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Identification of different chromatin classes in wheat using in situ hybridization with simple sequence repeat oligonucleotides

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Abstract Clusters of four simple sequence repeats (SSRs), AAC, AAG, AG and CAT, have been mapped physically to hexaploid wheat chromosomes; 15—24-bp synthetic oligonucleotides were labelled by random-primer labelling and used as probes for fluorescent in situ hybridization with standard formamide and low-salt conditions. AAC hybridized strongly to the pericentromeric regions and several intercalary sites of all seven chromosomes of the B-genome corresponding to N bands and enabling their identification. Most of the AAC sites also co-localize with AAG, although the strength of the AAC and AAG signal was often different at the same location. Not all heterochromatic bands showed AAC signals and a few AAC sites were detected that are neither AAG nor N band positive, revealing the complex and heterogeneous genome organization of wheat and identifying the four most frequent classes of banded chromatin. Clusters characterised by a high concentration of AG repeats were detected on chromosome arms 3BS, 4BL, 5BS and 5BL, adjacent to AAG sites. The only detectable CAT cluster was found on chromosome arm 3BL, making this oligonucleotide valuable in identifying this particular chromosome. SSR in situ hybridization is useful as a diagnostic tool in cytogenetics and for understanding genome organization in wheat.

Key words *Triticum aestivum* L. · Microsatellites · C banding · N banding · Genome organization

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Introduction

In addition to identifying chromosome number and the constitution of a species or line, detailed chromosome diagnosis is an important aspect of comparative genome analysis. Applications lie in: (1) detecting chromosomal aberrations or introgressions; (2) assigning genetic or molecular markers to chromosomal regions; (3) following chromosome pairing and segregation; and (4) inferring evolutionary relationships between species by a comparison of chromosome and genome structure. In most Triticeae species including hexaploid wheat, *Triticum aestivum*, it is difficult to identify the chromosomes and specific chromosomal regions due to their similar chromosome size and lack of reproducible morphological markers. Nevertheless, Giemsa C banding and N banding to demonstrate constitutive heterochromatin has been applied successfully, although only routinely in a few laboratories, and standard karyotypes have been published for the agronomically important species, rye, wheat and barley, and their close relatives (Linde-Laursen 1975; Schlegel and Gill 1984; Gill et al. 1991). C and N banding has been able to identify chromosome rearrangements, alien chromatin introgression, evolutionary relations and meiotic pairing (e.g. Gill and Kimber 1977; Friebe et al. 1991; Naranjo and Fernandez-Rueda 1991; Linde-Laursen and Baden 1994; Cuadrado and Jouve 1995).

As an alternative and very informative approach, fluorescence in situ hybridization (FISH), that enables the simultaneous or successive localization of one or more DNA probes along chromosomes, is becoming the strategy of choice for chromosome diagnosis and studying genome organization in plant species (see e.g. Schwarzacher 1996; Schmidt and Heslop-Harrison 1998). In situ hybridization using multi-copy genes, such as the 45 S and 5 S rRNA genes, and repetitive cloned DNA sequences has been very useful in Triticeae species producing chromosome- and genome-specific markers and banding patterns that can be used diagnostically to identify specific chromosomes and chromosome regions (e.g. Leitch and

Heslop-Harrison 1993; Mukai et al 1993; Castilho and Heslop-Harrison 1995, Cuadrado and Jouve 1996; Pedersen et al. 1996; Bardsley et al. 1999).

Simple sequence repeats (SSRs) or microsatellites are a widespread and highly abundant class of repeated DNA sequences within eukaryotic genomes (Tautz 1989; Powell et al. 1996). They are arranged in tandem arrays of identical, composite or degenerate short sequence motifs, generally less than five nucleotides and have been found to be dispersed throughout the genomes with additional clusters at heterochromatic regions (e.g. Stallinger et al. 1981; Schmidt and Heslop-Harrison 1996). The high abundance and frequency of variation in the number of repeats at a given locus in different individuals have made microsatellites valuable for fingerprinting individuals, species and varieties, and for the generation of genetic maps (Weber and May 1989; Weising et al 1989; Powell et al. 1996). A few reports are now available describing the physical organization of SSRs in plant chromosomes utilising in situ hybridization. Schmidt and Heslop-Harrison (1996) in beet and Gortner et al. (1998) in chickpea, used different SSRs and apart from the dispersed overall signal, found motif-specific patterns of distribution with enrichment or depletion of some motifs at centromeric or intercalary positions. Fuchs et al. (1998) found that some SSRs provided suitable physical landmarks in pea chromosomes. Pedersen et al. (1996) used a successive in situ hybridization procedure combining the 18S-5.8S-26 S rDNA and a GAA repeated motif to identify all barley chromosomes. We recently analysed the chromosomal distribution of clusters of ten different SSRs in wheat and rye genomes using FISH (Cuadrado and Schwarzacher 1998). While all motifs resulted in a dispersed hybridization along all chromosomes, some motifs, in particular GACA, gave additional strong bands that allowed the identification of all wheat B-genome and rye chromosomes and resembled N banding in wheat.

