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Applicability of inter-simple sequence repeat polymorphisms in wheat for use as DNA markers in comparison to RFLP and RAPD markers

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Abstract Inter-simple sequence repeat polymorphic DNA (ISSR) was evaluated for its applicability as a genetic marker system in wheat. PCR was carried out with primers that annealed to simple sequence repeats. The resultant products were subjected to agarose-gel electrophoresis, and the banding patterns were compared among six wheat accessions containing diploid, tetraploid, and hexaploid members. Out of 100 examined, 33 primers produced distinguishable as well as polymorphic bands in each of the six accessions. Although most of the primers that gave distinct bands (30 primers out of 33) contained dinucleotide repeats, each of the primers with tri-, tetra-, and penta-nucleotide motifs also yielded discrete bands. Primers based on $(AC)_n$ repeats gave the most polymorphic bands. In total, 224 polymorphic bands were found in the comparison between Einkorn wheats whereas, on the average, 120 polymorphic bands were detected between common wheats. ISSR primers produced several times more information than RAPD markers. The extent of band polymorphism was similar to that of RFLP markers, and greater than that of RAPDs. The genetic relationships of wheat accessions estimated by the polymorphism of ISSR markers were identical with those inferred by RFLP and RAPD markers, indicating the reliability of ISSR markers for estimation of genotypes. These polymorphic bands are potential candidates as novel markers for use in linkage-map construction in wheat. The characteristic features of ISSR markers, i.e. polymorphism, generation of information and ease of handling, suggest their applicability to the analysis of genotypes as well as to the construction of PCR-based genome maps of wheats.

Key words Microsatellite DNA · RAPD · PCR · Markers · Wheats

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Introduction

Simple sequence repeats (SSRs), also called microsatellites, are tandemly arranged arrays of short repeats comprising a few nucleotides (Hamada et al. 1982; Tautz 1989; Weber and May 1989), and there is estimated to be a total of 5×10^3 to 3×10^5 microsatellites per plant genome (Condit and Hubbell 1991). SSRs are known to be hypervariable and distributed throughout the genome even in plants (Lagercrantz et al. 1993; Morgante and Olivieri 1993; Senior and Heun 1993; Wu and Tanksley 1993; Bell and Ecker 1994). Furthermore, microsatellite regions can be amplified by the polymerase chain reaction (PCR) using flanking primers (e.g. Saghai Maroof et al. 1994). Because of their hypervariability and ease of handling in comparison to restriction fragment length polymorphisms (RFLPs), SSRs can provide a powerful tool to construct linkage maps (Senior and Heun 1993; Wu and Tanksley 1993; Bell and Ecker 1994). In contrast to the SSR-PCR method that amplifies with primers located on the flanking single-copy DNA, microsatellite-anchored primers that anneal to an SSR region can amplify regions between adjacent SSRs (inter-simple sequence repeats; ISSR). The ISSR markers are also useful for detecting genetic polymorphisms (Zietkiewicz et al. 1994). Thus, ISSR DNA has been proposed as a new source of genetic marker that overcomes many of the technical limitations of RFLP (Rafalski et al. 1991) and RAPD (Devos and Gale 1992) analyses, even in plants (Tsumura et al. 1996). In the present paper, we evaluate the

applicability of ISSR as a source of genetic markers in wheat.

Materials and methods

Plant materials

Diploid, tetraploid, and hexaploid wheat species were used in the present investigation, as shown in Table 1. These seed stocks are maintained at the genetic resource center of the Kihara Institute for Biological Research (KIBR), Yokohama City University.

DNA isolation and Southern hybridization

Total DNAs were extracted from green leaves by the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson 1980). Ten-microgram aliquots of the DNAs were digested with *Bam*HI and *Hin*dIII. A total of 46 TAG clones (Liu et al. 1990) were used as probes. Southern hybridization was carried out on the digested DNAs with DNA probes as previously described (Ogihara et al. 1994).

