INTERNATIONAL JOURNAL OF PLANT, ANIMAL AND ENVIRONMENTAL SCIENCES
IJPAES

Volume-3, Issue-4, Oct-Dec-2013
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ISSN 2231-4490
www.ijpaes.com

Received: 22nd Oct-2012 *Revised: 17th Nov-2012* *Accepted: 10th Oct -2013*

Research article

EVALUATION OF RAPD AND SSR MOLECULAR MARKERS IN BREAD WHEAT GENOTYPES

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ABSTRACT: In this study was evaluated the molecular analysis of 10 bread wheat genotypes using RAPD and SSR markers. In RAPD with 6 primers and in SSR with 5 primers were detected 33 and 17 polymorphism allels for genotypes. The most Polymorphic Information Content (PIC) value and polymorphism percentage was detected by UBC 350 and UBC 109 markers with values 0.53 and 0.50 respectively in RAPDs. In SSRs, Xgwm 469-6D marker detected 14 bands with 5 polymorphic alleles but Xgwm120-2B had the most PIC with 57%. For both markers UPGMA was the best method of clustering. Although markers could show genotypes in 2 clusters but that was very different in molecular analysis. In final results RAPDs could detect more polymorphism alleles than SSRs.

Key word: Wheat, Molecular marker, RAPD, SSR, Cluster

INTRODUCTION

Today, evaluation on wheat genotypes for knowing of genetic diversity by classic such as regression and cluster analysis is not enough. During the past two decades, molecular studies have helped in the identification of genotypes in a wide range of crop species. The significant finding by using of molecular markers has enabled scientists to analyze distinct genotypes genetically and have better insight for the next studies. Molecular wheat breeding is method of biotechnological tools in wheat breeding such marker assisted selection. Molecular marker-assisted breeding is defined as use of genetic and genomic analysis to identify DNA regions that linked to quantitative traits in crops. It can facilitate breeding programs for wheat improvement [5]. Molecular markers are very important materials for the evaluation of genetic diversity. These markers can show types of high and low polymorphism in wheat [2]. The new methods with molecular markers has been developed in many recent studies that most of these were based on PCR amplification of genomic DNA. Polymerase chain reactions (PCR) have been started as the most modern technique in molecular biology in 1980s. This tool was introduced as decreased data method to identify of relationships with together. Several molecular markers like random amplified polymorphic DNAs (RAPD) and simple sequence repeats (SSRs) are presently available to identify the variability , diversity and similarity in molecular levels [8]. Knowledge of genotypes diversity is a basic foundation in wheat plant breeding [4].

Singles Sequence Repeat (SSRs)

SSRs are locus specific and abundant. Besides, they are distributed over the genome and require only small amounts of genomic DNA for analysis. The SSRs are highly polymorphic, even among closely related cultivars. These are one of the most promising molecular marker types that are able to identify genotypes within a species.

RAPD primers

RAPD markers are decamer (10 nucleotide length) DNA fragments from PCR amplification of random segments of genomic DNA with single primer of arbitrary nucleotide sequence and which are able to differentiate between genetically distinct individuals, although not necessarily in a reproducible way.

Percentage of Polymorphism

One of the most basic steps is identify and calculation of percentage of polymorphism in used markers. Different polymorphism amounts show efficiency of each marker to separate and segregate in studied genotypes in normal and drought conditions. In previous study in Iranian wheat cultivars, 58% polymorphism was reported using 8 RAPD primers [1, 11] also revealed that out of tested ten RAPD primers, only five primers gave polymorphism in wheat genotypes whereas other five did not amplify any genotype. [13] studied the genetic variability among 14 wheat accessions using 39 RAPD primers. The total number of amplicons was 117, including 108 polymorphic amplicons.

MATERIALS AND METHODS

Plant materials

A total of 10 wheat genotypes were used (Table 1). All of them are hexaploid (*Triticum aestivum* L., AABBDD, 2n = 6x = 42) and known as materials of advanced trials in Mazandaran. These genotypes obtained from results of studied in 3 years and were sown in small pots in growth chamber. Leaf samples of all plant materials were harvested from 7 day-old seedlings.

