	

F: Forward; R: Reverse

Results

Microsatellite Polymorphism

Nine microsatellite markers used to evaluate genetic diversity in 35 wheat genotypes. The analysis of markers data of nine SSRs led to identification of 33 alleles. The number of alleles ranged from two to six, with mean allelic richness of 3.66. Maximum number of allele (six) amplified by marker Xgdm-62-3D with allele size 279 - 724 bp. The PIC value across the loci ranged from 0.14 for Xgwm609-2D to 0.72 for Xgdm109-5A with an average of 0.50. The correlation coefficient between number of alleles and PIC value was high with r = 0.87 (Fig 1). Out of nine markers, six markers had PIC value above 0.50, indicating these markers were highly informative. Major allele frequency varied from 0.375 to 0.920 with a mean value of 0.610. The average number of effective alleles was found to be 10.28. Number of effective alleles ranged from 1.41 to 40.83. The least number of effective alleles (1.41) found for marker Xgdm109-5A. Heterozygosity for nine markers ranged from 0.29 (Xgdm109-5A) to 0.97 (Xgdm93-4B) with a mean of 0.68.

In the A genome, total 11 alleles detected with an average of 3.66 alleles per locus. In the B genome, total 10 alleles with an average of 3.33 alleles per locus were detected. In comparison with A and B genome, 12 alleles were detected in D genome with an average of four alleles per locus. The most polymorphic locus located on chromosome 3D with highest number of alleles (six). Among the A, B and D genome, the mean value of PIC was 0.53, 0.48 and 0.49, respectively. The highest value of 0.72 and 0.70 were found for Xgdm109 and Xgdm62, which were located on chromosome 5A and 3D, respectively. The maximum number of effective alleles (40.83) was found for chromosome 4B.

Table 3: Details of microsatellite markers used for genetic diversity

Sr. No	SSR locus	No. of allele	Allele size (bp)	MAF	Ae	PIC	He
	A Genome						
1	Xgdm93-2A	5	110 - 130	0.382	3.52	0.69	0.71
2	Xgdm88-4A	2	68 - 130	0.900	14.93	0.18	0.93
3	Xgdm109-5A	4	74 - 165	0.375	1.41	0.72	0.29
	B Genome						
4	Xgwm148-2B	4	136 - 298	0.514	2.33	0.57	0.57
5	Xgwm340-3B	3	110 - 139	0.800	18.56	0.34	0.94
6	Xgdm93-4B	3	61 - 135	0.625	40.83	0.53	0.97
	D Genome						
7	Xgwm609-2D	2	104 - 156	0.920	2.29	0.14	0.56
8	Xgdm87-2D	4	85 - 208	0.520	1.49	0.64	0.33
9	Xgdm62-3D	6	279 - 724	0.458	7.20	0.70	0.86
Average		3.66		0.610	10.28	0.50	0.68

Ae: Effective alleles, bp: Base Pair, He: Heterozygosity, MAF: Major allele frequency, PIC: Polymorphic information content

Genotypic response

It was observed that genotypes *viz.*, IC0596737 followed by GAW-16-14 and GAW-16-21 were least responsive to primers. For the markers Xgdm-87-2D and Xgdm-93-2A all the genotypes responded except EC0597833 and IC0596737, respectively, whereas for Xgdm-109-5A only two genotypes *viz.*, GAW-16-14 and GAW-16-21 did not respond. All the other markers had response from limited genotypes only.

For a more precise inference, all the alleles were grouped into four groups *viz*, <105 bp, (110 - 210) bp, (210 - 310) bp and >310 bp. Each allelic group responded for 6, 20, 2 and 5 number of alleles, respectively. A graph was prepared to visualize the individual genotypic response for each allelic group (Fig 2). A large number of genotypes responded for group (110 - 210) bp as they had 20 alleles. For this group, the genotype IC0598590 responded most with eight alleles, but IC0596737 remained least responsive with only one allele. For group (<105 bp) six alleles responded. All the

genotypes showed response for that except the two genotypes *viz*., GW - 11 and GW - 322. On the other hand, five and two alleles amplified in the allele size range >310 bp and (210 - 310) bp, respectively. Majority of genotypes responded for former group with two alleles per genotypes whereas, only three genotypes *viz*., EC0635796, IC0598590 and IC059117 responded for allele group (210 - 310) bp with only one allele. The highest number of total alleles (11) across all nine markers were given by IC0598590, whereas the lowest alleles observed for genotype IC0596737 with three alleles. Total number of genotypes responding to all alleles was found to be 250 with an average of 7.57 alleles per genotype.