PCR amplification of ISSRs

One-hundred SSR primers from the University of British Columbia Biotechnology Laboratory (UBCBL) primer set #9 were tested for PCR amplification. These primers were mostly 16- to 17-mers; 33 primers that gave clearly distinguishable bands between two wheat accessions (Table 2) were then used for further experiments. Amplifications were carried out in 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM dNTPs, 0.1% Triton X-100, 2% formamide, 200 nM primer, 1 unit of *Taq* polymerase (Promega Co. Ltd.), and 5 ng of genomic DNA per 25-µl reaction. Initial denaturation was for 7 min at 94°C, followed by 45 cycles of 30 s at 94°C, 45 s at 52°C, 2 min at 72°C, and a final 7-min extension at 72°C.

RAPD amplification

Two-hundred combinations of two primers randomly selected from UBCBL primer sets #1 and #2 were examined for amplification of RAPD sequences; 25 combinations of primers (Table 3) that showed relatively clear band patterns were used for further investigation. PCR amplifications were performed in 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM dNTPs, 0.1% Triton X-100, 200 nM of each primer, 1 unit of *Taq* polymerase (Promega Co. Ltd.), and 50 ng of genomic DNA per 25-µl reaction. Initial denaturation was for 3 min at 94°C, followed by 45 cycles of 1 min at 94°C, 1 min at 36°C, 2 min at 72°C, and a final 7-min extension at 72°C.

PCR reactions was performed in a PCT-100 thermcycler (MJ Research). PCR products were electrophoresed on 2% agarose gels, and detected by staining with ethidium bromide.

Table 2 ISSR primers that gave distinguishable bands between two wheat species

UBC no.[a]	Sequence (5′–3′)	No. shared bands between Mn and Bt	No. discrete bands
807	AGAGAGAGAGAGAGAGT	2	10
808	AGAGAGAGAGAGAGAGC	8	3
810	GAGAGAGAGAGAGAGAT	8	4
811	GAGAGAGAGAGAGAGAC	10	5
815	CTCTCTCTCTCTCTCTG	10	3
818	CACACACACACACACAG	10	3
819	GTGTGTGTGTGTGTGTA	3	4
822	TCTCTCTCTCTCTCTCA	7	1
823	TCTCTCTCTCTCTCTCC	10	5
824	TCTCTCTCTCTCTCTCG	6	5
825	ACACACACACACACACT	6	5
826	ACACACACACACACACC	16	1
827	ACACACACACACACACG	6	8
830	TGTGTGTGTGTGTGTGG	6	7
834	AGAGAGAGAGAGAGAGYT	12	4
835	AGAGAGAGAGAGAGAGYC	4	6
840	GAGAGAGAGAGAGAGAYT	14	6
841	GAGAGAGAGAGAGAGAYC	12	4
842	GAGAGAGAGAGAGAGA	8	8
845	CTCTCTCTCTCTCTCTRG	8	10
847	CACACACACACACACARC	6	11
848	CACACACACACACACARG	8	4
849	GTGTGTGTGTGTGTGTYA	4	16
851	GTGTGTGTGTGTGTGTYG	12	16
853	TCTCTCTCTCTCTCTCRT	6	15
855	ACACACACACACACACYT	10	12
856	ACACACACACACACACYA	24	3
857	ACACACACACACACACYG	14	12
859	TGTGTGTGTGTGTGTGRC	8	8
860	TGTGTGTGTGTGTGTGRA	10	4
864	ATGATGATGATGATGATG	8	3
873	GACAGACAGACAGACA	14	6
880	GGAGAGGAGAGGAGA	8	12
Total		298	224

[a] Primers were supplied from University of British Columbia set #9
R, purine; Y, pyrimidine; Mn, *Triticum monococcum* Early mutant; Bt, *T. boeoticum* ssp. *boeoticum*

Table 1 Wheat accessions used in the present investigation

No.	Abbrev.	Accessions	Genome formula	Collection no. of KIBR[a]
1	Mn	*Triticum monococcum* Early mutant	AA	KT3-5
2	Bt	*T. boeoticum* ssp. *boeoticum*	AA	KT1-1
3	Dc	*T. dicoccum* var. *farrum*	AABB	KT7-2
4	CS	*T. aestivum* cv Chinese Spring	AABBDD	KT20-3
5	N26	*T. aestivum* cv Norin 26	AABBDD	KT20-29
6	Splt	*T. spelta* var. *duhamelianum*	AABBDD	KT19-1

[a] KIBR: Kihara Institute for Biological Research, Yokohama City University

Table 3 Combinations of RAPD primers that produced distinguishable bands in wheat species

