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Characterization of *Aegilops uniaristata* chromosomes by comparative DNA marker analysis and repetitive DNA sequence in situ hybridization

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Abstract RFLP analyses were performed on wheat-*Aegilops uniaristata* Vis. addition and translocation lines to confirm the identity of added N-genome chromosomes. Complete 1N, 3N, 4N, 5N and 7N chromosome additions were identified, while the complete long arm and only part of the short arm was identified for chromosome 2N. There were no wheat-like 4/5 and 4/7 translocations in the *Ae. uniaristata* chromosomes. Chromosome 3N carried an asymmetric pericentric inversion, and the translocation line was a product of centric fusion between the long arms of chromosomes 3B and 3N. Chromosome-specific RAPD and microsatellite markers were also identified for all the added *Ae. uniaristata* chromosomes available in this set of addition lines. A new genomic in situ hybridization protocol combining pre-annealing of probe and blocking DNA and prehybridization with blocking DNA was developed to differentiate the very closely related genomes of *Ae. uniaristata* and wheat. Hybridization sites for the repetitive DNA sequences pAs1, pSc119.2 and pTa71 were identified on the N-genome chromosomes of *Ae. uniaristata* using the fluorescent in situ hybridization technique. Results showed deviation from the previously published ideogram of this species. A new ideogram, which shows the hybridization sites for the above sequences, was produced in which the chromosomes are arranged according to their homoeologous group.

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Introduction

Alien chromosome addition and translocation lines are the starting point of breeding programmes aimed at the transfer of alien chromatin material to cultivated varieties (Feldman and Sears 1980). They also provide a basis for studying the homoeologous relationship between the chromosomes of different species (Miller and Reader 1987) and for locating genes on a particular chromosome (Riley et al. 1966). Comparative restriction fragment length polymorphism (RFLP) analyses have been utilized to identify inter- and intra-chromosomal translocations and rearrangements in alien chromosomes compared to the chromosomes of wheat (Liu et al. 1992; Devos et al. 1995). Random amplified polymorphic DNA (RAPD) and microsatellite (SSR, simple sequence repeat) markers have also been used for the detection of alien chromosome addition lines (Hernandez et al. 1996) and to confirm the presence of alien chromatin material (Francis et al. 1995).

The genus *Aegilops* is closely related to the genus *Triticum*, and its value as a source of genetic improvement for wheat is well documented (see Gale and Miller 1987 for review). *Ae. uniaristata* is of particular interest as it has been shown to be tolerant to high levels of soil aluminium (Berzonsky and Kimber 1986), and this tolerance has been shown to be effective when transferred to wheat (Miller et al. 1995, 1997).

The aims of the study reported here were to identify and characterize the *Ae. uniaristata* N-genome chromosomes in wheat-*Ae. uniaristata* addition and translocation lines and to find chromosome-specific polymerase chain reaction (PCR)-based markers for the rapid identification of *Ae. uniaristata*, chromosomes and chromosome segments. Also, fluorescence in situ hybridization (FISH) was used to map the position of repetitive DNA

sequences on the N-genome chromosomes so that individual chromosomes could be identified and characterized with respect to homoeology.

Materials and methods

The plant material consisted of the parental species *Aegilops uniaristata* Vis. accession 2120001 (2n=2x=14, NN) from the John Innes Centre collection, *Triticum aestivum* L. cv. Chinese Spring (CS; 2n=6x=42, AABBDD), eight disomic addition lines of *Ae. uniaristata* into CS (selected on the basis of morphology) and one wheat/*Ae. uniaristata* translocation line (Miller et al. 1997). Three substitution lines, 3A(3N), and 3B(3N) and 3D(3N) (Miller et al. 1995), were also used in this study.

The cDNA and genomic wheat RFLP probes were from the collection maintained at the John Innes Centre, Norwich. For microsatellite amplifications PSP primers from the John Innes Centre and WMS primers from Institut fur Pflanzengenetik und Kulturpflanzenforschung (IPK), Gatersleben, Germany were employed.

For RAPD analysis, oligonucleotide primers, the complete OPA and OPB series, four primers from the OPE series (3, 8, 11, 20), seven from the OPF series (5, 8, 9, 14, 15, 16, 20), nine from the OPL series (4, 9, 11, 13, 14, 15, 17, 18, 19), five from the OPR series (1, 2, 5, 6, 19) and four from the OPV series (4, 10, 18, 20) from Operon Technologies were used.

