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Comparative Efficiency of Functional Gene-based Markers, Start Codon Targeted Polymorphism (SCoT) and Conserved DNA-derived Polymorphism (CDDP) with ISSR Markers for Diagnostic Fingerprinting in Wheat (*Triticum aestivum* L.)

H. HAMIDI[1], R. TALEBI[2]* and F. KESHAVARZI[3]

[1]Department of Cellular and Molecular Biology, Kurdistan Science and Research Branch, Islamic Azad University, Sanandaj, Iran
[2]Department of Agronomy & Plant Breeding, College of Agriculture, Sanandaj Branch, Islamic Azad University, Sanandaj, P.O. Box 618, Iran
[3]Department of Biology, Sanandaj Branch, Islamic Azad University, Sanandaj, Iran

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Three molecular markering techniques: inter-simple sequence repeat (ISSR); start codon targeted (SCoT), conserved DNA-derived polymorphism (CDDP) markers were compared for fingerprinting of 40 varieties of bread wheat. The number of scoreable and polymorphic bands produced using the ISSR, SCoT and CDDP primers for varieties was more than that of genotypes. Average polymorphism information content (PIC) for ISSR, SCoT and CDDP markers was 0.39, 0.41 and 0.34, respectively, and this revealed that three different marker types were equal for the assessment of diversity amongst genotypes. Cluster analysis for three different molecular types revealed that genotypes taken for the analysis can be divided in three and four distinct clusters. There were no significant differences among these markers in terms of the evaluation of genotypes. These results suggest that efficiency of SCoT, CDDP and ISSR markers was relatively the same in fingerprinting of genotypes but SCoT and CDDP analysis are more effective in fingerprinting of wheat genotypes. To our knowledge, this was the first detailed report of a comparison of performance among two targeted DNA region molecular markers (SCoT and CDDP) in comparision with ISSR technique on a set of samples of wheat cultivars. Overall, our results indicate that SCoT, ISSR and CDDP fingerprinting could be used to detect polymorphism for genotypes of wheat.

Keywords: wheat, genetic diversity, ISSR, SCoT, CDDP

Introduction

During the last few decades, better progress has been made in increasing the unit area yield of wheat in the world. By comparing the yield of wheat per unit area of Iran with the per unit area yield of wheat of the advanced countries, we are still outlying at the rear. Iran, as

* Corresponding author; E-mail: srtalebi@yahoo.com; Phone: +98-8716387110; Fax: +98-8716387100

a developing country has less share in global wheat production. Its consumption is increasing day by day due to ever increasing population. Characterization of accessions for important traits will facilitate efficient synthesis of breeding populations that are designed to accomplish specific objectives (Fufa et al. 2005; Talebi et al. 2012). It is widely believed that the genetic diversity of major crops, including bread wheat (*Triticum aestivum* L.), has suffered an overall reduction with time, primarily as a consequence of domestication processes and, more recently, as a result of the recurrent use of adapted germplasm and the adoption of breeding schemes not favoring wide genetic recombination (Donini et al. 2000; Martos et al. 2005). Molecular markers have provided a powerful approach to analyze genetic relationships among accessions in many crop species (Maccaferri et al. 2003; Schuster et al. 2009). Genetic diversity in the Triticeae has been explored using a range of molecular markers such as SSR (Röder et al. 1998a, b; Song et al. 2005), AFLP (Talebi et al. 2012) and etc. (reviewed in Gupta et al. 1999). There is urgent need for new molecular marker techniques that do not require any genome sequence information as well as the above-mentioned marker techniques. Due to the tremendous growth in public biological databases, the development of functional markers that are located in or near the candidate genes have become considerably easy (Andersen and Lubberstedt 2003). With initiating a trend away from random DNA markers towards gene-targeted markers, a novel marker systems called conserved DNA-derived polymorphism (CDDP) (Collard and Mackill 2009a) and start codon targeted polymorphism (SCoT) (Collard and Mackill 2009b) were developed based on the conserved regions are typically functional domains which correspond to conserved DNA sequences within genes. Conserved DNA regions are often conserved across different plant species. Compared to the RAPD method, this method uses longer primers and higher annealing temperatures that should improve reproducibility. Furthermore, this method focuses on gene regions which may have advantages over random markers for applications in QTL mapping (Andersen and Lubberstedt 2003). In principle, CDDP and SCoT are similar to ISSR because the same single primer is used as the forward and reverse primer (Collard and Mackill 2009a; Gorji et al. 2011). Here, the use of CDDP and SCoT markers for studying genetic diversity was reported for the first time in wheat genotypes. Objectives of the present study are as follows: (1) to determine the potential of this methodologies to generate polymorphic markers in wheat; (2) to investigate whether SCoT and CDDP markers could be effectively used in determining genetic relationships among wheat genotypes compare to ISSR markers data; and (3) to identify the relationships of different types of molecular fingerprinting of genotypes.

