Detection of Various U and M Chromosomes in Wheat-*Aegilops biuncialis* Hybrids and Derivatives Using Fluorescence *in situ* Hybridisation and Molecular Markers

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Abstract: The aim of the study was to select wheat-*Aegilops biuncialis* addition lines carrying *Aegilops biuncialis* chromosomes differing from those which were introgressed into the wheat-*Ae. biuncialis* addition lines produced earlier in Martonvásár, Hungary. In the course of the experiments new wheat-*Ae. biuncialis* addition lines carrying chromosomes $2U^b$, $6M^b$, $6U^b$; $5U^b$, $3U^b$, $7U^b$; $5M^b$, $6M^b$ and $7M^b$ were selected. The $2U^b$ disomic addition line is relatively stable, as 91% of the progenies contain this chromosome pair. The $6M^b$ disomic addition line proved to be dwarf and sterile, but it still exists as a monosomic addition line. Progenies analysed from the $6U^b$ monosomic addition line did not carry the $6U^b$ chromosome. One plant containing the $5U^b$, $3U^b$ and $7U^b$ chromosomes and one plant carrying $5M^b$, $6M^b$ and $7M^b$ chromosomes showed very low fertility. Each of the plants produced a single seed, but seeds of the parent plants are still available. Line No. 49/00 carried a submetacentric *Ae. biuncialis* chromosome pair and the chromosome number 44 has been constant for several generations. After FISH no hybridisation site was observed on the *Ae. biuncialis* chromosome pair using the pSc119.2 and Afa family repetitive DNA probes, so it was not possible to identify the *Ae. biuncialis* chromosome pair. However, the use of wheat SSR markers and the $(GAA)_n$ microsatellite DNA probe allowed it to be characterised more accurately. These new lines facilitate gene transfer from *Ae. biuncialis* into cultivated wheat and the selection of U and M genome-specific wheat SSR markers.

Keywords: addition lines; FISH polymorphism; goatgrass; wheat SSR markers

Aegilops biuncialis ($2n = 4x = 28$, $U^bU^bM^bM^b$) could play an important role in the broadening of the cultivated wheat gene pool (Van Slageren 1994). *Ae. biuncialis* is a wild species closely related to cultivated wheat, showing a great number of agronomically useful features such as salt and drought tolerance, disease and rust resistance (Van Slageren 1994; Molnár *et al.* 2004; Colmer *et al.* 2006; for review see Schneider *et al.* 2008). These useful genes can be transferred into cultivated wheat by developing addition or substitution lines (Schneider *et al.* 2005) or by inducing intergenomic translocations (Molnár *et al.* 2009). The great genetic adaptibility of *Ae. biuncialis* may be due to the natural cross between *Ae. umbellulata* ($2n = 2x = 14$, UU) and *Ae. comosa* ($2n = 2x = 14$, MM), allowing it to carry useful traits from both ancestor species. The high genetic variability of *Ae. biuncialis* is manifested in differences in the locations and copy numbers of the repetitive sequences, causing relatively high variability in the fluorescence *in situ* hybridisation (FISH), C- and N-banding patterns of the U and M genomes (Landjeva & Ganeva 2000; Badaeva *et al.* 2004; Schneider *et al.* 2005; Molnár *et al.* 2011a). For this reason, it is advisable to charac-

terise *Ae. biuncialis* chromosomes using several DNA probes (Molnár *et al.* 2011a) and to confirm the FISH identification with the help of molecular (microsatellite, SSR) markers.

