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Identification of hybrids between triticale and *Aegilops juvenalis* (Thell.) Eig and determination of genetic similarity with ISSRs

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ABSTRACT. Wide hybridization expands the gene pool of cultivated species and can produce genotypes with practical value, often resistant to diseases. In plants, ISSRs are often used for hybrid identification as well as for diversity evaluation. We estimated genetic similarity among advanced genetic stocks of triticale hybrids and identified *Aegilops juvenalis* introgressions into triticale. Fourteen ISSR primers amplified from 12 to 23 DNA fragments. The number of polymorphic products per primer ranged from three to nine, with a mean of 5.1. The polymorphic information content values ranged from 0.39 to 0.66, with a mean of 0.52. Thirteen primers amplified products indicating presence of *A. juvenalis* chromatin. The largest mean genetic similarity (0.84) with all other forms was shown by a strain derived from an *A. juvenalis* 6x × [(Lanca × L506/79) × CZR142/79] hybrid, while *A. juvenalis* was the least similar (0.33). We conclude that ISSRs can reliably identify *A. juvenalis* chromatin in the triticale background and efficiently estimate genetic similarity of hybrids and parental forms.

Key words: Genetic similarity; Triticale; Hybrids; *Aegilops juvenalis*; ISSR

INTRODUCTION

Hexaploid triticale (x *Triticosecale* Wittmack), just as its rye parent, is resistant to abiotic stresses, such as frost and drought (Goyali et al., 2002; Woś et al., 2002). For many years, triticale was also one of the most resistant cereals to diseases, but in many instances, resistance has been broken by wheat or rye pathogens. Still, the disease symptoms tend to be milder relative to the parents (Zamorski and Schollenberger, 1995; Góral, 2006).

Crop resistance to stresses, both biotic and abiotic, can be improved by the introduction of genes from wild relatives. Moreover, wide hybridization expands the genetic diversity of a crop species, giving rise to new genotypes that may be of practical value (Gruszecka, 1998; Gradzielewska, 2006a,b; Vaccino et al., 2010).

Triticale's genetic diversity can be extended by hybridization with wild Triticeae, including *Aegilops* species, which carry resistance genes to fungal pathogens, such as leaf and stem rusts, powdery mildew, take-all, and numerous pests (nematodes and insects), as well as abiotic stresses such as drought, cold, frost, salinity, low soil pH and high soil aluminum levels (Kimber and Feldman, 1987; Holoubec et al., 1992, 1993; Masłowski et al., 1997). Earlier studies have shown that some qualitative and quantitative traits of triticale were improved in hybrids with *Aegilops* (Gruszecka, 1998; Gruszecka and Kowalczyk, 2002; Gruszecka et al., 2004).

Molecular markers are often used by plant breeders for diversity evaluation within or between populations, as well as for hybrid identification. In plants, the inter-simple sequence repeat (ISSR) technique is widely employed for analyses of genetic diversity (Ziętkiewicz et al., 1994; Reddy et al., 2002). These markers have been applied for polymorphism investigations in rye (Matos et al., 2001; Bolibok et al., 2005), wheat (Nagaoka and Ogihara, 1997), barley (Fernandez et al., 2002), and maize (Kantety et al., 1995).

In triticale, ISSR showed little polymorphism among cultivars, but still, the method was more effective than RAPD (random amplified polymorphic DNA) (Gonzales et al., 2002; Stojałowski and Góral, 2002; Tams et al., 2002). The ISSRs were shown to be effective in the development of the sequence-characterized amplified region markers and useful for detection of rye chromosome fragments in the triticale background (Vaillancourt et al., 2008).

The goal of this study was to estimate genetic similarity among hybrids of hexaploid triticale with *Aegilops juvenalis* (Thell.) Eig and the parental forms and to detect and confirm the presence of the wild parent's DNA among the hybrid derivatives.

MATERIAL AND METHODS

The material for this study consisted of seven advanced strains of hexaploid triticale derived from hybrids with *A. juvenalis* (Thell.) Eig, and the parents themselves. The F_1 hybrids of triticale with *Aegilops* were backcrossed two or four times to the triticale parent and subsequently self-pollinated (Table 1). The accession of *A. juvenalis* used here was obtained from the collection of the Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany.

Total genomic DNA was extracted from the coleoptiles of several-day-old seedlings, following the method of Milligan (1992), while ISSR analyses were conducted as described by Ziętkiewicz et al. (1994), with modifications. Fourteen primers were used (Table 2) selected from a group of 40 primers tested in two replications per genotype. For each primer, a negative control reaction with double-distilled water was included.

