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Genomic variation studies in durum wheat (*Triticum turgidum* ssp. *durum*) using CBDP, SCoT and ISSR markers

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Abstract

In the present study three DNA marker systems *viz.*, start codon targeted (SCoT), CAAT box-derived polymorphism (CBDP) and inter-simple sequence repeats (ISSR) markers were used to detect the genetic diversity and relationship among 17 durum wheat genotypes. The ISSR primers generated 130 bands across the genotypes with an average of 8.12 bands per primer, whereas CBDP and SCoT primers created 66 and 99 polymorphic bands with an average of 5.5 and 7.07 bands per primer, respectively. Values of MI and PIC obtained for all three marker systems indicated the high efficiency of these markers to detect the genetic variation in durum wheat. The results revealed that ISSR markers with an average of 83.46% polymorphism was a suitable marker system to detect genetic variation among genetic materials. On the other hand, gene targeted markers (SCoT and CBDP), similar to ISSR marker, were comparatively less efficient techniques to estimate the genetic variation. The present findings revealed that the genetic analyses using gene targeted markers such as CBDP and SCoT would be more useful for crop improvement programs.

Key words: Durum wheat, SCoT, ISSR, CBDP, genetic diversity

Introduction

Durum wheat (*Triticum turgidum*) is one of the most important cereals cultivated in semi-arid regions of the world and it has dedicated to itself approximately 10% of wheat area under cultivation (Naghdipour et al. 2012). The success of durum wheat in Iran is due to its good ability and capacity to yield well under diverse conditions (Heidari et al. 2016). Pasta industries development with increased demand for durum wheat requires more researches, particularly in the field of its breeding not only to highlight the genotypes with high yield and desired traits but also for the management of genetic resources and their use in applied breeding programs (Reeves et al. 1999). Information on genetic distance in a germplasm collection provides the ability to categorize the individuals within the germplasm, which is highly important in breeding programs (Pakseresht et al. 2013). Assessment of genetic diversity is very important to maximize the efficiency of breeding programs (Pour-Aboughadareh et al. 2017). Today, the determination of plant genetic diversity has exceeded the levels of morphology and phenology and is achieved using modern biotechnology methods at the level of plant DNA. DNA-based molecular markers specially gene targeted markers express a more detailed description of genotypes capacities independent from environmental effects (Nakamura 2000; Gianibelli et al. 2001). Recently, advances in the genomics and molecular biology has provided novel DNA-based marker systems such as, CAAT box-derived polymorphism (CBDP) and start codon targeted (SCoT) markers. Collard and Mackill (2009) described a new marker system that is produced using single primer design based on the short conserved region around ATG start codon as a forward and reverses primer. Singh et al. (2014) reported the polymorphism derived from the region of CAAT box of plant gene promoters that plays an important role during transcription. These two marker are novel, simple, and reliable gene-targeted marker systems. SCoT and CBDP markers have been used for molecular assessment of different plant species diversities (Amirmoradi et al. 2012; Al-Qurainy et al. 2015; Etminan et al. 2016; Hamidi et al. 2014). ISSR markers are amplified sequences of DNA

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regions that are located between the sequences of two micro-satellites. In this method the repeated fragments of SSR with selected bases at their ends are used as primer annealing site. ISSR primers usually show a high polymorphism and their main advantages are high repeatability, high accuracy, reliability, high levels of diversity and low cost in genetic diversity studies (Chawla 2002). Based on the results obtained from studies of AL-Ganeer et al. (2015), Shikhseidova et al. (2015) and Etminan et al. (2016), ISSR technique seem to be a powerful tool for the analysis of genetic diversity among genotypes of durum wheat. Different markers might reveal different classes of variation (Powell et al. 1996). The efficiency of DNA markers estimate through parameters such as polymorphism information content (PIC) and marker index (MI). These parameters have been used in different crops such as Kalmegh (*Andrographis paniculata*) (Tiwari et al. 2016), durum wheat (Seyedimoradi et al. 2016; Etminan et al. 2016), Jojoba (*Simmondsia chinensis*) (Heikrujam et al. 2015) and chickpea (*Cicer arietinum*) (Pakseresht et al. 2013)). The comparison of marker techniques efficiency for discovering the suitable marker in amplifying genome fragments will be more efficacious in utilization of these markers for future breeding researches (Powell et al. 1996). The main aim of the present study was to make comparison of CBDP, SCoT and ISSR molecular markers to determine the genetic diversity in durum wheat germplasm.

