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Studies of the transferability of microsatellites derived from *Triticum tauschii* to hexaploid wheat and to diploid related species using amplification, hybridization and sequence comparisons

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Abstract Hexaploid wheat (*Triticum aestivum* L em Thell) is derived from a complex hybridization procedure involving three diploid species carrying the A, B and D genomes, respectively. We recently isolated microsatellites from a *T. tauschii* library enriched for various motifs and evaluated the transferability of these markers to several diploid species carrying the A, B or D genomes. All of the primer pairs amplifying more than one locus on bread wheat and half of those giving D-genome-specific loci gave an amplification product on A-and/or B-diploid species. All of the markers giving a single amplification product for *T. tauschii* and no amplification on the other diploid species were D-genome-specific at the hexaploid level. The non-specific microsatellite markers (which gave an amplification product on diploid species carrying the A, B or D genome) gave either a complex amplification pattern on bread wheat (with several bands) or generated a single band which mapped to the D genome. Southern blot hybridizations with probes corresponding to the microsatellite flanking regions gave a signal on all diploid and hexaploid species, whatever the specificity of the microsatellite. The patterns observed on bread wheat were generally in accordance with those observed for diploid species, with slight rearrangements. This suggests that the specificity of microsatellite markers is probably due to mutations in microsatellite flanking regions rather than sequence elimination during polyploidization events and that genome stringency is higher at the polyploid than at the diploid level.

Keywords Wheat · Microsatellite flanking sequences · Evolution · Polyploidization · Genome-specificity

Primer sequences are available upon request.

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Introduction

Bread wheat (*Triticum aestivum*) is an allohexaploid species made up of three diploid genomes: A, B and D. It results from two successive intercrossings involving at least three different species (Kihara 1950). The A-diploid donor is related to *T. monococcum ssp urartu* (Riley et al. 1967). The B-genome donor remains unknown but is probably related to the *Sitopsis* section and most likely has a polyphyletic origin (Vardi 1973). The D genome was introduced during the last intercrossing, between the amphiploid AABB *T. turgidum* and *T. tauschii* (DD). Establishing molecular marker linkage maps in the hexaploid context for this latter genome is more difficult than for the A and B genomes. Therefore we recently isolated microsatellites from *T. tauschii* to enhance specifically map coverage of the D genome (Guyomarc'h et al. 2002).

Microsatellites are short elements consisting of tandem repeat units of one to six base pairs in length (Tautz 1989). They are widely present in eukaryotic genomes and randomly distributed all over the genome. They also constitute powerful genetic markers because of the high level of polymorphism they reveal. In polyploid species, restriction fragment length polymorphism (RFLP) probes often detect homoeologous loci (Ogihara et al. 1994; Nelson et al. 1995), whereas sequence tag site (STS) markers, including microsatellites, are more genome-specific (Wolff et al. 1994; Weising et al. 1996; Diwan et al. 1997; Buteler et al. 1999; Bryan et al. 1997; Röder et al. 1998) and, therefore, are particularly useful for mapping. A microsatellite primer pair is considered

Table 1 List of the different species and wheat lines used in the present work. Most of the diploid species were furnished by INRA, Rennes, France. Aneuploid lines were provided by S. Reader (John Innes Center, UK)

Species	Accession	Genome	Cultivar	Abbreviation	Genome
Triricum monoccocum ssp *urartu*	77097	AA	W-7984	Syc	AABBDD
	77098	AA	Opata	Op	AABBDD
T. monococcum ssp *monococcum*	81051	AA	Courtot	Ct	AABBDD
	81187	AA	Chinese Spring	CS	AABBDD
T. monococcum ssp *boeoticum*	68181	AA	Renan	Re	AABBDD
	94114	AA	Récital	Rec	AABBDD
Aegilops speltoides	37	BB	Arche	Ar	AABBDD
	38	BB	CS nulli-1D	N1DT1A	AABBDD
Ae. searsii	4	BB	CS nulli-2D	N2DT2A	AABBDD
T. longissimum	1	BB	CS nulli-3D	N3DT3A	AABBDD
	3	BB	CS nulli-4D	N4DT4B	AABBDD
T. tauschii	13	DD	CS nulli-5D	N5DT5B	AABBDD
	15	DD	CS nulli-6D	N6DT6A	AABBDD
	32	DD	CS nulli-7D	N7DT7A	AABBDD
	33	DD			

to be (1) genome-specific when it produces one or several bands which map to the same genome and (2) locus-specific when it produces one or several co-segregating bands which map to the same locus.