DNA for RFLP, RAPD and SSR was isolated following the method of Davis et al. (1986). RFLP analysis was performed according to Davis et al. (1986), and the method of Farooq et al. (1994) was employed for RAPD analysis. For microsatellite amplifications the methods described by Röder et al. (1995) were followed. Denaturing acrylamide gels and silver staining methods for visualization of microsatellites have been described by Tixier et al. (1997).

Total genomic *Ae. uniaristata* DNA was used as a probe to confirm the presence of a pair of alien chromosomes in the addition lines. Published techniques for genomic in situ hybridization (Schwarzacher et al. 1989; Leitch et al. 1994; Reader et al. 1994) proved ineffective in distinguising *Ae. uniaristata* chromosomes in a wheat background. The following protocol was devised to successfully differentiate the *Ae. uniaristata* N-genome chromosomes. In this protocol a pre-hybridization step is used to block the common sequences present on chromosomes in cytological preparations and, at the same time, pre-annealing of the probe with blocking DNA is performed to remove most of the highly repetitive common sequences. The pre-annealing and pre-hybridization steps were performed at 76°C to ensure that hybridization occurs only between sequences which have more than 90% homology. Higher concentrations of probes were used in the pre-hybridization mixtures so that enough genome-specific sequences were available after this step to hybridize with sequences present on chromosome preparations. Hybridization temperatures were increased to 74°C to eliminate cross hybridization and thus optimize genome differentiation.

Mitotic slides were prepared from root-tip meristems of the addition lines and the wheat/*Ae. uniaristata* translocation line using the enzyme digestion method of Schwarzacher et al. (1989). Total genomic *Ae. uniaristata* DNA was labelled by the nick translation method (Leitch et al. 1994) using fluorescent labelled nucleotides (NEN Life Sciences). The reaction was stopped by adding 1/10 volumes of 0.3 *M* EDTA (pH 8); the tubes were then wrapped in aluminium foil and stored at –20°C without an ethanol precipitation step (S.M. Reader unpublished). The rapid in situ hybridization method (Reader et al. 1994) was modified as described below. Total genomic wheat DNA was used for blocking.

Slides were treated with 0.01 *M* HCl for 2 min and incubated with pepsin (5 µg/ml) for 20 min at 37°C. The slides were then washed with water and 2×SSC (0.3 *M* NaCl, 30 m*M* trisodium citrate) for 2 min and 10 min, respectively. Slides were treated with RNase (5 µg/ml) for 30 min washed with 2× SSC and fixed with paraformaldehyde (4% in 2× SSC). The preparations were then denatured in formamide solution (70% formamide in 2× SSC) at 70°C for 2 min, followed by dehydration through an ice-cold ethanol series (70%, 90% and 100% for 2 min each).

Pre-annealing of probe

For pre-annealing, 200 ng of labelled probe DNA was mixed with an equal amount of blocking DNA in the hybridization mixture (10% dextran sulphate, 2× SSC, 0.25% SDS) in a total volume of 60 µl. The mixture was heated at 100°C in a hot block for 3 min, centrifuged briefly and incubated at 76°C for 2 h.

Pre-hybridization of slides

The pre-hybridization mixture was prepared as above but contained only blocking DNA (0.5 µg), i.e. omitting the probe DNA. The mixture was denatured by heating at 100°C for 3 min and chilled on ice for a further 3 min after a brief centrifugation. It was then put onto the previously denatured chromosome preparations, which were covered with plastic coverslips, and incubated for 2 h at 76°C.

Hybridization and washing

Slides which had been subjected to the pre-hybridization protocol above were then hybridized with the pre-annealed probe mixture at 74°C for 4 h. After hybridization, slides were washed twice (5 min each) with 2× SSC at 74°C, transferred to 37°C and then to room temperature. Slides were then stained with DAPI (0.125 µg/ml), mounted with Vectashield (Vector laboratories) and examined with a Nikon epifluorescent microscope.

Three repetitive sequences pAs1 (Rayburn and Gill 1986), pTa71 (Gerlach and Bedbrook 1979) and pSc119.2 (Bedbrook et al. 1980) were also used as probes for FISH again using the nick translation method of labelling of Leitch et al. (1994). With these probes the unchanged rapid fluorescence in situ hybridization method developed by Reader et al. (1994) was utilized for the hybridization of the labelled probes to the chromosome preparations.