Materials and methods

Plant materials and DNA extraction

The experimental materials consisted of 17 durum wheat genotypes, namely, Saji (G1), Zardak (G2), Sardari (G3), 19E-TOPDY (G4), 19E-Rascon (G5), 19E19E-M84859 (G6), 19E-M141979 (G7), 19E-M141982 (G8), 19E-M141994 (G9), 19E-M141995 (G10), 18E-M142005 (G11), 19E-M142017 (G12), 19E-M142025 (G13), 19E-M142038 (G14), 19E-M142045 (G15), 19E-M142069 (G16) and 19E-M142070 (G17), which were originated from the Sararoud Dryland Agricultural Research Institute (DARI) at Kermanshah, Iran. After the seed germination and growth, the total genomic DNA was isolated from the young leaves of glasshouse-grown plants according to the CTAB protocol (Saghai-Maroof 1984). The quality of the DNA was estimated by 0.8% agarose gel electrophoresis.

PCR amplification of different markers

Nucleotide sequence and annealing temperature of all the primers used in the study are given in Supplementary Table S1. A set of 14 SCoT primers, 12 CBDP primer sequences (Singh et al. 2014) and 16 ISSR primers were used to amplify the genomic DNA of all 17 genotypes.

The amplification was performed in a Bio-Rad (T100) thermal cycler. The PCRs were performed in the reaction mixture 20 µl volume, with 10 µl master mix 2XPCR (Ready to use PCR master mix 2X), 6 µl ddH_2O, 2 µl of isolated DNA from each sample and 2 µl of each primer. Amplification for SCoT marker was run at 94^oC for 5 min, followed by 45 cycles of denaturation at 94^oC for 45 seconds, primer annealing at 45^oC for 45 seconds and primer elongation at 72^oC for 90 seconds. The final extension was 10 min at 72^oC.

Amplification in respect of CBDP was carried out with 4 min initial denaturing at 95^oC (to activate Taq DNA polymerase), followed by 30 cycles of denaturing at 94^oC for 60 seconds, primer annealing at 50^oC for 60 seconds and primer elongation at 72^oC for 120 seconds. This was followed by a final extension stage at 72^oC for 7 min. For ISSR primers, the amplification was run at 94^oC for 4 min, followed by 30 cycles of denaturation at 94^oC for 30 seconds, primer annealing at primer's annealing temperature for 45 seconds and primer elongation at 72^oC for 2 min. The final extension was 7 min at 72^oC. Amplification reaction products were separated on 1.5% agarose gels stained with safe view II until the loading dye reached to the bottom of the gel.

Data analysis

Amplified fragments were constructed by scoring 0 and 1 for absence and presence of bands, respectively. The generated data matrixes were subjected to statistical analysis using the DARwin computer software (Perrier et al. 2006). For comparison the efficiency of marker systems, polymorphism information content (PIC) and marker index (MI) were calculated. PIC was calculated according to the formula of Anderson et al. (1993), as $PIC = 1 - Sp_i^2$, where p_i is the frequency of the *i*th allele of the locus. Marker index (MI) was calculated by the formula according to Kumar et al. (2009). Cluster analysis based on Jaccard's similarity coefficients (Jaccard 1908) matrix and principle coordinate analysis were constructed using DARwin5.0 software (Perrier and Jacquemoud-Collet 2006).