Table 1 - Pedigree of studied genotypes

No. Genotypes	Pedigree
1	MILAN/ATTILA//ATTILA-4Y
2	PASTOR/FINSI
3	ATTILA/3*BCN//BAV92/3/TILHI
4	OASIS/5*BORL95//SIRKKU/3/CHIBIA
5	BL2064//SW89-5124*2/FASAN/3/TILHI
6	NANJING2149/KAUZ/4/JUP/ALD"S"//KIT"S"/3/VEE"S"/5/SHA 7//HAHN"S"*2/PRL"S"
7	BABAX/LR42//BABAX*2/3/VIVITSI
8	MILAN CM75118/KA CM75118/K 1//TAJAN
9	MILAN/S87230//BABAX
10	ATTILA/3*BCN*2//BAV92

Table 2 . Used RAPD and SSR markers with GC%, Tm and sequences

Primer's name	Sequence	
SSR Primer		
Xgwm-133-6B(GC=50) Tm:50?C	F	5'-ATCTAAACAAGACGGCGGTG-3'
	R	5'-ATCTGTGACAACCGGTGTGA-3'
Xgwm-120-2B (GC=55%) Tm: 52?C	F	5'-GATCCACCTTCCTCTCTCTC-3'
	R	5'-GATTATACTGGTGCCGAAAC-3'
Xgwm-469-6D(GC=52%)T Tm50?C	F	CAACTCAGTGCTCACACAACG-3'-5'
	R	5'-CGATAACCACTCATCCACACC-3'
Xgwm-325-6D(GC=47%) Tm: 50?C	F	5'-TTTCTTCTGTCGTTCTCTTCCC-3'
	R	5'-TTTTTACGCGTCAACGACG-3'
Xgdm-109-5A(GC=52%) Tm: 50?C	F	5'-AAAGCTGCTCATCGTGGTG-3'
	R	5'-GGTCCGCCTGACAGACC-3'
RAPD primer		
UBC350,(GC=72%),Tm: 36?C		5'-TGACGCGCGTC-3'
UBC109,(GC=50%),Tm: 34?C		5'-TGTACGTGAC-3'
UBC104,(GC=50%),Tm:34?C		GGGCAATGAT-3'-5'
UBC600,(GC=66%), m:37?C		5'-GAAAGACCGC-3'
UBC13, (GC=69%),Tm:37?C		5'-CCTGGGTGGA-3'
UBC129.(GC=50%),Tm:37?C		5'-GCGGTATAGT-3'

RAPD and SSR markers

We bought 6 RAPD and 5 SSR from Nedaye fancorporation (Table 2).

DNA extraction

Total genomic DNA was extracted from leaves using CTAB method [7]. 20 seeds of each genotypes were planted in the laboratory of seed and plant improvement section with mean temperature 18ºc and after 8 days cut 5-7 cm long piece of fresh leaf material from the plants and the leaf tissues were ground in a preheated extraction buffer (100 mM Tris, pH 8, 1.4 M sodium chloride, and 20 mM EDTA, pH 8.0). Liquid nitrogen ground samples of 10 genotypes were also processed first with 600 µl extraction buffer (1 minute shaker) second add 30 µl SDS (30 seconds shaker) and then were incubated for 25 minutes in 65°C water bath. Then add to the samples 92 µl Nacl 5M (30 seconds shaker) and after that, add 75 µl CTAB buffer and shake tube. In next step add 30 µl sodium astate and shake by hand and were incubated 65°C water bath for 15 minutes. The samples were mixed gently after adding 500 µl of chloroform isoamyl alcohol and shaking by hand, were placed in centrifuge at 12000 rpm for 10 minute. Then, we removed limpid liquids of tubes and transferred them in to new tubes. In next step, add cold absolute isopropanol to free DNA and maintained in freezer for 1 hour. Tubes of samples were placed in centrifuge at 12000 rpm for 10 minute again. In this step. Sedimentary DNAs were washed by ethanol 70% in tubes and dried for 1 hour. To dilute we added 200-300 µl placed on an orbit shaker

for 20 minutes at room temperature The samples with occasional vigorous shaking.. After centrifugation at 5000 rpm, an equal volume of was added to the supernatant. The solution was well mixed and incubated for 60 minutes at 20°C. The sample was centrifuged for 5 minutes at 5000 rpm to pellet the DNA was followed by washing with 70% alcohol and then dried at 56oC for 5 minutes. In final, DNA were suspended by adding 300 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Then DNAs were maintained in freezer.(Fig 1 , 2)

Figures 1 , 2 – The processes of the firs and last DNA extraction

Polymorphism informative content (PIC)

Polymorphism informative content (PIC) is one of the most basic step in determining of genetic diversity among genotypes by markers or primers. This, help us that distinguish molecular difference in studied cases. PIC value was calculated using the formula

PIC = 1-∑pi2, where pi is the frequency of the ith allele. The concept of Polymorphic information content