UBC no.[a]	Sequence (5′–3′)	No. informative bands in Mn-Bt
1/2	CCTGGGCTTC/CCTGGGCTTG	3
29/30	CCGGCCTTAC/CCGGCCTTAG	1
39/40	TTAACCGGGC/TTACCTGGGC	6
49/50	TTCCCCGAGC/TTCCCCGCGC	5
51/52	CTACCCGTGC/TTCCCGGAGC	4
53/54	CTCCCTGAGC/GTCCCAGAGC	4
55/56	TCCCTCGTGC/TGCCCCGAGC	10
73/74	GGGCACGCGA/GAGCACCTGA	1
75/76	GAGGTCCAGA/GAGCACCAGT	3
2/52	CCTGGGCTTG/TTCCCGGAGC	4
12/62	CCTGGGTCCA/TTCCCCGTCG	6
13/63	CCTGGGTGGA/TTCCCCGCCC	5
23/73	CCCGCCTTCC/GGGCACGCGA	3
34/84	CCGGCCCCAA/GGGCGCGAGT	3
40/90	TTACCTGGGC/GGGGGTTAGG	3
50/100	TTCCCCGCGC/ATCGGGTCCG	3
117/118	TTAGCGGTCT/CCCGTTTTGT	1
143/144	TCGCAGAACG/AGAGGGTTCT	1
189/190	TGCTAGCCTC/AGAATCCGCC	1
111/161	AGTAGACGGG/CGTTATCTCG	2
118/168	CCCGTTTTGT/CTAGATGTGC	3
122/172	GTAGACGAGC/ACCGTCGTAG	2
133/183	GGAAACCTCT/CGTGATTGCT	5
136/186	TACGTCTTGC/GTGCGTCGCT	4
141/191	ATCCTGTTCG/CGATGGCTTT	5
Total		88

[a] A pair of primers obtained from the University of British Columbia were placed in one tube for the PCR reaction

Data analysis

Pair-wise comparisons of accessions, based on the presence or absence of unique and shared fragments produced by PCR amplifications and Southern hybridizations, were used to generate a simple matching coefficient (S_{sm}). The genetic distance (d) was calculated as:

$$d = \sqrt{1 - S_{sm}}$$

and was used to construct a dendrogram by an unweighted pair-group method with arithmetical averages (UPGMA; Sneath and Sokal 1973).

Results

ISSR band patterns in wheat species

Out of 100 primers examined, 33 of them gave distinguishable bands after PCR amplification. Typical band patterns on 2.0% agarose are presented in Fig. 1A. Most primers (30 out of 33) annealed to the dinucleotide repeats, whereas each of the remaining three annealed to the tri-, tetra- and penta-nucleotide repeats, respectively (Table 2). PCR amplification using ISSR sequences as the primer produced 7.6 bands, on average, for diploid wheat, 9.0 for tetraploids, and 10.8 for hexaploid. Amplified DNA fragments varied in size from approximately 100 bp to 2000 bp (Fig. 1A). The

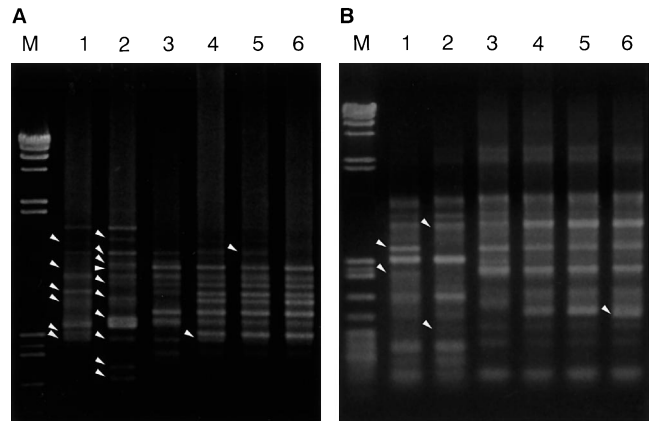

Fig. 1 PCR-amplified fragment patterns of six wheat accessions generated by primers UBC851 for ISSR (**A**), and UBC2/52 for RAPD (**B**). *Numbers 1–6* stand for the wheat accessions listed in Table 1. *M* indicates the molecular-size marker. Polymorphic bands in comparison between diploids (*nos. 1 and 2*), and hexaploids (*nos. 4–6*) are marked as *white triangles*