In all these studies, the synthetic oligonucleotide sequences (15—30-bp long) utilised as probes were end-labelled with the enzyme terminal deoxynucleotidyl transferase (TdT) attaching at the 3' end biotin- or digoxigenin- modified nucleotides. In situ hybridization was performed with no formamide and high salt conditions to cater for the low calculated melting temperatures of the oligonucleotides (see Wallace et al. 1981). A hybridization signal was observed over the whole genome indicative of the dispersed nature of SSRs and that the overlaying SSR clusters on specific chromosome sites were not always clearly visible. In the present paper, we aimed to develop an in situ hybridization procedure to use the synthetic oligonucleotide probes under standard hybridization and washing conditions allowing oligonucleotides and longer cloned sequences to be hybridized simultaneously in multi-colour experiments. We aimed to correlate the distribution of four simple sequence repeats in wheat under these conditions with each other, with the previously described $(GACA)_4$, and with heterochromatin distribution.

Materials and methods

Plant material and preparation for in situ hybridization

Seeds of *T. aestivum* L. 'Chinese Spring' (2n=42; genomic constitution AABBDD) were germinated in distiled water at 25°C until roots emerged, transferred to 4°C for 48 h and then back to 25°C for 24 h. Root tips were excised and treated in ice cold water (0°C) for 24 h and fixed in 3:1 (v/v) 100% ethanol: glacial acetic acid. Root-tip spreads were performed as described by Schwarzacher et al. (1989) using pectinase (15 U/ml) and cellulase (80 U/ml) digestion prior to squashing in 45% acetic acid onto acid-cleaned microscope slides.