Although SSR markers are extensively applied in wheat genome characterisation (for review see Landjeva *et al.* 2007), only very few SSR markers have been described for different *Aegilops* species (Lelley *et al.* 2000; Zhang *et al.* 2001; Adonina *et al.* 2005) and the number of microsatellite markers specific to the U and M genomes of *Aegilops* species is also limited (Dhaliwal *et al.* 2002; Schneider *et al.* 2010a; Molnár *et al.* 2011b). When wheat-*Ae. biuncialis* (Schneider *et al.* 2005) and wheat-*Ae. geniculata* addition lines (Friebe *et al.* 1999) were used to select U and M genome-specific wheat SSR markers, it was found that the results obtained for wheat-*Ae. geniculata* addition lines could not be adapted to wheat-*Ae. biuncialis* addition lines (Schneider *et al.* 2010a) due to the high genetic variability of *Ae. biuncialis* and *Ae. geniculata*. Therefore, a complete set of wheat-*Ae. biuncialis* addition lines would be useful for the selection and chromosomal localisation of U and M genome-specific wheat SSR markers. The $2M^b$, $3M^b$, $7M^b$, $1U^b$ and $3U^b$ wheat-*Ae. biuncialis* addition lines developed so far (Molnár-Láng *et al.* 2002; Schneider *et al.* 2005) do not provide full information about the localisation of the selected wheat SSR markers on the U and M genome chromosomes, so the aim of the experiments was to produce new wheat-*Ae. biuncialis* addition lines, promoting gene transfer from *Ae. biuncialis* into wheat and the selection of SSR markers specific to the U- and M-genome chromosomes.

MATERIAL AND METHODS

Plant material. The plant material consisted of selfed progenies of the BC_2 and BC_3 generations of wheat (*Triticum aestivum* cv. Martonvásári 9 *kr1*) × *Ae. biuncialis* (accession No. 642) hybrids (Logojan & Molnár-Láng 2000), the wheat genotype cv. Martonvásári 9 kr1 (Mv9kr1), and the *Ae. biuncialis* Martonvásár genebank accession No. 642 (MvGB642).

The progenies of the BC_2 and BC_3 generations of the wheat × *Ae. biuncialis* hybrids were analysed and selected using fluorescence *in situ* hybridisation (FISH). The plants were vernalized at 4°C for 6 weeks, and were grown in a phytotron under controlled environmental conditions in a Conviron PGR-15 cabinet until tillering at the initial temperature of 15°C by day and 10°C by night, 12 h light:12 h dark photoperiod (Tischner *et al.* 1997). The temperature rose by increments of 2°C after tillering (day length 14 h), stem elongation (16 h illumination), flowering, and 2 weeks after fertilization.

Fluorescence *in situ* hybridisation (FISH). The seeds of the BC_2 and BC_3 generations of the wheat × *Ae. biuncialis* hybrids were germinated at room temperature for 24 h, incubated at 4°C for 48 h and then at 25°C for 26 h. Root tips were collected and treated in ice-cold sterile water for 24 h and fixed in a 3:1 (v/v) mixture of 100% ethanol and acetic acid. Root-tip squash preparations were made in 45% acetic acid according to Jiang *et al.* (1994a). The coverslips were removed in liquid nitrogen and the preparations were air dried overnight. The slides were stored at –20°C. The repetitive DNA probes used for FISH were as follows: pSc119.2, a 120 bp highly repeated sequence amplified from rye genomic DNA and labelled with biotin-11-dUTP or digoxigenin-16-dUTP using PCR according to Contento *et al.* (2005), Afa family (Nagaki *et al.* 1995), a subclone of the pAs1 tandem repetitive sequences, labelled with biotin-11-dUTP or digoxigenin-16-dUTP using PCR, and pTa71 (Gerlach & Bedbrook 1979), labelled simultaneously with 50% digoxigenin-16-dUTP and 50% biotin-11-dUTP by nick translation. The $(GAA)_n$ microsatellite probe was amplified from *Ae. biuncialis* genomic DNA using a PCR reaction as described by Vrána *et al.* (2000) and labelled with digoxigenin-16-dUTP. Digoxigenin and biotin signals were detected simultaneously using anti-digoxigenin-rhodamine (Roche, Mannheim, Germany) and streptavidin-FITC (Roche, Mannheim, Germany). FISH was carried out according to Molnár-Láng *et al.* (2010). Images were taken using Image Pro plus 5.1 software (Media Cybernetics, Silver Spring, USA) and a Spot CCD camera (Diagnostic Instruments, Inc., Sterling Heights, USA) attached to a Zeiss Axioscope 2 epifluorescence microscope. The FISH patterns of the different wheat-*Ae. biuncialis* hybrid derivatives were compared to the karyotypes of wheat (Schneider *et al.* 2003) and *Ae. biuncialis* (Schneider *et al.* 2005; Molnár *et al.* 2011a).