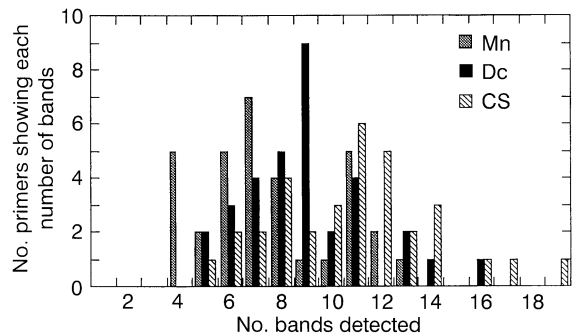

Fig. 2 Frequency of primers with each number of bands after PCR amplification at three ploidy levels of wheats. ▩ represents the number of primers that produced each number of bands in *T. monococcum* (diploid), ■ stands for *T. dicoccum* (tetraploid), and ▧ for *T. aestivum* cv Chinese Spring (hexaploid)

distribution patterns of the number of bands produced in each of the three ploidy levels are shown in Fig. 2. Although the number of bands increased along with the increase in ploidy level, the mean number of bands for each ploidy was not in proportion to the ploidy level (Fig. 2). It should be emphasized that all primers that gave amplification products included the polymorphic bands at all ploidy levels (Table 4). More than half of the simple dinucleotide repeat primers yielded discrete bands. On the other hand, only a few primers of tri-, tetra- and penta-nucleotides, and none of the other categories of primers, produced clear bands, suggesting that the intervening regions adjacent to the dinucleotide repeats were co-amplified by the PCR reaction. Dinucleotide repeats [$(AC)_8$] produced the most polymorphic bands in Einkorn wheats (Mn and Bt), whereas tetranucleotides yielded hypervariable fragments in common wheats (Table 4). The number of polymorphic

Table 4 Number of polymorphic bands detected by ISSR-PCR method in wheat lines. Subscripts attached to the parentheses indicate repetitive number of dinucleotides

	Simple sequence repeats									
	$(AG)_8$	$(GA)_8$	$(AC)_8$	$(CA)_8$	$(CT)_8$	$(TC)_8$	$(TG)_8$	$(GT)_8$	Others[a]	Total
Total no. primers	6	6	6	6	6	6	6	6	21	69
No. primers detecting polymorphisms	4	5	6	3	2	4	3	3	3	33
No. polymorphic bands between Mn-Bt comparison	23	27	41	18	37	19	25	13	21	224
Ave. no. polymorphic bands	5.8	5.4	6.8	6.0	18.5	4.8	13.0	3.3	7.0	6.8
No. polymorphic bands in										
CS-N26	11	8	14	5	5	7	2	4	25	81
CS-Splt	16	21	13	9	8	19	11	11	26	134
N26-Splt	17	21	21	10	7	22	11	9	25	143
Ave. no. polymorphic bands	3.7	3.3	2.6	2.7	3.3	4.0	2.7	2.7	8.4	3.7

[a] $(ATG)_6$, $(GACA)_4$, $(GGAGA)_3$ sequence repeats are included together

bands was greater in the comparison of Einkorn wheats than in that of hexaploid wheats, indicating the genetic diversity of the two Einkorn wheats (see below). In total, 224 polymorphic bands were detected with 33 ISSR primers in the comparison between *T. monococcum* and *T. boeoticum*, 81 between *T. aestivum* cv Chinese Spring and Norin 26, 134 between Chinese Spring and *T. spelta*, and 143 between Norin 26 and *T. spelta*. The frequency of polymorphic bands was approximately 40% in Einkorn wheats, and less than 20% in hexaploid wheats (Table 5). These polymorphic bands are candidates for DNA markers for use in the construction of genetic linkage maps in diploid and hexaploid wheat lines.