Results and discussion

RFLP studies

All probes used for the identification of each homoeologous group and those with polymorphic homoeoloci in the respective addition lines are listed in Table 1.

About 49% of the RFLP probes revealed polymorphism between CS and *Ae. uniaristata*. Homoeologous group 6 probes, although polymorphic, were unable to detect *Ae. uniaristata*-specific bands in any of the addition lines, thus indicating the absence of a 6N addition in this set.

A total of seven homoeologous group 1 RFLP probes, three for the short arm and four for the long arm, revealed *Ae. uniaristata*-specific homoeoloci in one addition line. This addition line therefore carries a pair of complete 1N chromosomes from *Ae. uniaristata.*

One addition line showed *Ae. uniaristata* specific hybridization signals with four long arm probes but only one short arm group-2 RFLP probe. This short arm probe is mapped close to the centromere on wheat group 2 chromosomes. These results indicate that the complete

Table 1 Loci used as RFLP probes for the identification of the homoeology of the *Ae. uniaristata* chromosomes

Homoeologous arm location in wheat	Locus	Polymorphic homoeolocus in *Ae. uniaristata*
1S	*Gli-1-1*[a]	–
	Glu-3[b]	+
	Xpsr963	+
	Xpsr596	–
	Xpsr634	–
	Xpsr161	+
1L	*Xpsr544*	+
	Xpsr653	–
	Xpsr330	+
	Lec[c]	+
	Xpsr953	+
	Xpsr313	–
2S	*Xpsr649*	–
	Xpsr335	–
	Xpsr566	–
	Xpsr928	–
	Xpsr108	–
	Xpsr666	–
	Xpsr109	–
	Xpsr131	–
	Xpsr146	+
	Xpsr126	–
	Xpsr137	–
2L	*Xpsr571*	+
	Xpsr388	–
	Xpsr102	+
	Xpsr901	–
	Xpsr630	+
	Xpsr540	–
	Xpsr934	–
	Xpsr609	+
3S	*Xpsr305*	+
	Xpsr1196	+
	Xpsr598	+
	Xpsr547	+
3L	*Xpsr394*	+
	Xpsr56	+
	Xpsr116	–
	Xpsr156	+
	Xpsr170	+
	Cxp1 (*Xpsr483*)[d]	+
4S	*Xpsr921*	+
	Xpsr922	+
	Xpsr144	+
4L	*Xpsr110*	+
	Xpsr920	–
	Xpsr163	–
	Xpsr914	+
	Xpsr648	–
	Cat[e]	+
	Xpsr164	–
	Xpsr567	–
5S	*Xpsr170*	+
	Xpsr618	–
	Xpsr118	–
	Xpsr929	+
5L	*Xpsr109*	–
	Xpsr120.1	–
	Xpsr128	–
	Xpsr150	+
	Xpsr911	–
	Xpsr120.2	–
	Xpsr115	+
	Xpsr580	+
	Xpsr1206	+
	Xpsr567	+
7S	*Xpsr160*	–
	Xpsr119	+
	SS1[f]	+
	Xpsr108	–
	Xpsr103	+
	Xpsr150	+
7L	*Xpsr169*	+
	Xpsr129	–
	Xpsr547	–
	Xpsr680	–

[a] Probe pTag1436, Bartels et al. (1986)
[b] Probe pTag544, Bartels et al. (1986)
[c] Probe pNVR1, Raikhel and Wilkins (1987)
[d] Probe pλc.3, Doan and Fincher (1988)
e Probe pCat2.1cf, Berthards et al. (1987)
[f] Probe pST8, Marana et al. (1988)

long arm of chromosome 2N is present but that part of the short arm may be absent in this addition line. The morphology of the added chromosome pair was the same as in *Ae. uniaristata*, suggesting that no breakage or translocation occurred during the production of this addition line. In wheat, a 2BS/6BS translocation in which part of the chromosome 2B short arm is transferred to chromosome 6B has been detected (Devos et al. 1993). A similar kind of translocation may be present in *Ae. uniaristata*, and the subterminal centromeric morphology of the 2N chromosome might be the result of this translocational event. Unfortunately, the 6N addition line was not available in this set and this hypothesis, therefore, could not be confirmed. Moreover, no group 6 RFLPs were revealed on the 2N addition chromosome.

