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EVALUATION OF ISSR MARKERS TO ASSESS GENETIC VARIABILITY AND RELATIONSHIP AMONG WINTER TRITICALE (X TRITICOSECALE WITTMACK) CULTIVARS

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Abstract

The ISSR technique was used to identify genetic relationships in 11 winter hexaploid triticale cultivars "Lasko, Stan-1, Malno, Purdy, AN-34, Tatlicak-97, Karma-2000, Presto, Melez-2001, Mikham-2002, Samur Sorti". Twenty ISSR primers were tested and twelve of them amplified clear and reproducible bands. The number of ISSR fragments generated per primer set ranged from 5 to 31 with fragment sizes varying from 320 to 2700bp. A total of 209 ISSR fragments were detected, of which 159 were polymorphic (76.07%). All cultivars were clearly differentiated by their ISSR fingerprints. Based on UPGMA analysis a dendrogram was constructed and 11 triticale cultivars were grouped in two clusters. Cluster I was the largest, comprising 10 cultivars which can be divided into four subclusters. Only one cultivar, "Stan-1" was positioned in Cluster II. The polymorphic patterns generated by ISSR profiles showed different degrees of genetic relationship among the cultivars studied. Similarity values between cultivars ranged from 0.59 to 0.89. The results indicate that ISSRs may constitute a relatively simple and efficient method for analysing genetic variation in triticale.

Introduction

Triticale is a crop species resulting from crosses between wheat (*Triticum* spp. L*)* and rye (*Secale cereale* L.). It is produced by doubling the chromosomes of the sterile hybrid that results when crossing wheat and rye. Depending on the ploidy level of the parental species, triticale can be tetraploid, hexaploid, or octoploid (Varughese *et al*., 1996). Durum wheat, the donor of A and B genomes, is known for its high yield potential and adaptation to relatively dry environments (Cantrell, 1987). On the other hand, rye, the R genome donor, has lower yield potential but is well adapted to extreme cold, drought, and acidic soils. The yield potential of triticale under optimum crop production environments has reached nearly the same level of wheat while outperforming wheat under marginal environments (Varughese *et al*., 1996). For this reason, triticale seems to be an interesting alternative to other cereals, particularly bread wheat, in environments where growing conditions are unfavorable or in low-input systems (Erekul & Köhn, 2006).

Triticale is used as food material for human and feed for animals throughout the world because its flour is rich in proteins (average 14-15%) and contains high level of essential amino acids and does not accumulate NO_3 to the critical level of intoxication as a forage crop (Gulmezoglu *et al.,* 2010). Furthermore, triticale has become a valuable genetic resource for transferring desirable genes; particularly disease-resistance genes, from rye to wheat (Kuleung *et al*., 2004).

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The characterization of germplasm is essential for identification of various species, varieties and cultivars and also to determine their genetic relationships. Traditionally, morphological characters are used for establishing the identity of cultivars. But these characters are under the influence of environmental changes, epistatic interactions, pleitropic effects. Therefore, DNA fingerprinting techniques have been developed for measuring genetic variability and cultivar identification. The most common techniques include isozymes and PCR-based assays such as Randomly Amplified Polymorphic DNA (RAPD; Williams *et al*., 1990), Simple Sequence Repeats (SSR; Akkaya *et al*., 1992), Amplified Fragment Length Polymorphism (AFLP; Vos *et al*., 1995) and Inter Simple Sequence Repeats (ISSR; Zietkiewicz *et al*., 1994). Among these ISSR markers are more and more in demand, because they are known to be abundant, very reproducible, highly polymorphic, highly informative and quick to use (Zietkiewicz *et al*., 1994, Bornet & Branchard, 2001). ISSR analysis involves the PCR amplification of regions between inversely oriented microsatellites. Neither sequence information nor prior genetic studies were required for these analyses (Zietkiewicz *et al*., 1994). ISSR markers are considered to be more reproducible than RAPD markers due to high annealing temperature (Bornet & Branchard, 2001; Chowdhury *et al*., 2002). In addition, the ISSR method has been reported to produce more complex marker patterns than the RAPD assay Chowdhury *et al*., 2002), which is advantageous when differentiating closely related cultivars. ISSR markers have been used to measure genetic diversity in barley (Fernández *et al*., 2002), rice (Joshi *et al*., 2000), as well as to identify cultivars in maize (Pejic *et al*., 1998), wheat (Nagaoka & Ogihara, 1997), potato (Prevost & Wilkinson, 1999) and bean (Métais *et al*., 2000), apple (Goulao & Oliveira, 2001), peanut (Raina *et al*., 2001), strawberry (Arnau *et al*., 2003) and cicer (Sudupak, 2004).