Result and discussion

Molecular analysis of different markers

Fourteen SCoT primers created a total of 118 bands with an average of 8.42 bands per primer which 99 bands were shown polymorphism (Table 1). The average number of polymorphic bands was estimated at 7.07 bands per primer. Number of amplified fragments with different primers was varied ranging from 4 bands for SC-14 and SC-7 to 12 bands for SC-4. Polymorphism percentage was varied for each primer from 57.14% for SC-14 and SC-7 to 100% for SC-4, SC-1, SC-12, SC-21 and SC-8. The average of polymorphism percentage was 83.24. The average of MI and PIC for 14 SCoT primers was 2.43 and 0.34, respectively. The highest and lowest value of MI was observed for SC-4 (4.3) and SC-15 (1.17), respectively. SC-14 and SC-2 showed the high values of PIC (0.46 and 0.42 respectively) but the lowest value was observed for SC-3 (0.22).

Table 1. Efficiency and number of polymorphic markers produced by SCoT primers used in durum wheat genoypes

Primers	No. of total bands	No. of polymor-phic bands	Polymor-phism (%)	Resol-ving power index (Rp)	PIC	MI
SC-2*	10	8	80.00	7.54	0.42	3.39
SC-3	9	7	77.77	3.22	0.22	1.60
SC-4	12	12	100.00	7.81	0.35	4.30
SC-5	9	7	77.77	4.10	0.38	2.72
SC-7	7	4	57.14	2.49	0.31	1.26
SC-8	9	9	100.00	6.76	0.37	3.35
SC-9	11	10	90.90	7.16	0. 36	3.66
SC-11	9	9	100.00	9.85	0.33	2.97
SC-12	6	6	100.00	1.88	0.25	1.52
SC-14	7	4	57.14	4.50	0.46	1.84
SC-15	7	5	71.42	1.76	0.23	1.17
SC-21	6	6	100.00	9.09	0.36	2.20
SC-24	10	7	70.00	4.76	0.34	2.41
SC-26	6	5	83.33	5.21	0.32	1.61
Mean	8.42	7.07	83.24	5.44	0.34	2.43

SC-Number* SCoT primes

All CBDP primers created 93 bands with an average of 7.75 bands per primer which 66 bands were polymorphic (Table 2). The average number of polymorphic bands was estimated at 5.5 bands per primer. Number of amplified fragments with different primers was varied ranging from one band for CB-1 to ten bands for CB-2. The average of MI and PIC for CBDP primers were 2.06 and 0.28 respectively. CB-1 showed the lowest value of MI (0.05), but CB-3 and CB-9 showed the highest values of MI (4.19 and 3.46, respectively). The highest and lowest value of PIC was observed for CB-3 (0.46) and CB-1 (0.05) respectively. The polymorphism information content and marker index of each primer help to determine the efficiency of primer in the analysis of genetic diversity (Heikrujam et al. 2015). In general MI index can be used as a general index for predicting the efficiency of a marker in a germplasm (Powell et al. 1996).

Table 2. Efficiency and number of polymorphic markers produced by CBDP primers

Primers	No. of total bands	No. of polymor-phic bands	Polymor-phism (%)	Resol-ving power index (Rp)	PIC	MI
CB-1*	9	1	11.11	0.05	0.05	0.05
CB-2	10	10	100.00	8.48	0.32	3.27
CB-3	9	9	100.00	9.92	0.46	4.19
CB-4	7	7	100.00	7. 95	0.30	2.16
CB-5	9	5	55.55	2.27	0.29	1.48
CB-7	9	9	100.00	11.69	0.37	3.36
CB-9	8	8	100.00	6.53	0.43	3.46
CB-10	7	6	85.71	8.11	0.41	2.46
CB-12	6	6	100.00	8.68	0.39	2.37
CB-13	5	5	100.00	7.21	0.39	1.90
CB-14	9	0	0.00	0.00	0.00	0.00
CB-15	5	0	0.00	0.00	0.00	0.00
Mean	7.75	5.5	71.03	5.91	0.28	2.06