Microsatellites should thus enable a better understanding of bread wheat genome evolution, from diploid to hexaploid species. Microsatellites from hexaploid wheat have been shown to give amplification products in many diploid related species and allow accurate classifications (Sourdille et al. 2001a). Alternatively, Pestsova et al. (2000) demonstrated the transferability of microsatellites from *T. tauschii* to bread wheat. In a previous paper (Guyomarc'h et al. 2002), we confirmed this transferability and found that all microsatellite markers isolated from *T. tauschii* amplify at least one sequence assigned to the D genome of hexaploid wheat. In this paper, we report our findings on the transferability of these microsatellites to the A and B genomes of bread wheat and to related diploid species. When no amplification on diploid species was observed, we tested both hypothesis: (1) mutations in microsatellite flanking sequences or (2) deletion of this locus on the genome. In order to analyse the evolution of microsatellite flanking sequences after polyploidization, we chose a primer pair that gave an amplification product using all diploid donors and which gave three homoeologous loci in bread wheat. The amplification products were then sequenced and compared.

Materials and methods

Plant material

Plant material used in this study is summarized in Table 1. DNA extractions were performed from fresh leaves using a modified potassium acetate protocol (Lu et al. 1994).

For the evaluation of Nei's diversity index, also referred to as polymorphism information content (PIC), seven wheat lines were used: W-7984 (synthetic wheat,) (Van Deynze et al. 1995), Opata, Courtot, Chinese Spring (CS), Renan, Arche, and Récital. *Triticum tauschii* accession no. 15 was used as an amplification control. Chinese Spring nulli-tetrasomic lines (N1DT1A, N2DT2A, N3DT3A, N4DT4B, N5DT5B, N6DT6A, N7DT7A) were used for loci assignment.

Microsatellite markers

Microsatellite markers were isolated from a *T. tauschii* enriched library (Guyomarc'h et al. 2002; sequences available upon request). Eighty-four markers were used in this study. Polymerase chain reactions (PCR) were performed as described by Tixier et al. (1998) at an annealing temperature of 60 °C. Microsatellite amplification was visualized by silver staining (Tixier et al. 1997).

Southern blot hybridization

The presence of microsatellite loci on the diploid and hexaploid genomes was checked by Southern hybridization on restricted DNA with probes corresponding to microsatellite flanking regions. Total plant DNA was digested with *Rsa*I, a methylation-sensitive enzyme which was used for the construction of the enriched library (Edwards et al. 1996). Restricted fragments were separated on a 1.2% agarose gel (1 × TAE) at 50 V for 7 h. DNA was then blotted onto a Hybond N+ membrane (Amersham Pharmacia Biotech) as described in Sambrook et al. (1989).

Probes containing the microsatellites were synthesized by means of PCR using a universal primer pair (pUC/M13 forward and reverse) and the corresponding plasmid as DNA template. PCR reactions were performed in a final volume of 100 µL in a Perkin-Elmer 9600 thermocycler. The reaction buffer contained 100 ng of DNA template, 10% (v:v) glycerol, 0.5 µ*M* of each primer, 0.2 m*M* of each dNTP and 2.5 *Taq*-DNA polymerase (Roche). One cycle of 5 min at 95 °C, was followed by 35 cycles of 30 s at 94 °C, 30 s at 49 °C and 30 s at 72 °C, with a final elongation step of 4 min at 72 °C. Similar probes, but containing only the microsatellite flanking sequences, were synthesized from the same DNA template using primer pairs specific for the flanking regions.

The probes were [^{32}P]-labelled by random priming with the Megaprime DNA labelling kit (Amersham Pharmacia Biotech) and hybridized as described in Lu et al. (1994).

Recovery and re-amplification of DNA fragments from silver-stained polyacrylamide gels and sequencing

The portion of the silver-stained acrylamide gel containing the band of interest was cut out, and the gel slice was transferred into a microfuge tube containing 30 µL H_2O. The slice was crushed

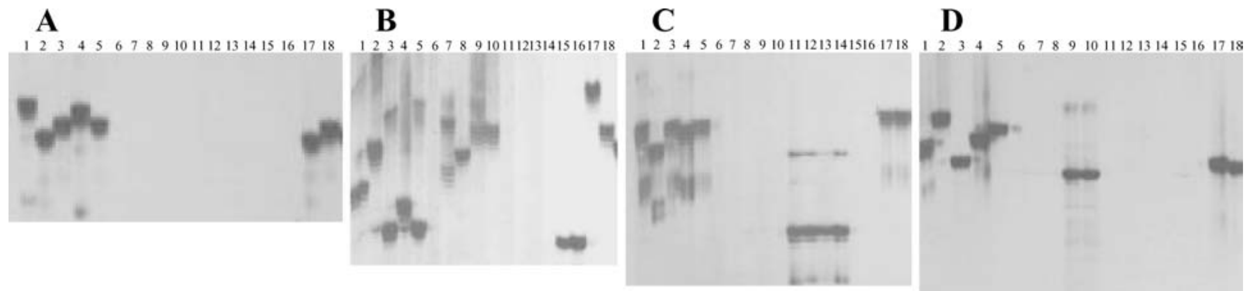

Fig. 1A–D Examples of microsatellite transferability: cfd8 (**A**), cfd29 (**B**), cfd47 (**C**), cfd57 (**D**). *Lanes 1–5* D diploids (*Triticum tauschii* 42, 33, 32, 15, 13), *lanes 6–10* B diploids (*Aegilops longissima* 3, 1, *Ae. searsii* 4, *Ae. speltoides* 38, 37), *lanes 11–16* A diploids (*T. m* ssp *boeoticum* 68181, 90194; *T. m* ssp *monococcum* 81187, 81051; *T. m* ssp *urartu* 77098, 77097) *lane 17* CS, *lane 18* Syc