To date, genetic variation in different triticale genotypes has been studied by SSR (Tams *et al*., 2004, Kuleung *et al*., 2004, 2006) and RAPD markers (Atak *et al*., 2005). The objective of this study was to determine the genetic variation between 11 winter triticale cultivars by using ISSR technique.

Materials and Methods

Plant materials: The 11 registered winter hexaploid triticale cultivars were used in this study. Seeds of Lasko, Stan-1, Malno, Purdy, AN-34 were obtained from International Maize and Wheat Improvement Center, Mexico (CIMMYT) and the seeds of Tatlicak-97, Karma-2000, Presto, Melez-2001, Mikham-2002, Samur Sorti were obtained from Bahri Dagdas International Agricultural Research Institute, Konya, Turkey. Country origins and available pedigree data of these cultivars are provided in Table 1. The seeds of each cultivar were germinated in a growth chamber (Sanyo, MLR350H) at 25°C and a continuous light. Germinated seeds were transplanted into a ready-to-use potting mixture (VSB, Holland) and after ten days of growth, under controlled conditions, the seedlings were cut and frozen in liquid nitrogen for DNA extraction.

DNA extraction: Total DNA was isolated from bulk samples using a modified CTAB protocol (Doyle & Doyle, 1990). In order to remove RNA, DNA sample was treated with 4 µl RNase A (50 mg/ml) at 37°C for overnight. DNA samples were quantified in a Nanodrop® ND-1000 spectrophotometer (Wilmington, Delaware, USA) at 260 nm and the quality estimated by the ratio A260/A280. DNA samples were diluted to a uniform concentration of 4 ng/µl.

Table 1. Registration year, country origin and available pedigree information of the winter triticale genotypes used in this study.

Genotype	Year release	Country origin	Pedigree
Lasko	1982	Poland	(T57 x wheat C1203/97) x 6 TA 206
Malno	1987	Poland	Mo 4107 x A-8-1
Purdy	1992	Poland	Salvo x Dagro
Stan-1	1988	USA	Unavailable
AN-34	1992	Mexico	274/320//244.KISS
Tatlicak-97	1997	Turkey	Unknown, Eucarpia
Karma-2000	2000	Turkey	150-83/SHETLAND1 SWTY89.62-1 MI-OMI-OE
Presto	2000	Turkey	Unavailable
Melez-2001	2001	Turkey	GT-AD-1/91//CWT1988/79/10 BDKT910018-3F4BD-0BD
Mikham-2002	2002	Turkey	BDMT-19/JGS1 BDKT910036-3F4BD-0BD
Samur Sorti	Unknown	Azerbaijan	Unavailable

Table 2. List of ISSR primers used in this study.