Microsatellite marker analysis. The PCR reaction was carried out with the wheat SSR markers GWM44 (Röder *et al.* 1998) and GDM61 (Pestsova *et al.* 2000) in an Eppendorf Mastercycler

(Eppendorf-Netheler-Hinc Inc., Hamburg, Germany) according to Schneider *et al.* (2010a, b). Agarose gel electrophoresis was carried out using 2% agarose gels with 200V voltage for approx. 40 min. The bands were visualised by ethidium bromide staining. Images were taken with the help of a Syngene G Box gel documentation system.

RESULTS

Fluorescence *in situ* hybridisation was used to determine the karyotypic constitution of four wheat-*Ae. biuncialis* addition lines and two other promising lines, both containing three different *Ae. biuncialis* chromosomes, selected from selfed progenies in the BC_2 and BC_3 generation (Figures 1–3). The FISH patterns of the *Ae. biuncialis* chromosomes in the wheat-*Ae. biuncialis* addition lines were compared to the *Ae. biuncialis* karyotypes published earlier (Schneider *et al.* 2005; Molnár *et al.* 2011a) and to *Ae. biuncialis* accession No. MvGB642, used for the production of the wheat-*Ae. biuncialis* addition lines (Figure 1a). All fourteen chromosome pairs of *Ae. biuncialis* accession No. MvGB642 can be identified on the basis of their diagnostic FISH patterns using pSc119.2, Afa family and pTa71 DNA probes (Figure 1a), enabling the exact FISH characterisation of the *Ae. biuncialis* chromosomes in the wheat-*Ae. biuncialis* addition lines.

The $2U^b$ and $6M^b$ disomic addition lines were identified according to their specific FISH patterns (Figures 1a–c). In the $2U^b$ wheat-*Ae. biuncialis* disomic addition line a slight difference was observed in the intensity of the terminal pSc119.2 site and an additional subterminal Afa family site was also detected on the long arm compared to the parental *Ae. biuncialis* accession (Figures 1a, b), but the arm ratio corresponded to that of the $2U^b$ chromosome. The hybridisation pattern of the $6M^b$ chromosome was identical to that of *Ae. biuncialis* accession No. MvGB642 (Figures 1a, c). The latter is characteristic, as it is the smallest *Ae. biuncialis* chromosome with a terminal pSc119.2 and a subterminal Afa family site on the long arm. The intercalary pSc119.2 site detected on the long arm in *Ae. biuncialis* MvGB642 was absent from the $6U^b$ monosomic addition line. Instead of the intercalary Afa family site detected on the long arm in *Ae. biuncialis* MvGB642 a subterminal one was visible on the long arm in the $6U^b$ monosomic addition line (Figures 1a, d). The arm ratio of the latter corresponded to that of the $6U^b$ chromosome of *Ae. biuncialis* (Figures 1a, d), as it is the most acrocentric one (Figure 1a). The overall distributions of pSc119.2 and Afa signals along the chromosomes in the line carrying the $5U^b$, $3U^b$ and $7U^b$ chromosomes (Figure 1e) and that containing the $5M^b$, $6M^b$ and $7M^b$ chromosomes (Figure 1f) were mostly identical to those of parental *Ae. biuncialis* accession No. MvGB642 (Figures 1a, e, f). An additional terminal pSc119.2 site was observed on the long arm of the $7U^b$ chromosome in the line carrying the $5U^b$, $3U^b$ and $7U^b$ chromosomes (Figure 1e), so a total of three pSc119.2 sites could be detected on the long arm of this chromosome (Figure 1e). The *Ae. biuncialis* $7U^b$ chromosome can be easily recognized due to the intercalary and subterminal pSc119.2 hybridisation sites on the long arm (Figures 1a, e). A slight difference was detected in the location of the pSc119.2 site on the short arm of the $7M^b$ chromosome (Figures 1a, f). The latter is the one of the most characteristic *Ae. biuncialis* chromosomes, as it is the only metacentric one with a (sub)terminal pSc119.2 site on the short arm and with a terminal Afa family site on the long arm (Figures 1a, f).