Table 1. Pedigrees of triticale lines used in this study.

	No. of the strain	Designation	Generation	Pedigree
Combination 1	1	CZR350/9	B_4/F_7	*Aegilops juvenalis* 6x x [(Lanca x L506/79) x CZR142/79]
	2	CZR350/833/3/95	B_2/F_9	
	3	CZR350/833/3/95	B_2/F_9	
	4	CZR350/833/3/95	B_2/F_9	
Combination 2	5	CZR700/9	B_2/F_8	[(Jana x Tempo) x Jana] x *Aegilops juvenalis* 6x
	6	CZR700/VI/4/1/01	B_2/F_4	
	7	CZR700/VIII/1/3/01	B_2/F_2	
Parental forms				
Combination 1	Aj	♀ *Aegilops juvenalis* (Thell.) Eig		
	A	♂ (Lanca x L506/79) x CZR142/79		
Combination 2	B	♀ (Jana x Tempo) x Jana		
	Aj	♂ *Aegilops juvenalis* (Thell.) Eig		

The total reaction volume was 15 µL and contained 1X PCR buffer with $(NH_4)_2SO_4$ (75 mM Tris, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20; Fermentas), 1.5 mM $MgCl_2$, 160 µM of each dNTP, 0.47 µM primer, 40 ng DNA template and 0.5 U *Taq* DNA Polymerase (Fermentas), run on the T1 thermocycler (Biometra). The cycling pattern was primer-specific. For SR16, SR27, SR28, SR31, SR37, and SR38, the thermal profile was initial denaturation at 95°C for 7 min, followed by 38 cycles of denaturation at 95°C for 30 s, annealing for 45 s with the temperature for the first 3 cycles being 54°C, 53°C for the next 3 cycles, and 52°C for the remaining 32 cycles, and extension at 72°C for 2 min. The last cycle was followed by a final incubation for 7 min at 72°C. For SR14, SR17, SR23, and SR34 annealing was as follows: the first 3 cycles at 57°C, the following 3 cycles at 56°C and remaining cycles at 55°C. For SR33, the annealing temperatures were 52°, 51° and 50°C, respectively.

The amplification products were loaded onto a 2% agarose gel with 0.01% ethidium bromide and run in TBE buffer (89 mM Tris-borate, 2.5 mM EDTA) for 2 h at 120 V. The fragment sizes were determined relative to the GeneRuler™ 100-bp DNA Ladder Plus (Fermentas). Separated DNA fragments were visualized under UV light and photographed.

Based on the results obtained, the indices - the polymorphism information content (PIC) (Nei, 1973) and the assay efficiency index (AEI, mean number of polymorphic fragments) (Pejic et al., 1998) - of the ISSR method were calculated. Each band was considered an ISSR locus, and only reproducible bands were scored for the construction of binary matrices. ISSR bands with the same molecular weight were considered to be the same locus. The data matrix was used to calculate the genetic similarity (GS) index between pairs of all genotypes analyzed, using the Dice formula (Nei and Li, 1979). Genetic relationships were estimated using the unweighted pair-group method with arithmetic mean (UPGMA) cluster analysis based on the GS indices. The NTSYS-pc 2.10q software was used for calculations (Rohlf, 2001).

RESULTS

The 14 primers used amplified a total number of 240 DNA fragments of various sizes. The number of fragments amplified per primer ranged from 12 (SR38) to 23 (SR16) (Table 2), averaging 17.1 per primer. Of the total of 240 fragments, 72 (30%) were polymorphic (Table 2). On average, a single primer generated between three (SR17, SR34, SR36, SR38) and nine (SR14, SR16) polymorphic fragments, and the AEI index was calculated at 5.1. The values

of the PIC of the ISSR method ranged from 0.39 to 0.66, with an average of 0.52 (Table 2).

Of the 14 primers, 13 amplified fragments specific for *A. juvenalis* (Tables 2 and 3). These products confirmed the presence of *A. juvenalis* DNA in triticale. The reaction carried out with two primers (SR17 and SR23) allowed the identification of wild species DNA in all seven strains investigated. Overall, *A. juvenalis* DNA was identified by four to eleven specific ISSR fragments (Table 3). On the other hand, all 14 primers tested amplified 72 products in *A. juvenalis* itself. These fragments were considered specific to this wild species (Table 2).

Table 2. Polymorphism of inter-simple sequence repeat markers.