Materials and Methods

Plant materials and DNA extraction

Forty hexaploid wheat cultivars – mainly registered in Iranian catalogue – were used to analyze the genetic diversity using three different marker types (ISSR, SCoT and CDDP). All these cultivars, which were grown at the seed and Plant Improvement Institute of Iran

(SPII), were obtained and used by the majority of public wheat breeders and farmers between 1950 and 2000. The varieties are listed in Table S1*. DNA was extracted from 2 g of young leaves collected from 10 days old seedling plants of each accession using the CTAB method (Lassner et al. 1989) with the modification described by Torres et al. (1993).

ISSR markers analysis

For ISSR analysis, a set of 10 primers representing di, tri, tetra and pentamer repeats (UBC set # 9) was procured from the Biotechnology Laboratory, University of British Columbia, Canada. Used primers are listed in Table S2. PCR amplification was performed in 20 µl reaction containing 1× PCR buffer, 30 ng sample DNA, 2.5 µM primer, 200 µM of each dNTP, 1.5–2.5 mM $MgCl_2$ and 1.5 unit of Taq DNA polymerase (Cinnagen, Iran). All amplification were carried out in a Eppendorf thermocyclers as follows: 94°C for 3 min, followed by 35 cycles of denaturation at 93°C for 45 s, annealing at optimum *Ta* for 45 s, and extension at 72°C for 90 s. A final extension cycle at 72°C for 10 min followed. PCR products were separated on 1.2% agarose gels, stained with ethidium bromide and scored for the presence or absence of bands.

SCoT markers analysis

Primer sequences employed in the present study were designed by Collard and Mackill (2009b) based on the consensus sequences of translation initiation codon region in higher plants with ATG codon at positions +1, +2, +3; 'G' at position +4; and 'A', 'C', and 'C' at positions +7, +8, and +9, respectively (Table S2). PCR was optimized for 10 primers as described previously by Collard and Mackill (2009b). PCR amplification was performed in 20 µl reaction containing 1× PCR buffer, 50 ng sample DNA, 2.5 µM primer, 200 µM of each dNTP, 3 mM $MgCl_2$ and 1.5 unit of Taq DNA polymerase (Cinnagen, Iran). All amplification were carried out in a Eppendorf thermocyclers as follows: 94°C for 3 min, followed by 35 cycles of denaturation at 93°C for 1 min, annealing at 48 °C for 1 min, and extension at 72°C for 2 min. A final extension cycle at 72°C for 10 min was followed. PCR products were separated on 1.5% agarose gels, stained with ethidium bromide and scored for the presence or absence of bands.

CDDP markers analysis

Primer sequences employed in the present study were designed by Collard and Mackill (2009a) based on the protein sequences of well-characterized genes from diverse plant species (Table S3). Sequences of these genes were scanned for short conserved amino acid regions with the low permutations of possible codons. Up to three degenerate nucleotides were included in a single primer. Since plant exons are typically guanine–cytosine (GC) rich, some degeneracies were incorporated into primers corresponding to the third nucleotide position of a codon (i.e. G or C in the primer sequence was designed as an "S"). Prim-

* Further details about the Electronic Supplementary Material (ESM) can be found at the end of the article.

ers were 15 to 19 nucleotides in length and had >60% GC content. PCRs were performed within a total volume of 20 µL using a Eppendorf thermocyclers. PCR mixtures contained: PCR buffer (Cinnagen; 20 mM Tris-HCl [pH 8.4], 50 mM KCl), 1.5 mM $MgCl_2$, 0.24 mM of each dNTP, 1 U of Taq polymerase (Cinnagen), and 0.8 µm of primer. Each reaction contained 25 ng of template DNA. A standard PCR cycle was used: an initial denaturation step at 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min; the final extension was held for 5 min. All PCR amplification products were separated on 1.2% agarose gels in Tris-borate-ethylenediaminetetraacetic acid buffer stained with ethidium bromide and visualized under UV light.