CB-Number* CBDP primes

Sixteen ISSR primers generated 156 clearly bands across 17 genotypes with an average of 9.75 bands per primer (Table 3). Totally, 130 bands were shown polymorphism and the average of polymorphic bands was estimated at 8.12 bands per primer. The number of amplified fragments with different primers was varied from seven bands for IS-9 and IS-15 primers up to fifteen bands for IS-25 primer. The polymorphism

Table 3 . Efficiency and number of polymorphic markers produced by ISSR primers used in durum wheat

Primers	No. of total bands	No. of polymor-phic bands	Polymor-phism (%)	Resol-ving power index (Rp)	PIC	MI
IS-1*	13	8	61.53	4.58	0.32	2.58
IS-3	9	8	88.89	7.04	0.35	2.84
IS-4	8	8	100.00	6.34	0.36	2.95
IS-5	8	6	75.00	4.95	0.37	2.22
IS-6	12	11	91.67	13.75	0.44	4.84
IS-7	9	8	88.89	9.18	0.36	2.93
IS-9	7	6	85.71	4.42	0.34	2.04
IS-11	9	9	100.00	12.24	0.36	3.28
IS-12	9	8	88.89	4.11	0.28	2.24
IS-13	10	7	70.00	5.21	0.34	2.43
IS-14	10	10	100.00	10.55	0.39	3.91
IS-15	7	4	57.14	4.47	0.41	1.64
IS-23	8	6	75.00	2.66	0.22	1.33
IS-25	15	12	80.00	11.28	0.39	4.70
IS-27	11	9	81.81	6.42	0.38	3.45
IS-28	11	10	90.90	4.49	0.30	3.03
Mean	9.75	8.12	83.46	6.98	0.35	2.90

IS-Number*ISSR primes

percentage for each primer was varied from 57.14% for IS-15 to 100% for IS-4 and IS-14. Al-Ganeer et al. (2015), Shikhseidova et al. (2015), Etminan et al. (2016) and Hajiyev et al. (2015) successfully applied ISSR primers for genetic relationships study of durum wheat genotypes. The PIC value of the sixteen primers varied between 0.22 (IS-23) to 0.44 (IS-6) distinguishing the different genotypes. IS-6, IS-25, IS-14 and IS-27 showed the highest values of MI (4.84, 4.70, 3.91, and 3.45 respectively) that revealed higher resolving power of these primers in comparison with other primers. Among sixteen ISSR primers, the lowest value of MI was observed for IS-23. The average of MI and PIC for ISSR primers were 2.90 and 0.35, respectively. Relatively high values of polymorphism information content index has been reported in many genetic diversity studies on cereals using ISSR markers (Muthusamy et al. 2008; Etminan et al. 2016; Shirnasabian et al. 2015; Heidari et al. 2017). High values of MI and PIC obtained for SCoT (SC-9, SC-8, SC-2 and SC-4), CBDP(CB-3, CB-9) and ISSR (IS-6, IS-25, IS-14 and IS-27) primers indicate the correct selection and high efficiency of these primers in assessment of genetic variation in durum wheat germplasm. So these primers can be used to investigate the genetic relationships in durum wheat germplasms. In previous studies carried out by Paliwal et al. (2013) on 21 Giloe accessions, Yadav et al. (2016) on 14 accessions of Fennel (*Foeniculum vulgare* Mill.) by using SCoT markers and Heikrujam et al. (2015) on Jojoba by using CBDP markers, some of primers could amplify reproducible bands showing the accuracy of these markers for molecular characterization of plants. Sivaprakash et al. (2004) suggested that the ability of a marker system for the analysis of genetic diversity could be more related to the degree of its polymorphism. The present investigation proved that ISSR marker with an average of 83.46% polymorphism was a suitable marker to detect genetic variation among genetic materials. On the other hand, gene targeted markers (SCoT and CBDP), similar to ISSR marker, were efficient techniques to estimate the genetic variation. The large polymorphic fragment percentage and number of polymorphic bands obtained in our study indicates the power of SCoT marker in fingerprinting and diversity analyses. Additionally, the genetic diversity explained by gene targeted markers showed that SCoT and CBDP markers can be very effective for characterization of genetic diversity in plants.