• Measures the usefulness of a marker , • Informativeness in specific families

For RAPD analysis, a total 6 primer were used for polymerase chain reaction (PCR) amplification. PCR in RAPD was carried out in a 25-µL reaction volume, containing 16 µL double-distilled deionized H2O, 2.5 µL 10X buffer, 2.5 µL MgCl2, 0.5 µL dNTPs, 0.3 µL Taq polymerase, 1 µL primer, and 1.2 µL DNA(50ng). The SSR (PCR) amplification of genomic DNA was done by incubating the DNA samples at 94°C for 10 min, then 45 cycles comprising 94°C for 1 min, annealing of primer at 34°-37°C for 2 min and then extension at 72°C for 1 min. The final extension was carried out at 72°C for 10 min. [12] described that RAPD- PCR is a powerful tool for the selection of genetic variation of population/organisms.

Data analysis

Marker index for RAPD and SSR markers was calculated in order to characterize the capacity of each primer to detect polymorphic loci among the genotypes. It is the sum total of the polymorphism information content (PIC) values of all the markers produced by a particular primer.

The data obtained by scoring the RAPD and ISSR profiles with different primers individually as well as collectively were subjected to the construction of similarity matrix using Jaccard's (Jaccard, 1908) coefficients. The similarity values were used for cluster analysis. Data analysis was done using NTSYSpc software 2.02 (Rohlf, 1998). The product-moment correlation (r) based on Mantel Z-value (Mantel, 1967) was computed to measure the degree of relationship between similarity index matrices produced by any two-marker systems. The PCR products were electrophoresed on 2.5% agarose gels containing 7 µL ethidium bromide, at 80 V for 1.5 h, and observed under a UV transilluminator. **Bands** were counted and the presence and absence of bands were scored as 1 and 0, respectively. The data were collected and aligned for the construction of cluster analysis. The cluster analysis of 10 genotypes was performed using the SPSS software version 16 to determine genetic diversity and similarity among genotypes. The allele counts were scored for the presence or absence in each sample, based on allele size measured with DNA Marker 500 bp and compared with the fragment sizes reported in available literature. The presence or absence was coded by 1 or 0, respectively, to generate the raw data matrix. Null alleles are common in wheat (Plashke 1995). Meanwhile, the genetic similarity coefficient was separately estimated for markers of the A and B genomes. To determine whether the genetic differentiation on the A genome was consistent with the B genome, the Mantel-test was carried out by comparing the genetic similarity data matrix of the A genome with that of the B genome.

RESULTS

A total of 33 polymorphic alleles were detected for genotypes by RAPD markers. The number of alleles ranged from 3 to 8 (Table3). The most Polymorphic Information Content (PIC) value and percentage was detected by UBC 350 and UBC 109 with values 0.53 and 0.50 respectively. Markers UBC350 and UBC104 were able to distinguish drought tolerance genotype No. 7 with high product bp.This markers detected 3 and 8 polymorphic alleles and 22 and 16 bands (Figures 3-8). 17 polymorphic alleles were detected for genotypes by SSR markers. The number of alleles ranged from 2 to 5. One SSR marker (Xgwm 325-6D) could not detect polymorphic allele (Table 4). The most (PIC) value was detected by Xgwm 469-6D. This marker detected 14 bands and 5 polymorphic alleles (Figures 9-13). These tables showed polymorphic bands among the 10 genotypes with an average of 3.66 polymorphic bands per primer for SSR and 5.5 polymorphic bands per primer for RAPD. Thus RAPD could detect more than SSR. Todorovska et al. (2009) detected 322 alleles with a mean number 10.063 alleles/ locus by SSRs primers (Xgwm). They resulted that the highest genetic Distance among 32 SSR loci had Xgwm 484-2D (0.901) and Xgwm-282-7A (0.901). Saffdar et al., 2009 revealed that out of tested ten RAPD primers, only five primers gave polymorphism in wheat genotypes whereas other five did not amplify any genotype. Molecular sizes of amplified fragments ranged from approximately 70 to 600 bp by SSRs and 50 to 3000 bp by RAPDs. The most product size was seen in Xgwm 120- 2B in SSRs and UBC104 in RAPDs.