Primer	Sequence	Number of bands				PIC	Band size range (bp)	
		Total	Polymorphic	From *Aegilops juvenalis*	Specific only for *A. juvenalis*		Total	Polymorphic
SR14	$(GA)_7YG$	21	9	1	6	0.61	180-2050	180-2050
SR16	$(GA)_8C$	23	9	3	5	0.50	260-1570	260-900
SR17	$(GA)_8YC$	16	3	1	6	0.59	210-1210	640-710
SR22	$(CA)_8G$	15	5	1	3	0.55	480-1830	560-1830
SR23	$(CA)_8GC$	18	6	1	6	0.61	310-1880	530-1880
SR27	$(TC)_8G$	18	8	2	6	0.62	400-1710	400-1710
SR28	$(TG)_8G$	17	4	2	4	0.49	310-1700	460-1450
SR31	$(AG)_8YC$	15	4	1	7	0.66	210-1410	320-1270
SR32	$(AG)_8YT$	16	5	1	5	0.51	300-1860	460-1170
SR33	$(AG)_8T$	17	6	2	5	0.53	360-1650	460-1020
SR34	$(TC)_8CC$	17	3	0	6	0.44	430-2400	700-1600
SR36	$(AC)_8CG$	19	3	2	7	0.51	480-2540	580-1070
SR37	$(AC)_8C$	16	4	3	3	0.39	470-1280	780-1280
SR38	$(CT)_8G$	12	3	2	3	0.49	420-1780	620-1010
Sum/size range	-	240	72	22	72	-	180-2540	180-2050
Mean	-	17.1	5.1	1.6	5.1	0.52	-	

PIC = polymorphic information content.

Table 3. Inter-simple sequence repeat markers identifying *Aegilops juvenalis* (Thell.) Eig chromatin in *Triticale* hybrids.

Hybrid	Band size [bp]
1	$SR23^{1880}$ $SR28^{1445}$ $SR32^{860}$ $SR37^{1170}$
2	$SR17^{710}$ $SR23^{1880}$ $SR27^{970}$ $SR27^{790}$ $SR32^{860}$ $SR33^{610}$ $SR37^{780}$ $SR38^{780}$ $SR38^{625}$
3	$SR17^{710}$ $SR23^{1880}$ $SR27^{970}$ $SR27^{790}$ $SR32^{860}$ $SR33^{610}$ $SR36^{670}$ $SR37^{1280}$ $SR37^{1170}$ $SR37^{780}$ $SR38^{780}$
4	$SR17^{710}$ $SR27^{970}$ $SR27^{790}$ $SR33^{610}$ $SR36^{670}$ $SR37^{1280}$ $SR37^{1170}$ $SR37^{780}$ $SR38^{780}$
5	$SR14^{290}$ $SR16^{690}$ $SR16^{390}$ $SR17^{710}$ $SR22^{730}$ $SR28^{1445}$ $SR28^{970}$ $SR31^{320}$ $SR33^{1020}$ $SR33^{610}$ $SR36^{1070}$
6	$SR16^{620}$ $SR17^{710}$ $SR22^{730}$ $SR28^{1445}$ $SR31^{320}$ $SR36^{670}$ $SR33^{1020}$ $SR33^{610}$
7	$SR14^{290}$ $SR16^{620}$ $SR16^{390}$ $SR17^{710}$ $SR22^{730}$ $SR28^{1445}$ $SR28^{970}$ $SR31^{320}$ $SR33^{1020}$ $SR36^{1070}$ $SR36^{670}$

Based on the polymorphism of ISSR markers, a matrix of Dice similarity indices was constructed (Table 4). Among hybrid progenies and the parental forms (excluding *A. juvenalis*), the greatest genetic similarity of 0.96 was between entries #2 and #3 (see Table 4) and the lowest (0.84) between the paternal line (Lanca x L506/79) x CZR142/79 of the first hybrid combination and entry #6 from the second hybrid combination. Taking into account all hybrids and parents (including *A. juvenalis*), the least genetic similarity (0.30) was between *A. juvenalis* and the triticale paternal hybrid form (Lanca x L506/79) x CZR142/79 (Table 4).

A. juvenalis had the least genetic similarity to all accessions studied: it ranged from 0.30 to 0.35 (Table 4), with an average of 0.33. The greatest average genetic similarity to all

the other accessions was for entry #4. The mean genetic similarity of all the materials studied was 0.77.