Cluster analysis

To investigate genetic relationships among durum wheat genotypes, cluster analysis based on Jaccard's similarity coefficients and UPGMA algorithm was used. According to the clustering pattern obtained by SCoT (Fig. 1a), CBDP (Fig. 1b) and ISSR (Fig. 1c), the 17 durum wheat genotypes classified into different groups. It was found that all three marker techniques separated genotypes differently, that it could be due to differences in the separation of different regions of the genome targeted as suggested by Gajera et al. (2010). Heikrujam et al. (2015) in investigation the genetic diversity of Jojoba genotypes using SCoT and CBDP markers showed that both markers generated dendrograms clustered genotypes into two distinguishing groups. Based on SCoT data, the maximum distance (0.69) was between genotype G6 and G10. Using CBDP marker, the maximum genetic distance (0.64) was recorded between genotypes G9 and G6 and considering ISSR data, G17 and G6 with 0.31similarity had the highest genetic distance. In present study, according to the dendrogram (Fig. 1),

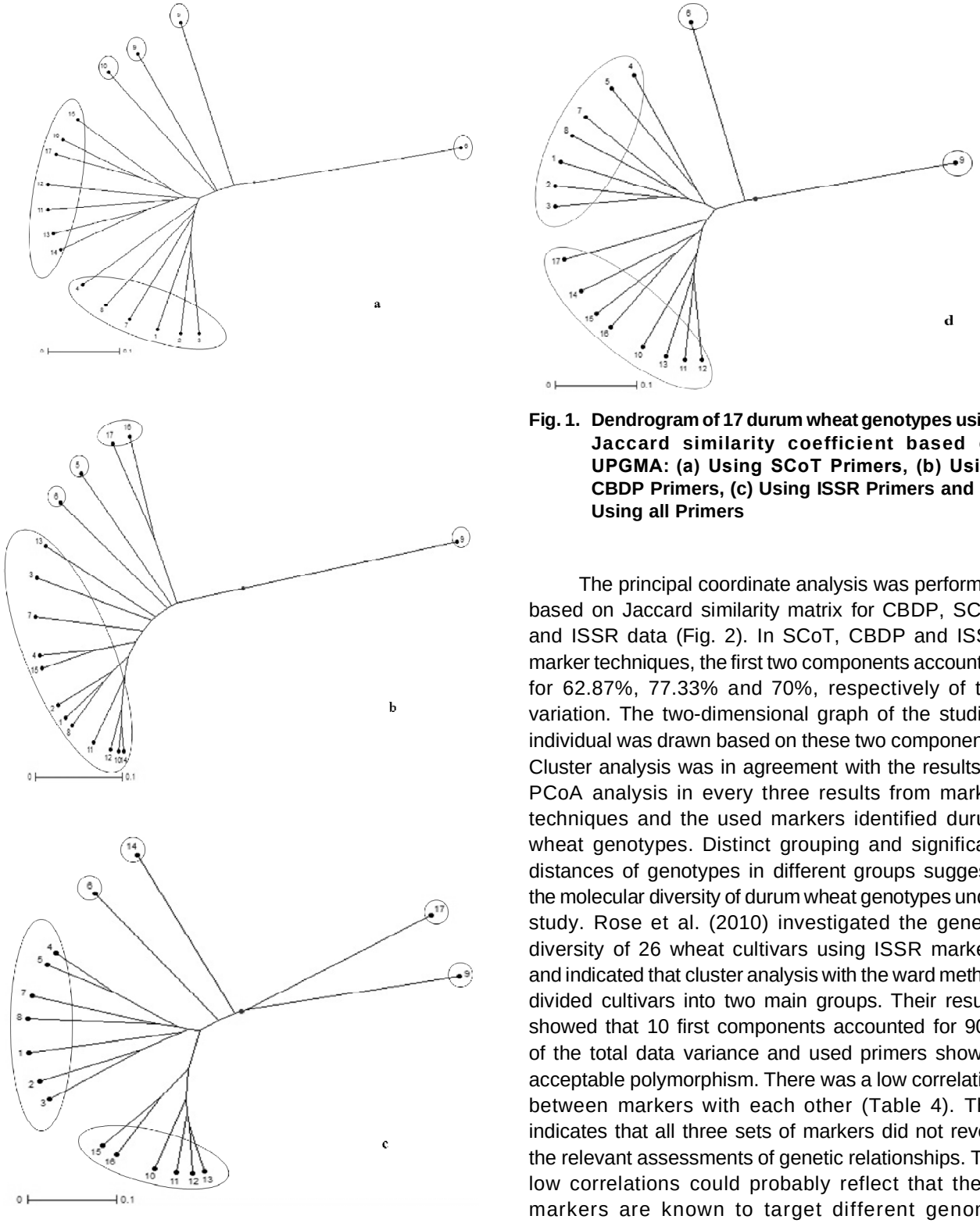
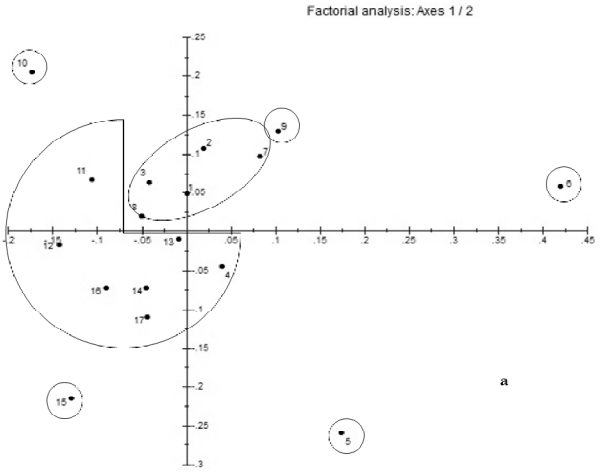
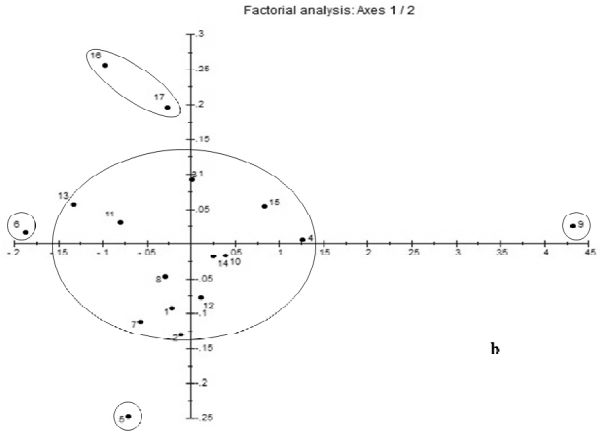


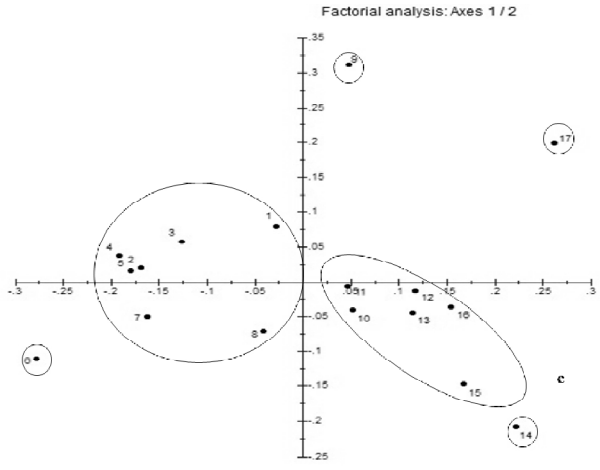
Fig. 1. Dendrogram of 17 durum wheat genotypes using Jaccard similarity coefficient based on UPGMA: (a) Using SCoT Primers, (b) Using CBDP Primers, (c) Using ISSR Primers and (d) Using all Primers

G6 and G9 were classified separately based on all three marker techniques indicating the difference of these genotypes.