Table 5 Number of ISSR-bands showing polymorphism (above the diagonal) and the percentages of differential fragments (below the diagonal) between all six pairs of wheat lines. Total number of bands in each accession are given in parentheses. For abbreviations of wheat accessions, see Table 1

Wheat accessions	Mn	Bt	Dc	CS	N26	Splt
Mn	(252)	224	407	479	486	459
Bt	42.9	(270)	421	475	480	457
Dc	74.1	74.3	(297)	254	255	214
CS	78.7	75.8	38.8	(357)	81	134
N26	79.4	76.2	38.8	11.3	(360)	143
Splt	76.6	74.1	33.2	19.0	20.2	(347)

RAPD fragment patterns

Two-hundred combinations of a pair of 10-mer primers (Monna et al. 1994) were tested in wheat species to examine RAPD patterns; 25 combinations of primers produced informative bands (Table 4), consequently these primers were used for further experiments. The total numbers of bands produced after the PCR reaction were similar to those for the ISSR bands, approximately 220 bands in diploid wheats, 240 in tetraploid wheat, and 260 in hexaploid wheats (Fig. 1B and Table 6). The number of bands was not proportional to the increase in ploidy level, probably because of their redundancy. In comparison to those of ISSR and RFLP bands, fewer polymorphisms of RAPD bands were detected between the pairs of wheat lines examined. The frequency of polymorphic bands was approximately 20% in the comparison between *T. monococcum* and *T. boeoticum*, and less than 10% between hexaploid wheats (Table 6), being less than half those of ISSRs.

Table 6 Number of RAPD-bands showing polymorphism (above the diagonal) and the percentages of differential fragments (below the diagonal) between all six pairs of wheat accessions. Total number of bands in each accession are given in parentheses. For abbreviations of wheat accessions, see Table 1.

Wheat accessions	Mn	Bt	Dc	CS	N26	Splt
Mn	(223)	88	283	303	303	302
Bt	19.1	(237)	291	307	309	308
Dc	60.6	60.5	(244)	114	122	103
CS	62.7	61.8	22.6	(260)	36	51
N26	62.7	62.2	24.2	6.9	(260)	49
Splt	62.1	61.6	20.3	9.8	9.4	(263)

RFLPs in the six wheat accessions

Of the 92 probe-enzyme combinations (46 probes × 2 enzymes) examined, 88 produced informative autoradiograms in the six wheat accessions. The total number of RFLP bands produced, and the numbers of polymorphic bands between all six pairs of wheat accessions were similar to those of ISSR bands (data not shown). Our results confirmed the previous data reported by Liu et al. (1990) and Takumi et al. (1993).

Cluster analysis

Based on the data given in Tables 5 and 6, and those for RFLPs, cluster analysis was performed to generate dendrograms constructed by each of three markers, showing the genetic relationships among the six wheat accessions (Fig. 3). The genetic relationships of the six wheat accessions estimated by the use of the three DNA markers were the same; CS and N26 were most closely related. Splt was relatively distant from the CS-N26 complex. Dc (tetraploid) was distant from the hexaploid wheat group. Mn and Bt (both diploids) were distant from each other. The genetic distance between Einkorn wheats was similar to that of tetraploid-hexaploids, indicating the genetic divergence between cultivated and wild Einkorn wheats. The ISSR markers were more variable than the RAPD markers, but similar to the RFLPs, in terms of the presence/absence of bands (Fig. 3).

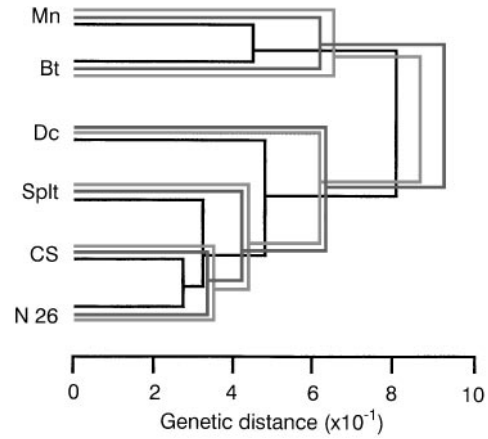

Fig. 3 Dendrograms showing genetic relationships among the six wheat accessions constructed by the UPGMA method based on ISSR (—), RAPD (—) and RFLP (—) markers. For abbreviations of wheat accessions, see Table 1. The scale indicates the genetic distance

Discussion

ISSRs in wheat species

We examined the optimal condition for detecting ISSRs in wheat species, and set up the following condition: 2% formamide is critical to avoid a high background and smearing of the PCR products. Additionally, 5 ng of template wheat DNA yielded stable and reproducible PCR fragments, although higher amounts of templates (about 50 ng) have often been used (Saghai Maroof et al. 1994; Zietkiewicz et al. 1994). A total of 45 cycles of PCR amplification gave bands of higher intensity and stability than did a lower number of cycles, e.g. 25 or 30. The annealing temperature of 52°C gave the best banding patterns. We tried an annealing temperature of 37°C for $(AT)_n$ primers, but these primers did not produce the distinct bands at all temperatures examined (Gupta et al. 1994).