Table 3- RAPD based study of genetic divergence of 10 wheat genotypes

Marker's name	Base pairs	Numbers of bands	Numbers of Polymorphism allels	Polymorphism %	PIC
UBC350 (GC=72%)36 нуқтаи ^{3}C нав6	50-100-280-700-1000-1360	22	6	0.28	**0.53**
UBC109 (GC=50%) Tm: 34^{3}C	100-145-245-350-425-550--800-1750	16	8	0.50	**0.42**
UBC104 (GC=50%) Tm:34^{3}C	400-500-700-1000-1250-3000	18	6	0.33	**0.24**
UBC600 (GC=66%) Tm:37^{3}C	460-550-690-890-1800	17	5	0.29	**0.27**
UBC13 (GC=69%) Tm:37^{3}C	145-245-400-500-700	19	5	0.26	**0.19**
UBC129 (GC=50%) Tm:37^{3}C	80-100--150-250-350-420	18	6	0.33	**0.36**
Total		110	33	2.00	**2.01**
Means in Markers			5.5	0.34	**0.34**

Figures 3-8- PCR products of the RAPD markers for 10 wheat genotypes (Genotypes 1-10 as in Table 1, M100= band size marker

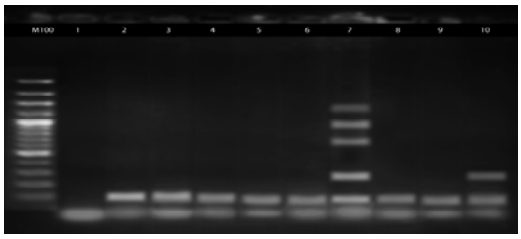

Fig 3. RAPD marker UBC350 for 10 wheat genotypes (Genotypes 1-10 as as in Table 1, M100= band size marker)

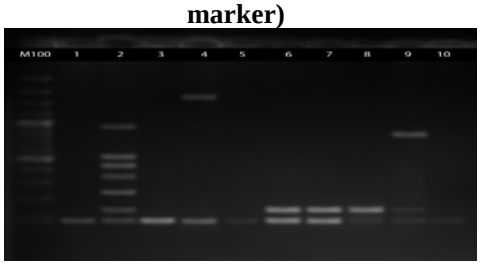

Fig 4. RAPD marker UBC109 for 10 wheat genotypes (Genotypes 1-10 as in Table 1, M100= band size marker

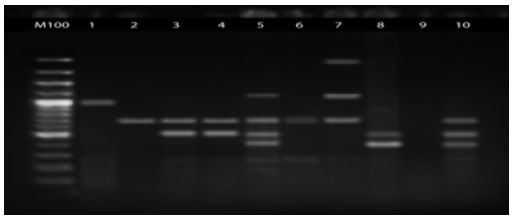

Fig 5. RAPD marker UBC104 for 10 wheat genotypes (Genotypes 1-10 as in Table 1, M100= band size marker

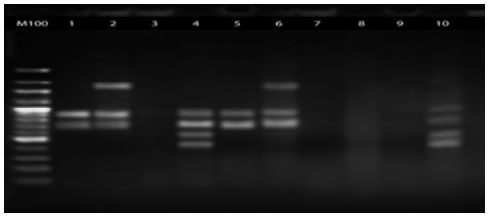

Fig 6. RAPD marker UBC600 for 10 wheat genotypes (Genotypes 1-10 as in Table 1, M100= band size marker

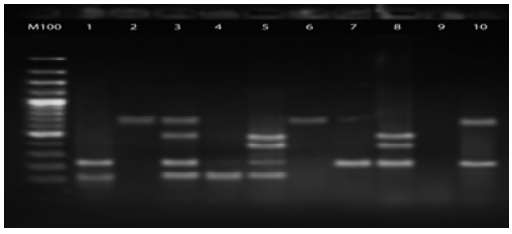

Fig 7. RAPD marker UBC13 for 10 wheat genotypes (Genotypes 1-10 as in Table 1, M100= band size marker

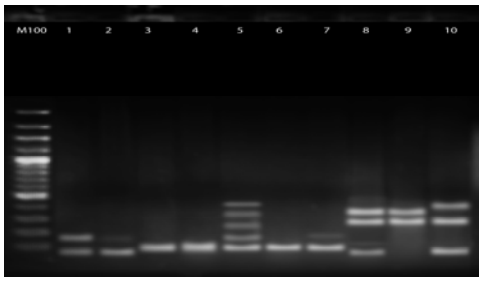

Fig 8 RAPD marker UBC129 for 10 wheat genotypes(Genotypes 1-10 as in Table 1, M100= band size marker

Figures 9-13- PCR products of the ssr markers for 10 wheat genotypes (Genotypes 1-10 as in Table 1, M100= band size marker