Primer	Sequence*	No. of scorable bands	No. of polymorphic bands
ISSR2	$(CAG)_5$	16	13
ISSR4	$(GA)_8YC$	5	2
ISSR5	$(AG)_8G$	10	6
ISSR6	$(GA)_8T$	11	8
ISSR7	$(AC)_8YT$	21	14
ISSR8	$(GT)_8YC$	22	17
ISSR9	$(GA)_8A$	18	11
ISSR10	$(AG)_8T$	28	21
ISSR11	$(AG)_8C$	31	27
ISSR12	$(AC)_8C$	14	12
ISSR17	$(AGC)_6G$	18	15
ISSR18	$(AGC)_6C$	15	13
	Total	**209**	**159**

DNA amplification: The ISSR primers used in this study were synthesized by Bio Basic Inc. (Ontario, Canada). Twenty primers were screened on genomic DNA from six randomly chosen cultivars. Only the primers which showed diversity among the varieties were used for analyzing all the samples. The nucleotide sequences of the primers used in this study are listed in Table 2.

Amplification of ISSR fragments from genomic DNA was carried out in a total reaction volume of 25 µl containing 10 ng of DNA, 1X *Taq* polymerase reaction buffer, 2 mM $MgCl_2$, 0.1 mM each of dNTPs (dATP, dCTP, dGTP, and dTTP), 0.2 mM primer and 1 U of *Taq* DNA polymerase (Fermentas, Maryland, USA). Amplifications were performed in a Progene thermocycler (Techne, Burlington, USA) programmed for a 3 min denaturation at 94°C and 40 cycles of 1 min denaturation at 94°C, 1 min annealing at 50- 54°C (Table 2), and a 2 min extension at 72°C, followed by a final extension at 72°C for 7 min. PCR amplifications were repeated at least twice for each ISSR primer to evaluate the reproducibility of the bands obtained. A negative control which contained all PCR components except DNA was also included in every PCR reaction.

Amplification products were separated on 1.4% agarose gel containing ethidium bromide (0.5 μg/ml). As molecular size standards we included a 100bp and 1kb ladder (Fermentas).Gels were visualized under UV light and digitally photographed with UVIpro gel documentation system (UVItec, Cambridge, UK).

Data analysis: The presence (1) or absence (0) of the amplified bands were scored in all 11 cultivars for each primer. Faintly stained bands were not considered. The genetic similarity between all cultivars was calculated according to the Dice coefficient (Dice, 1945) and a dendrogram was generated by Unweighted Pair Group Method with Arithmetic Mean (UPGMA; Sneath & Sokal, 1973) and the SAHN clustering analysis. Mantel's test (Mantel, 1967) was performed to check the goodness of fit of a cluster analysis to the matrix on which it was based. All analyses were done using the NTSYS-pc 2.01 software package (Rohlf, 1998).

Results

Selection of primers and ISSR diversity: Initially, 20 ISSR primers were screened against genomic DNA from randomly chosen six triticale cultivars (AN-34, Mikham-2002, Presto, Purdy, Samur Sorti and Stan-1) for their ability to amplify DNA fragments. Of the 20 primers, two produced no distinct bands on a smeary background and six resulted in very faint bands upon a highly smeared background. The remaining 12 primers (Table 2) produced clear amplification patterns. As an example, the pattern obtained for each cultivar with ISSR-10 and ISSR-18 primers is shown in Fig. 1.

Twelve ISSR primers, including 9 di-nucleotide repeats and 3 tri-nucleotide repeats, yielded a total of 209 bands from the 11 winter triticale cultivars tested. Primer ISSR-4 resulted in the smallest number of bands (5) and primer ISSR-11 generated the largest number of bands (31) (Table 2). The average number of bands per primer was 17.41. Band size ranged from 320 bp to 2.7 kb. Among the 11 triticale cultivars, 159 (76.07 %) of the ISSR bands were polymorphic. The percentage of polymorphic fragments per primer was 40-87.09%.

Genetic relationships among triticale cultivars: The similarity matrix values for 11 winter triticale cultivars ranged from 0.59 to 0.89 (Table 3). The highest similarity was observed between Tatlıcak-97 and Presto (0.89) and the lowest between Stan-1 and AN-34 (0.59). A dendrogram resulting from UPGMA analysis of the similarity matrix showed two clusters (Fig. 2). Cluster I is the largest, comprising 10 cultivars which can be divided into two main subclusters. First subcluster includes Purdy, AN-34, Malno and Lasko cultivars. Second subcluster contains 6 cultivars, which can be divided into two groups. The first group includes Mikham-2002, Samur Sorti and Melez-2001, and the second group contains Karma-2000, Tatlicak-97 and Presto cultivars. Cluster II includes only cultivar Stan-1.