The principal coordinate analysis was performed based on Jaccard similarity matrix for CBDP, SCoT and ISSR data (Fig. 2). In SCoT, CBDP and ISSR marker techniques, the first two components accounted for 62.87%, 77.33% and 70%, respectively of the variation. The two-dimensional graph of the studied individual was drawn based on these two components. Cluster analysis was in agreement with the results of PCoA analysis in every three results from marker techniques and the used markers identified durum wheat genotypes. Distinct grouping and significant distances of genotypes in different groups suggests the molecular diversity of durum wheat genotypes under study. Rose et al. (2010) investigated the genetic diversity of 26 wheat cultivars using ISSR markers and indicated that cluster analysis with the ward method divided cultivars into two main groups. Their results showed that 10 first components accounted for 90% of the total data variance and used primers showed acceptable polymorphism. There was a low correlation between markers with each other (Table 4). This indicates that all three sets of markers did not reveal the relevant assessments of genetic relationships. The low correlations could probably reflect that these markers are known to target different genome fragments involving repeat or unique sequences, which may have differentially evolved or been present during the stage of neutral or artificial selection as has been described by Pakseresht et al. (2013). The results revealed that all of the marker data together, can be







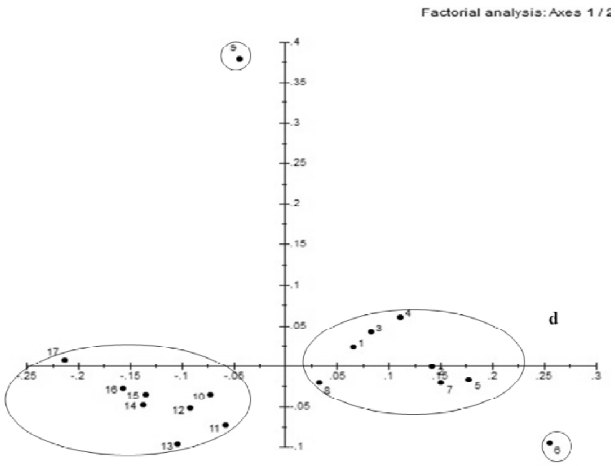


Fig. 2. Two-dimensional graph from principal coordinate component analysis based on Jaccard similarity matrix: a, b, c and d are related to two-dimensional graph from PCOA ISSR, CBDP, SCoT Primers and the whole Primers under study, respectively

Table 4. Mantel test (Mantel 1967) correlation coefficients among similarity matrices

SCoT	CBDP	ISSR	TOTAL	Markers
SCoT	_			
CBDP	0.33	_		
ISSR	0.33	0.34	_	
TOTAL markers	0.67*	0.70*	0.84*	_

effectively used in determination of genetic relationships among durum wheat genotypes.

The ISSR technique uses microsatellites as primers to amplify mainly, the inter- SSR sequences. Since evolutionary rate of change within microsatellites is considerably higher than most other types of DNA, so the likelihood of polymorphism in these sequences is greater (Fang and Roose 1997). Another advantage in the use of ISSR markers lies in their linkage to SSR loci. Although microsatellites themselves are probably nonfunctional and selectively neutral, they are known to be linked to coding regions and, hence, ISSRs are likely to mark gene rich regions (Kojima et al. 1998). The main advantages of using gene targeted markers such as CBDP and SCoT techniques compared to ISSR is that these markers are derived from gene regions of the genome while ISSR amplifies the markers from neutral genetic regions. As a result, any assessment can reflect the functional diversity among genotypes using CBDP and SCoT markers. In addition, the probability of finding a marker with a particular trait using CBDP and SCoT markers derived from genetic regions may be very high. CBDP technique is not plant species-specific thus, it can be

employed to any plant groups or species, also easy to apply and does not require prior knowledge of genome sequences (Singh et al. 2014). The present findings revealed that the genetic analyses using gene targeted markers such as CBDP and SCoT would be more useful for crop improvement programs, such as genotype identification, assessing genetic diversity, hybridization between diverse genotypes, construction of linkage maps and QTL mapping.

