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Study of interspecific SSR polymorphism among 14 species from *Triticum-Aegilops* group

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Summary

In the present study, using in-gel hybridization and PCR based approaches, interspecific SSR polymorphism was studied among 14 species of *Triticum-Aegilops* group. The material represented seven different genomes and three ploidy levels (2x, 4x, 6x). In-gel hybridization involved 13 probe-enzyme combinations (four SSR oligonucleotide probes in combination with 2-4 enzymes) and resolved 5 to 20 bands (0.40kb to >23kb) in each of the 14 individual species. This suggested ubiquitous distribution and interspecific polymorphism of SSRs among different species of *Triticum-Aegilops* group. The available polymorphism also proved helpful in discriminating not only the species with different ploidy levels and possessing different genomes, but also those possessing similar or very closely related genomes. The amplification of SSR loci using 15 primer pairs derived from hexaploid wheat was also carried out in all the 14 species. The primer pairs, each amplified SSR loci not only in species containing A, B and D genomes, but also in 2 to 10 of the remaining species that contained other genomes. This suggested that wheat SSRs might have been derived from the corresponding SSRs in an ancestral genome and are conserved across a number of species in the *Triticum-Aegilops* group. Also, two pairs of SSRs (one consisting of WMC243 and WMC415 and the other consisting of WMC35 and WMC404) each discriminated all the 14 species examined during the present study. Therefore, one can infer from the present study that SSR primers can be used in studies on DNA polymorphism, genetic diversity, gene mapping and synteny conservation across different species of *Triticum-Aegilops* group.

Key words: in-gel hybridization, microsatellite, polymorphism, *Triticum*, *Aegilops*

Introduction

Molecular markers have been extensively utilized for the study of genetic diversity and genomic constitutions in a number of species of the tribe Triticeae. Genome-specific molecular markers have also been identified in several crops including wheat (Roder et al. 1998; Pestsova et al. 2000; Gupta et al. 2002). Among the different types of molecular markers, microsatellites or simple sequence repeats (SSRs)/simple

tandem repeats (STRs) have become the markers of choice due to their abundance and ubiquitous distribution in both the nuclear and organellar genomes. It has also been shown that SSRs are frequent in both repetitive and unique sequences of the nuclear genome (for a review, see Gupta and Varshney 2000; Morgante et al. 2002).

SSR markers can also be used for distinguishing related genomes, since often homoeoloci in related genomes that are characteristic of RFLPs are infrequent among SSR loci. Wheat SSRs, therefore, facilitated a variety of studies that involved mapping and gene tagging (for review see Gupta et al. 1999; Varshney et al. 2000b; Varshney et al. 2001; Gupta et al. 2002), genetic diversity (Plaschke et al. 1995; Roder et al. 1995; Prasad et al. 2000), in-gel hybridization (Varshney et al. 1998) and in-situ hybridization (Cuadrado and Schwararchzer 1998). They have also been used for studying the role of natural selection in differentiation (Li et al. 1999, 2000). A proportion of SSRs derived from wheat and *Ae. tauschii* have also been used in related species containing A, B and D genomes (Sourdille et al. 2001; Guyomarc'h et al. 2002). In the present study, we examined the distribution and organization of SSRs in 14 different diploid and polyploid species of *Triticum-Aegilops* group through in-gel hybridization and PCR based amplification of SSR loci. This facilitated an assessment of the potential of SSR oligonucleotide probes and SSR primers for a study of molecular marker- based studies in the tribe Triticeae.

Materials and methods

Plant material: Seed of 19 accessions belonging to 14 species from *Triticum-Aegilops* group was procured from PAU, Ludhiana (India) and used for the present study (see Table 1).

Table 1. Summary of the results of amplification of DNA by 15 wheat microsatellite primers in 14 different species from *Triticum-Aegilops* group. Accession numbers of different species used are given in parentheses against the names of the species in the footnote.