against the wall of the tube with a pipette tip. The microfuge tube was then incubated for 1 h at 37 °C. After centrifugation (15,000 *g* for 2 min), the supernatant was transferred into a fresh PCR tube and used as template. PCR was performed as described above, and the products were loaded onto an acrylamide gel in order to control the size of the amplified fragment. After verification of the size, the PCR products were sequenced (Genome Express, Paris, France). Multiple alignment of sequences was realised using CLUSTAL (http://www.infobiogen.fr) with default settings.

Results

Genomic specificity of microsatellite markers on hexaploid wheat

For each of the 84 primer pairs used in this study, at least one band was mapped on the D genome of bread wheat. Thirty-nine (46%) primer pairs were locus-specific and mapped to the D genome of bread wheat; 33 of these (39%) gave a single product (only one band) on hexaploid wheat, while six others (7%) produced several co-segregating bands. The remaining 45 primer pairs produced several loci mapping on different genomes, of which 24 (29%) produced one polymorphic and one or more monomorphic bands, but with molecular weights generally lower than that of the fragment expected, and 21 (25%) amplified several polymorphic fragments.

Microsatellite markers transferability on A- and B-diploid species and on synthetic wheats

Transferability was tested using 40 primer pairs that were chosen in order to represent all types of microsatellite motifs. We distinguished two groups based on our results from bread wheat: Set no. 1, which produced only one band on bread wheat (21 cases) and Set no. 2, which produced several but non-cosegregating bands on bread wheat (19 cases). The results are summarized in Table 2, and examples are given in Fig. 1. For all microsatellite markers, the synthetic wheat gave the same number of bands as cultivated wheats except for primer pairs cfd18 and cfd61, which produced one additional band on the synthetic wheat, and cfd36, which produced one additional band on cultivated wheats. For cfd61, this may be explained by the occurrence of null alleles at one of the two loci.

In the transferability studies, different PCR responses were observed within the groups of diploid species (Fig. 1). All primer pairs of both sets gave an amplification product with all *T. tauschii* accessions. Within the group of species carrying the A genome (A-diploids, Table 2), amplification results were relatively homogeneous with 10 of the 40 primer pairs giving different responses between species i.e. 10 primer pairs did not produce any amplification product on at least one species while the others produced one amplification product. Within the group of species carrying the B genome (B-diploids, Table 2), responses contrasted between species. Similar to that observed for the A-diploids, 15 primer pairs gave a differential response, but differences were also detected within some species. For example, the microsatellite cfd40 amplified only one fragment in *Ae. speltoides* 37 but none in *Ae. speltoides* 38 nor in the other B-diploids.

Another point to be mentioned is the amplification results with respect to the group of species (Fig. 1). Using a given microsatellite, when at least one species gave one amplification product within a group of diploid donors, we declared that this microsatellite exhibited transferability. Among Set no. 1, 11 of the 21 (52%) primer pairs gave an amplification product only on those species carrying the D genome (D-diploids, Table 2), while 4 of the 21 (19%) primer pairs amplified simultaneously on B- and D-diploids, 3 of the 21 (14%) simultaneously on A- and D-diploids and only 3 of the 21 (14%) on the A-, B- and D-diploids. This contrasted with Set no. 2 where 14 of the 19 primer pairs (74%) produced an amplification product on the A-, B- and D-diploids, 5 of the 19 (26%) on B- and D-diploids, none of the primer pairs on A- and D-diploids, and none produced D-genome-specific products.

Presence of microsatellite loci in the diploid genomes as revealed by Southern Blot

The lack of PCR amplification could be due either to mutations in microsatellite flanking sequences or to an absence of the locus in the genome. In order to test both hypotheses, we carried out Southern blot hybridizations using total restricted genomic DNA. Membranes were

Table 2 Results of amplification of 40 microsatellites (µsat.) on hexaploid cultivars (Hexa.) and on diploid species carrying either the A (A-diploids), B (B-diploids) or D (D-diploids) genome. The number of amplification products is specified for each sample