In line No. 49/00 no FISH site was observed on the *Ae. biuncialis* chromosome pair using the pSc119.2 and Afa family repetitive DNA probes (Figure 1g), although all the chromosomes of *Ae. biuncialis* accession No. MvGB642 had diagnostic FISH patterns using the pSc119.2, Afa family and pTa71 DNA probes (Figure 1a). It proved to be possible to characterise this addition line through a combination of wheat SSR markers and FISH using a microsatellite DNA. Wheat SSR markers GWM44 and GDM61 were polymorphic between the Mv9kr1 wheat genotype and *Ae. biuncialis* MvGB642 and were located on the $2M^b$ and $3M^b$ chromosomes of the $2M^b$ and $3M^b$ wheat-*Ae. biuncialis* addition lines (Schneider *et al.* 2010a, Figure 3). These two SSR markers showed a diagnostic *Ae. biuncialis*-specific PCR fragment in line No. 49/00, the same size as in the $2M^b$ and $3M^b$ addition lines (Figure 3), suggesting that this line carried chromatin homoeologous to the $2M^b$ and $3M^b$ chromosomes of *Ae. biuncialis* (Figure 3). Hybridisation with the $(GAA)_n$ microsatellite DNA sequence on the $2M^b$ and $3M^b$ additions and on line No. 49/00 showed that the hybridisation pattern and arm ratio of the *Ae. biuncialis* chromosomes in line No. 49/00 showed greater resemblance to

