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ORIGINAL PAPER

Flow cytometric chromosome sorting from diploid progenitors of bread wheat, *T. urartu*, *Ae. speltoides* and *Ae. tauschii*

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Abstract

Key message **Chromosomes 5A^u, 5S and 5D can be isolated from wild progenitors, providing a chromosome-based approach to develop tools for breeding and to study the genome evolution of wheat.**

Abstract The three subgenomes of hexaploid bread wheat originated from *Triticum urartu* (A^{u}A^u), from a species similar to *Aegilops speltoides* (SS) (progenitor of the B genome), and from *Ae. tauschii* (DD). Earlier studies indicated the potential of chromosome genomics to assist gene transfer from wild relatives of wheat and discover novel genes for wheat improvement. This study evaluates the potential of flow cytometric chromosome sorting in the diploid progenitors of bread wheat. Flow karyotypes obtained by analysing DAPI-stained chromosomes were characterized and the contents of the chromosome peaks were determined. FISH analysis with repetitive DNA probes proved that chromosomes 5A^u, 5S and 5D could be sorted with purities of 78–90 %, while the remaining chromosomes could be sorted in groups of three. Twenty-five conserved orthologous set (COS) markers covering wheat homoeologous chromosome groups 1–7 were used for PCR with DNA amplified from flow-sorted chromosomes and genomic DNA. These assays validated the cytomolecular results as follows: peak I on flow karyotypes contained chromosome groups 1, 4 and 6, peak II represented homoeologous group 5, while peak III consisted of groups 2, 3 and 7. The isolation of individual chromosomes of wild progenitors provides an attractive opportunity to investigate the structure and evolution of the polyploid genome and to deliver tools for wheat improvement.

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Introduction

Bread wheat (*Triticum aestivum* L.) is the second most widely grown crop and is the primary cereal in the temperate region, serving as a staple food for about 40 % of the world's population (http://faostat.fao.org). Bread wheat has a large genome of about 17,000 Mb with three constituent subgenomes, A, B and D (Devos and Gale 2000). The genome architecture of wheat originated from two successive interspecific hybridizations and polyploidizations. The first hybridization occurred approximately 0.3–0.5 million years ago between the A genome progenitor *Triticum urartu* Thum. ex Gandil. (A^{u}A^u, $2n = 2x = 14$) and a B genome progenitor species which is considered to be similar to *Aegilops speltoides* Tausch. (SS, $2n = 2x = 14$). This resulted in the evolution of wild emmer wheat *T. turgidum ssp. dicoccoides* (Körn.) Thell. (A^{u}A^{u}BB, $2n = 4x = 28$) (Dvorak et al. 1993; Maestra and Naranjo 1998). Human selection then created cultivated emmer wheat *T. turgidum ssp. dicoccon* (Schrank) Thell., which hybridized spontaneously with *Ae. tauschii* (DD, $2n = 2x = 14$) around

9,000 years ago to produce allohexaploid wheat, *T. aestivum* L. ($2n = 6x = 42$, A^uA^uBBDD) (Dvorak et al. 1998).

Domestication and thousands of years of cultivation narrowed down the genetic variation within bread wheat. The narrow gene pool of wheat relative to that of its wild diploid progenitor species was demonstrated by Lubbers et al. (1991), who reported a polymorphic index of 0.41 after investigating 102 *Ae. tauschii* accessions at 20 RFLP loci. This was significantly higher than the index determined for hexaploid wheat (0.04) after investigating 21 wheat cultivars originating from different location around the world using 33 genomic probes (Cox 1998). Thus, the genetically diverse diploid progenitors represent a huge reservoir of alleles and genes, which could be used to increase the genetic variation of hexaploid wheat through interspecific hybridization (Cox 1998; Friebe et al. 1996). Several agronomic traits, including resistance to pests and diseases, tolerance to abiotic stresses and traits affecting nutritional and bread making quality or yield components, were identified in accessions of wild diploid progenitors of wheat. Rouse and Jin (2011), for example, identified 86 accessions of *T. urartu* conferring resistance against the stem rust race TTKSK (Ug99) of *Puccinia graminis* f. sp. *tritici*. Diploid *T. urartu* is also recognized as a source of traits affecting bread-making quality (Guzmán and Alvarez 2012). *Ae. speltoides* serves as a source of biotic stress tolerance genes, which have already been transferred to wheat (*Lr28, Lr35, Lr36, Lr47, Lr51, Sr32, Sr39, Pm12, Pm32, Gb5*) (see Kilian et al. 2011). Moreover, this species is a potential source of other important traits such as resistance against pests and diseases (Fusarium head blight, Hessian fly, *Septoria tritici*), grain hardness protein, heat tolerance and tolerance to manganese toxicity (see Kilian et al. 2011). *Ae. tauschii* is probably the most frequently studied diploid progenitor. Many resistance genes against pests and diseases have already been transferred from *Ae. tauschii* to wheat (see Schneider et al. 2008; Kilian et al. 2011) and accessions of *Ae. tauschii* are also attractive sources of adaptive traits, yield components, bread-making quality and abiotic stress tolerance (Fritz et al. 1995; Cox et al. 1990; Schachtman et al. 1992; Limin and Fowler 1993; Xiu-Jin et al. 1997).

Despite the extensive research efforts, the introgression of favourable agronomic traits from diploid progenitor species to cultivated wheat via interspecific hybridization remains difficult due to the considerable number of undesirable genes located in the targeted genomic regions (Klindworth et al. 2013). A better knowledge of the diploid progenitors of the wheat genomes, including their homology to bread wheat subgenomes, and the application of molecular tools could accelerate the targeted introgression of favourable traits into wheat, as well as providing an invaluable resource for studying the structure and evolution of the wheat genome. Molecular genetic studies on diploid progenitors, including the creation of genetic linkage maps and large-insert bacterial artificial chromosome (BAC) libraries, have already been reported (Gill et al. 1991; Boyko et al. 1999; Akhunov et al. 2005).

The advent of next-generation sequencing (NGS) platforms paved the way for cost-effective large-scale DNA sequencing in plants (Margulies et al. 2005; Schatz et al. 2010; Edwards and Batley 2010). These methods are increasingly being applied in plant genomic research and in breeding major cereal crops and related species, including bread wheat, *Ae. tauschii* and *T. urartu* (Brenchley et al. 2012; You et al. 2011; Ling et al. 2013), thus facilitating SNP discovery, physical mapping and whole genome shotgun sequencing (Kumar et al. 2012). However, the successful application of NGS technology for de novo sequencing in species with large and complex genomes may be problematic, as the high proportion of repetitive sequences (57, 61 and 57 % for the A^u, S and D genomes, respectively, in diploid wheats) hampers the assembly of short NGS reads (Kilian et al. 2011; Shangguan et al. 2013).