Data analysis

Amplified bands obtained with all the molecular markers were scored visually for the presence (1) and absence (0) of bands for all the 40 accessions. Nei's genetic distance (Nei 1973) was determined among the genotypes and used for grouping of the genotypes by UNJ (Un-weighted Neighbor Joining) cluster method (Perrier et al. 2003). The fit of dendrograms obtained were checked by bootstrapping using 100 replications. NTSYS ver 2.02 (Rohlf 1998) and DARwin ver 5.0 (Perrier and Jacquemoud-Collet 2006) were used for clustering. Mantel statistic was used to compare the dissimilarity matrices as well as the dendrograms produced by the ISSR, CDDP and SCoT techniques through NTSYS software. Polymorphic information content (PIC) values were calculated for each ISSR, CDDP and SCoT primers according to the formula: $PIC = 1 - \Sigma(Pij)^2$, where P_{ij} is the frequency of the i^{th} pattern revealed by the j^{th} primer summed across all patterns revealed by the primers (Botstein et al. 1980).

Results

DNA fingerprint database has been reported using the three different PCR-based molecular markers (ISSR, SCoT and CDDP) systems for 40 Iranian bread wheat cultivars. Our results indicated that primers which were obtained from the different regions of genomic DNA, successfully amplified accessions template DNAs (Fig. S1). All the three molecular markers used in this study were able to distinguish and identify each of 40 cultivars. Salient features of fingerprint database obtained using different markers are given below:

ISSR analysis

A set of 10 ISSR primers were used to fingerprint 40 wheat cultivars. Only primers that exhibited unambiguous and reproducible band patterns were selected for further analysis. Thus, a total of 6 primers that exhibit distinct and reliable band patterns were utilized for bands scoring, following genetic similarity analysis and cluster analysis.

The total number of markers (amplified bands) observed among the wheat genotypes based on ISSR analysis with six primers was 57, which 47 were polymorphic. The number of polymorphic bands produced per primer ranged from 3 to 12 and size of the products ranged from 150 bp to 2700 bp (Table 1). The PIC values, a reflection of allele diversity and frequency among the varieties, were uniformly high for all the ISSR loci tested. The

Table 1. Polymorphism and PIC values in bead wheat cultivars as revealed by ISSR, SCoT and CDDP markers

Marker	Primer	No. of amplified bands	No. of polymorphic bands	Polymorphism (%)	PIC value
ISSR	UBC807	6	6	100%	0.41
	UBC815	16	12	75%	0.49
	UBC816	4	3	75%	0.21
	UBC818	13	12	92%	0.48
	UBC828	8	5	62.5%	0.37
	UBC880	10	9	90%	0.41
SCoT	SCoT1	7	6	85.7	0.48
	SCoT11	10	9	90	0.49
	SCoT13	6	3	50	0.34
	SCoT19	8	7	87.5	0.39
	SCoT22	13	10	76.9	0.44
	SCoT28	10	9	90	0.49
	SCoT35	6	5	83.3	0.39
	SCoT36	9	4	44.4	0.32
CDDP	*Knox1*	7	5	71.4	0.42
	Knox2	12	10	83.3	0.49
	Myb1	7	2	28.7	0.19
	Myb2	6	4	66.6	0.39
	ERF1	8	2	25	0.21
	ERF2	10	3	30	0.25
	WRKYR1	8	6	75	0.40
	WRKYF1	4	3	75	0.41
	ABP1-1	7	4	57.1	0.35
	ABP1-3	12	4	33.3	0.31

PIC value ranged from 0.21 (UBC816) to 0.49 (UBC815) with a mean of 0.39 (Table 1). From the dendrogram constructed by DARwin using un-weighted neighbour joining method, it was discernible in to four major clusters (Fig. 1). Clusters I, III and IV contained maximum number of genotypes. Correlation coefficient between similarity matrix and coefenitic matrix was significant (r = 0.67**), which demonstrated that 67% of variation between genotypes revealed by ISSR analysis

SCoT analysis

A set of 10 SCoT primers were used to fingerprint 40 wheat cultivars. Among them, eight primers showed reliable polymorphiv patterns were utilized for bands scoring, following genetic similarity analysis and cluster analysis. A total of 69 bands were detected among 40 wheat genotypes using 8 SCoT markers out of which 53 were polymorphic (Table 1). Number of polymorphic bands were ranged from 4 (SCoT36) to 10 (SCoT22) with an average 6.6 per primers. Percent polymorphism ranged from 44.4% to as high as 90% with an average polymorphism of 75.9% across all accessions. PIC values ranged from 0.32 to 0.49, with an average value of 0.41 per primer (Table 1). Based on un-weighted neighbour-joining method, a dendrogram demonstrating the genetic relationships among the

wheat cultivars were constructed. The 40 wheat cultivars fell under three major groups (Fig. 2). Clusters II and III divided into two sub-clusters. Cluster III contained maximum numbers of genotypes. In general, similarity between genotypes clustering in SCoT analysis and ISSR based clustering was low, also in some cases genotypes grouped with each other in same cluster.