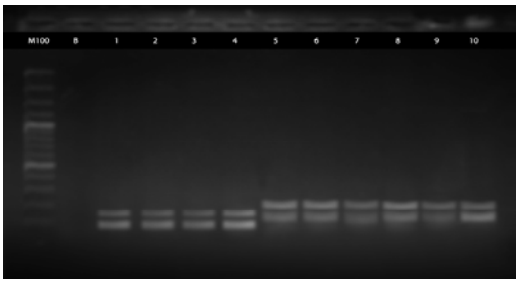

Fig 9. SSR marker Xgwm 133-6B for 10 wheat genotypes (B= water and Genotypes 1-10 as in Table 1, M100= band size marker

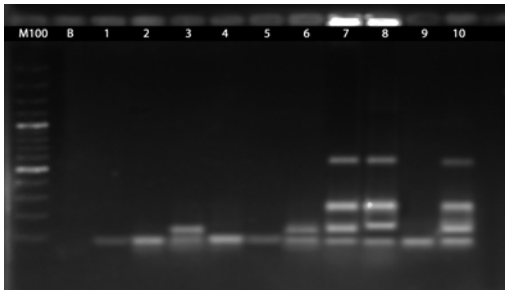

Fig 10. SSR marker Xgwm 120-2B for 10 wheat genotypes (B= water and Genotypes 1-10 as in Table 1, M100= band size marker

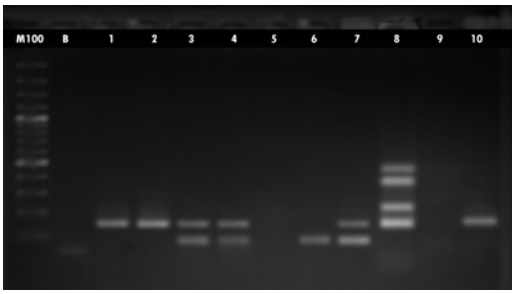

Fig 11. SSR marker Xgwm 469-6D for 10 wheat genotypes (B= water and Genotypes 1-10 as in Table 1, M100= band size marker

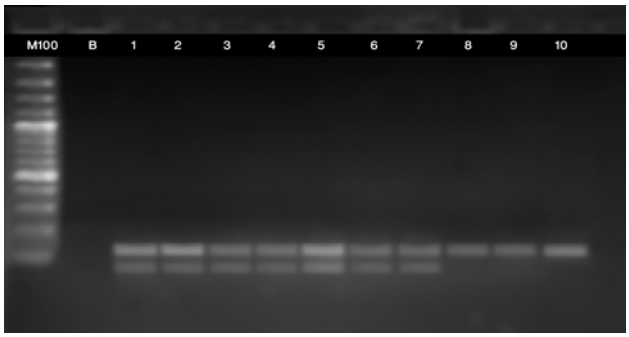

Fig 12. SSR marker Xgwm 325-6D for 10 wheat genotypes (B= water and Genotypes 1-10 as in Table 1, M100= band size marker

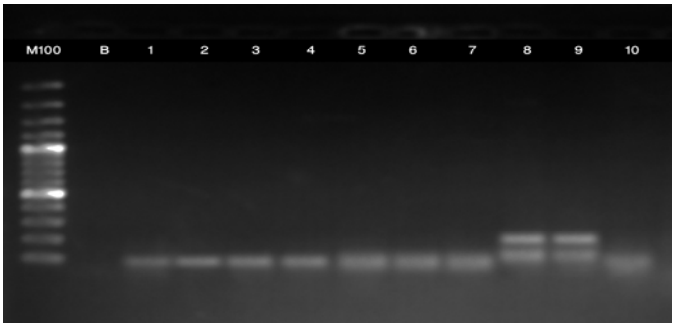

Fig 13. SSR marker Xgwm 109-5A for 10 wheat genotypes (B= water and Genotypes 1-10 as in Table 1, M100= band size marker

Marker's name	Base pairs	Numbers of bands	Numbers of Polymorphism allels	Polymorphism%	PIC
Xgwm-133-6B(GC=50) 50°Cнуфтаи нав6	100-150-200	20	3	15	**0,48**
Xgwm-120-2B (GC=55%) Tm: 52°C	70-150-220-600	20	4	20	**0,57**
Xgwm-469-6D(GC=52%) Tm50°C	70-150-220-400-500	14	5	36	**0,32**
Xgwm-325-6D(GC=47%) Tm: 50°C	70-150	17	2	12	**0,51**
Xgdm-109-5A(GC=52%) Tm: 50°C	100-145-200	12	3	25	**0,28**
Total Means in Markers		83 16,6	17 3,4	108 21,6	**216** **0,432**