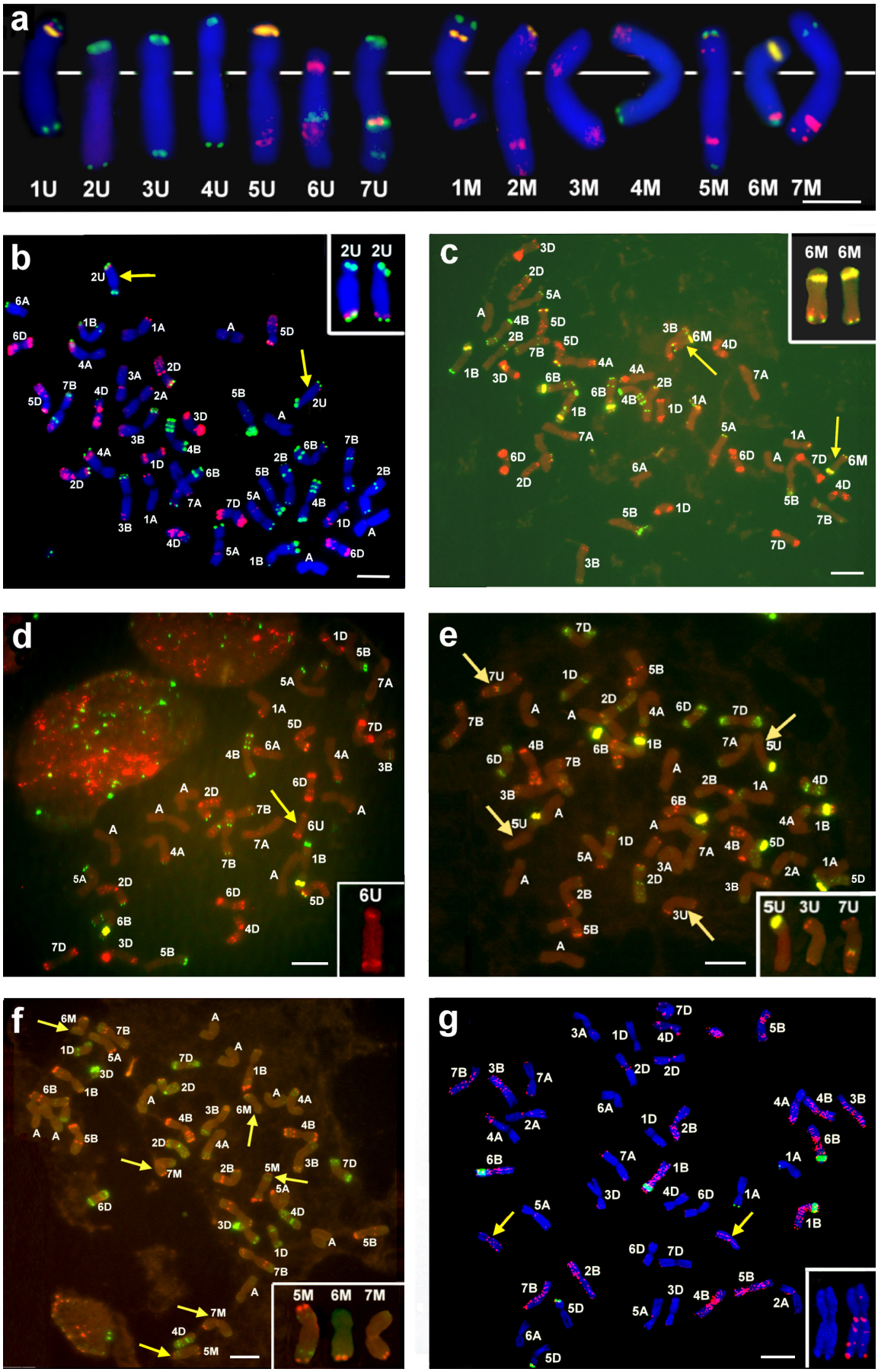
a
1U 2U 3U 4U 5U 6U 7U 1M 2M 3M 4M 5M 6M 7M
b
2U 2U
c
6M 6M
d
6U
e
5U 3U 7U
f
5M 6M 7M
g

Figure 2. From left to right, spikes of the Mv9kr1 wheat genotype, *Ae. biuncialis* accession No. MvGB642, wheat-*Ae. biuncialis* disomic addition lines $2U^b$ and $6M^b$, the plant containing chromosomes $5U^b$, $3U^b$ and $7U^b$, the plant carrying chromosomes $5M^b$, $6M^b$ and $7M^b$ and wheat-*Ae. biuncialis* disomic addition line No. 49/00 containing a modified $3M^b$ chromosome

those of the $3M^b$ wheat-*Ae. biuncialis* addition line (Figure 4), suggesting that this line included a modified $3M^b$ chromosome.

The spike morphology of the addition lines was unique, depending on the *Ae. biuncialis* chromosomes they carried (Figure 2). The spikes of the $2U^b$ wheat-*Ae. biuncialis* addition line differed from those of the Mv9kr1 wheat genotype. The spikes of the line containing the $7U^b$, $5U^b$ and $3U^b$ chromosomes and of that carrying the $5M^b$, $6M^b$ and $7M^b$ chromosomes showed greater resemblance to those of *Ae. biuncialis* (Figure 2). The present results showed that the $2U^b$ wheat-*Ae. biuncialis* disomic addition line was mostly stable, as 91% of the progenies carried the alien chromosome pair (Table 1). The $6M^b$ disomic addition line was dwarf and all the spikes were sterile, but this line still exists as a monosomic addition (Table 1). Progenies analysed from the $6U^b$ monosomic addition line did not contain the $6U^b$ chromosome (Table 1). One plant containing the $5M^b$, $6M^b$ and $7M^b$ chromosomes and one carrying the $5U^b$, $3U^b$ and $7U^b$ chromosomes had very low fertility, each plant producing a single seed. The progenies of the latter two plants exhibited