The analysis of large genomes can be simplified by reducing the sample complexity by isolating single chromosomes and analysing them instead of the whole genome. In recent decades, the development of flow cytometric chromosome sorting in plants has opened the way for the application of genomics tools to chromosomes to obtain shotgun sequences, to develop markers and to construct sequence-ready physical maps (Doležel et al. 2012, 2014). The application of the NGS technology (Edwards and Batley 2010) allowed the sequencing of wheat chromosome arm 7DS to 34× coverage using Illumina and the assembly of low copy and genic regions of this chromosome, representing approximately 40 % of the chromosome arm and all known 7DS genes (Berkman et al. 2011). The Roche 454 technology was applied for the sequencing of barley chromosome 1H and the arms of barley chromosomes 2H–7H to about 2× coverage and 21,766 barley genes were assigned to individual chromosome arms. Using the conserved synteny with the genomes of rice, sorghum and *Brachypodium,* the barley genes were arranged in a putative linear order on the individual chromosome arms (Mayer et al. 2009, 2011). In a similar study, Martis et al. (2013) sequenced flow-sorted chromosomes of rye and established linear gene order model (genome zipper) comprising 22,426 or 72 % of the detected set of 31,008 rye genes. Moreover, the study indicated that introgressive hybridizations and/or a series of whole-genome or chromosome duplications played a role in rye speciation and genome evolution.

Chromosome genomics relies on the ability to isolate chromosomes via flow-cytometric sorting. The flow-cytometric analysis of mitotic chromosomes has been reported in 24 plant species (Doležel et al. 2014), including

hexaploid and tetraploid wheat and their wild relatives in the genus *Aegilops* and *Dasypyrum* (Molnár et al. 2011b; Grosso et al. 2012). To date, flow cytometric chromosome analysis and sorting has not been reported for the diploid progenitors of bread wheat. As the technology could greatly aid the transfer of genes from wild relatives to cultivated wheat and the study of the evolution of polyploid wheat, we set out to explore the possibility of isolating individual chromosomes from diploid *T. urartu*, *Ae. speltoides* and *Ae. tauschii* by means of flow sorting. Chromosomes were sorted from the individual peaks of flow karyotypes and were identified by FISH with a set of repetitive DNA probes. DNA amplified from isolated chromosomes was used as a template for PCR using conserved orthologous set (COS) markers with the aim of identifying their genomic location in the diploid progenitors of wheat and of confirming the cytological identification of sorted chromosomes. The results of the present work provide an important step towards analysing the molecular organization of chromosomes in the diploid progenitors of wheat and towards developing tools to support alien gene transfer in wheat improvement programmes.

Materials and methods

Plant material

Triticum urartu accession MvGB115, *Ae. speltoides* accession MvGB905 and *Ae. tauschii* accession MvGB605, maintained in the Martonvásár Cereal Gene Bank, were used for flow cytometric chromosome analysis and sorting for in situ hybridization experiments and for COS marker analysis.

Preparation of liquid suspensions of chromosomes

The synchronization of the cell cycle of root tip meristem cells using 2 mM hydroxyurea and their accumulation in metaphase using 2.5 μM amiprohos-methyl were carried out as described by Kubaláková et al. (2005). Suspensions of intact chromosomes were prepared from synchronized root tips according to Vrána et al. (2000). Briefly, 50 roots were cut 1 cm from the root tip and fixed in 2 % (v/v) formaldehyde in Tris buffer (10 mM Tris, 10 mM Na_2EDTA, 100 mM NaCl, 0.1 % Triton X-100, pH 7.5) at 5 °C for 20 min. After washing in Tris buffer, the meristem tips were excised and transferred to a tube containing 1 ml of LB01 buffer (15 mM Tris, 2 mM Na_2EDTA, 80 mM KCl, 20 mM NaCl, 0.5 mM spermine, 15 mM β-mercaptoethanol, 0.1 % Triton X-100) (Doležel et al. 1989) at pH 9. Metaphase chromosomes were released after homogenization with a Polytron PT1300 homogenizer (Kinematica AG, Littau, Switzerland) at 20,000 rpm for 13 s. The crude suspension was passed through a 50-μm pore size nylon mesh to remove large cellular debris and stored on ice until analysis on the same day.

Flow cytometric chromosome analysis and sorting

The samples were analysed using a FACSVantage SE flow cytometer (Becton–Dickinson, San José, USA) equipped with an argon ion laser set to multiline UV and 300 mW output power. Chromosome suspensions were stained with DAPI (4′,6-diamidino-2-phenylindole) at a final concentration of 2 μg/ml and analysed at rates of 200–400 particles per second. DAPI fluorescence was acquired through a 424/44 band-pass filter. Approximately 30, 000 chromosomes were analysed from each sample and the results were displayed as histograms of relative fluorescence intensity (flow karyotypes). In order to verify the chromosome content of individual peaks on the flow karyotypes, 1, 000 chromosomes were sorted from each peak at rates of approximately 5–10 per second into a 15 μl drops of PRINS buffer (100 mM Tris, 50 mM KCl, 2 mM $MgCl_2 \times 6H_2O$) supplemented with 5 % sucrose on a microscope slide (Kubaláková et al. 1997), air-dried and used for FISH with probes for DNA repeats that give chromosome-specific fluorescent labelling patterns.

Fluorescence in situ hybridization

Total genomic DNA was extracted from fresh leaves of *Ae. tauschii* (D genome), *S. cereale* and *O. sativa* using Quick Gene-Mini80 (FujiFilm, Tokyo, Japan) according to the manufacturer's instructions. The repetitive DNA sequences *Afa* family, pSc119.2 and the 18S unit of the 45S ribosomal RNA gene were amplified using PCR from the genomic DNA of *Ae. tauschii, S. cereale* and rice, respectively, as described by Nagaki et al. (1995), Contento et al. (2005) and Chang et al. (2010). *Afa*, pSc119.2 and the 18S rRNA unit were labelled with digoxigenin-11-dUTP (Roche, Manheim, Germany), biotin-16-dUTP (Roche) and a mix of biotin-11-dUTP (50 %) and digoxigenin-11-dUTP (50 %), respectively, by nick-translation using standard kits from Roche following the manufacturer's instructions. Digoxigenin and biotin were detected using anti-digoxigenin-rhodamine Fab fragments (Roche) and streptavidin-FITC (Roche), respectively.