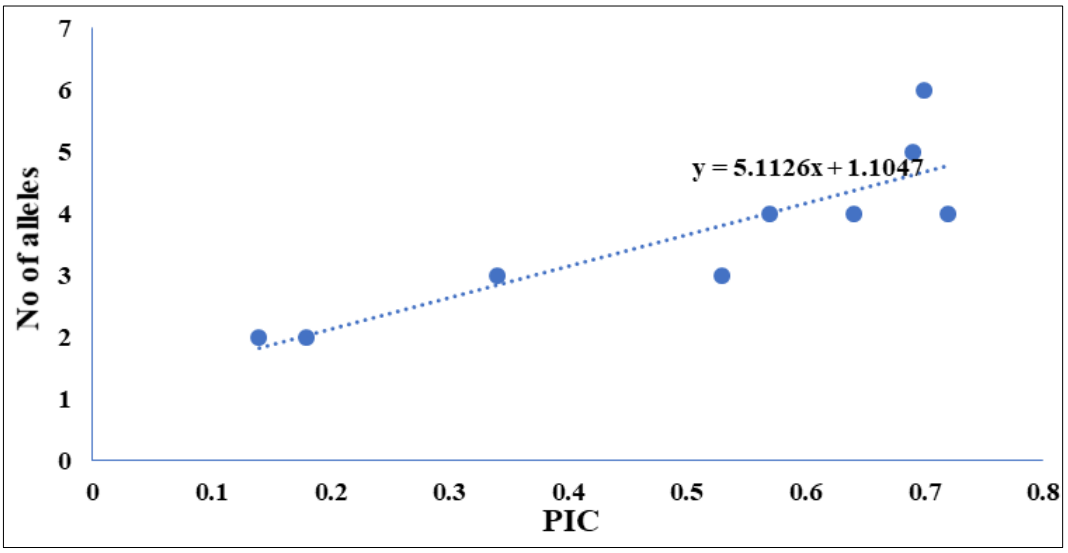
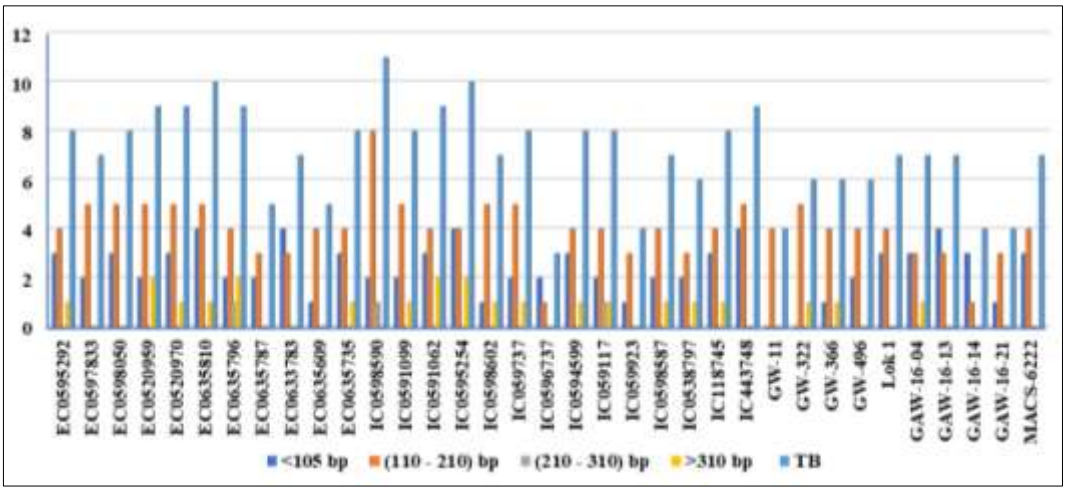


Fig 1: Correlation coefficient between no of alleles and PIC value



TB = Total number of bands (alleles) offered by a single genotype across all markers

Fig 2: Individual genotypic response for different allelic group

Phylogenetic analysis

Genetic dissimilarity index and cluster analysis done by Euclidean method using R software. A dendrogram was constructed based on the genetic dissimilarities index values. The dissimilarity index value among genotypes ranged from 2.013 to 4.721. The highest dissimilarity index was 4.721 found between the genotypes IC0598590 and IC0595254. The lowest dissimilarity index of 2.013 was observed between the genotypes GW - 496 and EC0635787, and also between IC059923 and GW - 366.