The dendrogram of genetic relationships between the genotypes studied was constructed on the basis of the similarity matrix obtained by the UPGMA method (Figure 1). The dendrogram showed three main clusters. Entries from two cross-combinations were separated and formed individual clusters. Of the hybrid strains studied, only accession #1 did not cluster with the other three accessions from this combination or with the accessions of the second hybrid combination, but it associated with its own paternal form (Lanca x L506/79) x CZR142/79, indicating that the backcross process was successful. Predictably, *A. juvenalis* was the least similar to all other entries tested (Figure 1).

Table 4. Dice coefficient similarity matrix based on the polymorphism of ISSR markers.

	Aj	A	1	2	3	4	B	5	6
A	**0.297**								
1	0.317	0.940							
2	0.325	0.883	0.871						
3	0.339	0.893	0.880	**0.958**					
4	0.336	0.895	0.875	0.940	0.948				
B	0.328	0.864	0.873	0.851	0.861	0.863			
5	0.343	0.886	0.902	0.858	0.868	0.899	0.875		
6	0.338	**0.842**	0.850	0.850	0.853	0.884	0.889	0.911	
7	0.349	0.868	0.877	0.855	0.858	0.882	0.879	0.923	0.945

Aj = *Aegilops juvenalis* (Thell.) Eig; A = paternal form (Lanca x L506/79) x CZR142/79; B = maternal form (Jana x Tempo) x Jana; 1-7 = hybrid strains.

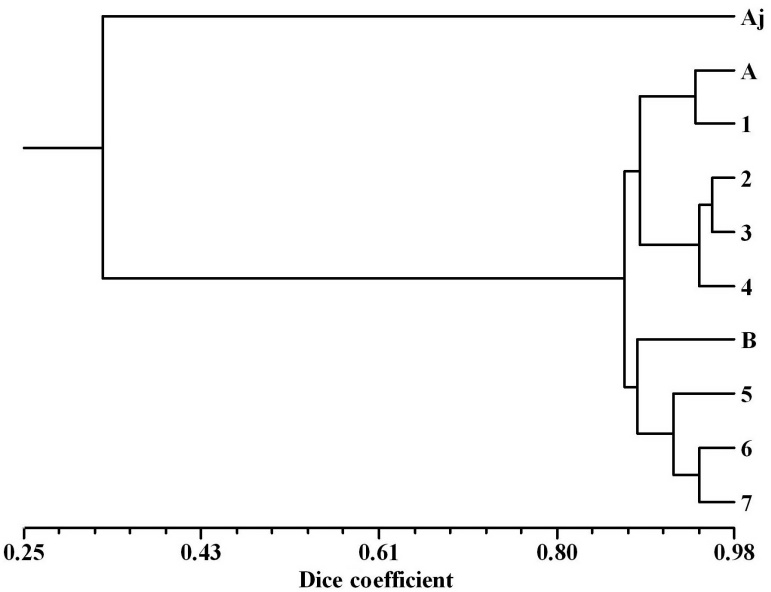

Figure 1. UPGMA dendrogram of *Triticale* hybrids based on the ISSR markers. Aj = *Aegilops juvenalis* (Thell.) Eig; A = paternal form (Lanca x L506/79) x CZR142/79; B = maternal form (Jana x Tempo) x Jana; 1-7 = hybrid strains.

DISCUSSION

The ISSR method is often used in plant genetics. It is relatively easy and as fast as RAPD, but in contrast to the latter, it is highly reproducible and tends to uncover far more polymorphism. This method has often been applied to studies of genetic diversity in triticale, rye and wheat, including the identification of the intergeneric hybrids.

Stojałowski and Góral (2002) identified triticale cultivars and male sterile lines using only seven ISSR primers. These generated 15 polymorphic fragments (12.2% of the total fragments generated), which clearly identified all genotypes tested. Matos et al. (2001), using polyacrylamide gels in a study of 10 rye cultivars, observed an abundance of 280 ISSR markers with only 9 primers. Similarly, a great number of polymorphic ISSRs on a polyacrylamide gel were shown by Galaev et al. (2004), who sought intergeneric polymorphism among hybrids of *Triticum aestivum* with *Aegilops cylindrica*. Again, just seven primers generated 216 polymorphic fragments, and that was 40.6% of all markers (fragments) obtained. High polymorphism of ISSRs was also shown by Bolibok et al. (2005) in rye. In 30 rye cultivars screened, up to 95% of all markers amplified using just 14 primers were polymorphic on an agarose gel, a low-resolution medium.