The presence of a complete chromosome 3N was confirmed by eight homoeologous group 3 RFLP probes. RFLP results for the translocation line showed the absence of the 3B short arm, but all the 3B long arm specific probes hybridized to this line. The line also showed the presence of 3N short arm as well as long arm probes, although two long arm homoeoloci, close to the centromere, were absent. The morphology of the translocated chromosome indicated a centric fusion between the long arms of chromosome 3B of wheat and chromosome 3N of *Ae. uniaristata*. Figure 1 shows a model based on the results for chromosome 3N of the addition line and chromosome 3BL.3NL of the translocation line. The pres-

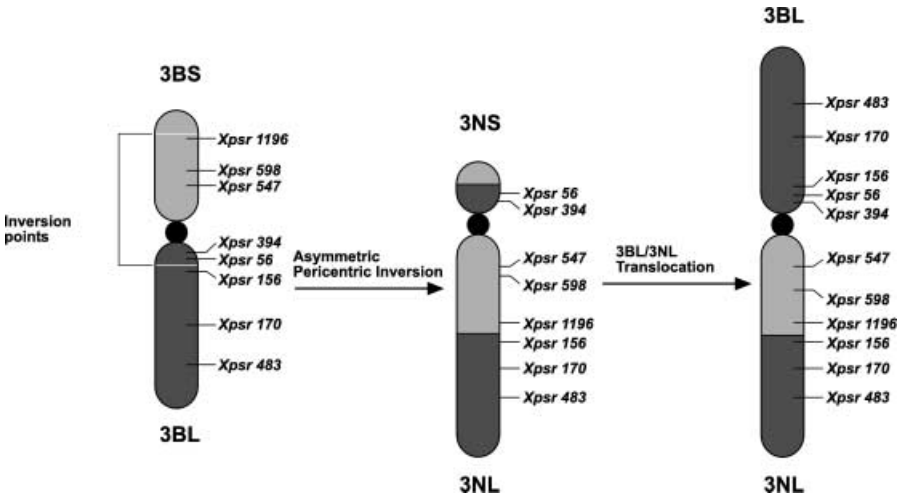

Fig. 1 Diagrammatic representation of the RFLP-based evidence that the highly heterobrachial morphology of chromosome 3N is the product of a pericentric inversion

ence of short arm specific homoeoloci and absence of some long arm loci in the translocation line can be explained on the basis of the pericentric inversion in 3N. The inversion involved more than 80% of the short arm and less than 20% of the long arm. The physical distances, reported by Delaney et al. (1995), of the suggested homoeoloci involved in this inversion also explain the highly heterobrachial morphology of chromosome 3N.

The 4N addition was confirmed by six group 4 homoeologous RFLP probes, three for each arm. The chromosome 5N addition line was characterized by two short arm probes and five long arm probes. Wheat carries a 4/5 translocation and probes PSR115 and PSR1206 detect the 5AL segment transferred to the long arm of chromosome 4A. The *Ae. uniaristata* homoeoloci detected by these probes were present in the 5N addition line but not in the 4N addition line. *Ae. uniaristata*, therefore, does not carry a 4/5 translocation like wheat, although a similar type of translocation has been reported in *Triticum urartu*, *Aegilops umbellulata* and *Thinopyrum bessarabicum* (King et al. 1994).

The chromosome 7N addition line was identified by four long arm specific and one short arm specific homoeologous group 7 RFLP probes. These results also indicated that *Ae. uniaristata* does not carry a wheat-like 4/7 translocation. The PSR119 probe which detects the presence of the chromosome 7B fragment on chromosome 4A in wheat detected an *Ae. uniaristata* homoeolocus in the 7N addition line and not in the 4N addition line.

RAPD and SSR analysis

RAPD and SSR analyses were performed on the addition lines when the identity of the added *Ae. uniaristata* chromosomes had been established by RFLP analysis. A total of 69 RAPD and 78 SSR primers were utilized to identify PCR-based chromosome specific markers for *Ae. uniaristata*. Only about 16% of the RAPD and 19% of the SSR primers were able to amplify polymorphic products that could be used as markers to detect *Ae. uniaristata* chromosomes (Table 2). Figure 2 shows examples of SSR and RAPD *Ae. uniaristata* specific products in the 7N addition line.