Primer designation	Species and their genome constitution													
	Ae.ta (D)	*Ae.sp* (S)	*Ae.bi* (S^b)	*Ae.lo* (S^l)	*Ae.sh* (S^{sh})	*T. ur* (A)	*T. bo* (A)	*Ae.ko* (US)	*Ae.ov* (UM)	*Ae.ti* (UC)	*Ae.cy* (CD)	*Ae.cr* (DM)	*T. di* (AB)	*T. ae* (ABD)
WMC243	+	+	-	+	+	+	-	+	-	-	+	+	+	+
WMC 415	+	+	+	+	+	+	+	+	+	-	+	+	+	+
WMC 35	+	+	+	+	+	-	-	+	-	-	+	+	+	+
WMC 404	+	+	+	+	+	-	-	+	-	+	+	+	+	+
WMC 120	-	-	-	+	-	+	+	-	-	-	-	+	+	+
WMC 256	+	+	-	-	-	+	+	+	+	+	+	+	+	+
WMC 25	+	+	+	+	+	+	+	+	+	+	+	+	+	+
WMC 254	-	+	+	+	+	+	-	-	-	-	-	-	+	+
WMC 245	+	+	-	-	-	-	-	-	+	-	+	+	-	+
WMC 44	-	-	+	+	+	-	-	-	-	-	-	-	+	+
WMC 250	-	-	-	-	-	+	-	+	+	+	+	-	-	+
WMC 263	-	+	+	+	-	+	-	+	+	+	+	+	+	+
WMC 265	+	-	+	+	-	+	+	+	+	+	+	+	+	+
WMC 149	-	+	+	+	+	+	+	+	+	+	+	+	+	+
WMC 284	+	+	-	-	-	+	-	-	-	+	+	-	+	+

Ae. ta= Ae. tauschii (3727), *Ae. sp= Ae. speltoides* (743), *Ae. bi= Ae. bicornis* (3997), *Ae. lo= Ae longissima* (3506, 3770), *Ae. sh= Ae. sharonensis* (3513), *T. ur= T. urartu* (5324, 5338), *T. bo=T. boeoticum* (4856, 4866), *Ae. ko= Ae. kotschyi* (753), *Ae. ov= Ae. ovata* (3548), *Ae. ti= Ae. triuncialis* (750), *Ae. cy= Ae. cylindrica* (3472, 3486), *Ae. cr= Ae. crassa* (3509), *T. di= T. dicoccoides* (4634, 4640), *T. ae= T. aestivum* (anonymous)
+: Successful amplification, -: No amplification

SSR probes: Four synthetic SSR oligonucleotide probes including three tri-nucleotide repeats {$(CAC)_5$, $(CAG)_5$, $(TCC)_5$} and one di-nucleotide repeat {$(GA)_8$} were used for in-gel hybridization.

SSR primers: For 13 of the 15 SSR primers, the details on sequences, repeat motifs, locus designations and the expected product sizes are published elsewhere (Prasad et al. 2000; Varshney et al. 2000a). The remaining two primer pairs belonging to WMC404 and WMC415 were developed by Romestant (RAGT, France) and are proprietary in nature (mromestant@ragt.fr).

DNA isolation, digestion, electrophoresis, in-gel hybridization and PCR: The details of methods for DNA isolation, restriction digestion (using *Alu*I and *Hinf*I, *Hin*dIII, *Dra*I and *Eco*RI), electrophoresis, in-gel hybridization, PCR, and PAGE (polyacrylamide gel electrophoresis) are described elsewhere (Varshney et al. 1998; Prasad et al. 2000).

Statistical analysis: Polymorphic information content (PIC) for each SSR locus was calculated using the formula: PIC = $1-\Sigma(P_i)^2$, where P_i is the frequency of i^{th} allele at a locus (Botstein et al. 1980).