DNA Probes and labelling

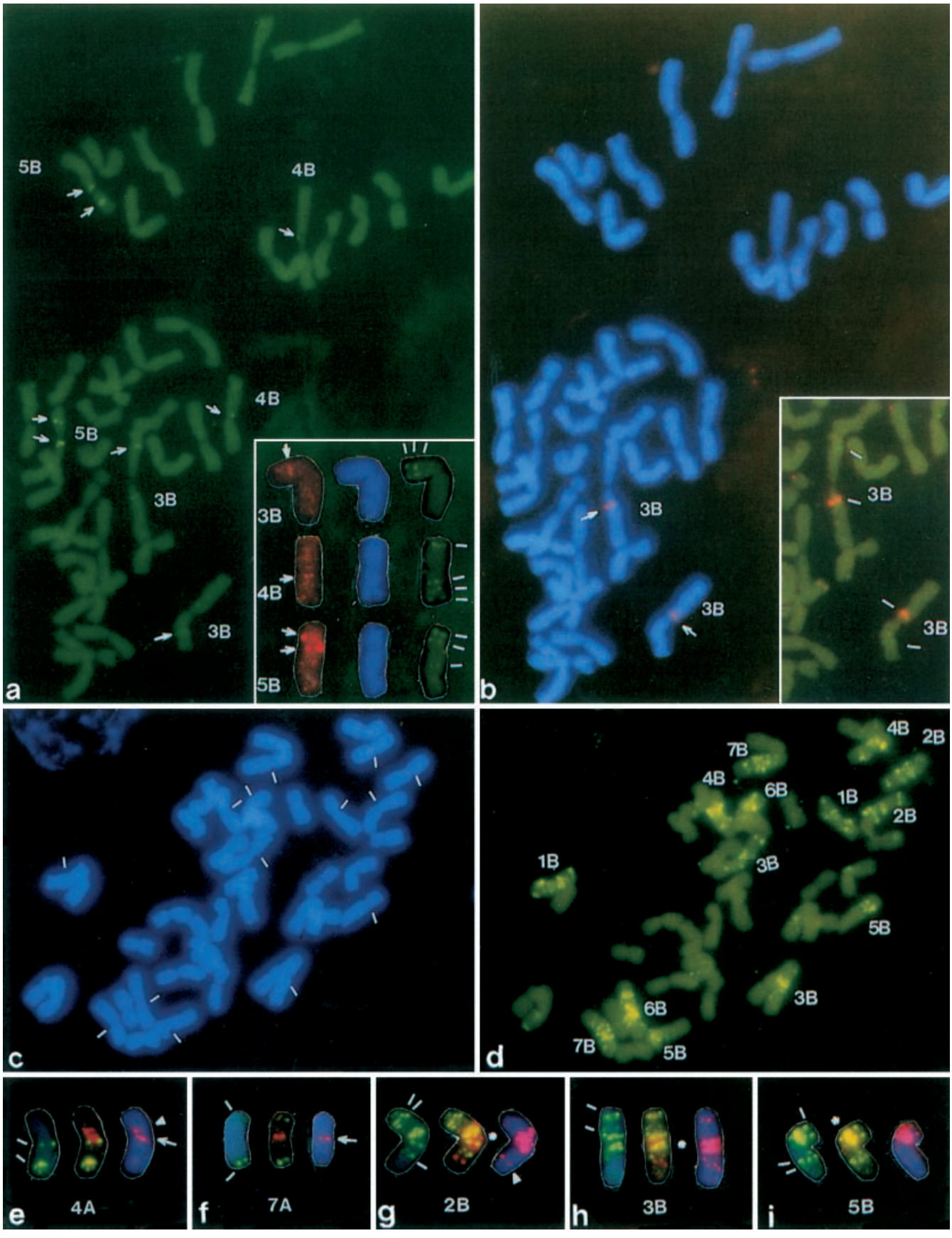
The probes were four synthetic SSR-oligonucleotides, $(AG)_{12}$, $(CAT)_5$, $(AAC)_5$ and $(AAG)_5$, and two different repetitive clones: pSc119.2 which contains a 120-bp tandem repeated DNA sequence from rye, *Secale cereale* L. (Bedbrook et al. 1980; McIntyre et al. 1990), and pAs1 which contains a 1-kb repetitive DNA sequence from *Aegilops squarrosa* L., with a D-genome specific distribution (Rayburn and Gill 1986).

SSR-oligonucleotides were labelled with biotin or digoxigenin by random primer labelling using 2.5 µg of SSR-oligonucleotides in a 20-µl standard reaction (e.g. Roche) containing 0.125 A260 units of a random hexanucleotide mixture, 0.7 nM biotin-16-dUTP or digoxigenin-11-dUTP, 1.4 nM TTP, 2 nM dATP, 2 nM dCTP, 2 nM dGTP, 1 µM Tris-HCl pH 7.2, 0.2 µM $MgCl_2$, 0.02 µM dithiothreitol and 2 U of the Klenow fragment of *Eschericha coli* DNA polymerase. The plasmid clones pSc119.2 and pAs1 were amplified and labelled with digoxigenin-11-dUTP or rhodamine-4-dUTP (AmershamPharmacia) using PCR with universal forward and reverse sequencing primers, 30-s annealing at 55°C and a 90-s extension at 72°C.

In situ hybridization

Chromosome slide preparations were pre-treated with RNase and fixed with 4% paraformaldehyde, and in situ hybridization was carried out as described by Heslop-Harrison et al. (1991) and Cuadrado and Jouve (1994). Slide preparations were denatured prior to in situ hybridization using a programmable thermal cycler (PT-100, M-J Research Inc). The hybridization mixture, 30 µl per slide, was prepared by adding 50% de-ionized formamide, 10% dextran sulphate, 2 × SSC (0.3 M NaCl, 0.03 M Na_3-citrate), 0.1%