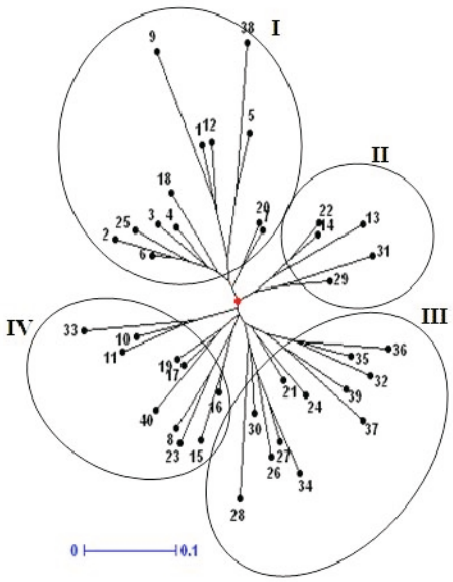

Figure 1. Dendrogram of the 40 Iranian wheat cultivars based on the dissimilarity matrix developed using ISSR markers

CDDP analysis

The CDDP primers were used to fingerprint a diverse set of improved wheat cultivars. Ten primers generated a total of 81 bands out of which 43 were polymorphic (Table 1). Number of polymorphic bands were ranged from 2 (Myb1) to 10 (Knox2) with an average 4.3 per primers. Percent polymorphism ranged from 25% to as high as 83.3% with an average polymorphism of 54.5% across all genotypes. PIC values ranged from 0.19 to 0.49, with an average value of 0.34 per primer (Table 1). Based on un-weighted neighbour-joining method, the 40 wheat genotypes grouped in three distinct clusters (Fig. 3). Clusters I, II and III contained 12, 17 and 11 genotypes, respectively. Cluster II contained maximum numbers of genotypes and showed relatively similar grouping pattern with clusters III and IV that obtained by ISSR and cluster III that obtained by SCoT markers.

Correlation between different marker systems

Cophenetic coefficient were acceptable in all three molecular markers systems (ISSR = 0.64; SCoT = 0.73 and CDDP = 0.79) indicating good fit for clustering. The values of

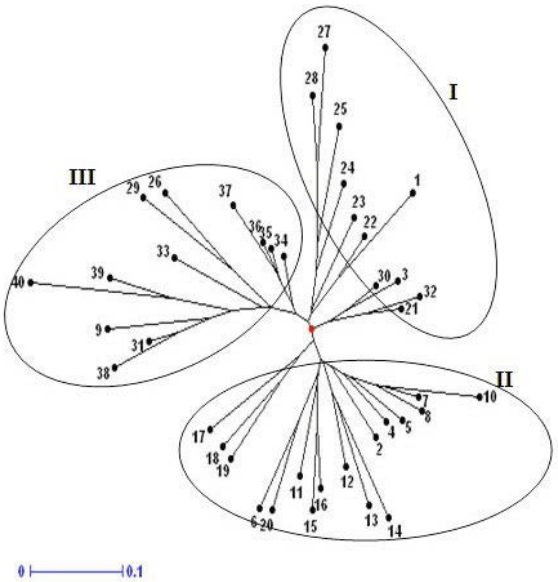

Figure 2. Dendrogram of the 40 Iranian wheat cultivars based on the dissimilarity matrix developed using SCoT markers

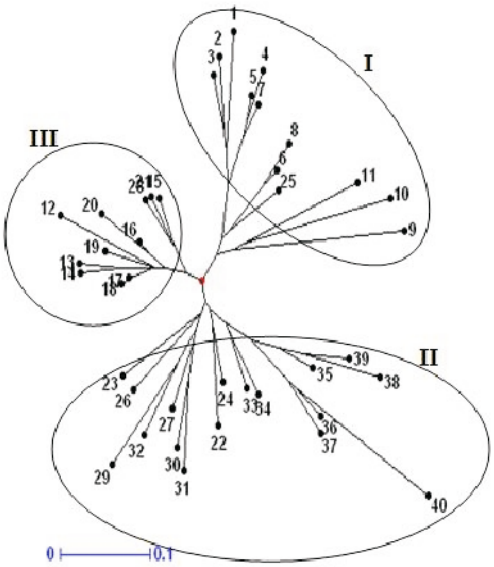

Figure 3. Dendrogram of the 40 Iranian wheat cultivars based on the dissimilarity matrix developed using CDDP markers

mantel test correlation showed a positive correlation between the three marker types. The correlation coefficient (r) was 0.32 between ISSR and CDDP, 0.19 between ISSR and SCoT and 0.34 between CDDP and SCoTs. All three molecular marker types showed positive but non-significant correlation with each other (Table 2). In order to estimate the genetic distance among genotypes, the dissimilarity matrix was computed. In order to estimate the genetic distance among genotypes, the dissimilarity matrix was computed. The mean value of genetic distance obtained by ISSR, DAMD and SCoT markers were 0.39, 0.49 and 0.43, respectively.