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Figure 1. (a) Fluorescence *in situ* hybridization (FISH) patterns of the individual somatic chromosomes of parental *Aegilops biuncialis* accession No. MvGB642 using pSc119.2 (green), Afa family (red) and pTa71 (yellow) repetitive DNA probes; b–g FISH patterns on the metaphase chromosome spreads of different progenies of the wheat-*Ae. biuncialis* BC_2 and BC_3 generations; the *Ae. biuncialis* chromosomes are indicated by arrows; the *Ae. biuncialis* chromosomes can be seen enlarged in the top or bottom right-hand corner; (b) FISH patterns of the $2U^b$ wheat-*Ae. biuncialis* disomic addition line using pSc119.2 (green) and Afa family (red) repetitive DNA probes; (c, d) FISH patterns of the $6M^b$ disomic and $6U^b$ monosomic wheat-*Ae. biuncialis* addition lines using pSc119.2 (green), Afa family (red) and pTa71 (yellow) DNA probes; (e) FISH patterns of the line containing the $5U^b$, $3U^b$ and $7U^b$ chromosomes using pSc119.2 (red), Afa family (green) and pTa71 (yellow) DNA probes; (f) FISH patterns of the line containing the $5M^b$, $6M^b$ and $7M^b$ chromosomes using pSc119.2 (red) and Afa family (green) DNA probes; (g) FISH patterns of disomic addition line No. 49/00 using $(GAA)_n$ (red) and pTa71 (green) DNA probes; the FISH patterns of one of the additioned *Ae. biuncialis* chromosomes can be seen enlarged in the bottom right-hand corner using the repetitive DNA probe combinations pSc119.2, Afa family (on the left) and $(GAA)_n$, pTa71 (on the right); a, b, g: Chromosomes are stained with DAPI (blue); c, d, e, f: FISH patterns are demonstrated without DAPI contrast staining. Scale bars are indicated in the bottom right-hand corner; a: 5 μm, b–g: 10 μm

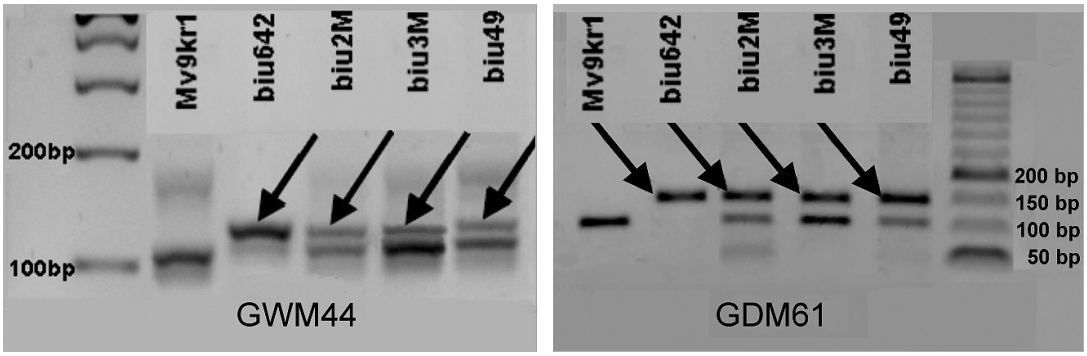

Figure 3. Electrophoretic patterns obtained after PCR for the wheat SSR markers GWM44 and GDM61 on the Mv9kr1 wheat genotype, on *Ae. biuncialis* gene bank accession No. MvGB642 (biu642), and on the wheat-*Ae. biuncialis* addition lines $2M^b$ (biu2M), $3M^b$ (biu3M) and No. 49/00 (biu49); bands specific to *Ae. biuncialis* chromosomes are indicated by arrows

