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<sup>b</sup>, 6M<sup>b</sup>, 6U<sup>b</sup>; 5U<sup>b</sup>, 7U<sup>b</sup>; 5M<sup>b</sup>, 6M<sup>b</sup> and 7M<sup>b</sup> were selected. The 2U<sup>b</sup> disomic addition line is relatively stable, as 91% of the progenies contain this chromosome pair. The 6M<sup>b</sup> disomic addition line proved to be dwarf and sterile, but it still exists as a monosomic addition line. Progenies analysed from the 6U<sup>b</sup> monosomic addition line did not carry the 6U<sup>b</sup> chromosome. One plant containing the 5U<sup>b</sup>, 3U<sup>b</sup> and 7U<sup>b</sup> chromosomes and one plant carrying 5M<sup>b</sup>, 6M<sup>b</sup> and 7M<sup>b</sup> 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)<sub>n</sub> 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* (2<sub>n</sub> = 4<sub>x</sub> = 28, U<sup>b</sup>U<sup>b</sup>M<sup>b</sup>M<sup>b</sup>) 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* (2<sub>n</sub> = 2<sub>x</sub> = 14, UU) and *Ae. comosa* (2<sub>n</sub> = 2<sub>x</sub> = 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. 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 Landieva et al. 2007), only very few SSR markers have been described for different Aegilops species (Leelley 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<sup>b</sup>, 3M<sup>b</sup>, 7M<sup>b</sup>, 1U<sup>b</sup> and 3U<sup>b</sup> 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<sub>2</sub> and BC<sub>3</sub> 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<sub>2</sub> and BC<sub>3</sub> 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<sub>2</sub> and BC<sub>3</sub> 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 Contenko 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)<sub>8</sub> microsatellite probe was amplified from Ae. biuncialis genomic DNA using a PCR reaction as described by Vráná 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...
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 BC₂ and BC₃ 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 2UB and 6MB disomic addition lines were identified according to their specific FISH patterns (Figures 1a–c). In the 2UB 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 2UB chromosome. The hybridisation pattern of the 6MB chromosome was identical to that of Ae. biuncialis accession No. MvGB642 (Figures 1a, c). The latter is characteristic, as it is the smallest chromosome of a Syngene G Box gel documentation system.

The hybridisation pattern of the 6MB chromosome was identical to that of the 6UB 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 5UB, 3UB and 7UB chromosomes (Figure 1e) and that containing the 5MB, 6MB and 7MB 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 7UB chromosome in the line carrying the 5UB, 3UB and 7UB 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 7UB 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 7MB 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 2MB and 3MB chromosomes of the 2MB and 3MB 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 2MB and 3MB addition lines (Figure 3), suggesting that this line carried chromatin homoeologous to the 2MB and 3MB chromosomes of Ae. biuncialis (Figure 3). Hybridisation with the (GAA)_n microsatellite DNA sequence on the 2MB and 3MB 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
those of the 3M<sup>b</sup> wheat-Ae. biuncialis addition line (Figure 4), suggesting that this line included a modified 3M<sup>b</sup> 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<sup>b</sup> wheat-Ae. biuncialis addition line differed from those of the Mv9kr1 wheat genotype. The spikes of the line containing the 7U<sup>b</sup>, 5U<sup>b</sup> and 3U<sup>b</sup> chromosomes and of that carrying the 5M<sup>b</sup>, 6M<sup>b</sup> and 7M<sup>b</sup> chromosomes showed greater resemblance to those of Ae. biuncialis (Figure 2). The present results showed that the 2U<sup>b</sup> wheat-Ae. biuncialis disomic addition line was mostly stable, as 91% of the progenies carried the alien chromosome pair (Table 1). The 6M<sup>b</sup> 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<sup>b</sup> monosomic addition line did not contain the 6U<sup>b</sup> chromosome (Table 1). One plant containing the 5M<sup>b</sup>, 6M<sup>b</sup> and 7M<sup>b</sup> chromosomes and one carrying the 5U<sup>b</sup>, 3U<sup>b</sup> and 7U<sup>b</sup> chromosomes had very low fertility, each plant producing a single seed. The progenies of the latter two plants exhibited...
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).

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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