Table 2 Microsatellite (SSR) and RAPD primers that amplified polymorphic products detecting *Ae. uniaristata* chromosomes

Ae. uniaristata chromosome	Primers	
	SSR	RAPD
1N	WMS194 WMS299 PSP3001	OPB18 OPE3
2N	PSP3001	OPB12 OPB15 OPB08
3N	WMS161 WMS325 PSP3029 PSP3047 PSP3144	OPB10 OPB13
4N	WMS325 WMS194	OPE20
5N	WMS5325 WMS5631 PSP3030 PSP3101	OPB15 OPV18
7N	WMS437 PSP3037	OPE03 OPV10

Successful amplification of DNA by PCR requires good homology between primer and template DNA. The low polymorphism in RAPD profiles of CS and *Ae. uniaristata* indicates the highly repetitive nature of the genomes. On the other hand, the failure of most of the microsatellite primers to amplify any products from *Ae. uniaristata* DNA indicates differences in the nucleotide sequences across these genomes. SSR primers amplifying D genome specific products in wheat were, in general, more successful in amplifying the *Ae. uniaristata*-specific products. Some SSR products, already mapped in wheat, produced homoeologous results for *Ae. uniaristata* and, in such cases, these markers can be used to identify specific loci on N genome chromosomes. How-

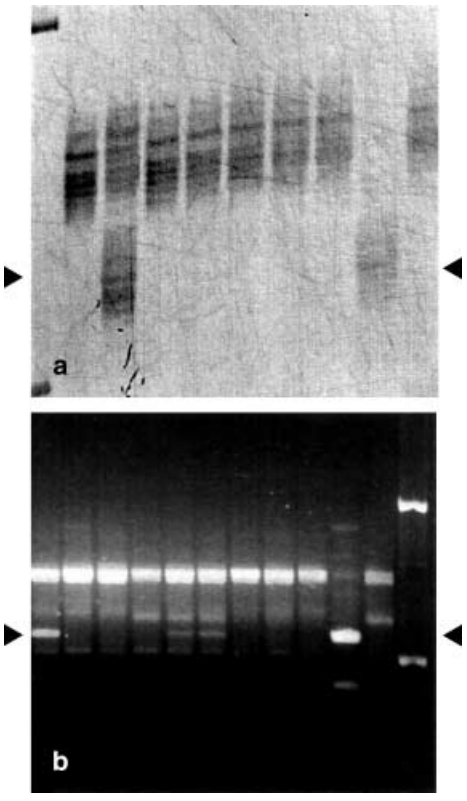

Fig. 2 a Microsatellite primer WMS437 product amplified (*arrowheads*) in the 7N addition line and *Ae. uniaristata*. **b** RAPD profiles produced by primer OPE03 showing a specific fragment (*arrowheads*) in the 7N addition line and *Ae. uniaristata*

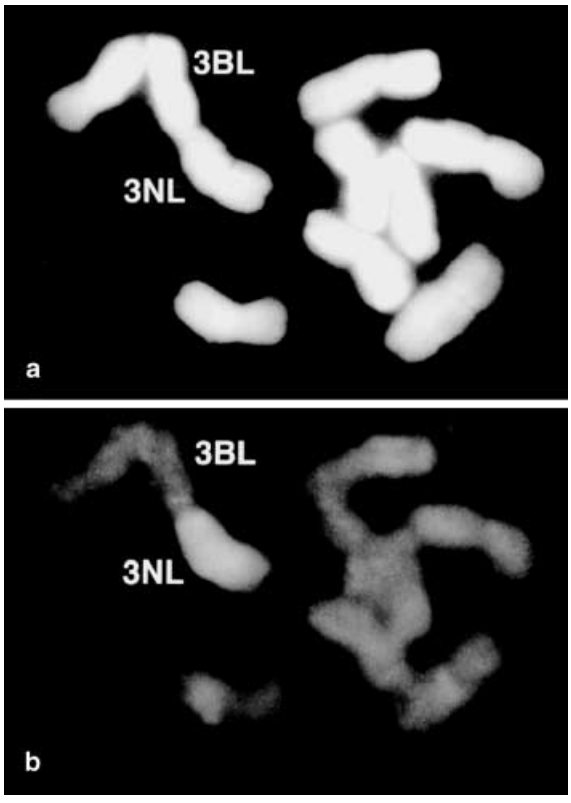

Fig. 3a, b Chromosomes of the 3B.3N translocation line. **a** DAPI-stained chromosomes and **b** the same chromosomes probed with labelled *Ae. uniaristata* DNA. The two arms of the 3BL.3NL translocated chromosome can be distinguished by differences in the intensity of the hybridization signal

ever, in many cases the amplified products were not of a homoeologous nature and can only be used to detect the presence or absence of a particular chromosome.