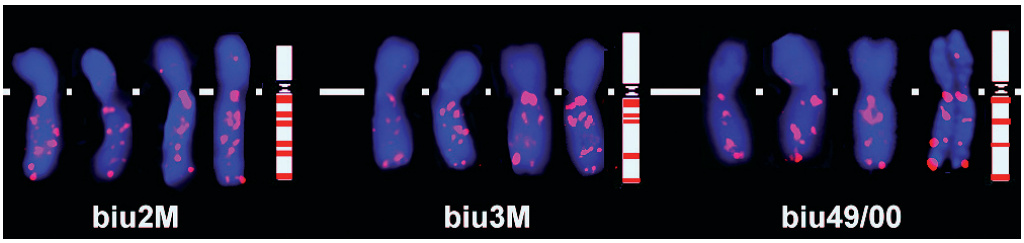

Figure 4. Fluorescence *in situ* hybridization (FISH) patterns (on the left) and graphical representation (on the right) of the individual somatic *Aegilops biuncialis* chromosomes of the wheat-*Ae. biuncialis* $2M^b$, $3M^b$ and 49/00 disomic addition lines using the $(GAA)_n$ microsatellite DNA probe; chromosomes are stained with DAPI (blue), while $(GAA)_n$ hybridisation sites show red fluorescence; biu2M, biu3M and biu49/00 indicate the individual *Ae. biuncialis* chromosomes of the $2M^b$, $3M^b$ and 49/00 wheat-*Ae. biuncialis* addition lines

Table 1. Numerical data of the fluorescence *in situ* hybridisation (FISH) analysis, fertility and alien chromosome transmission rate in the progenies of wheat-*Ae. biuncialis* BC_2 and BC_3 generations

Identification number	Wheat-*Ae. biuncialis* addition lines identified using FISH	Generations used for FISH analysis	No. of plants available	No. of seeds available	No. of seeds analysed in the progenies	*Ae. biuncialis* chromosomes detected in the progenies (chromosome, percentage)
1575/08	$2U^b$ disomic	BC_3F_3	25	175	34	$2U^b$, 91.17%
1564/08	$6M^b$ disomic	BC_3F_2	1	0*	0	0
33/01	$6U^b$ mono	BC_2F_2	1	100	39	0
1583/09	$5U^b$, $3U^b$, $7U^b$	BC_2F_1	1	1	0**	0**
1581/09	$5M^b$, $6M^b$, $7M^b$	BC_2F_1	1	1	1	$6M^b$, $7M^b$, 100%***
49/00	modified $3M^b$ disomic	BC_3F_4	> 300	> 3000	70	modified $3M^b$, 99%

*no seed set; **no root tips available due to poor germination; the original plant died during vernalization; ***the plant showed reduced growth habit and died before heading

a dwarfism defect and later died, but their parents are still available, allowing the selection of new lines carrying different *Ae. biuncialis* chromosomes (Table 1). Line No. 49/00 has been grown for several years, and its chromosome number has been 44 for several generations (Table 1).