Fig. 1 a–i FISH to metaphase chromosomes of wheat 'Chinese Spring' with SSR oligonucleotides. **a** A somatic metaphase with the $(AG)_{12}$ motif detected with green fluorescence. In the inset to **a**, *arrows* indicate the hybridization sites with $(AG)_{12}$ in *red* and the pSc119.2 probe in *green* on chromosomes from a different metaphase. The pSc119.2 signal permits the unequivocal identification of the three AG sites on chromosomes 3B, 4B and 5B**; b** the same metaphase as **a** with the $(CAT)_5$ motif, detected with *red fluorescence*. Chromosomes show the counterstaining with DAPI in blue. Arrows on the long arm of chromosome 3B (inset, without DAPI counterstain**)** indicate the different locations of the $(AG)_{12}$ oligonucleotide (*yellow*) and $(CAT)_5$ (*orange*). **c, d** A metaphase after DAPI staining: *bars* indicate all B-genome chromosomes **c** and FISH with the $(AAC)_5$ oligonucleotide detected with FITC (yellow green, **d).** All B-genome chromosomes can be identified. **e–i** Individual chromosomes (4 A, 7 A, 2B, 3B and 5B) after FISH using $(AAG)_5$ (*green signal* on the left), $(AAC)_5$ (*pink signal* on the right) and the simultaneous location of both nucleotides (middle). *Asteriks* C+ N+ AAC+ AAG+; *bars* C+ N+ AAC- AAG+ (Class 2). *Arrows* C+ N- AAC+ AAG- (Class 3). *Arrowhead* C- N- AAC+ AAG- (Class 4)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sodium dodecyl sulphate, 50 µg/ml de-natured *Escherichia coli* DNA and 50–100 ng of probe. When two probes were used in the same hybridization mixture, the two different labelled probes were mixed 1:1 prior to use. After overnight hybridization at 37°C, stringent washes were in 20% formamide/0.1 × SSC at 40°C, followed by washes in 2 × SSC at 42°C and room temperature. Detection of biotin and digoxigenin hybridization sites was carried out simultaneously by incubating the slides in a solution of 4 µg/ml of anti-digoxigenin-FITC (Roche) and 5 µg/ml of streptavidin-Cy3 (Sigma) in 5% (w/v) bovine serum albumin in 4 × SSC/Tween-20 for 1 h at 37°C.

Slides were washed in 4 × SSC/Tween before staining the DNA with 4 µg/ml of DAPI (4',6'-diamidino-2-phenylindole). The preparations were mounted in Vectashield mounting medium for fluorescence H-1000 (Vector Lab, Burlingame, Calif., USA). Slides were examined with a Zeiss Axiophot epifluorescence microscope following excitation with UV, blue and green light. Photographs were taken on Fujicolor Super G 400 film. The $(AAC)_5$ and $(AAG)_5$ distribution pattern of ''Chinese Spring'' was analysed in six metaphase cells and superimposed onto the standard wheat karyotype of Gill et al. (1991)

Results

We adapted a protocol of random primer labelling for generating probes labelled with biotin-16-dUTP or digoxigenin-11-dUTP from short synthetic oligonucleotides, $(AAC)_5$, $(AAG)_5$, $(AG)_{12}$ and $(CAT)_5$, that can be hybridized with standard hybridization (50% formamide/2 × SSC at 37°C) and washing (20%formamide/0.1 × SSC at 40°C) conditions allowing multi-target FISH experiments, mixing oligonucleotide probes with longer cloned probes. In our experiments, we combined the synthetic oligonucleotide probes with the clones pAs1 and pSc119.2 for identification of the wheat chromosomes.

Using the formula from Meinkoth and Wahl (1984), the above conditions imply hybridization at 70% stringency (allowing 30% mismatch) and washing at 76% stringency for the cloned probe pSc119.2 (600-bp fragment and 55% GC content) and 78% and 83% stringency, respectively for pAs1 (a 1-kb fragment with a 35% GC content). For the short oligonucleotides $(AAC)_5$, $(AAG)_5$, $(CAT)_5$ and $(AG)_{12}$ the conditions are extremely stringent.

The repetitive DNA sequence probes gave the characteristic patterns described by Mukai et al. (1993) and allowed the identification of all the B-genome, D-genome and some A-genome chromosomes (Fig. 1a inset and data not shown). Hybridization signals with the random primer-labelled SSR probes were very strong, and well-defined bands with little dispersed signal along the chromosomes were detected with all four SSR motifs (Fig. 1). Different batches of random primer labelling, some carried out in different laboratories, resulted in reproducible banding patterns, indicating that the probes generated are specific. AAC and AAG clusters showed the same distribution patterns as previously described (Cuadrado and Schwarzacher 1998) using the same oligonucleotides labelled with terminal transferase (TdT) and hybridization with low stringency conditions; but AG and CAT showed significant differences.