Pretreatments and stringency washes (Schneider et al. 2005) were applied only for slides containing root tip metaphase cells. These steps were omitted in experiments on flow-sorted chromosomes. The hybridization mix (30 μl per slide), containing 50 % formamide, 2 × SSC, 10 % dextran sulphate, 20 ng of 18S rDNA and 70 ng each of the pSc119.2 and *Afa* family probes in the presence of

6.25 μg Salmon sperm DNA, was denatured at 80 °C for 10 min and stored on ice for 5 min. The chromosome DNA was denatured in the presence of the hybridization mix at 75 °C for 6 min and allowed to hybridize overnight at 37 °C. The detection of the hybridization signals involved the use of 10 μg ml^{-1} each of streptavidin-FITC and anti-digoxigenin-Rhodamin. Finally, the slides were counterstained with 2 μg ml^{-1} DAPI and examined under a Zeiss Axioskop-2 fluorescence microscope using a Plan Neofluar oil objective 63×, N.A. 1.25 (Zeiss, Oberkochen, Germany) equipped with filter sets appropriate for DAPI (Zeiss filter set 02) and for FITC and Rhodamin (Zeiss filter set 24). Images were acquired with a Spot CCD camera (Diagnostic Instruments, Sterling Heights, USA) and compiled with Image Pro Plus software (Media Cybernetics, Silver Spring, USA). The relative fraction of group 5 chromosomes as a percentage of the whole genome was determined by measuring the lengths of at least 20 chromosomes per homoeologous group on DAPI-stained pictures of *T. urartu* MvGB115, *Ae. speltoides* MvGB905 and *Ae. tauschii* MvGB605.

Amplification of chromosomal DNA

Chromosomes were sorted from each peak on the flow karyotype in batches of 25–50,000 (equivalent to 20–40 ng) into PCR tubes containing 40 μl of sterile deionized water. The chromosomes were treated with proteinase K and their DNA was amplified by multiple displacement amplification (MDA) using an Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Chalfont St. Giles, United Kingdom) as described by Šimková et al. (2008). The amplified DNA was used as a template for PCR with microsatellite markers.

COS marker analysis

Genomic DNA was prepared from the accessions of *T. urartu* MvGB115, *Ae. speltoides* MvGB905 and *Ae. tauschii* MvGB605, used for the flow-cytometric analysis and from the wheat (*T. aestivum* L.) genotype Mv9kr1 as described by Cseh et al. (2013). A total of 25 conserved orthologous set (COS) markers (Online Resource 1) specific for wheat homoeologous groups I-VII were chosen from publicly available COS marker collections (the Wheat Genetic Improvement Network: http://www.wgin.org.uk/resources/Markers/TAmarkers.php; Tools and Resources (TR) collections: http://www.modelcrop.org/cgi-bin/gbrowse/brachyv1/; Quraishi et al. 2009).

PCR reactions were performed in a 12-μl reaction volume in a reaction mix consisting of 1 × PerfectTaq Plus PCR Buffer (5 Prime GmbH, Hamburg, Germany) and 0.4 μM primers; 50 ng genomic DNA or 1.5 ng of MDA DNA from flow-sorted chromosomes was used as a template. PCR was carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the reaction profiles WGIN: 95 °C (15 min), 39 cycles of [95 °C (0.5 min), 58 °C (0.5 min), 72 °C (0.5 min)], hold at 72 °C (5 min) then at 10 °C and TR: 94 °C (10 min), 16 cycles of [95 °C (0.5 min), 58 °C (1 min), decreasing by 0.5 °C per cycle to 50 °C, 72 °C (1 min)], 25 cycles of [94 °C (0.5 min), 50 °C (1 min), 72 °C (1 min)], hold at 15 °C. The annealing temperature and PCR reaction profiles are summarized together with the primer sequences in Online Resource 1. The PCR amplicons were separated with a Fragment Analyzer™ Automated CE System equipped with a 12-Capillary Array Cartridge (effective length 33 cm) (Advanced Analytical Technologies, Ames, USA). The results were analysed using PROsize v2.0 software.

Results

Chromosome analysis using flow cytometry (flow karyotyping)

The analysis of DAPI-stained chromosome suspensions prepared from the diploid progenitors of hexaploid wheat resulted in flow karyotypes with three peaks (Fig. 1). However, there were differences between the species in the degree of resolution of the individual peaks and their position on the flow karyotype. While the second peak on the flow karyotypes of *T. urartu* and *Ae. speltoides* could only partially be resolved from the composite peaks I and III (Fig. 1a, b), in *Ae. tauschii* peak II was clearly discriminated (Fig. 1c). Moreover, the chromosome peaks on the flow karyotype of *T. urartu* were observed at higher fluorescence intensity channels (115–160) than those of *Ae. speltoides* (channels 105–140), while the peak positions in *Ae. tauschii* were located at the lowest fluorescence interval (channels 95–135) (Fig. 1).

FISH on mitotic metaphase spreads

In order to obtain a reference karyotype for the identification of flow-sorted chromosomes, root tip mitotic metaphase spreads from accessions of the diploid progenitor species were investigated using fluorescence in situ hybridization (FISH) with repetitive DNA probes (*Afa* family, pSc119.2, 18S rDNA), which allowed the whole set of chromosomes in *T. urartu* MvGB115, *Ae. speltoides* MvGB905 and *Ae. tauschii* MvGB605 to be identified (Fig. 2). Differences were observed in the FISH hybridization patterns of the genotypes used in this study compared with those used previously or those of durum and hexaploid wheat (Badaeva et al. 1996a, b; Kubaláková et al.