In situ hybridization studies

Anamthawat-Jónsson and Reader (1995) showed the value of pre-annealing DNA probes for genomic in situ hybridization, and Hassani (1998) used pre-hybridization blocking to improve genome differentiation. Using the new combined pre-anealing and prehybridization technique clearly enabled the N genome chromosomes to be differentiated from the A, B and D genome chromosomes. This was particularly successful in the case of the D genome, which was virtually impossible to separate from the N genome using the previously published techniques. Thus, it was possible to confirm the presence of an alien pair of chromosomes in each of the addition lines and to confirm the Robertsonian nature of the 3B.3N translocation (Fig. 3).

In situ hybridization studies with the repetitive sequences proved very informative in the identification and differentiation of individual chromosomes. The probe pAs1 hybridized strongly with seven pairs of chromosomes of wheat, while only two pairs of wheat chromosomes did not show sites for pSc119.2. The repetitive sequence pTa71 hybridized strongly with two pairs of satellited wheat chromosomes, and two weak signals could also be seen. These results are in accord with those of Mukai and Nakahara (1993), which also showed strongly hybridization signals for pAs1 on the D genome chromosomes and the absence of pSc119.2 hybridization sites on only 4 of the 42 chromosomes.

Six pairs of *Ae. uniaristata* chromosomes showed strong hybridization signals with pAs1, while one pair showed weak signals with this probe. The probe pAs1 is a D genome specific probe isolated from *Ae. squarrosa* (Rayburn and Gill 1986) and shows strong hybridization sites with the D genome of wheat. These results, therefore, indicate the relatedness of the N genome to the D genome of wheat. For pSc119.2, three pairs of chromosomes showed one signal at the telomeres of their short arms, while two pairs had signals at both the short and the long arm telomeres. Two pairs of chromosomes showed three sites on each chromosome, one at the telomere of the short arm and two on the long arm.

A generalized ideogram of *Ae. uniaristata*, based on C-banding and FISH studies, has been published by Badaeva et al. (1996). In another study, Friebe et al. (1996) assigned homoeologous groups to three of the seven chromosomes on the basis of their C-banding pat-

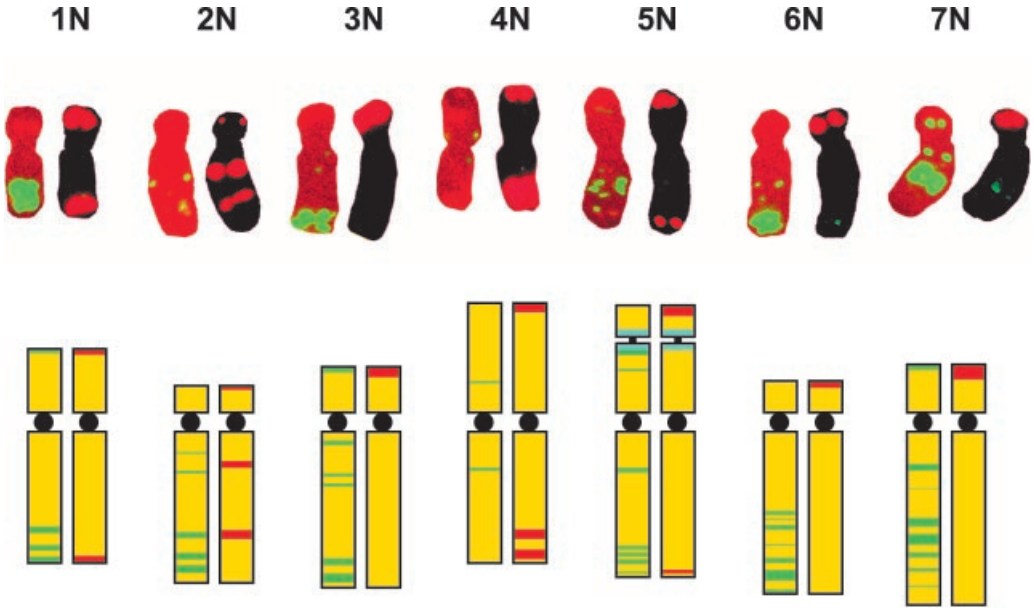

Fig. 4 Representative *Ae. uniaristata* chromosomes showing morphology and pSc119.2 and pAs1 repetitive DNA sequence locations. *Below* an ideogram of the karyotype based on the results. *Red* pSc119.2, *green* pAs1, *blue* pTa71