Results and discussion

In-gel hybridization using synthetic oligonueleotide (SSR) probes: Thirteen (13) probe-enzyme combinations (involving four synthetic SSR probes each in combination with 2-4 enzymes) were used for in-gel hybridization. SSR sequences, homologous to each of the four synthetic probes, were available in all the 19 accessions belonging to 14 different species from *Triticum-Aegilops* group. This is in agreement with earlier reports suggesting ubiquitous distribution of SSRs in different plant and animal species (for a review, see Gupta et al. 1996). No intraspecific polymorphism was detected in the present study. Even in an earlier study, we found that such intraspecific polymorphism was rather rare (Varshney et al. 1998). This suggested that in-gel hybridization involving synthetic SSRs as probes may not be a suitable technique for detection of intraspecific polymorphism in members of the tribe Triticeae; however, in several other plant species, a high level of intraspecific polymorphism was actually observed (see Weising et al. 1995).

In the present study, 12 of the 13 probe-enzyme combinations hybridized with 1-4 bands against a clear background while the remaining solitary probe-enzyme combination {$(GA)_8$-*Eco*RI} gave bands on in-lane smear background. In our earlier study on bread wheat also, out of the 142 probe-enzyme combinations, bands were available in 107 cases and a smear with high in-lane background was observed in 35 cases (Varshney et al. 1998). Using 12 different probe-enzyme combinations, a total of 20 bands were visualized (1-4 fragments per probe-enzyme combination) in 14 species. Only hexaploid wheat (*T. aestivum*, ABD genomes) and no other species gave all the bands, so that there was no unique fragment which wag absent in wheat and present in another species, although as many as four additional genomes occurred in these alien species. In the remaining 13 individual species, a total of 5 to 15 bands were visualized with all the probe-enzyme combinations considered together. In 9 of the above 12 probe-enzyme combinations, a solitary band of >23 kb was observed in 9 of the 14 species examined; in the five remaining species (*Ae. kotschyii, Ae. sharonensis, Ae. cylindrica, T. boeoticum* and *T. urartu*), this band was not observed in a few of these 9 probe-enzyme combinations, but other bands were available. In the remaining three of the 12 probe-enzyme combinations {*Hin*dIII-$(CAC)_5$ *Alu*I-$(CAC)_5$ *Alu*I-$(CAG)_5$}, in accessions of several species, this characteristic band of>23 kb was observed in association with other bands ranging in size from 7

kb to 0.4 kb; in few species, however, one or more bands of smaller size were present. Similar high molecular weight bands (>21 kb to 30 kb) were also reported in earlier studies on barley, sugar beet and wheat (Beyermarin et al. 1992; Schmidt and Heslop-Harrison 1996; Varshney et al. 1998). The high molecular weight band (>23 kb) that was visualized with different probe- enzyme combinations, may represent same or different repeat sequences of >23 kb harboring SSRs. In a recent study in barley, characterization of clones harboring dinucleotide SSR repeats revealed that a high percentage of such clones are associated with retrotransposon-like and other dispersed repetitive elements (Ramsay et al. 1999). SSRs are, however. now known to be frequent in unique DNA sequences also (Morgante et al. 2002).