Cluster analysis led to grouping of 35 wheat genotypes in to two major cluster *viz.*, cluster I and cluster II (Fig 3). One wheat genotype IC0595254 indicating its genetic distance from all other genotypes and remained separate so it is considered as outlier. Cluster I was further divided in to two sub-clusters, IA and IB. Sub cluster IA comprised of four genotypes *viz.*, IC0598590, GW-322, GW-11 and GAW-16-21, whereas Sub-cluster IB comprised of 17 wheat genotypes *viz.*, IC059117, EC0635796, EC0633783, IC059587, MACS-6222, GAW-16-04, GAW-16-13, GAW-16-14, EC063575, EC0520970, IC059923, GW-366, Lok 1, IC0538797, EC0635609, EC0635787 and GW-496. Cluster II was also bifurcated in to two sub-clusters, IIA and IIB. Sub-cluster IIA included three wheat genotypes *viz.*, IC0591062, IC0591099 and EC059592, while Sub-cluster IIB included 10 wheat genotypes *viz.*, IC059737, IC0598602, EC0520959, IC0596737, EC0635810, IC0594599, IC118745, IC443748, EC0597833 and EC0598050.

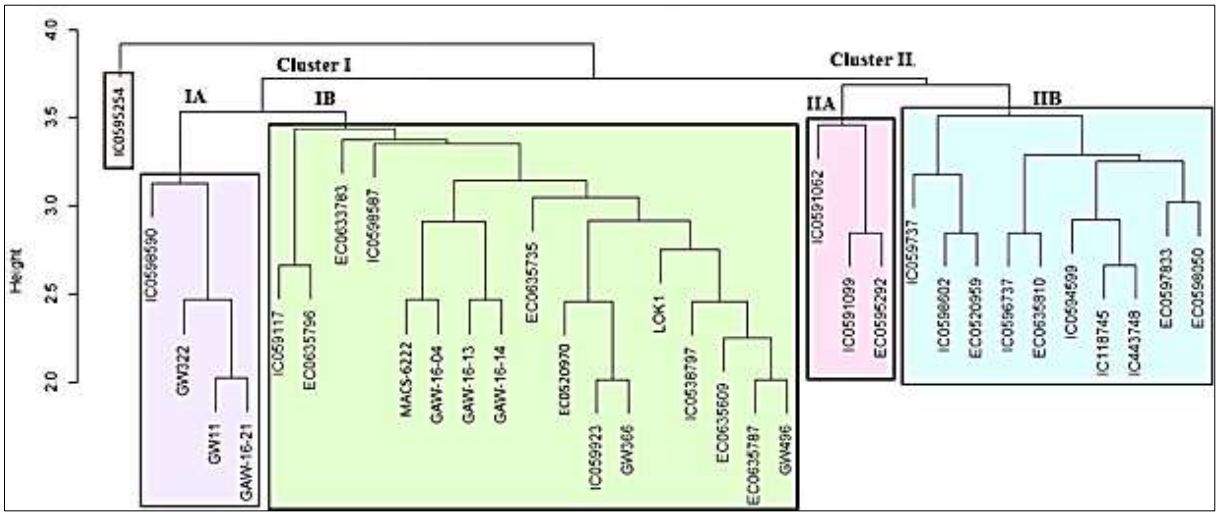


Fig 3: Dendrogram of 35 wheat genotypes based on SSR markers

Discussion

The results obtained from microsatellite analysis of these 35 hexaploid wheat showed that microsatellites are extremely useful for determining genetic diversity. In a present study, total 33 alleles were detected by nine microsatellite markers. The number of alleles ranged from as low as two for Xgdm-88-4A and Xgwm 609-2D, to six for primer Xgdm-62-3D with an average of 3.66 allele per locus. In comparison to earlier studies by Liu *et al*., (2007) [11] reported 4.6 alleles per locus and Haung *et al*., observed 18.1 alleles per locus so that these markers produced lesser number of alleles per locus whereas similar result was also reported by Ahmad (2002) [1]. Moreover, out of nine six markers (Xgdm-62-3D, Xgdm-87-2D, Xgdm-88-4A, Xgdm-93-2A, Xgdm-93-4B and Xgdm-109-5A) studied by Zeb *et al*., (2009) [25]. They reported 32.71 alleles per locus using 14 SSR markers which was higher compared to present study. Marker Xgwm-148-2B studied by Chen and Li (2007) [5], they reported five alleles for marker, that was comparable to present result obtained for this marker and also studied by Salehi *et al*., (2018) [19]. They reported major allele frequency of 0.19 which was lower compared to 0.519 and obtained higher number of alleles (10) and PIC value (0.87).