µsat.	Hexa.		A-diploids						B-diploids					D-diploids				
	W-7984	*Chinese Spring*	*T. m.* ssp *urartu* 77097	*T. m.* ssp *urartu* 77098	*T. m.* ssp *monoc-cocum* 81051	*T. m.* ssp *monoc-cocum* 81187	*T. m.* ssp *boeoticum* 90194	*T. m.* ssp *boeoticum* 68181	*Ae. speltoides* 37	*Ae. speltoides* 38	*Ae. searsii* 4	*Ae. longissima* 1	*Ae. longissima* 3	*T. tauschii* 13	*T. tauschii* 15	*T. tauschii* 32	*T. tauschii* 33	*T. tauschii* 42
cfd8	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
cfd9	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
cfd33	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
cfd35[a]	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
cfd38	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
cfd42	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
cfd44	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
cfd45	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
cfd72	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
cfd78	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
cfd161	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
cfd3	1	1	0	0	0	0	0	0	0	0	1	0	0	1	1	1	1	1
cfd26	1	1	0	0	0	0	0	0	2[b]	2[b]	0	0	0	1	1	1	1	1
cfd40	1	1	0	0	0	0	0	0	1	0	0	0	0	1	1	1	1	1
cfd57	1	1	0	0	0	0	0	0	1	1	0	0	0	1	1	1	1	1
cfd47	1	1	0	0	2[b]	2[b]	2[b]	2[b]	0	0	0	0	0	1	1	1	1	1
cfd66	1	1	1[b]	1[b]	1[b]	1[b]	1[b]	1[b]	0	0	0	0	0	1	1	1	1	1
cfd68	1	1	0	0	1	1	1	1	0	0	0	0	0	1	1	1	1	1
cfd5	1	1	0	0	1	0	1	0	0	0	0	0	0	1	1	1	1	1
cfd27	1	1	1	1	0	0	0	1	1	1	0	0	0	1	1	1	1	1
cfd29	1	1	1	1	0	0	0	0	1	1	1	2	0	1	1	1	1	1
cfd14	2	2	0	0	0	0	0	0	0	0	1	0	0	1	1	1	1	1
cfd18	2	1	0	0	0	0	0	0	4	3	1	2	1	1	1	1	1	2
cfd37	3[b]	3[b]	0	0	0	0	0	0	0	0	2[b]	0	0	2[b]	2[b]	2[b]	2[b]	2[b]
cfd75	2	2	0	0	0	0	0	0	0	1	2	2	1	2	2	2	2	2
cfd81	2	2	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1
cfd4	2	2	1[b]	1[b]	1	1	1	1	2	3	1	1	1	1	1	1	1	1
cfd7	2	2	1[b]	1[b]	1[b]	1[b]	1[b]	1[b]	1[b]	1[b]	1[b]	1[b]	1[b]	1	1	1	1	1
cfd12	3	3	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1
cfd21	3	3	0	1	1	1	1	1	1	0	0	1	1	0	2	1	2	2
cfd34	2	2	1	1	1	1	1	1	1[b]	1[b]	1[b]	1[b]	1[b]	2	2	2	2	2
cfd36	1	2	0	0	1	1	1	1	0	1	1	0	1	1	1	1	1	1
cfd39	3	3	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
cfd60	2	2	1	1	1	1	1	1	1	1	1[b]	1[b]	1[b]	1	1	1	1	1
cfd61[a]	2	1	1	1	1	1	1	0	1	1	1	0	0	1	1	1	1	1
cfd63	3	3	1	1	0	0	0	0	1	1	0	1	1	1	1	1	1	1
cfd67	3	3	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2
Cfd71	2	2	3	3	1	1	1	1	3[b]	3[b]	0	0	0	1	1	1	1	1
Cfd79	3	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Cfd80	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