Table 4 - SSR based study of genetic divergence of 10 wheat genotypes

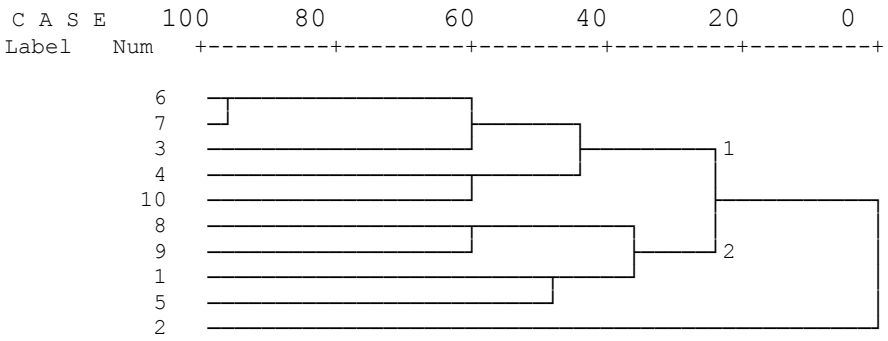

Fig 14. Dendrogram of total RAPD markers

In RAPDs dendrogram shows for genotypes ranged from 0.24 to 0.96 using this marker. The UPGMA-based dendrogram grouped genotypes into 2 clusters (Fig.14). The lowest genetic diversity is related to genotypes 6 and 7 in cluster I. These two genotypes had genetic distance to others. Most genetic similarity is seen for 8 and 9 in cluster II. Genotype 2 appeared as unique, having no commonality with other genotypes.

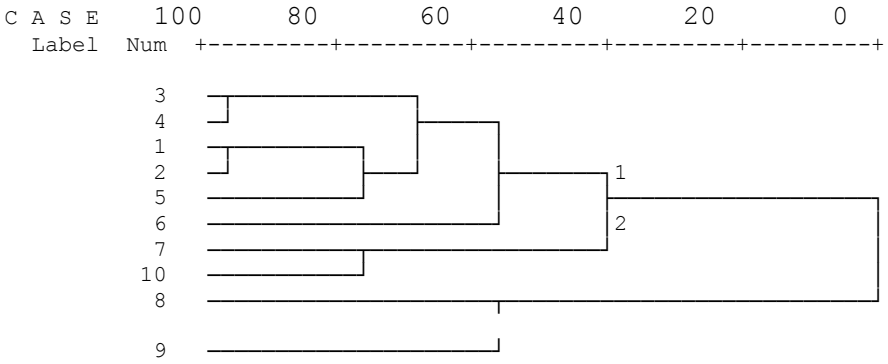

Fig 15. Dendrogram of total SSR markers

In SSRs dendrogram shows for genotypes ranged from 0.40 to 0.96 using this marker. The UPGMA-based dendrogram grouped genotypes into 2 clusters. The lowest genetic diversity is related to genotypes 3, 4 and 1, 2 with 5%. The highest genetic diversity is shown among 8 and 9 with the other genotypes 98% (Fig 15). These two genotypes appeared as unique, having no commonality with other genotypes. Our results points to the need to adopt different strategies for selecting markers and choosing an upper number of SSR and RAPD markers. Our results indicate that, RAPD and SSR markers can be identified more carefully and provided consistent information for diversity studies and were used on drought condition in order to estimate of genetic distance. In contrary, which SSR markers were promising in terms of the polymorphism and information content revealed, but RAPD showed good view The results also suggest that the number of loci evaluated should be increased. Our results suggest that RAPD markers are the good choice for the evaluation of diversity and assessing the genetic relationships between spring wheat genotypes in drought levels. Although SSR markers showed acceptable results. RAPDs also correlates moderately to lowly with results obtained using the SSRs system and is a fast and reliable system capable of supporting a multiplex approach not requiring previous knowledge of DNA sequencing. It was assumed that such a high level of genetic similarity may be the result of biased selection of the material in the previous breeding programs, which ultimately narrowed the genetic base of the wheat germplasm in the country. It is further suggested that more polymorphic wheat RAPD and microsatellites could be used for efficient screening of the germplasm by saturating more regions of the wheat genome.

ACKNOWLEDGEMENT

Authors are thanlful to Dr. Amini for laboratory of biotechnology in Mazandaran Agricultural research Centre

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