Authors' contribution

Conceptualization of research (PH, AE, RA, MK); Designing of the experiments (AE, RA, MK); Contribution of experimental materials (AE); Execution of field/lab experiments and data collection (PH, AE, RA, MK); Analysis of data and interpretation (PH, AE, RA, MK); Preparation of manuscript (PH, AE, RA, MK).

Declaration

The authors declare no conflict of interest.

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Supplementary Table S1. Nucleotide sequence and annealing temperature of the used primers

Primer name	Primer sequence	Annealing temperature (^{o}C)
SCoT2	CAACAATGGCTACCACCC(18)	
SCoT3	CAACAATGGCTACCACCG(18)	
SCoT4	CAACAATGGCTACCACCT(18)	
SCoT5	CAACAATGGCTACCACGA(18)	
SCoT7	CAACAATGGCTACCACGG(18)	
SCoT8	CAACAATGGCTACCACGT(18)	
SCoT9	CAACAATGGCTACCAGCA(18)	45
SCoT11	AAGCAATGGCTACCACCA(18)	
SCoT12	ACGACATGGCGACCAACG(18)	
SCoT14	ACGACATGGCGACCACGC(18)	
SCoT15	ACGACATGGCGACCGCGA(18)	
SCoT21	CACCATGGCTACCACCAT(18)	
SCoT24	CCATGGCTACCACCGCCA(18)	
SCoT26	ACAATGGCTACCACCATC(18)	
CBDP1	TGAGCACGATCCAATAGC(18)	
CBDP2	TGAGCACGATCCAATAAT(18)	
CBDP3	TGAGCACGATCCAATACC(18)	
CBDP4	TGAGCACGATCCAATAAG(18)	
CBDP5	TGAGCACGATCCAATCTA(18)	50
CBDP7	TGAGCACGATCCAATCGA(18)	
CBDP9	TGAGCACGATCCAATGAT(18)	
CBDP10	TGAGCACGATCCAATGTT(18)	
CBDP12	TGAGCACGATCCAATATA(18)	
CBDP13	TGAGCACGATCCAATGAG(18)	
CBDP14	TGAGCACGATCCAATGCG(18)	
CBDP15	TGAGCACGATCCAATTGA(18)	
ISSR1	DBDACACACACACACACA(18)	52,1
ISSR3	GACAGACAGACAGACA(16)	49,2
ISSR4	AGAGAGAGAGAGAGAGYT(18)	52,5
ISSR5	ACACACACACACACACC(17)	52,8
ISSR6	GAGAGAGAGAGAGAGARC(18)	54,8
ISSR7	CTCTCTCTCTCTCTCTG(17)	52,8
ISSR9	CACACACACACACACAG(17)	52,8
ISSR11	ACACACACACACACACYA(18)	52,5
ISSR12	GTGTGTGTGTGTGTGTYG(18)	54,8
ISSR13	GAGAGAGAGAGAGAGAYC(18)	54,8
ISSR14	AGAGAGAGAGAGAGAGT(17)	50,4
ISSR15	ACACACACACACACACYG(18)	54,8
ISSR23	CTCTCTCTCTCTCTCTRC(18)	54,8
ISSR25	CACACACACACACACARG(18)	54,8
ISSR27	TGTGTGTGTGTGTGTGRC(18)	54,8
ISSR28	TCTCTCTCTCTCTCTCG(17)	52,8

(D) indicates bases of A, G and T, (B), bases of C, G and T, (Y), bases of C and T and (R), bases of A and G.