[a] Null alleles can be observed
[b] One minor band can be observed

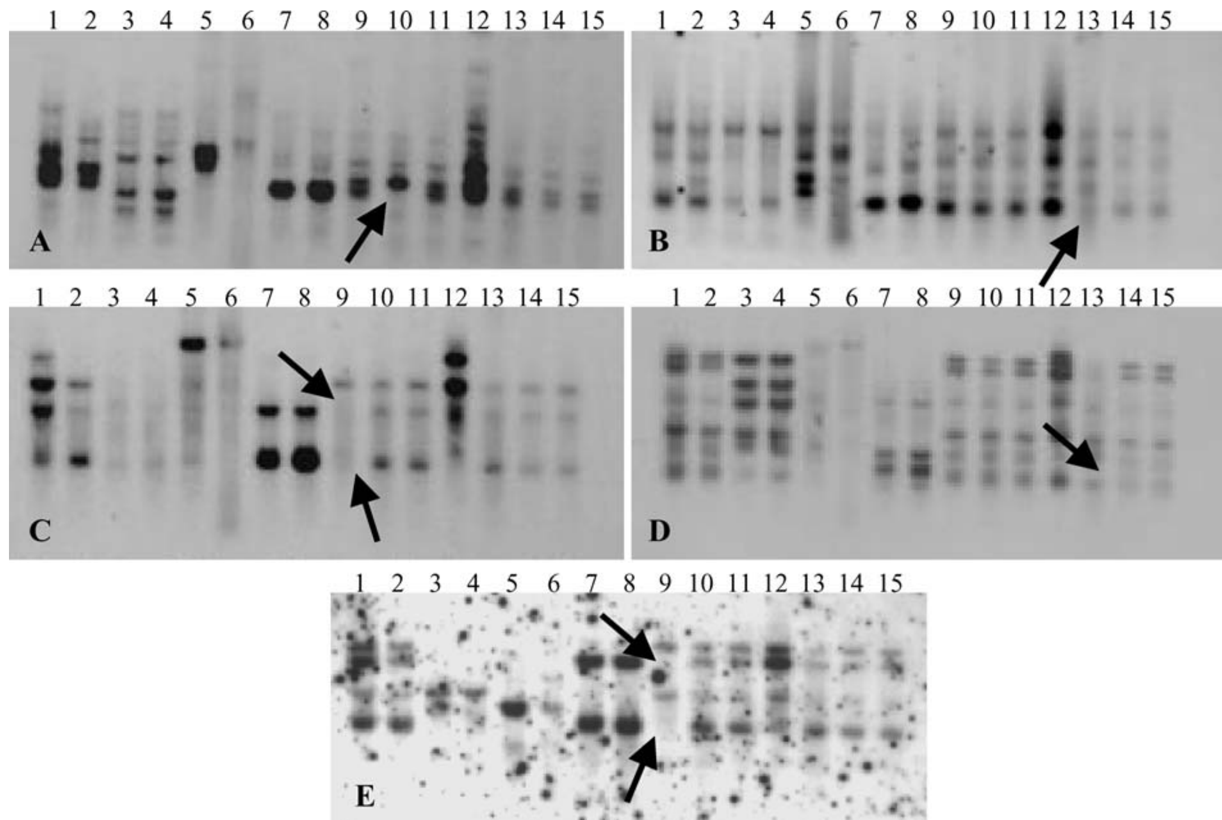

Fig. 2A–E Hybridization patterns of probes cfd161 (**A**), cfd57 (**B**), cfd72 (**C**), cfd78 (**D**), and cfd63 (**E**). *Arrows* indicate the bands corresponding to *T. tauschii* and missing in nulli-tetrasomic lines. *Lanes*: *1* Ct, *2* Syc, *3 T. m* ssp *urartu* 77097, *4 T. m* ssp *urartu* 77098, *5 Ae. speltoides* 37, *6 Ae. speltoides* 38, *7 T. tauschii* 15, *8 T. tauschii* 35, *9–15* CS aneuploid lines N1DT1A, N2DT2A, N3DT3A, N4DT4B, N5DT5B, N6DT6A, N7DT7A

hybridized with five different probes corresponding to flanking regions of four microsatellites from Set no. 1 and one from Set no. 2. Three of those from Set no. 1 were strictly D-genome-specific (cfd72, cfd78 and cfd161, Table 2), and one amplified simultaneously *T. tauschii* and *Ae. speltoides* (cfd57, Table 2); the microsatellite from Set no. 2 (cfd63) amplified all diploid genomes. An hybridization signal was detected for all genotypes and all five probes (Fig. 2). All of the probes showed the same type of hybridization pattern, although some of them gave a weak signal on diploid genotypes carrying either the A (probe cfd72, Fig. 2C) or the B genome (probe cfd78, Fig. 2D). Surprisingly, most of the genotypes, including diploids, exhibited multiple banding patterns. In order to verify that this result was not due to the presence of the microsatellite within the probe, we used probes corresponding only to the flanking regions, which were synthesized by PCR (see Materials and methods). These probes showed the same results, suggesting that the complexity of the patterns was not due to the microsatellite.

For most of the probes, the pattern detected for the hexaploid wheat corresponded to the juxtaposition of the patterns of the diploid genotypes, with minor changes (Fig. 2B–E). For all of the probes, some bands were assigned using nulli-tetrasomic lines, and this assignment was in accordance with the mapping position of the corresponding microsatellite: cfd72 and cfd63 on 1D (two bands); cfd161 on 2D; cfd57 and cfd78 on 5D. The bands that were assigned (indicated by arrows on Fig. 2) were observed only on *T. tauschii* and hexaploid wheat patterns, and not on those from *T. m.* ssp *urartu* or *Ae. speltoides*.

Band sequencing of multiple amplification patterns

The primer pair cfd79 (Set no. 2) produced an amplification product on each diploid species, *T. m.* ssp *urartu*, *Ae. speltoides* and *T. tauschii*, and three bands on hexaploid wheat (Fig. 3). Using Chinese Spring nulli-tetrasomic lines, we assigned, the upper band (no. 4, Fig. 3) to chromosome 3A, the middle band (no. 5) to chromosome 3B and the lower band (no. 6) to chromosome 3D, thereby indicating that the three loci were clearly homoeologous. In order to study any differences that may exist at these three loci, we amplified all of these bands separately and sequenced them. A multiple alignment of these sequences (Fig. 4) showed that there were two different mutations. The first one at the level of nucleotide 136 changed a C into a T. This mutation was detected both in the sequence issued from *T. tauschii* (band no. 1) and in those issued from the hexaploids (nos. 6 and 8). Band no. 6 from Chinese Spring was assigned to chro-