In this study, 72 ISSR fragments were polymorphic, amounting to 30% of all the fragments identified, also on an agarose gel. We observed a similar level of polymorphism (34%) with both ISSRs and RAPDs, in hybrids between triticale and *Aegilops crassa* 4x Boiss (Gradzielewska et al., 2010). The ISSR primers amplified three to nine polymorphic ISSR fragments, with AEI of 5.1. This index was similar to that of 3.7 for RAPD and 4.4 for ISSR, when we tested triticale hybrids with *A. crassa* (Gradzielewska et al., 2010). This is in line with the results of Bolibok et al. (2005), who obtained on average 4.6 polymorphic ISSR markers amplified per primer. In turn, Stojałowski and Góral (2002) obtained between one and four polymorphic products with each ISSR primer used.

The distribution of identified polymorphisms was reflected in the PIC values. The mean value of this index for ISSR method was calculated at 0.52. This is essentially identical to the PIC value of 0.53 that we obtained with ISSRs when testing triticale-*A. crassa* hybrids (Gradzielewska et al., 2010) and comfortably close to the highest PIC value that Powell et al. (1996) observed using four screening methods in soybean (AFLP, RAPD, RFLP, and SSR; SSRs had a PIC of 0.60). On the other hand, Sarla et al. (2005) with ISSRs and Li et al. (2000) with SSRs determined PIC values similar to ours: 0.50 for a group of rice cultivars and 0.57 and 0.51 for *Avena* species and cultivars, respectively. Among winter triticale cultivars and breeding lines, the average PIC calculated on the basis of polymorphic SSR markers was also similar to the one we obtained in our study (0.54) (Tams et al., 2004). In Brazilian triticale, PIC of SSRs was calculated at 0.36, indicating low variability in the set of 54 genotypes studied (Da Costa et al., 2007). Thus, the ISSR technique seems to reveal a similar or even higher level of polymorphism in triticale as SSRs.

In this study, the ISSRs were useful for the identification of the *A. juvenalis* DNA in triticale hybrids, as we have shown earlier with RAPD (Gradzielewska et al., 2009). Among 94 ISSR fragments specific to *A. juvenalis*, 22 confirmed the presence of its genetic material in all seven hybrid derivatives. With RAPD, only four such fragments were amplified (Gradzielewska et al., 2009). In hybrids of triticale with *A. crassa*, we obtained 20 (ISSR) and 14 (RAPD) markers identifying the genetic material of the wild parent (Gradzielewska et al.,

2010). Similarly, Galaev et al. (2004), in advanced progenies from hybrids of *T. aestivum* with *A. cylindrica*, with just seven ISSR primers, identified an impressive 19 polymorphic loci, which confirmed the introgression of *A. cylindrica* DNA into *T. aestivum*. In contrast, 40 pairs of primers for SSRs identified only seven introgressive SSR alleles Thus, ISSRs seem to be more effective than SSRs and RAPDs for detecting variability caused by introgression of alien genetic material into the genomes of wheat and triticale.

Our results confirm genetic affinity within the two groups of derivatives. The progenies from any of the *A. juvenalis* 6x x [(Lanca x L506/79) x CZR142/79] or [(Jana x Tempo) x Jana] x *A. juvenalis* 6x cross-combinations formed separate clusters on the dendrogram and joined with their maternal triticale parents. Interestingly, such close association could not be shown with RAPDs (Gradzielewska et al., 2009). Emel (2010) confirmed the utility of ISSRs for analyzing of genetic variation in triticale, and Stojałowski and Góral (2002) also found their advantage over ISSRs.

On the basis of the matrix of Dice genetic similarities, *A. juvenalis* was on average the least similar to the hybrids and the triticale parents, with GS of 0.33, as expected. A similar value was calculated for RAPDs (0.39; Gradzielewska et al., 2009). On the other hand, the highest mean affinity estimated in this study by means of ISSR and in another study by means of RAPDs (Gradzielewska et al., 2009) was shown by entry #4 from *A. juvenalis* 6x x [(Lanca x L506/79) x CZR142/79] with GS at 0.84 and 0.85, respectively. Both techniques showed the greatest genetic similarity calculated at 0.96 (ISSRs, in this study) and 0.97 (RAPD, Gradzielewska et al., 2009) between entries #2 and #3.

Based on the results of this study, the utility of ISSRs for estimations of genetic similarity of hybrids between triticale and *A. juvenalis* and for identification of interspecific (intergeneric, actually) introgressions was confirmed. Just two ISSR primers were capable of identifying the *A. juvenalis* DNA fragments in each of the seven hybrid progenies, a feat impossible with RAPDs (Gradzielewska et al., 2009).

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