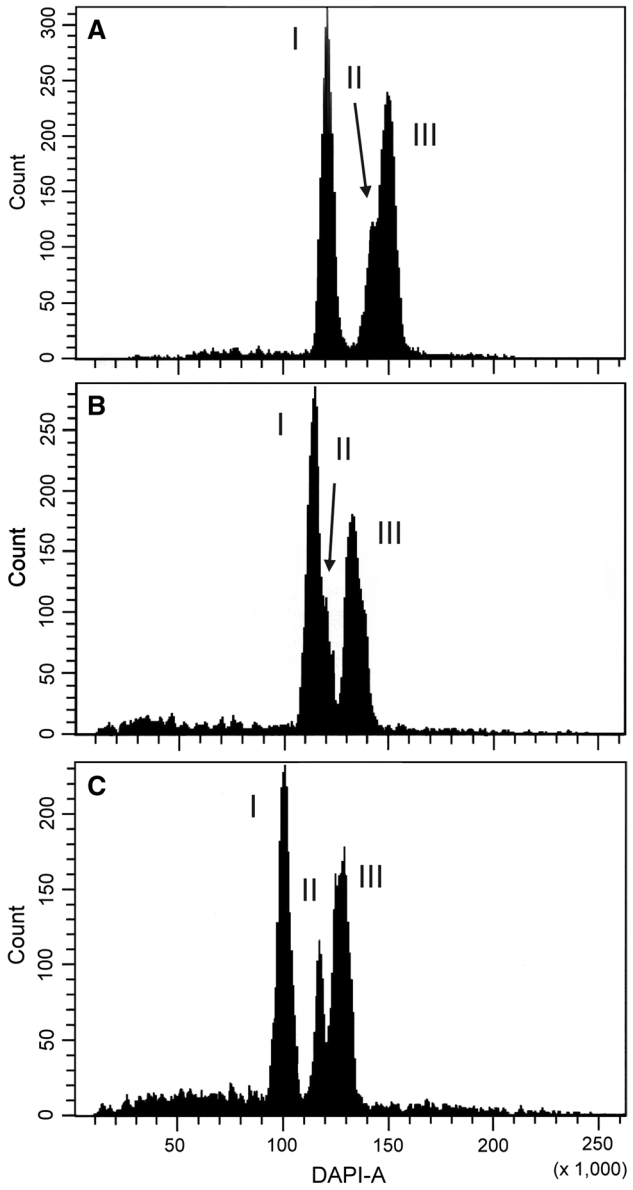


Fig. 1 Flow karyotypes obtained after the analysis of DAPI-stained chromosome suspensions prepared from *T. urartu* MvGB115 ($2n = 2x = 14$, A^uA^u) (**a**), *Ae. speltoides* MvGB905 ($2n = 2x = 14$, SS) (**b**) and *Ae. tauschii* MvGB605 ($2n = 2x = 14$, DD) (**c**). Each flow karyotype consists of two composite peaks I and III, each of them representing a group of three chromosomes. Chromosome 5 is represented by peak II, which is well resolved only in *Ae. tauschii*. In *T. urartu* and *Ae. speltoides* the peak of chromosome 5 is not well resolved, appearing as a shoulder on peak III in *T. urartu* and on peak I in *Ae. speltoides*

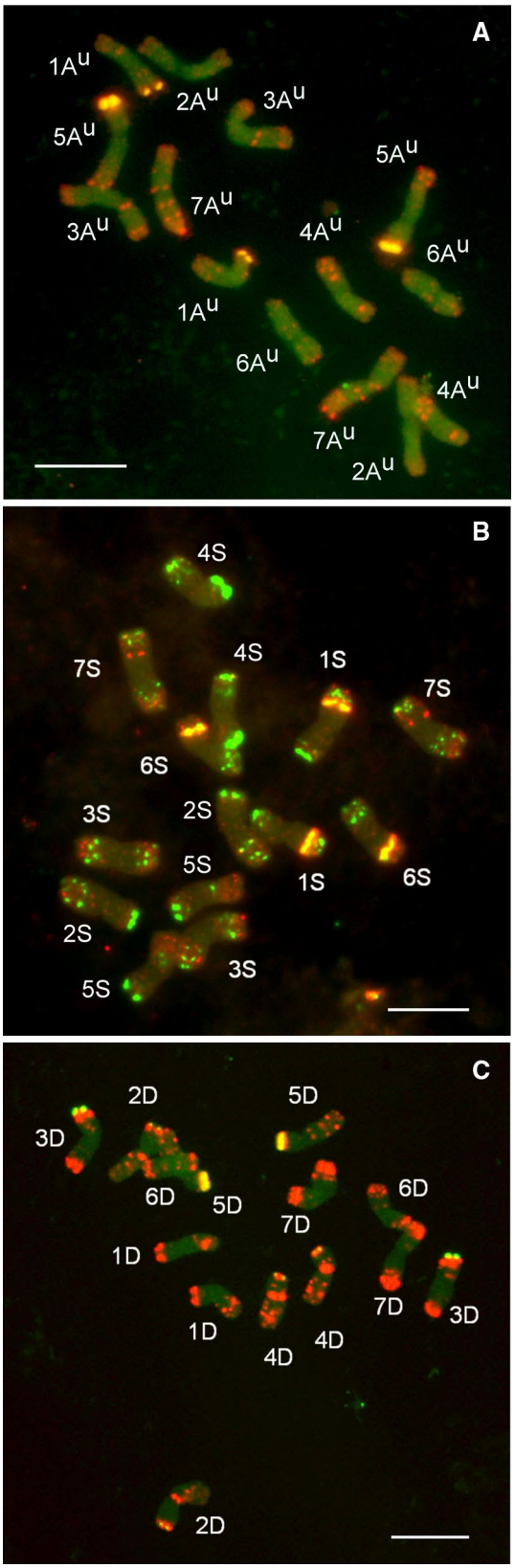
2005; Sepsi et al. 2008). Strong 18S rDNA signals were observed on the short arms of $1A^u$ and $5A^u$, which together with the *Afa* hybridization patterns (diagnostic bands on the chromosome arms $1A^uS$ and $5A^uL$) allowed the discrimination of the two chromosomes (Fig. 2a). Distinctive *Afa* labelling patterns were also observed on chromosome $2A^u$ and chromosome arm $4A^uS$. Chromosomes $3A^u$, $6A^u$ and $7A^u$ could be identified from their hybridization patterns, which were similar to those of the wheat chromosomes. The chromosomes of *Ae. speltoides* MvGB905 showed hybridization patterns similar to those of hexaploid wheat, which allowed the identification of the whole set of chromosomes (Fig. 2b).

The idiogram representing the genomic distribution of the *Afa* family, pSc119.2 and 18S rDNA sequences in *T. urartu* MvGB115, *Ae. speltoides* MvGB905 and *Ae. tauschii* MvGB605 is shown in Fig. 3. Differences in the fluorescence hybridization patterns facilitated the identification of each of the seven chromosomes of the diploid progenitors in the wheat background.

The chromosomes that showed different hybridization patterns relative to wheat were 2S, where a weak subtelomeric signal was detected relative to wheat 2B, 3S, where strong pSc119.2 subtelomeric signals were detected on the long arm, 4S, where the subtelomeric region of the long arm had only one weak pSc119.2 signal, and 7S, which showed a weak telomeric pSc119.2 band on the long arm relative to the corresponding wheat chromosomes. Only minor differences were found on chromosomes 3D and 4D (weak telomeric 18S rDNA and pSc119.2 signals, respectively, on the short arms) in *Ae. tauschii* relative to the corresponding chromosomes of wheat (Fig. 2c). However, the identification of the chromosomes was not hindered by these differences and all chromosomes of the diploid progenitor species could be distinguished on the basis of their fluorescence labelling patterns.