Fig. 3 Results of PCR amplification using microsatellite cfd79. *Lane A T. tauschii*, *lane B Ae. speltoides*, *lane C T. m* ssp *urartu*, *lane D*: CS, *lane E* Syc. *Numbers* refer to bands that were sequenced

mosome 3D. This placement suggests that this mutation was specifically from the D genome and that band no. 8 from Synthetic wheat is also probably located on chromosome 3D. The second mutation occurred at the level of nucleotide 158 and changed a C into a G. This mutation appeared both in the sequence issued from *Ae. speltoides* (no. 2) and in those from the hexaploid (nos. 5 and 9). Band no. 5 from Chinese Spring was assigned to chromosome 3B, which indicates that this mutation was specifically from the B genome and that band no. 9 from Synthetic wheat is also probably located on chromosome 3B. The "ancestor" sequence may thus correspond to the one from the A genome (nos. 3, 4 and 7). It also suggests that the mutations occurred before polyploidization since they are also observed in the diploid species.

With respect to the number of (AG) repetitions (n) for this particular microsatellite, sequences from *T. m.* ssp *urartu* (n=15) and *Ae. speltoides* (n=26) showed very different numbers of repetitions compared to that observed in corresponding sequences from the hexaploid (n=29 and n=19 for the A and B sequences, respectively). However, for the D genome, the numbers of repeats were quite similar with n=16, 14 and 15 for *T. tauschii*,

Fig. 4 Multiple sequence alignment of the different amplification products obtained with the microsatellite cfd79 using CLUSTAL. Sequences correspond to the bands that were numbered in Fig. 3 *1*/*T. tauschii* (*T.t*), *2*/*Ae. speltoides* (*Ae.s*), *3*/*T. m* ssp *urartu* (*T.u*); *4*/CS (upper band: *CSu-3A*), *5*/CS (middle band: *CSm-3B*), *6*/CS (lower band: *CSl-3D*), *7*/Syc (upper band: *Su*), *8*/Syc (middle band: *Sm*); *9*/Syc (lower band: *Sl*). Mutations are indicated in *bold*

```
                1                                                          60
1/ T.t      ------------------GGAAATCAAAAGCTGGTGAGATGTTTCAAAGCTTCTTTCGTT
2/ Ae.s     ---------------------AATCAAAAGCTGGTGAGATGTTTCAAAGCTTCTTTGGTT
3/ T.u      ----------GTGTAGTTGGAAATCAAAAGCTGGTGAGATGTTTCAAAGCTTCTTTCGTT
4/ CSu-3A   -----CAAGTGTGTAGTTGGAAATCAAAAGCTGGTGAGATGTTTCAAAGCTTCTTTCGTT
5/ CSm-3B   --------------AGTTGGACATCAAAAGCTGGTGAGATGTTTCAAAGCTTCTTTCGTT
6/ CSl-3D   ---GGCAAGTGTGTAGTTGGAAATCAAAAGCTGGTGAGATGTTTCAAAGCTTCTTTCGTT
7/ Su       ----------GTGTAGTTGGAAATCAAAAGCTGGTGAGATGTTTCAAAGCTTCTTTCGTT
8/ Sm       -----------------TGGAAATCAAAAGCTGGTGAGATGTTTCAAAGCTTCTTTCGTT
9/ Sl       CAGGGCAAGTGTGTAGTTGGAAATCAAAAGCTGGTGAGATGTTTCAAAGCTTCTTTCGTT
            61                                                        120
1/ T.t      TTTAATGGGGATGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG---------------
2/ Ae.s     TTTAATGGGGATGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA
3/ T.u      TTTAATGGGGATGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG-----------------
4/ CSu-3A   TTTAATGGGGATGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA
5/ CSm-3B   TTTAATGGGGATGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG---------
6/ CSl-3D   TTTAATGGGGATGAGAGAGAGAGAGAGAGAGAGAGAGAGAG-------------------
7/ Su       TTTAATGGGGATGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA
8/ Sm       TTTAATGGGGATGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG-----------------
9/ Sl       TTTAATGGGGATGAGAGAGAGAGAGAGAGAG-----------------------------
           121                                                        180
1/ T.t      -----------GCTTTGTGGGAAGAAATAGTGGCCTACTGATGTTTAGATTCTTTCCTAC
2/ Ae.s     GAGAG------GCTTCGTGGGAAGAAATAGTGGCCTAGTGATGTTTAGATTCTTTCCTAC
3/ T.u      -----------GCTTCGTGGGAAGAAATAGTGGCCTACTGATGTTTAGATTCTTTCCTAC
4/ CSu-3A   GAGAGAGAGAGGCTTCGTGGGAAGAAATAGTGGCCTACTGATGTTTAGATTCTTTCCTAC
5/ CSm-3B   -----------GCTTCGTGGGAAGAAATAGTGGCCTAGTGATGTTTAGATTCTTTCCTAC
6/ CSl-3D   -----------GCTTTGTGGGAAGAAATAGTGGCCTACTGATGTTTAGATTCTTTCCTAC
7/ Su       GAGAGAGAG--GCTTCGTGGGAAGAAATAGTGGCCTACTGATGTTTAGATTCTTTCCTAC
8/ Sm       -----------GCTTTGTGGGAAGAAATAGTGGCCTACTGATGTTTAGATTCTTTCCTAC
9/ Sl       -----------GCTTCGTGGGAAGAAATAGTGGCCTAGTGATGTTTAGATTCTTTCCTAC
           181                                                      238
1/ T.t      ATGAAGCGTGTTTGACTGAACAATTTGTCACCCTTGC---------------------
2/ Ae.s     ATGAAGCGTGTTTGACTGAACAATTTGTCACCCTTGCACTATTAGGA-----------
3/ T.u      ATGAAGCGTGTTTGACTGAACAATTTGTCACCCTTGCACTATTAGGATTAGACGAGAG
4/ CSu-3A   ATGAAGCGTGTTTGACTGAACAATTTGTCACCCTTGCACTATTAGG------------
5/ CSm-3B   ATGAAGCGTGTTTGACTGAACAATTTGTCACCCTTGCACTATTAGGATT---------
6/ CSl-3D   ATGAAGCGTGTTTGACTGAACAATTTGTCACCCTTGCACTATTAGGATT---------
7/ Su       ATGAAGCGTGTTTGACTGAACAATTTGTCACCCTTGCACTATTAGGATT---------
8/ Sm       ATGAAGCGTGTTTGACTGAACAATTTGTCACCCTTGCACTATTAGGATT---------
9/ Sl       ATGAAGCGTGTTTGACTGAACAATTTGTCACCCTTGCACTATTAGGATT---------
```