DISCUSSION

In this study new wheat-*Ae. biuncialis* addition lines carrying chromosomes $2U^b$, $6M^b$, "$6U^b$", $5U^b$, $3U^b$, $7U^b$, $5M^b$, $6M^b$ and $7M^b$ were selected. The development of a complete set of addition lines is limited by several factors, including gametocidal genes, which are located on various chromosomes in the *Aegilops* species (for review see Schneider *et al.* 2008). When introduced into the wheat background, these *Gc* genes induce chromosome breakage, mainly in gametes lacking them, ensuring their preferential transmission into the progenies (Finch *et al.* 1984). The production of addition series is also restricted by the different transmission rates of the individual *Aegilops* chromosomes, as in this study the transferability of the $3M^b$, $2U^b$ and $6U^b$ chromosomes of *Ae. biuncialis* chromosomes varied greatly (99, 91 and 0%), which is possibly due to deviations in the centromere structure of the alien chromosomes (Chang *et al.* 2005). The selection of disomic plants from the selfing progenies of monosomic plants is very labour-intensive, due to the rapid elimination of single added chromosomes in wheat (Jiang *et al.* 1994b). In this study none of the thirty-nine progenies analysed from the $6U^b$ monosomic addition line carried the alien chromosome. The other limitation to the production of addition series is that some alien chromosomes, such as barley chromosome 1H, cause sterility when added to the wheat genome (Islam *et al.* 1978; Jiang *et al.* 1994b). Genes which are lethal when present in the disomic state in the wheat background may be located on the $6M^b$ *Ae. biuncialis* chromosome, as both the $6M^b$ disomic addition line and the progeny of the plant carrying $5M^b$, $6M^b$ and $7M^b$ chromosome pairs showed dwarfism and died.

A relatively high level of FISH polymorphism was observed in various *Aegilops* species, including *Ae. biuncialis* (Badaeva *et al.* 2004; Molnár *et al.* 2011a). The FISH sites of the $2U^b$, $6M^b$, $6U^b$, $5U^b$, $3U^b$, $7U^b$, $5M^b$, $6M^b$ and $7M^b$ *Ae. biuncialis* chromosomes in the wheat-*Ae. biuncialis* addition lines mostly corresponded to those of parental *Ae. biuncialis* accession No. MvGB642 (Schneider *et al.* 2005). No hybridisation sites were observed on the *Ae. biuncialis* chromosomes in wheat-*Ae. biuncialis* disomic addition line No. 49/00 using the pSc119.2 and Afa family DNA probes, which could be due to the decreased copy number and intensity of the hybridisation signals. A similar significant reduction in the copy number of various repetitive DNA sequences was observed in the amphiploids and synthetic allopolyploid forms of a number of *Triticum* and *Aegilops* species (Salina *et al.* 2004; Shcherban *et al.* 2008; Baum & Feldman 2010; Molnár *et al.* 2011a). The sequence elimination observed in allopolyploid wheat species suggests that the further differentiation of homoeologous chromosomes via the elimination of low-copy DNA sequences occurred soon after allopolyploidisation (Feldman & Levy 2005; Ma & Gustafson 2005). The experiments of Molnár *et al.* (2011a) showed that in tetraploid *Ae. biuncialis* and *Ae. geniculata* accessions, some of the NOR (pTa71) signals were eliminated from the satellite M chromosomes due to the polyploidisation process. The production of wheat-*Ae. biuncialis* addition lines may also lead to a reduction in the copy number of the repetitive sequences pSc119.2 and Afa family, increasing in the physical divergence between the wheat and *Ae. biuncialis* chromosomes, thus complicating the FISH characterisation of the alien chromosomes. The present results showed that a combination of the FISH technique, using several DNA probes, and SSR markers leads to the more accurate identification of *Ae. biuncialis* chromosomes in the wheat background.

The conservation of the genetic variability of wild species and the utilization of available accessions are important for the future of wheat production. Pre-breeding is a promising alternative for the inclusion of *Aegilops* genetic resources in wheat breeding programmes. Alien chromosome additions are useful tools in plant genetics research and breeding, as they serve as a bridge for the transfer of agronomically useful traits from wild species into cultivated wheat (Jiang *et al.* 1994b; Chang & De Jong 2005; Benavente *et al.* 2008). The $2U^b$ wheat-*Ae. biuncialis* addition line produced in the present work may play an important role in pre-breeding, because the kernels of a 2U wheat-*Ae. umbellulata* line produced earlier showed higher iron and zinc content than those of Chinese Spring wheat (Wang *et al.* 2011). This suggests that the $2U^b$ wheat-*Ae. biuncialis* addition line produced in this work could also contain high levels of microelements.

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