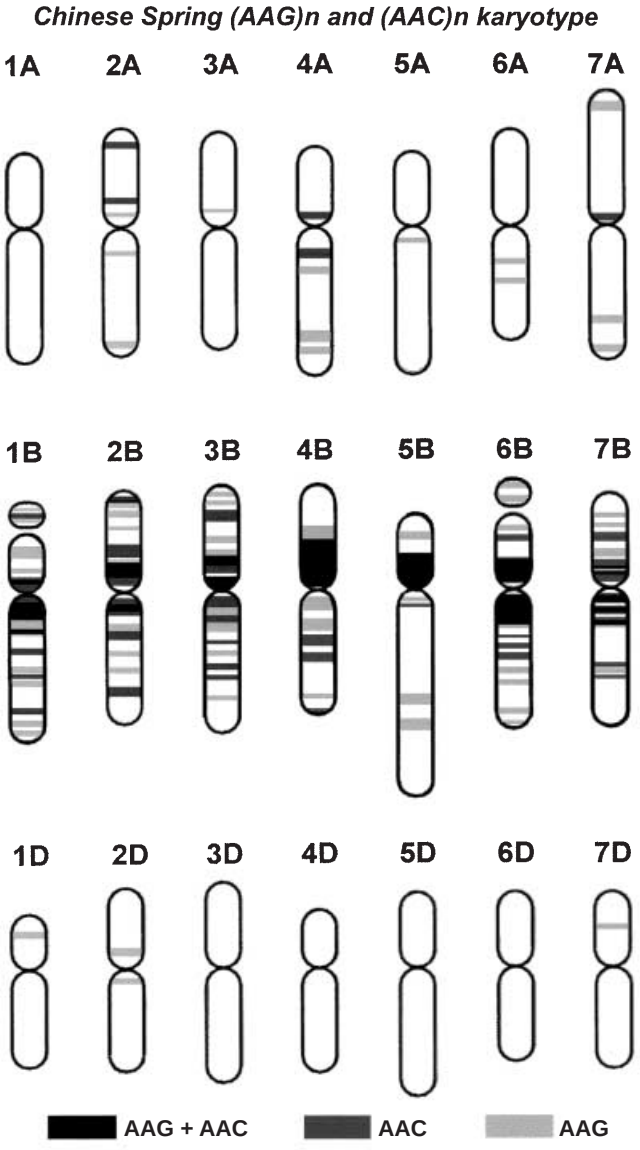

Fig. 2 Idiogram of chromosomes of ''Chinese Spring'' wheat showing the distribution patterns of $(AAG)_5$ (*grey*) and $(AAC)_5$ (*dark grey*). Sites where $(AAC)_5$ and $(AAG)_5$ co-localize are drawn in black. Chromosomes lengths and arm ratios are taken from Gill et al. (1991)

In situ hybridization using the random primer-labelled $(AG)_{12}$ probe at high stringency yielded four sites on B-genome chromosomes that were identified using the probe pSc119.2 (Fig. 1a): two sites occupied a position near the centromeres on 5BL and 4BL respectively, and two were found in the middle of 5BS and 3BS respectively. The $(CAT)_5$ probe hybridized at only one site close to the centromere on chromosome arm 3BL (Fig. 1b). Both, AG and CAT produced many more bands at low stringency (Cuadrado and Schwarzacher 1998).

Using an in situ hybridization pattern with the $(AAC)_5$ probe alone, it was possible to distinguish and identify the B-genome chromosomes (Figs. 1c, d) and in most cases several A genome chromosomes, i.e. 2 A, 4 A and 7 A. The results of the probe $(AAC)_5$, in general

resembled the pattern of strong C bands or N bands of wheat as described by Gill et al. (1991) and, with some exceptions, the $(GACA)_4$ SSR patterns found by Cuadrado and Schwarzacher (1998). The distribution of hybridization sites of the $(AAG)_5$ probe was very similar, although not identical, to the $(AAC)_5$ probe and together the two probes provide a rich banding pattern along most chromosome arms (Figs. 1e–i, 2). Most $(AAC)_5$ bands are concentrated near the centromeres of all B-genome chromosomes, while the $(AAG)_5$ bands are generally more distally located and are also found in additional A-genome (3 A, 5 A, 6 A) and some D-genome (1D, 2D, 7D) chromosomes. (Fig. 2).