Description of flow karyotypes

The chromosome content of the individual peaks was determined after FISH on flow-sorted chromosomes with probes for *Afa* family, pSc119.2 and 18S rDNA repeats (Fig. 4; Table 1). The strong fluorescent signals given by the FISH probes were similar to those observed on mitotic metaphase spreads and allowed the unambiguous identification of the sorted chromosomes. In *T. urartu*, peaks I and III contained chromosomes $1A^u$, $4A^u$ and $6A^u$, and $2A^u$, $3A^u$ and $7A^u$, respectively. Peak II corresponded to chromosome $5A^u$, representing 15.23 % of the *T. urartu* genome, which could be sorted at a purity of >78.53 % (Fig. 4; Table 1). Peaks I and III were also composite in *Ae. speltoides*, containing chromosomes 1S, 4S and 6S, and 2S, 3S and 7S, respectively (Fig 4; Table 1). As expected, the partially resolved peak II corresponded to chromosome 5S (~13.88 % of the S genome), which was sorted at a purity of 89.8 %. The distribution of the D genome chromosomes in *Ae. tauschii* between the peaks of the flow karyotypes was shown by FISH analysis to be similar to that observed in *T. urartu* and *Ae. speltoides*.



◀**Fig. 2** Fluorescence in situ hybridization (FISH) with probes for repetitive DNA sequences pSc119.2 (*green*), *Afa* family (*red*) and 18S rDNA (*yellow*) on mitotic metaphase spreads of *T. urartu* MvGB115 (**a**), *Ae. speltoides* MvGB905 (**b**) and *Ae. tauschii* MvGB605 (**c**). *Scale bars* 10 μm

The composite peaks I and III contained the chromosome groups 1D, 4D and 6D, and 2D, 3D and 7D, respectively. The well-resolved peak II corresponded to chromosome 5D (~15.56 % of the D genome), which represented 87.3 % of the sorted particles (Fig. 4; Table 1).

Assignment of COS markers to peaks on flow karyotypes

A set of conserved orthologous set (COS) markers specific for wheat homoeologous groups 1–7 were mapped to subgenomic DNA samples from individual peaks on the flow karyotypes to confirm the cytological assignment of chromosomes to the flow karyotype peaks. Of the 25 COS markers investigated, all 25 gave PCR products in wheat (genotype Mv9kr1), 24 in *T. urartu* MvGB115 and *Ae. tauschii* MvGB605 and 23 in *Ae. speltoides* MvGB905 (Online Resource 2). The 25 markers resulted in a total of 68 PCR products (range 1–7 PCR products/marker/genotype, mean 2.72 PCR products) in wheat (genotype Mv9kr1), while 137 products were amplified in the diploid progenitors (49, 42 and 46 products in the *T. urartu*, *Ae. speltoides* and *Ae. tauschii* genotypes, respectively). Of the 137 PCR products detected in the diploid species, 49 (35.8 %) were non-polymorphic, while 88 (64.2 %) were polymorphic relative to hexaploid wheat (the proportion of non-polymorphic amplicons was 38.8, 33.3 and 34.8 % in *T. urartu*, *Ae. speltoides* and *Ae. tauschii*, respectively).

Because each chromosome of the wild progenitors has a major location in one of the peaks on the flow karyotype (Table 1), the yield of PCR products differed between the peaks and the highest amount of PCR products was observed in the peak where the locus-carrying chromosome had its major location (Fig. 5). For example, the marker $X_{GPI:C:731424}$, specific for group 4 chromosomes of wheat, produced a 260-bp PCR amplicon with continuously decreasing yield in the *T. urartu* flow karyotype peaks I, II and III (no amplicon in peak III) (Fig. 5, Online Resource 2), where the 4A chromosome contents were 38.61, 0.56 and 0 %, respectively (Table 1). With this marker a similar relationship could be observed in *Ae. speltoides* and *Ae. tauschii* between the yield of group 4 chromosome-specific PCR amplicons and the relative content of group 4 chromosomes determined by cytomolecular methods for the flow karyotype peaks.