The comparison of Dice's similarity matrix with the matrix of cophenetic values of ISSR dendrogram using the Mantel test (Mantel, 1967) showed that the correlation between these two matrices was very high (r = 0.99), suggesting that data in the Dice's similarity matrix was represented very well by the UPGMA dendrogram.

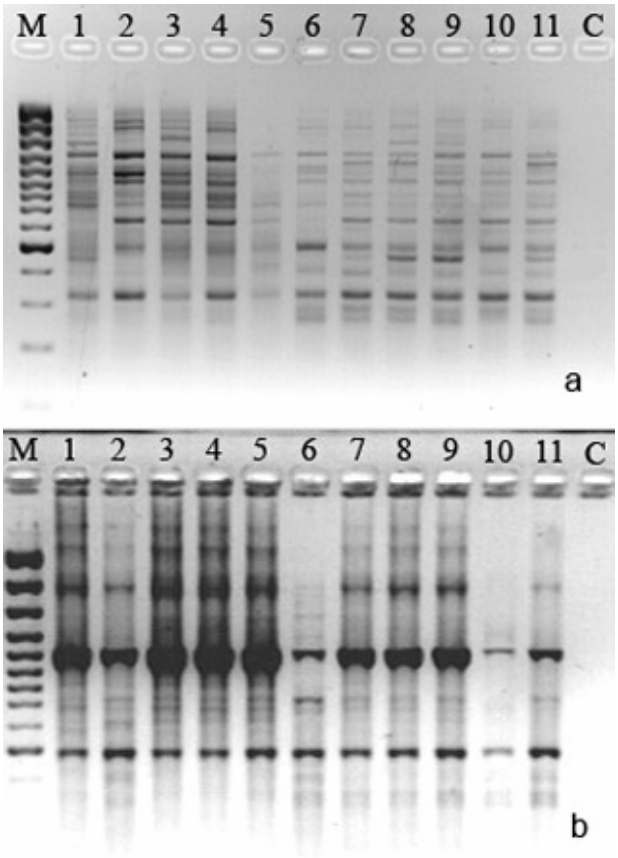

Fig. 1. Agarose gel showing the amplified product using ISSR-10 (a) and ISSR-18 (b) primers. M; 100 bp DNA ladder, Lane 1–11; Purdy, Stan-1, AN-34, Malno, Lasko, Mikham-2002, Karma-2000, Tatlicak-97, Presto, Samur Sorti and Melez-2001, C; Negative control.