Discussion

Classes of banded chromatin

On the basis of in situ hybridization with SSR probes (Figs. 1–2) and comparison of the detected SSR clusters with the C banding and N banding pattern (as described by Gill et al. 1991) in wheat, we are able to distinguish different classes of banded chromatin. In wheat, N banding only reveals bands observed by C banding (C+N+), although certain N bands may be stained more intensely; but for many C bands there are no corresponding N bands (C+N-). Of the 11 possible classes with different combinations of AAC sites, AAG sites and C bands (C+N+ and C+N-), four classes were substantially more abundant than the others in wheat. The $(GACA)_4$ distribution of Cuadrado and Schwarzacher (1998) is also indicated in the classification below.

Class 1, C+ N+ AAC+ AAG+: major $(AAC)_5$ probe hybridization sites (AAC+) that coincide with the heterochromatin distribution as revealed by C banding (C+), N banding (N+) and the $(AAG)_5$ probe distribution (AAG+). These are the majority of bands on the B-genome chromosomes, e.g. the pericentromeric sites of 2B, 3B and 5B (Fig. 1g,h and i), that also showed a hybridization signal with $(GACA)_4$. This is by far the most-frequent class.

Class 2, C+ N+ AAC- AAG+: sites revealed with the $(AAG)_5$ probe that match C and N bands, but do not hybridize with the $(AAC)_5$ probe; they are mainly interstitial heterochromatin present e.g. on the long arm of chromosomes 4 A and 5B (Figs. 1e and i). These sites were also $(GACA)_4$ positive.

Class 3, C+ N- AAC+ AAG-: $(AAC)_5$ probe hybridization sites that coincide with the heterochromatin distribution as revealed by C banding but are neither observed by N banding nor hybridization with the $(AAG)_5$ or $(GACA)_4$ oligonucleotide, e.g. the centromeric site on 7 A (Fig. 1f).

Class 4, C- N- AAC+ AAG-: sites specifically labelled when using the $(AAC)_5$ probe and lacking any type of other banding, e.g. the interstitial sites on chromosome arms 4AS and 2BL (Fig. 1e and g). These sites were also negative with $(GACA)_4$.

In summary, all N bands coincide with the presence of SSRs and we detect no sites with $(GACA)_4$ hybridization alone. The sites detected with the $(AG)_{12}$ and $(CAT)_5$ probe (Fig. 1a and b) are separate, but very close to AAG sites in the same heterochromatic block. Division of the C+N+ bands comes from our results showing that they are associated with different combinations of single sequence repeats, either AAC^+/AAG^+ or AAC^-/AAG^+. It is not surprising to find such a heterogeneity of the euchromatin and heterochromatin in wheat, as similarly different, but related satellites have been found distributed in many categories of mammalian, animal and plant heterochromatin (e.g. Schwarzacher et al. 1988; Lohe et al. 1993). Also, it is now well accepted that although C banding stains regions containing high amounts of satellite sequences it does not depend on the molecular nature of the DNA, but more on its protein-binding ability and secondary structure (Holmquist 1979).

The nature of SSR clusters and their relation to the heterogeneity of chromatin in wheat

It has been previously demonstrated that in cereals the GAA-satellite sequence hybridizes preferentially to the heterochromatic sites stained by N banding (Schlegel and Gill 1984; Pedersen et al. 1996; Pedersen and Langridge 1997) and, hence, has been suggested to reveal only heterochromatic polypyrimidine/polypurine DNA sequences. Our results (Fig. 1d) on the distribution pattern of AAG confirms that AAG is a major component of N bands (Classes 1 and 2), but since other SSRs, including AAC and GACA, are also located in N bands, the polypyrimidine/polypurine conformation is not on its own responsible for N banding (Cuadrado and Schwarzacher 1998).

Although AAC frequently associated with the AAG sequence in the pericentromeric regions of the B genome chromosomes, the strength of signal with the two motifs was often different and several AAG bands are devoid of the AAC motif while some regions are AAC+ and AAG- (Classes 3 and 4). The molecular structure of the GAA-satellite sequence in predominantly GAA tracts of 150 kb (Dennis et al. 1980; Pederson et al. 1996), mean that when AAC and AAG signals are located in the same physical position, they are probably not intermingled, but most likely form closely adjacent blocks. Such an organization in wheat, would agree with the localization of different satellite arrays of closely related sequences near one another in the same heterochromatic band of *Drosophila* (Lohe et al. 1993) and rye (Vershinin et al. 1995). Further, the molecular analysis showing that different GAA-satellite clones from wheat and barley, although primarily composed of GAA repeats, included the other triplets GAG, GCA and GGA, but rarely AAC (Dennis et al. 1980; Pederson et al. 1996), support the interpretation of separate AAC and AAG clusters.