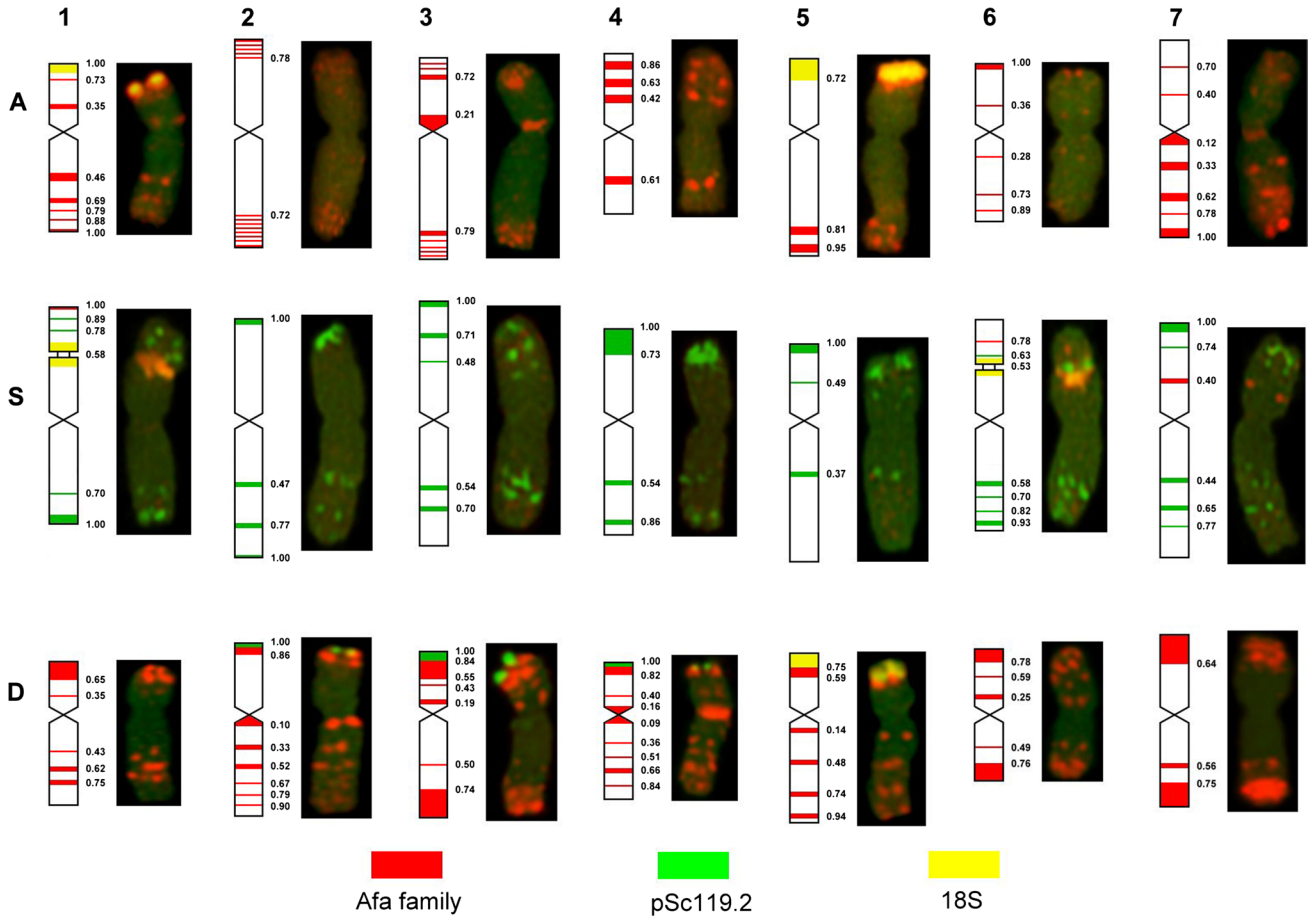


Fig. 3 Idiogram and karyotype of the chromosomes of *T. urartu* MvGB115 (**a**), *Ae. speltoides* MvGB905 (**s**) and *Ae. tauschii* MvGB605 (**d**) showing genomic distribution of repetitive DNA sequences pSc119.2 (*green*), Afa family (*red*) and 18S rDNA (*yellow*). Fraction lengths were determined as means of 15–20 measurements for each band

Based on the differences in the yield of PCR amplicons between the flow karyotype peaks, all the PCR amplicons could be assigned to peaks on flow karyotypes of the diploid species (Table 2, Online Resource 2). The COS markers specific for different ESTs and representing the group 1–7 chromosomes of hexaploid wheat showed similar results to the cytomolecular determination of the chromosome content of the flow karyotype peaks. In the first flow karyotype peak of *T. urartu*, *Ae. speltoides* and *Ae. tauschii*, the highest yield was detected for the PCR amplicons of markers specific for group 1, 4 and 6 chromosomes, while the markers specific for group 2, 3 and 7 chromosomes were assigned to peak III in the diploid progenitors. The markers representing the group 5 chromosomes were assigned unambiguously to peak II of *T. urartu*, *Ae. speltoides* and *Ae. tauschii* and with four exceptions (group 3-specific $X_{BM134465}$ in *Ae. tauschii*, group 4-specific X_{4S} and group 6-specific X_{6A} in *Ae. speltoides* and group 7-specific $X_{GPI:C:767323}$ in *T. urartu*) no markers specific for other homoeologous groups were assigned to peak II in any of the three species. These results indicate that flow karyotype peak II represents only the group 5 chromosomes of the diploid progenitors, consistently with the cytological results.

Discussion

Flow cytometric chromosome analysis and sorting has been developed in species with high socio-economic importance, such as bread wheat, durum wheat, barley and rye (Vrána et al. 2000; Kubaláková et al. 2003, 2005; Lysák et al. 1999). Chromosome genomics, which has been described as the application of genomic tools to flow-sorted chromosomes (Doležel et al. 2007), facilitates the molecular analysis of chromosome structure, the high-throughput development of markers, the construction of ready-to-sequence physical maps and positional gene cloning. The present work extends the potential of chromosome genomics to the diploid progenitors of bread wheat: *T. urartu*, *Ae. speltoides* and *Ae. tauschii*.

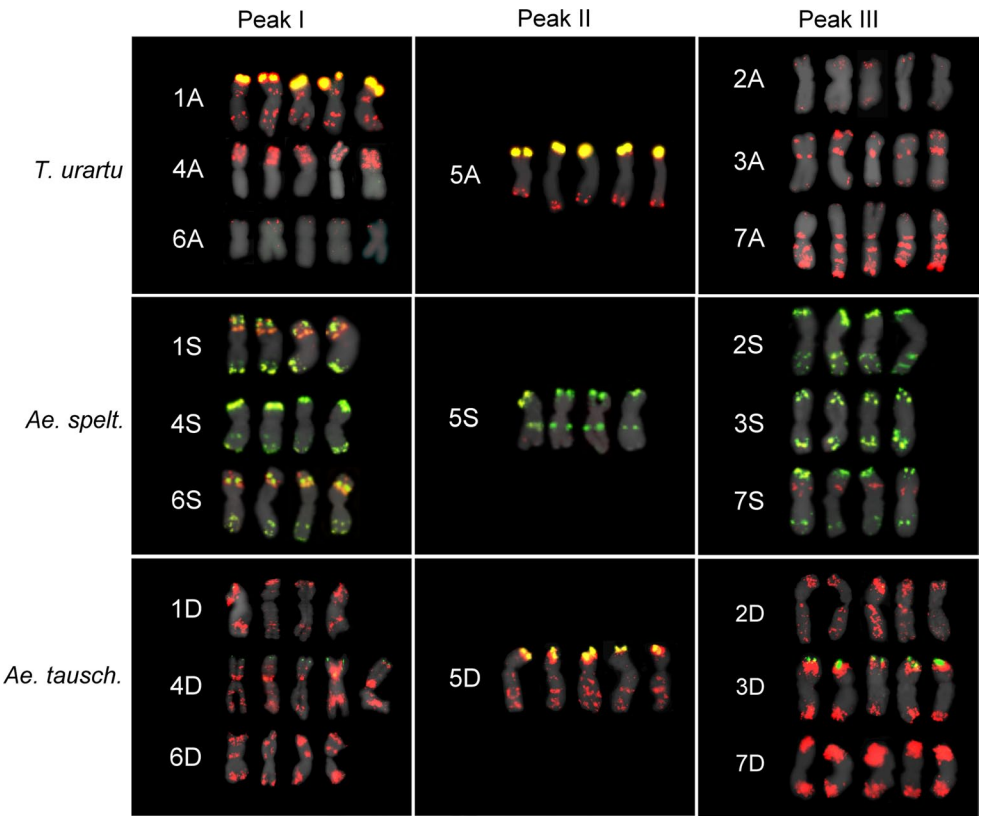


Fig. 4 Mitotic metaphase chromosomes sorted from individual peaks of flow karyotypes of *T. urartu* MvGB115, *Ae. speltoides* MvGB905 and *Ae. tauschii* MvGB605. Sorted chromosomes were identified after FISH with probes for repetitive DNA sequences pSc119.2 (*green*), *Afa* family (*red*) and 18S rDNA (*yellow*). Chromosomes were assigned to the peaks where their frequencies were the highest. Four or five representative examples are given for each chromosome