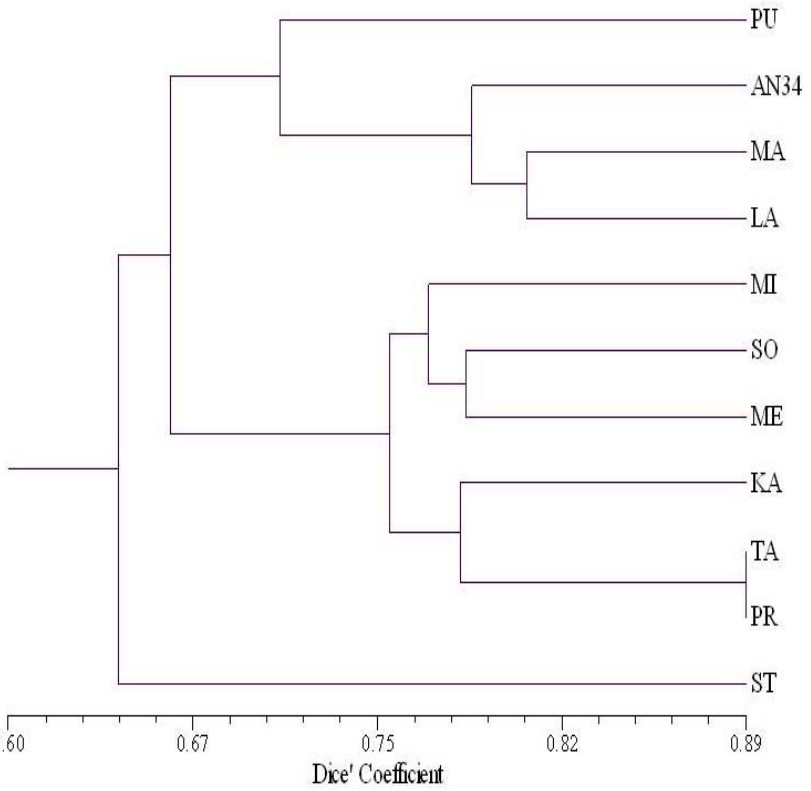

Fig. 2. UPGMA dendrogram showing the genetic relationships among eleven winter triticale cultivars. PU, Purdy; AN34, AN-34; MA, Malno, LA, Lasko; MI, Mikham-2002, SO, Samur Sorti, ME, Melez-2001, KA, Karma-2000, PR, Presto, TA, Tatlicak-97, ST, Stan-1.

Discussion

Determination of genetic variability among genotypes is useful for both practical applications in a breeding program and cultivar protection. A variety of approaches, from morphological data to molecular markers including RAPD (Yuzbasioglu *et al*., 2006; Bibi *et al*., 2009), AFLP (Ipek *et al*., 2008; An *et al*., 2009), SSR (Kuleung *et al*., 2006; Hina *et al*., 2008) and ISSR (Pharmawati *et al*., 2005; Guasmi *et al*., 2006) markers have been applied for assessment of genetic variability among genotypes. In the present study, we assessed usefulness of ISSR markers to investigate genetic variability and relationships among 11 winter triticale cultivars. This is the first report of the analysis of genetic relationships among winter triticale cultivars by using ISSR markers.

In this study, high level of polymorphism in 11 tested triticale cultivars (76.07%) was observed. Previously, Kuleung *et al*., (2004) identified polymorphism in 31% of the used markers by analyzing the genome of five triticales from Russia, Mexico and the United States, using 176 microsatellites developed for wheat and rye. Tams *et al*., (2004) investigated 128 European winter triticale varieties and breeding lines using SSR markers, and indicated an average of 0.54 polymorphism. The same value (0.54) was also found by Kuleung *et al*., (2006), when 80 hexaploid triticales were analysed by employing wheat and rye microsatellites. On the other hand, in a RAPD study of 25 triticale genotypes (24 breeding lines and a cultivar, Tatlicak) polymorphism rate was found as 61.6% (Atak *et al*., 2005). Different triticale genotypes and different type of markers used in these studies could be the reason of obtaining different polymorphism levels.

The similarity coefficient values between 11 winter triticale cultivars ranged from 0.59 to 0.89. The highest similarity value (0.89) was obtained between cultivars Tatlıcak-97 and Presto. It has been reported that cultivar Tatlicak-97 was developed from a cross of Presto originated from CIMMYT (Atak *et al*., 2005). An average genetic similarity coefficient among triticale genotypes was 0.6244. However, genetic similarity among some cultivar pairs are relatively low and these lines could be used as parents to produce new breeding lines (Table 3). Breeders usually share breeding material with other breeding stations or may use common breeding lines with different names. Consequently, identical breeding material at different breeding stations creates a problem of close kinship (Belaj *et al*., 2003) which may result in confused grouping. In our study, we observed no similarity coefficient values close to 1 between any two cultivars, indicating that there were no redundant accessions among those sampled in our study.