<table>
<thead>
<tr>
<th>Identification number</th>
<th>Wheat-<em>Ae. biuncialis</em> addition lines identified using FISH</th>
<th>Generations used for FISH analysis</th>
<th>No. of plants available</th>
<th>No. of seeds available</th>
<th>No. of seeds analysed in the progenies</th>
<th><em>Ae. biuncialis</em> chromosomes detected in the progenies (chromosome, percentage)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1575/08</td>
<td>2U$_b$ disomic</td>
<td>BC$_2$ F$_3$</td>
<td>25</td>
<td>175</td>
<td>34</td>
<td>2U$_b$, 91.17%</td>
</tr>
<tr>
<td>1564/08</td>
<td>6M$_b$ disomic</td>
<td>BC$_1$ F$_2$</td>
<td>1</td>
<td>0*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>33/01</td>
<td>6U$_b$ mono</td>
<td>BC$_2$ F$_2$</td>
<td>1</td>
<td>100</td>
<td>39</td>
<td>0</td>
</tr>
<tr>
<td>1583/09</td>
<td>5U$_b$, 3U$_b$, 7U$_b$</td>
<td>BC$_2$ F$_1$</td>
<td>1</td>
<td>1</td>
<td>0**</td>
<td>0**</td>
</tr>
<tr>
<td>1581/09</td>
<td>5M$_b$, 6M$_b$, 7M$_b$</td>
<td>BC$_2$ F$_1$</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>6M$_b$, 7M$_b$, 100%***</td>
</tr>
<tr>
<td>49/00</td>
<td>modified 3M$_b$ disomic</td>
<td>BC$_2$ F$_4$</td>
<td>&gt; 300</td>
<td>&gt; 3000</td>
<td>70</td>
<td>modified 3M$_b$, 99%</td>
</tr>
</tbody>
</table>

*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

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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

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DISCUSSION

In this study new wheat-\emph{Ae. biuncialis} addition lines carrying chromosomes 2\textsuperscript{Ub}, 6\textsuperscript{Mb}, "6U\textsuperscript{Ub}"; 5\textsuperscript{Ub}, 3\textsuperscript{Ub}, 7\textsuperscript{Ub}, 5\textsuperscript{Mb}, 6\textsuperscript{Mb} and 7\textsuperscript{Mb} 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 \emph{Aegilops} species (for review see Schneider \emph{et al.} 2008). When introduced into the wheat background, these \emph{Gc} genes induce chromosome breakage, mainly in gametes lacking them, ensuring their preferential transmission into the progenies (Finch \emph{et al.} 1984). The production of addition series is also restricted by the different transmission rates of the individual \emph{Aegilops} chromosomes, as in this study the transferability of the 3\textsuperscript{Mb}, 2\textsuperscript{Ub} and 6\textsuperscript{Ub} chromosomes of \emph{Ae. biuncialis} chromosomes varied greatly (99, 91 and 0\%), which is possibly due to deviations in the centromere structure of the alien chromosomes (Chang \emph{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 \emph{et al.} 1994b). In this study none of the thirty-nine progenies analysed from the 6\textsuperscript{Ub} 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 \emph{et al.} 1978; Jiang \emph{et al.} 1994b). Genes which are lethal when present in the disomic state in the wheat background may be located on the 6\textsuperscript{Mb} \emph{Ae. biuncialis} chromosome, as both the 6\textsuperscript{Mb} disomic addition line and the progeny of the plant carrying 5\textsuperscript{Mb}, 6\textsuperscript{Mb} and 7\textsuperscript{Mb} chromosome pairs showed dwarfism and died.

A relatively high level of FISH polymorphism was observed in various \emph{Aegilops} species, including \emph{Ae. biuncialis} (Badaeva \emph{et al.} 2004; Molnár \emph{et al.} 2011a). The FISH sites of the 2\textsuperscript{Ub}, 6\textsuperscript{Mb}, 6\textsuperscript{Ub}, 5\textsuperscript{Ub}, 3\textsuperscript{Ub}, 7\textsuperscript{Ub}, 5\textsuperscript{Mb}, 6\textsuperscript{Mb} and 7\textsuperscript{Mb} \emph{Ae. biuncialis} chromosomes in the wheat-\emph{Ae. biuncialis} addition lines mostly corresponded to those of parental \emph{Ae. biuncialis} accession No. MvGB642 (Schneider \emph{et al.} 2005). No hybridisation sites were observed on the \emph{Ae. biuncialis} chromosomes in wheat-\emph{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 \emph{Triticum} and \emph{Aegilops} species (Salina \emph{et al.} 2004; Scherban \emph{et al.} 2008; Baum & Feldman 2010; Molnár \emph{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 \emph{et al.} (2011a) showed that in tetraploid \emph{Ae. biuncialis} and \emph{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-\emph{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 \emph{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 \emph{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 \emph{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 \emph{et al.} 1994b; Chang & De Jong 2005; Benavente \emph{et al.} 2008). The 2\textsuperscript{Ub} wheat-\emph{Ae. biuncialis} addition line produced in the present work may play an important role in pre-breeding, because the kernels of a 2U wheat-\emph{Ae. umbellulata} line produced earlier showed higher iron and zinc content than those of Chinese Spring wheat (Wang \emph{et al.} 2011). This suggests that the 2\textsuperscript{Ub} wheat-\emph{Ae. biuncialis} addition line produced in this work could also contain high levels of microelements.

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**References**


the recessive crossability alleles \( kr1kr1kr2kr2 \) and the 1BL.1RS translocation, for the rapid enrichment of 1RS with new allelic variation. Theoretical and Applied Genetics, 120: 1535–1545.


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