The fact that the chromosome peaks on the flow karyotype of *T. urartu* were shifted towards higher fluorescence channels as compared to *Ae. speltoides* and *Ae. tauschii* suggests that the average size of the A^u genome chromosomes, and hence the size of the A^u genome is larger than that of the S and D genomes. These results agree with the 1C values published for *T. urartu* (5.784 and 5.88 pg DNA), *Ae. speltoides* (5.15 pg DNA) and *Ae. tauschii* (5.08 and 5.1 pg DNA) (Özkan et al. 2003, 2010; Furuta et al. 1986; Rees and Walters 1965) and indicate that their genomes underwent different evolutionary changes. As the number of genes per monoploid genome is similar in plant species, differences in genome size are mainly due to repetitive DNA sequences, of which retroelements are considered to play a dominant role in genome expansion (Bennetzen 2007; Feuillet and Keller 2002; Lisch 2009). Charles et al. (2008) showed that specific types of transposable elements underwent differential proliferation in various wheat genomes during their evolution. The activation of *Copia* and *CACTA* transposable elements has occurred in the wheat A genome, while the proliferation of *Gypsy* elements was observed in the B genome. The differential proliferation of these retroelements in the A and B genomes of wheat occurred prior to allopolyploidization events (Charles et al. 2008) and hence in the diploid wheat progenitors.

Differences in the molecular organization of the A, S and D genomes of *T. urartu*, *Ae. speltoides* and *Ae. tauschii* relative to tetraploid and hexaploid wheat have been demonstrated at the chromosome level using FISH. The FISH analysis performed in the present work involved probes for pSc119.2, *Afa* family and 18S rDNA repeats, whose genomic distribution was previously described in tetraploid and hexaploid wheat as well as in their diploid progenitors (Badaeva et al. 1996a, b; Kubaláková et al. 2005; Molnár et al. 2009). The most pronounced differences in the chromosomal structure concern the A genome of *T. urartu* relative to bread wheat, including the intensively labelled NOR regions of chromosomes 1 and 5, which were eliminated in durum and bread wheat after polyploidization (Jiang and Gill 1994). The inactivation of major NORs was also observed after the formation of the allopolyploid *Aegilops* species (Badaeva et al. 2004; Molnár et al. 2011a; Feldman et al. 2012). The difference in the hybridization pattern of chromosome 4A between *T. urartu* and bread wheat is due to the pericentric inversion of 4A and translocations involving chromosomes 5A and 7B, which occurred at the polyploid level (Hernandez et al. 2012). Smaller differences in the FISH karyotype were detected between *Ae. speltoides* and the B genome of wheat, while almost the same karyotype was observed in *Ae. tauschii* relative to the D genome chromosomes of wheat.

Genomic asymmetry, i.e. the alteration of wheat subgenomes relative to the diploid progenitors, may be related to the time that passed between the two polyploidization

Table 1 Assignment of chromosomes to peaks of flow karyotypes for *T. urartu* MvGB115, *Ae. speltoides* MvGB905 and *Ae. tauschii.* MvGB605. The numbers represent the percentage of the chromosome type in the whole peak content

Species	Genome	Chromosome	Peak I	Peak II	Peak III
T. urartu speltoides	A	1	25.64	1.41	–
		2	–	0.28	32.69
		3	–	2.25	34.39
		4	38.61	0.56	–
		5	0.28	78.53	1.69
		6	35.44	0.84	–
		7	–	16.1	31.21
No. of chromosomes analysed			347	354	471
Ae. speltoides	S	1	29.69	5.19	0.19
		2	–	–	36.59
		3	–	–	36.39
		4	31.51	2.28	–
		5	11.31	89.8	0.19
		6	27.47	2.7	0.38
		7	–	–	26.24
No. of chromosomes analysed			495	481	522
Ae. tauschii	D	1	34.17	0.82	–
		2	–	0.13	31.02
		3	–	11.0	29.67
		4	36.46	0.68	–
		5	–	87.3	0.84
		6	29.35	–	–
		7	–	–	38.44
No. of chromosomes analysed			436	727	593

events of hexaploid wheat (the formation of *T. dicoccoides* occurred approximately 0.3–0.5 million years ago, while hexaploid wheat evolved around 9,000 years ago) and to the process of genetic diploidization (Dvorak et al. 1998). Genetic diploidization causes non-random elimination of coding and non-coding sequences in parental genomes (Özkan et al. 2001; Feldman and Levy 2005) and could explain the observation of strong *Afa* FISH signals in the A genome of *T. urartu* and the lower number and size of *Afa* loci in hexaploid wheat. Diploidization affects both the expression of rRNA encoding genes, storage protein genes and other genes related to plant morphology, and the adaptation to environmental and biological factors, leading to the unequivocal partition of tasks between the constituent genomes of durum and bread wheat (Feldman et al. 2012). While the A genome of hexaploid wheat preferentially controls morphological traits, the B and D genomes control the reactions to biotic and abiotic factors (Feldman et al. 2012).

The finding that the $5A^u$, 5S and 5D chromosomes can be isolated from the diploid progenitors of bread wheat may facilitate the dissection of many important traits. The group 5 chromosomes harbour loci ensuring genetically stable inheritance and determining the morphological traits responsible for widespread cultivation and adaptation to different agroecological conditions and abiotic stresses (Cattivelli et al. 2002). Among them, the *Ph1* locus, the dominant factor in the genetic system ensuring diploid-like meiotic chromosome pairing in tetra- and hexaploid wheat, is located on the long arm of chromosome 5B (Riley and Chapman 1958). The *Q* gene, a predominant domestication gene in bread and durum wheat, conferring the free-threshing (naked grain) phenotype and pleiotropically affecting spike morphology, rachis fragility, glume tenacity, plant height and heading characters (Leighty and Boshnakian 1921; Mackey 1954; Muramatsu 1986; Kato et al. 1999; Faris et al. 2003; Simons et al. 2006) has been located on chromosome 5A. The *VRN1* gene, which is critical for the adaptation of polyploid wheats to autumn sowing and divides wheat varieties into the winter and spring market classes, has been mapped to the colinear regions of the long arm of chromosomes 5A, 5B and 5D (Galiba et al. 1995; Snape et al. 1997). The group 5 chromosomes of hexaploid wheat also carry major loci affecting winter hardiness and freezing tolerance, designated as *FROST RESISTANCE-1* (*FR-1*; Sutka and Snape 1989) and *FR-2,* which is approximately 30 centimorgans proximal to *VRN-1* and includes a cluster of 11 (or more) *C-REPEAT BINDING FACTOR* (*CBF*) genes (Vágújfalvi et al. 2003; Dhillon et al. 2010). Other loci affecting drought and salt tolerance and the crossability of wheat have also been mapped to group 5 chromosomes (Quarrie et al. 1994; Koebner et al. 1996; Riley and Chapman 1967; Sitch et al. 1985; Krolow 1970).