Assessments on the genetic relationships of these 11 triticale cultivars based on their ISSR similarities revealed that cultivar Stan-1 was more genetically distinct from the others. The relatively distant relationships between Stan-1 and the other cultivars (0.59-0.70) may be attributed to their originating geographical locations and pedigrees. The most of cultivars used in this study do not share a parent in their pedigree, except for Tatlicak-97 and Presto (Atak *et al*., 2005). These two cultivars were grouped together with high similarity value in the dendrogram. Interestingly, it was observed that cultivars sharing either country of origin or year released were usually clustered together. For example, first subcluster of Cluster I consisted of triticale lines that were released after 1980. The 3 cultivars in this subcluster, viz., Purdy, Malno and Lasko, were developed in Poland. However, cultivar AN-34 originated from Mexico was also grouped with Polish cultivars. It was suggested that most breeding programs around the world have used Polish materials in their crosses and this might be the reason why the Mexican cultivar AN-34 clusters with some Polish cultivars (pers. com. K. Ammar, CIMMYT personel). Second subcluster contained cultivars that were released from 1997 to 2002. This subcluster also included cultivars developed in Turkey except Samur Sorti (from Azerbaijan).

Table 3. Similarity matrix obtained from 209 ISSR markers for 11 winter triticale cultivars.

Code (*)	Purdy	Stan1	AN34	Malno	Lasko	Mikham 2002	Karma 2000	Tatlicak 97	Presto	Samur Sorti	Melez 2001
PU	1.00										
ST	0.607	1.00									
AN34	0.727	0.593	1.00								
MA	0.689	0.689	0.799	1.00							
LA	0.703	0.617	0.765	0.803	1.00						
MI	0.650	0.612	0.655	0.636	0.689	1.00					
KA	0.655	0.598	0.679	0.650	0.684	0.756	1.00				
TA	0.617	0.665	0.650	0.679	0.674	0.784	0.808	1.00			
PR	0.641	0.679	0.665	0.674	0.689	0.741	0.746	0.890	1.00		
SO	0.693	0.703	0.660	0.660	0.645	0.765	0.760	0.779	0.746	1.00	
ME	0.722	0.665	0.669	0.622	0.655	0.765	0.760	0.703	0.717	0.779	1.00

(*): PU, Purdy; AN34, AN-34; MA, Malno, LA, Lasko; MI, Mikham-2002, SO, Samur Sorti, ME, Melez-2001, KA, Karma-2000, PR, Presto, TA, Tatlicak-97, ST, Stan-1.

On the other hand, it can be seen from the dendrogram that except Stan-1, cultivars obtained from CIMMYT (Mexico) viz., Purdy, AN-34, Malno and Lasko appeared to be in same subcluster, and cultivars obtained from Bahri Dagdas International Agricultural Research Institute (Turkey) grouped in other subcluster. This could be explained to some extent by occurrence of cross pollination between cultivars. Triticale is known to be autogamous, however, considerable outcrossing rates between triticales were reported by several researchers (Yeung & Larter, 1972; Gülmezoğlu, 2004). Therefore, there may be possibility that the cultivars obtained from either institute were grown in same growing season and cross pollination occurred between them to some degree. Similarly, clustering of genotypes according to their centre and/or year of release has been reported for cotton (Iqbal *et al*., 1997), Basmati rice cultivars (Bligh *et al*., 1999) and wheat cultivars (Pecetti & Anicchiarico, 1998; Bhutta, 2006). Therefore, it is very important to define identity, purity and stability of such crop varieties including triticale for protection of breeders' rights.

Conclusion

Our results showed that ISSR analysis is less time consuming and less expensive than SSR procedure. Also, it is more reliable, reproducible and generate more polymorphic markers than RAPDs. ISSR analysis can generate sufficient polymorphisms and has potential for large-scale DNA fingerprinting purposes of triticale cultivars. Identification of unique DNA markers characterizing the various triticale genotypes by unique ISSR fingerprints could be useful for determination of cultivar purity and efficient use and management of genetic resources collection.

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