The above interspecific polymorphism among the 14 species of *Triticum-Aegilops* group proved helpful in discriminating not only species with different ploidy levels and possessing different genomes, but also those possessing similar or very closely related genomes. For instance, the patterns due to different probe-enzyme combinations discriminated among hexaploid wheat (ABD genomes), tetraploid wheat (AB genomes) and the two diploid species, *T. urartu* (A genome) and *Ae. tauschii* (D genome),which are the progenitors of hexaploid wheat. It was also noticed that three bands that were always present in hexaploid wheat (ABD genomes) were absent in *Ae. tauschii* (D genome) and one of these fragments was also absent in several other species including tetraploid wheat (AB genomes). Similarly, the trinucleotide probes $(CAC)_5$, and $(CAG)_5$ in combination with *Hin*dIII detected three bands that were common in *T. urartu* (A genome), the tetraploid wheat (AB genomes) and the hexaploid wheat (ABD genomes) suggesting that these bands could be specific to the A genome. However, these bands were not available in another A genome diploid species *T. boeoticum,* suggesting differentiation even within A genome during the course of evolution. Sometimes, a single probe-enzyme combination {$(CAC)_5$- *Hin*dIII} also proved adequate to discriminate between the three tetraploid species including *Ae. kotschyii (US), Ae. ovata* (UM) and *Ae. triuncialis* (UC) sharing U genome. Further, the trinucleotide probe $(CAC)_5$ in combination with four restriction enzymes (*Hin*dIII, *Eco*RI, *Hinf*I and *Alu*I) gave characteristic banding patterns that were adequate to discriminate the four S-genome containing species (*Ae. speltoides*, *Ae. bicornis*, *Ae. longissima* and *Ae. sharonensis*).

PCR amplification using SSR primers: The results of PCR amplification with 15 wheat SSR primers are summarized in Table 1 and a representative amplification profile is shown in Fig.1. In hexaploid wheat, 12 of these primer pairs amplified each a single locus, while each of the three remaining primer pairs amplified two different loci located on two different chromosomes. The chromosomes carrying the above 18 different loci were distributed in all the three genomes of hexaploid wheat (Prasad et al. 2000; Varshney et al. 2000a).

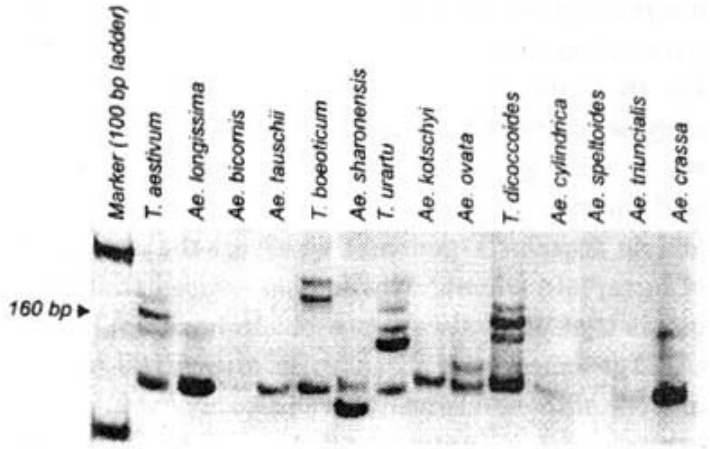

Fig. 1. DNA amplification patterns in accessions of 14 species of *Triticum-Aegilops* group using a microsatellite primer pair WMC 120 derived from hexaploid wheat. The arrowhead indicates the expected fragment size in hexaploid wheat (*T. aestivum*). Fragments in the expected size range were available in only six (*T. aestivum, Ae. longissima, T. boeoticum, T. urartu, T. dicoccoides and Ae. crassa*) of the 14 species. No fragment in the expected size range was available in the remaining eight species. Additional nonspecific fragments of varying sizes were observed in each of the 14 species.

It may be noted that an individual wheat SSR primer pair amplified loci in 2 to 10 of the 14 species, which also included species containing genomes other than those present in bread wheat (A, B and D). Therefore, each of the wheat SSR seems to be derived from a corresponding SSR in the presumed ancestral Triticeae genome and is conserved in several diploid and tetraploid species of Triticeae having varied genomic constitutions. In the past, a high proportion of SSRs developed. from *T. aestivum* and *Ae. tauschii* were also shown to be functional in related diploid species containing either A or B or D genome confirming their transferability and conservation across Triticeae species (Sourdille et al. 2001; Guyomarc'h et al. 2002). Such conserved wheat SSRs may be used in studies on polymorphism, genetic diversity, gene mapping and synteny conservation across different species of Triticeae.