Although the dinucleotide repeat primers revealed the most useful bands (30 primers gave discrete bands, as shown in Tables 2 and 4), tri-, tetra-, and pentanucleotide primers produced distinguishable bands in wheat species, in contrast to the case for conifers (Tsumura et al. 1996). The abundant $(AG)_n$ repeat sequences were most useful as in the case of conifers, but $(CT)_n$ and $(GT)_n$ also proved useful in wheats (Table 4). These results suggest the unique architecture of the wheat genome, it contains abundant $(AT)_n$ repeats (Wang et al. 1994). It should be emphasized that ISSR primers that gave discrete bands always produced polymorphic bands, even between two strains of the same species, namely *T. aestivum* (Table 4). Once the PCR conditions were set up, the higher annealing temperature and the length of ISSR primers, compared with those of RAPD primers, produced more reliable and reproducible bands. This indicates that ISSRs are superior to the RAPD system in terms of reliability.

Variability of ISSRs in wheat species

Although it is well recognized that RFLPs are suitable to estimate phylogenetic relationships among related taxa, the detection of RFLPs is both laborious and tedious, especially in plants with a great genome size such as wheat, and more than 40 RFLP markers are required to estimate accurate genetic relationships (Liu et al. 1990). On the other hand, RAPD markers are more easily handled, and thus are becoming more desirable to estimate genetic relationships among related taxa. Analysis of allelic variations in RAPD markers, however, is difficult, because most RAPD loci can not be mapped even in diploid chromosomes (e.g. Devos and Gale 1992). Taking these situations into account, the variability of ISSR markers in wheat species has been investigated. Thirty three primers yielded discrete, as well as polymorphic, bands out of a total of 100 primers examined. After PCR amplification with the ISSR primers, approximately 260 bands were produced in diploid wheats, 300 bands in tetraploids, and 350 bands in hexaploids (Table 5). Moreover, out of the

bands produced, 224 were polymorphic in the comparison between Mn and Bt, 81 between CS and N26, 134 between CS and Splt, and 143 between N26 and Splt. Although RAPD primers gave similar numbers of total bands with 200 combinations of primer pairs, i.e. 220 bands in diploid wheats, 240 in tetraploids and 260 in hexaploids (Table 6), out of the bands produced, only 88 polymorphisms in the comparison between Mn and Bt, 36 between CS and N26, 51 between CS and Splt, and 49 between N26 and Splt were detected. This result clearly indicates the ISSRs are highly polymorphic and especially informative for estimating genetic relationships. But, since genetic relationships estimated by the repetitive markers are often skewed from the real ones, the dendrogram constructed by use of the ISSR markers was compared with that constructed with the RFLP or RAPD markers. In the present investigation, 46 TAG markers, which are considered to be sufficient to estimate accurate genetic relationships among wheat accessions (Liu et al. 1990), were used; under these conditions, approximately 240 RFLP bands were detected in diploid wheats, 320 bands in tetraploids, and 380 bands in hexaploid. The number of total bands produced and the number of polymorphic bands in comparisons between each pair of wheat accessions were consistent with the case of ISSRs. Genetic relationships among the six wheat accessions estimated by the three different kinds of DNA markers were identical, as shown in Fig. 3. It should be noted that the genetic distance values among them calculated by the ISSR markers were almost same as those obtained with the RFLP markers. This strongly suggests that in the estimation of genetic relationships ISSR markers provide the same level of accuracy as RFLP markers.

In conclusion, the ISSR markers were easily handled, reliable once optimized, informative with one agarose-gel electrophoresis, highly polymorphic even for intraspecific variations, and reflected real genetic relationships among wheat accessions, indicating them to be quite useful markers to characterize wheat strains and to be applicable for use with other plant groups. The distribution of the ISSRs throughout the wheat chromosomes will now be investigated by mapping the ISSR markers.

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