terns. Some differences have been found between the FISH data from this study and that of Badaeva et al. (1996). The latter reported one telomeric pScq19.2 site on the short arm and two sites on the long arm of chromosome 1N; in the present study (Fig. 4) only one site was observed at the telomere of each arm. Hybridization sites for pAs1 were the same in both of the studies, with one weak site at the telomere of the short arm and three sites on the distal end of the long arm.

Three pSc119.2 sites were identified on the added chromosomes of the 2N addition line, one at the telomere of the short arm and two intercalary sites on the long arm. The probe pAs1 hybridized to four sites on the long arm, but no sites were present on the short arm. When the FISH results for chromosome 2N were compared with the previously published data it was found that the intercalary locations of the long arm pSc119.2 sites corresponded to those of the chromosome put into group six by Badaeva et al. (1996), while the pAs1 sites were the same as described for the 2N chromosome.

The pAs1 hybridization sites for chromosome 3N, one weak signal at the short arm telomere and four to five signals on the long arm, were also different from the previous study and more comparable with the sites described there for the group 6 chromosome. Location of the pSc119.2 hybridization site was the same as described before with only one site on the short arm of the chromosome.

The pAs1 repetitive sequence sites for chromosome 4N were the same as reported by Badaeva et al. (1996), with two weak hybridization signals close to the centromere on each arm. Chromosome 4N also showed three sites for pSc119.2, where two sites were present in the telomeric region of the long arm and one site at the telomere of the short arm. In the previous study, three sites for pSc119.2 were reported on the long arm of chromosome 4N.

The pair of *Ae. uniaristata* chromosomes in the 5N addition line was easily identified on the basis of the two additional pTa71 sites. Wheat chromosomes 1B and 6B have pTa71 hybridization sites, and four strong signals can be seen in CS. The 5N addition line showed six strong signals with this probe, and the presence of two extra strong sites was, therefore, due to the added pair of 5N chromosomes. Two sites for pTa71 had been reported earlier on the satellited group-5 chromosomes of *Ae. uniaristata*. The results for the pAs1 and pSc119.2 hybridization sites were also the same as described before.

The results for the 7N addition line with the pAs1 probe corresponded to those for chromosome number 3 in the ideogram published by Badaeva et al. (1996), with one site at the telomere of the short arm and six different sites on the long arm. The pSc119.2 result for chromosome 7N was the same as described earlier, i.e. one site at the telomere of the short arm.

In *Ae. uniaristata* chromosomes, 3N and 7N show only one pSc119.2 site at the telomeres of their short arms and they also have similar morphologies. Similarly, the morphologies of chromosome 2N and 6N are almost the same and are very difficult to differentiate correctly in cytological preparations of *Ae. uniaristata*. It is, therefore, possible that in the previous studies these chromosomes may have been confused and assigned to the wrong groups, especially as different probes were applied to different cytological preparations of the parental species. However, in this study disomic addition lines were used, where only a single chromosome pair from *Ae. uniaristata* was present, and the homoeology of each added pair had been previously determined on the basis of adult plant morphology and confirmed by RFLP analysis. Also, each labelled probe was applied to the same chromosome preparation, thus removing the chance of assigning the results of one chromosome to another.

On the basis of these studies a new ideogram for *Ae. uniaristata* chromosomes is presented (Fig. 4) in which the chromosomes are arranged according to their homoeologous group, and the location of hybridization sites for pAs1, pSc119.2 and pTa71 probes are indicated on these chromosomes.

This study has increased the amount of information available about the *Ae. uniaristata* chromosomes and chromosomal rearrangements compared to wheat. SSR markers have demonstrated the close homoeology of the N. genome to the D genome of wheat. The PCR markers identified in this study will be very helpful for the quick identification of N genome chromosomes and chromosome segments in a wheat background. The new genomic in situ hybridization protocol may be a useful tool for differentiating very closely related genomes in a polyploid situation.

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