The cases of failures of amplification of microsatellite loci in some of the species examined during the present study were classified as null alleles. It was assumed that such null alleles might have resulted either due to modification of the primer-binding site or to the loss of corresponding SSR during the course of evolution. Fourteen of the 15 microsatellite primer pairs detected null alleles in one or more (1 to 10) different species containing different genomes. Interestingly, some of the

primer pairs that amplified loci on chromosomes of A/B genomes of bread wheat, were found to amplify loci in *Ae. tauschii*, which is the diploid progenitor of D genome. Similarly, there were SSR loci that belonged to the D genome of bread wheat, but were amplified in diploid species with an A genome (*T. urartu*) (Tables 1 and 2). This means that during the evolution of bread wheat, some of the SSR loci found in a particular genome of a diploid progenitor species were either lost, or carried a mutation in the primer binding site leading to the origin of a null allele. In several recent studies involving artificially synthesized and naturally occurring allopolyploids of Triticeae, it was shown that allopolyploidization either induced elimination or caused cytosine methylation of certain unique and repetitive DNA sequences (Ozkan et al. 2001; Shaked et al. 2001). However, in studies conducted in hexaploid wheat and related species, Southern hybridization with probes carrying sequences corresponding to SSR primers gave a positive signal in related species having null alleles. This suggested that the locus specificity of SSRs in bread wheat probably originated due to mutations in primer binding sites rather than due to loss of SSRs themselves in related genomes during polyploidization (Guyomarc'h et al. 2002).

Table 2. Microsatellite primers used along with locus designation, number of alleles and PIC values

Primer designation	Locus designation	No. of alleles	PIC
WMC25	*Xwmc25-2D*	11	0.616
WMC35	*Xwmc35-4B*	6	0.528
WMC44	*Xwmc44-1B*	4	0.386
WMC120	*Xwmc120-1A*	7	0.562
WMC149	*Xwmc149-2B*	10	0.780
WMC243	*Xwmc243-3A*	10	0.576
WMC245	*Xwmc245-2D*	8	0.466
WMC250	*Xwmc250-6D*	3	0.406
WMC254	*Xwmc254-4B*	7	0.614
WMC256	*Xwmc256-6A*	4	0.556†
	Xwmc256-6D	6	
WMC263	*Xwmc263-6B*	4	0.752
WMC264	*Xwmc264-3A*	10	0.615
WMC265	*Xwmc265-7D*	4	0.650
WMC404	*Xwmc404-1B*	7	0.562†
	Xwmc404-1D	4	
WMC415	*Xwmc415-5A*	8	0.643†
	Xwmc415-5B	5	

† PIC was estimated using alleles from both the loci

The average number of alleles per locus in 14 species was 6.6 with a range of 4 to 11 (Table 2). The maximum number of 11 alleles (120 bp to 177 bp) was observed at *Xwmc25-2D* carrying (GT)n. The polymorphic information content (PIC) varied from 0.386 for WMC44 to 0.780 for WMC149 (Table 2). The average number of alleles in the present study was slightly lower than an average of 7.5 alleles per locus reported by us within bread wheat in an earlier study, where 20 WMC SSRs were tried on 55 genotypes (Prasad et al. 2000). We believe that fewer alleles per locus in the present study could be due to small sample, so that a bigger sample having many more species each represented by several accessions should resolve many more alleles on each SSR locus.

In the present study no single primer pair was adequate to discriminate all the 14 species studied. However, two different pairs of SSRs, one consisting of WMC243 and WMC415, and the other consisting of WMC35 and WMC404, each discriminated all the 14 species. In our earlier study on bread wheat it was shown that 12 microsatellite markers discriminated. 48 genotypes out of 55 genotypes studied (Prasad et al. 2000). The present study thus suggested that in addition to their use in discriminating accessions belonging to a particular species like bread wheat, SSR markers can also be used for discriminating different species of Triticeae.

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