In addition to that, as a measure of the in formativeness of microsatellites, the PIC values ranged from a low of 0.14 for Xgwm-609-2D to a high of 0.72 for Xgdm-109-5A with an average value of PIC was 0.50. PIC values of the markers used in present study was lower compared to earlier studies (Spanic *et al*., 2012; Salem *et al*., 2008 and Prasad *et al*., 2000) [21, 20, 16] and ranged from 0.21 to 0.90. Low PIC value is an indicator of low level of genetic diversity in the wheat genotypes studied. Apart from this, Salehi *et al*., (2018) [19] reported lower major allele frequency ranged from 0.14 to 0.39 with an average of 0.27 in comparison to present result, which offered higher major allele frequency range of 0.37 to 0.92 with an average of 0.61.

Additionally, contribution of A, B and D genome for genetic diversity revealed by SSR markers can be ranked as D>A>B, on the basis of number of alleles, indicating that three genomes have different level of genetic diversity. This is not in agreement with results from Peng *et al*., (2009) [14] they ranked the genome as A>D>B for SSR alleles. The highest PIC value was found for genome A (0.53) followed by genome D and B (0.49 and 0.48, respectively) which is not accordance with result Chen and Li (2007) [5], they obtained highest value of PIC for genome D (0.77) followed by genome B (0.70) and A (0.65). Similar result was also recorded by Wang *et al*., (2012) for mean number of alleles and mean PIC values for three genomes.

In cluster analysis, 35 genotypes were grouped into two major clusters (I and II) on the basis of Euclidean distance. The value of dissimilarity coefficients by Euclidean method ranged from 2.013 to 4.722. Higher coefficient value indicated greater diversity in wheat genotypes. It was observed that most of varieties of Gujarat falls in one cluster *i.e.* Cluster I, which was quite acceptable as most of them are released from a single state only. It was also noticed that wheat varieties released by public sector in Gujarat other than Anand are come in either cluster IA or IB, whereas varieties released in Gujarat from Anand are cluster in IB along with some other genotypes confirming their close genetic background. All Exotic and Indigenous collections of wheat are come in either cluster I or cluster II, whereas in cluster I some of them are mingled with Gujarat state varieties. One genotype IC0595254 remained solitary, which may possess diverse gene pool from rest of the genotypes and utilized in heterosis breeding programme for getting widely adaptable and agronomical superior hybrids.

Interestingly, two released variety Lok 1 and MACS-6222 released from Gramvidyapith, Gujarat and ARI, Pune respectively, are clustered in IB along with two variety GW-496 and GW-366 developed by Junagadh Agricultural University, Junagadh, indicating its genetic relatedness.

This outcome surely can be exploited in hybridization programme where two released variety can be crossed, or any Gujarat variety can be crossed with other belonging two different major clusters hence ensuring considerable divergence.

The dendrogram generated from the data did not show any correlation between place of development or recommendation of any genotypes and its clustering pattern which re-established the fact that due to continuous gene flow, naturally and artificially, geographical distribution of genotypes does not correlate at molecular level except for the varieties released in Gujarat grouped in major Cluster I. The present result of cluster analysis is in accordance with Haung *et al*., which suggested that genetic diversity of *T. aestivum* is not related with geographic distribution.

Conclusion

SSR marker system was effective in studying genetic diversity in inter and intra-specific wheat genotypes. Genetic dissimilarity or distance estimates among genotypes are helpful in the selection of parents to be used in subsequent breeding programmes. The present molecular genetic assay managed to shed more light on the genetic relatedness of wheat cultivars. This might assist breeder to set up the appropriate guidelines for successful breeding of wheat cultivars based on the established relationships. It also contributes further to develop suitable science-based approaches for molecular technique in wheat. It offers an alternative and reliable monitoring of wheat genetic diversity which should be starting point for further selection of parents.

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