Chinese Spring and the Synthetic wheat, respectively. This may be due to the fact that the D genome from *T. tauschii* was introduced only recently in bread wheat.

Similar experiments were assayed for several microsatellites from Set no. 1 in order to get sequences from the three homoeoloci of bread wheat. We attempted to amplify products using a lower annealing temperatures (55 °C and 50 °C), but only non-specific bands were obtained (results not shown).

Table 3 Definition of Types I and II and Set no. 1 and Set no. 2 based on the PCR amplification results on different genomes of hexaploids and on diploid donors of the A, B and D gemones

Genomes	A		B	D	Types	Set
Diploids	–		–	+	I	
Hexaploids	–		–	+		1
Diploids	+	and/or	+	+	II	
Hexaploids	–		–	+		
Diploids	+	and/or	+	+		2
Hexaploids	+	and/or	+	+		

Discussion

During the course of developing microsatellite markers from *T. tauschii*, the purpose of which was to increase the D-genome mapping possibilities, some distinctive features appeared with respect to the generality of these markers within *T. tauschii*, the possibility of their use on the D genome of bread wheat and their transferability to other genomes.

On bread wheat, primer pairs producing one band gave loci that systematically mapped to the D genome. We found that 39% of the microsatellites developed here gave a single amplification product, which was close to the results of Röder et al. (1998) (40%). When multiple banding patterns were detected, polymorphic bands were not always mapped soley on the D genome, but there was at least one band assigned to the latter. Of the primer pairs, 7% showed co-segregating bands. This was probably due to a duplication of one of the flanking sequences at the same locus. Additional monomorphic bands, observed for 24 among the 84 primer pairs (29%), were often of a lower molecular weight. Using wheat, Bryan et al. (1997) showed that 29% of the primer pairs generated supplementary products that were generally shorter and less polymorphic than expected. These could result from the amplification of a shorter microsatellite with similar flanking sequences. The hypothesis that polyploidy could lead to shorter microsatellites than those observed in a diploid context was already proposed in *Barbus* (Chenuil et al. 1997).

With respect to the additional polymorphic bands, only half mapped at homoeologous loci (48%), with the other half mapping either to different chromosomes but of the same genome (20%) or to a duplicate locus on the same chromosome (24%) (Guyomarc'h et al. 2002; Sourdille et al. 2001b, 2002). Only 6% of the loci mapped at different positions on a different genome. This suggests that multiple banding patterns correspond either to homoeoloci or to duplications. Testing the primers on Chinese Spring nulli-tetrasomic lines confirmed both hypotheses.

PCR amplification results on the different genomes of both hexaploid and diploid species carrying either the A, B or D genomes is summarized in Table 3. All of the primer pairs isolated from *T. tauschii* amplified all of the accessions of *T. tauschii* analysed, giving high levels of polymorphism, and also amplified loci located on the D genome of bread wheat. This suggests highly conserved microsatellite flanking sequences between the D genome of *T. tauschii* and the one from *T. aestivum*.