Table 2. Mantel test correlation coefficients among similarity matrices obtained using ISSR, SCoT and CDDP markers

	ISSR	SCoT	CDDP
ISSR	–		
SCoT	0.19	–	
CDDP	0.32	0.34	–

Discussion

The knowledge about the genetic relationships of genotypes provides useful information to address breeding programmes and germplasm resource management (Roldan-Ruiz et al. 2001; Eivazi et al. 2008). Traditional plant breeding utilizes natural variation present in germplasm for crop improvement. However, repeated use of germplasm lines with better yield and quality led to narrowing genetic base of modern cultivars (Ullah 2009; Talebi et al. 2012). Although wheat has a broad genetic base, with the discovery of diverse molecular markers from different targets regions of the genome, it is now possible to conduct extensive molecular diversity study in this important crop to identify genetically diverse germplasm with beneficial traits for use in crop improvement programs. In this study, we have compared the marker data-sets produced three different marker systems, ISSR, SCoT and CDDP, to define genetic relationships within a set of 40 Iranian bread wheat cultivars to know if these marker systems can be effectively used in breeding programme. The genotypes showed diverse pattern and distinct ISSR, SCoT and CDDP diversity profiles. The range of genetic distance based on these three different markers, which may reflect the fact of that as each technique targets different regions of the genome. The SCoT and CDDP marker techniques were employed in the present study for several reasons. Firstly, these are types of targeted molecular marker techniques characterized by simplicity and reproducibility. Their PCR products were resolved by performing agarose gel electrophoresis. Compared to arbitrary markers such as RAPD markers are highly reproducible due to the use of longer primers (Collard and Mackill 2009a, b). In other hand, PCR amplification using gene-specific primers targets conserved DNA regions within genes. Gene-targeted markers are preferable for numerous applications in plant molecular genetics especially QTL mapping since recombination levels between marker and gene or QTL are lower compared to “indirect random markers” such as

RAPDs, ISSRs, or SSRs (Andersen and Lubberstedt 2003). In this study, the amount of polymorphisms and efficiency of generating polymorphism observed by SCoT and CDDP markers were compared ISSR (Table 1). Evidently, the SCoT and CDDP markers technique can detect more polymorphic primers and moderate polymorphism compared with ISSR technique used in wheat (Table 1). However, it is difficult to compare the amount of SCoT and CDDP polymorphisms in the current study with previous studies that employed other molecular marker techniques because of the difference in the number of primers, accessions, and varieties used. This study has implications not just for the origin of this crop, but also for the management of genetic resources and their uses in applied breeding programmes, particularly for the development of a core collection. Information about current genetic diversity permits the classification of our available germplasm into various/heterotic groups, which is particularly important to hybrid/cross-breeding programs in wheat. The previous crossing programs in most of the research institutes of Iran were based on only phenotypic characters. The current study confirmed the importance of molecular studies (cheap, fast and informative markers) that can be used beside the morphological data in detecting genetic variation among genotypes in selecting diverse parents to carry out a new crossing programme successfully. In summary, a novel DNA marker method that targets certain parts of plant genome or candidate plant genes by primer design were used for diversity analysis in wheat. It is agarose-based and, therefore, simple and relatively cheap to use. these markers should provide an additional option to RAPD or ISSR techniques or other recently developed techniques such as CoRAP (Wang et al. 2009) for QTL mapping, bulked segregant analysis, and genetic diversity applications in plant genetic analysis. CDDP may be particularly useful for targeted QTL mapping when conserved DNA regions can be identified from candidate genes relevant to the QTL mapping experiment.

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Electronic Supplementary Material (ESM)

Electronic Supplementary Material (ESM) associated with this article can be found at the website of CRC at http://www.akademiai.com/content/120427/

Electronic Supplementary *Table S1*. List of Iranian bread wheat cultivars used in this study

Electronic Supplementary *Table S2*. Primers used in ISSR and SCoT analyses

Electronic Supplementary *Table S3*. Conserved DNA sequence targets and CDDPs primer sequences and details

Electronic Supplementary *Figure S1*. Amplification profile obtained with UBC828 (a), SCoT13 (b) and ABP1-1 (c) primers detected in different bread wheat cultivars