The GAA-satellite sequence was first isolated by Dennis et al. (1980) using ultracentrifugation with Ag^+-Cs_2SO_4 buoyant density gradients, and appeared as a

1.699 g/cc peak. Interestingly, the motif (AAC)*n* that has the same base pair composition as (GAA)n, two AT and one GC pairs, and hence the same molecular weight, was not isolated using buoyant density gradients. This indicates that important differences exist in the higher-order organization of these two SSR motifs although both motifs give bands when used as FISH probes (Figs. 1e–i, 2). While the described organization of the GAA satellite in long arrays in the heterochromatin is typical for satellite DNA (see above), AAC is also found in C-band and N band-negative chromatin (Class 4), and could be present as smaller arrays interspersed with other repeats or unique sequences to form extended regions of several kilobases that are required to produce consistent in situ hybridization signals. Such an arrangement would have similarities with the beta-heterochromatin of *Drosophila* and the syrian hamster where few unique sequences, and scrambled repeats of mobile elements without tandem repetition, were described (Holmquist et al. 1998).

SSRs as markers for chromosome identification

It is now clear that microsatellites are major components of many, if not all, plant genomes (Powell et al. 1996). Moreover, the chromosome localization in wheat, described here, and previously published for rye, beet and peas (Schmidt and Heslop-Harrison 1996; Cuadrado and Schwarzacher 1998; Fuchs et al 1998; Gortner et al. 1998), indicate that SSRs used as FISH probes have many advantages to make them a suitable and versatile tool in plant cytogenetics in general, namely: (1) as oligonucleotides are used, the cloning and maintaining of selected clones is not required, (2) as SSRs are ubiquitous in plant genomes, their use as probes will be applicable to most plant species with probably only a small effort in finding the most informative motifs, (3) it is expected that a variety of banding patterns depending on the motifs chosen will be available for each species.

The immediate advantage of some SSRs (e.g. AAC, AAG and CAGA in wheat) used as probes for in situ hybridization lies in identifying many chromosomes in a complement at once. However, as their diagnostic value is in the combination of patterns, and often depends on having all chromosomes of a complement available for comparison, it could be difficult to trace the origin of a signal to a particular source chromosome. In contrast, CAT and AG, hybridized at high stringency as used here (Fig. 1a and b), detect signals in only one or a few locations in the Chinese Spring wheat genome (at low stringency, more sites are visible resembling the GACA distribution; see Cuadrado and Schwarzacher 1998). CAT and AG provide the means to distinguish few chromosomes from each other and from chromosomes of other species easily, even if the quality of metaphase spreads is not ideal. This ease of identification offers direct diagnostic advantages of certain SSR probes and the possibility to screen large populations of plants for the presence of particular chromosomes or even small segments. On the other hand, genome-specific SSR motifs, such as AAC with a mainly B-genome distribution, provides a rapid means of establishing whether or not an interspecific hybridization has been effective.

Cloned long tandem repeat DNA sequences and genes (e.g. pSc119.2, pAs1 and the 45 S and 5 S rRNA genes) have proven useful as cytological markers in identifying both chromosomes and genomes in wheat (e.g. Friebe et al. 1991; Mukai et al 1993; Castilho and Heslop-Harrison 1995; Cuadrado and Jouve 1995, 1996; Pedersen and Langridge 1997; Bardsley et al. 1999). In wheat and also rye, the SSR sites do not correspond to sites of any of these repetitive cloned sequences and they occupy many proximal chromosome locations (Cuadardo and Schwarzacher 1998; and see Fig. 1a). Thus, by combining SSR probes with long tandem repeat sequences, we are now able to construct physical maps of wheat with multiple landmarks along most chromosome arms (e.g. Figs 1e–i, 2). These will form the basis for the identification of not only whole chromosomes, but also chromosome segments to describe chromosome rearrangements, to follow specific chromosomes in breeding programmes, when mapping clones by FISH, and to study genome organization, karyotype relations, diversity and evolution.

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