Regarding transferability, the following characteristics became apparent.

(1) Microsatellites isolated from *T. tauschii* generally showed quite a good transferability toward the diploid species carrying the A and B genomes, but a lower one for the A and B genomes of the hexaploid wheat. We verified that the sequences bearing the microsatellite motifs existed in all species, even if the microsatellite was not amplified by a particular combination of primers. Moreover, these sequences appeared to exist in several copies in the genomes, even in the diploid species. Finally, the RFLP patterns observed in the polyploids are roughly the juxtaposition of the patterns observed in the diploids, with some modifications. The slight differences observed could be due to evolution events following polyploidization. The elimination of non-coding sequences or the appearance of new fragments occurring after polyploidization was described in the earliest generations of self pollination in raw amphiploids like wheat (Feldman et al. 1997; Liu et al. 1998a) or rapeseed (Song et al. 1995) (for a review on evolution of sequences in polyploids, see Wendel 2000). These modifications appeared randomly and were due, in part, to changes in methylation. For low-copy coding sequences, sequence rearrangements seemed not to be randomly distributed and no elimination of sequences was observed (Liu et al. 1998b). Such rearrangements could explain the slight differences observed between the addition of the diploids patterns and those observed in polyploids. With respect to possible insertion events, no clear conclusion could be drawn from the size of the RFLP fragments.

(2) For those sequences which can be PCR-amplified in all genomes (diploid and hexaploid), point mutations seem to exist which are specific to each of the elementary genomes. A systematic study of such mutations in microsatellite flanking sequences should be carried out. This may lead to the development of specific SNP (single nucleotide polymorphism) markers which could provide more information in phylogenetic studies than microsatellite length polymorphisms.

(3) When the amplification is not genome-specific at the diploid level, this specificity may be acquired or not at the polyploid level. But when there is a genome specificity at the diploid level, this specificity also exists and is compulsory at the polyploid level. Conversely, the genome specificity observed at the polyploid level does not imply genome specificity at the diploid level. This confirms previous observations from Sourdille et al. (2001a): studying the transferability of microsatellites isolated from bread wheat towards diploid species, they showed that microsatellites specific for the D genome in hexaploid wheat have a rather high transferability to the A- and B-diploid genomes.

It is clear that PCR markers are more specific than markers obtained using hybridization technology because point mutations in primer sequences could prevent amplification, while hybridizations succeed even in cases of partial homology between probe and DNA template. Therefore, we can even observe multiple banding RFLP patterns corresponding to different loci not visible by PCR. The importance of mutation events in microsatellite flanking sequences was recently pointed out in different species: cattle (Brohede and Ellengren 1999), fishes (Van Oppen et al. 2000), pines (Karhu et al. 2000), wasps (Zhu et al. 2000) and primates (Clisson et al. 2000).

These results suggest that genome specificity is acquired through point mutations rather than by other modification events. The first step consists of particular point mutations specific to each elementary genome, whatever the ploidy level. A second step is the amplification of sequence divergence, which is sufficient to inhibit the possibility of designing primers allowing PCR amplification on the different genomes. We tried to amplify microsatellites belonging to Set no. 1/type II (specific in 6*x*, non-specific in 2*x*) on the different genomes by decreasing the annealing temperature (up to 50 °C) in order to obtain sequences and compare them. We were unable to amplify these sequences, which suggests an important degree of divergence.

The speed of divergence is clearly greater at the hexaploid level than at the diploid level, indicating that the evolution pressure is higher at the former than at the latter. This suggests that particular sequences like those containing microsatellites may strongly contribute to the molecular differentiation of genome.

For primer pairs generating multiple products in hexaploid wheat, transferability seemed to be greater from the D-diploid to the B-diploid species than to the A-diploid species, which suggests a closer genetic proximity between B- and D-diploid genomes than between A and D genomes, as previously shown by Thiellement et al. (1989) and Monte et al. (1993). We also noted that the response was more heterogeneous within the B-diploid group than within the A diploid group, reflecting the fact that accessions from the A group belong to a single species (*T. monococcum*), whereas accessions from the B group represent three different species.

Conclusions

It is clear that sequence point mutation was a powerful mechanism for evolution in sequences flanking microsatellites during the speciation process from the common ancestor of A-, B- and D-diploid genomes and the present hexaploid and diploid species. When mutations confer genome specificity at the diploid level, the amplification remains genome-specific at the hexaploid level. For mutations which do not confer compulsory genome specificity at the diploid level, then amplification either remains non-genome-specific at the hexaploid level (i.e., cfd79), or it becomes genome-specific in the polyploid context (cfd29). Therefore, such a mutation process may contribute to genomic differentiation and in conjunction with the *Ph1* gene and other rearrangement events in the genome following polyploidization, to the genetic and physical bases for the diploid-like meiotic behaviour of polyploid wheat, as pointed out by Feldman et al. (1997).

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