The identification of novel alleles of important genes in the wild diploid wheat species could facilitate their use for coping with the pleiotropic effects of climate change (Feuillet et al. 2008). The production of synthetic wheat by crossing tetraploid durum wheat and *Ae. tauschii* is an important pathway for the utilization of wild genetic diversity in breeding programmes. As reported by Dreisigacker et al. (2008), 1,100 synthetic wheat lines were produced by CIMMYT from a collection of ~900 *Ae. tauschii* accessions and showed significant variation for morphological and agronomic traits and for tolerance to biotic and abiotic stresses. As the draft genome sequences of *T. urartu* and *Ae. tauschii* became available (Ling et al. 2013; Jia et al. 2013), the isolation of group 5 chromosomes from various diploid wheat species will provide a cost-effective means for the targeted re-sequencing of the chromosomes using next generation technology (Vitulo et al. 2011). This will support comprehensive gene content analysis and allele discovery, and facilitate the development of gene-based markers for

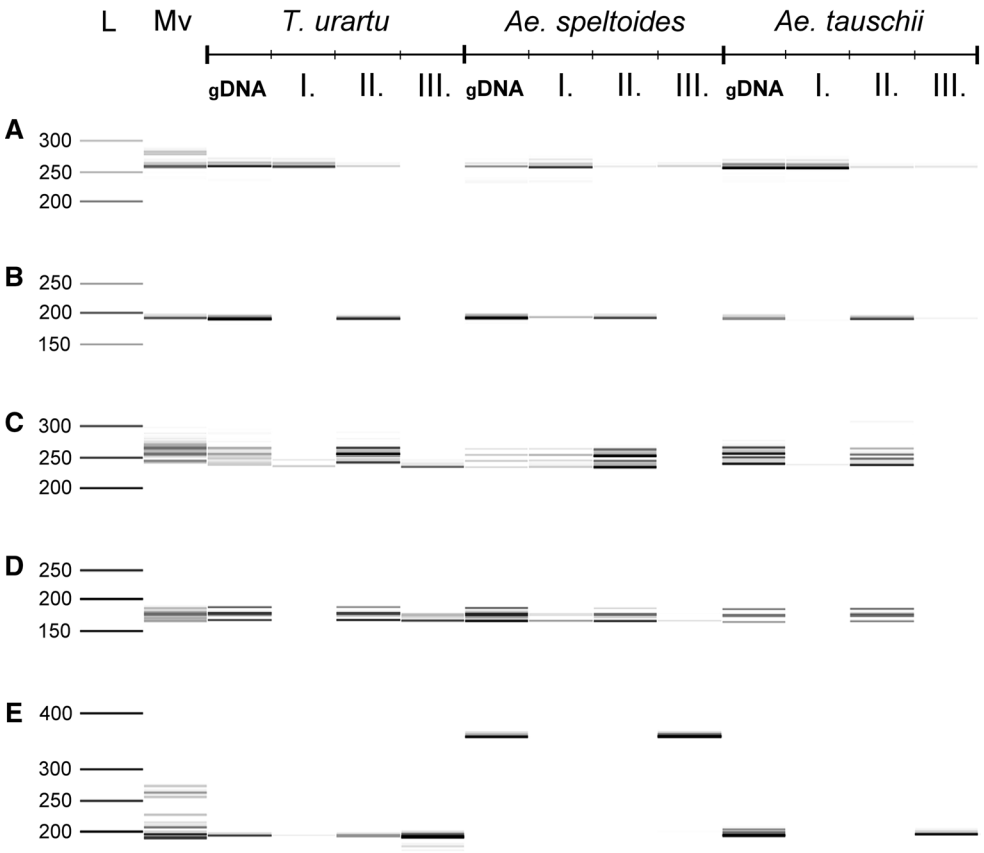


Fig. 5 PCR products of conserved orthologous set (COS) markers amplified from the wheat genotype Mv9kr1 (Mv), from total genomic DNA (gDNA) and from subgenomic DNA samples derived from chromosomes of particular peaks (I, II, III) on flow karyotypes of *T. urartu* MvGB115, *Ae. speltoides* MvGB905 and *Ae. tauschii.* MvGB605. The pictures represent markers amplified from flow karyotype peak I ($X_{GPI:C:731424}$) (**a**), peak II ($X_{GPI:C:726959}$, X_{5A}, $X_{GPI:C:729592}$) (**b**–**d**) and peak III (X_{tr150}) and those specific for homoeologous group 4 ($X_{GPI:C:731424}$), group 5 ($X_{GPI:C:726959}$, X_{5A} and $X_{GPI:C:729592}$) and group 2 (X_{tr150})

specific genomic regions (Wenzl et al. 2010; Berkman et al. 2011; Mayer et al. 2009, 2011; Wicker et al. 2011). Moreover, the availability of chromosome sequences will permit the validation of genome assemblies at the chromosome level.

Our findings confirm and expand the results of previous studies indicating the high transferability of COS markers between species (Parida et al. 2006; Burt and Nicholson 2011; Howard et al. 2011; Molnár et al. 2013). The fact that 64.2 % of the products obtained with chromosomes isolated from wild relatives were polymorphic relative to those obtained in hexaploid wheat suggested that the substantial genetic diversity of wild progenitors is due to the variability of intron regions (Yu et al. 2005). Thus, COS markers can be used to identify the chromatin of wild progenitors in the wheat background and for marker-assisted selection to facilitate the transfer of useful agronomic traits (Quraishi et al. 2009). The 24 COS markers assigned to chromosomes of the diploid progenitors of wheat can be used in pre-breeding programmes to facilitate gene introgression. In a previous study, 100 COS markers were assigned to the U and M genome chromosomes of diploid and tetraploid *Aegilops* species (Molnár et al. 2013) and used for the analysis of macrosynteny between *Aegilops*, wheat and the model species *Brachypodium* and rice. These results indicate that the markers used in the present study and the COS markers that are assigned to the chromosomes of the diploid progenitors of wheat in the future will be suitable tools to study the genome rearrangements and synteny perturbations that occurred during the polyploidisation and domestication of bread wheat.

The FISH analysis of flow-sorted chromosome fractions showed that chromosomes $5A^u$, 5S and 5D could be isolated at high purity (78.5, 89.8 and 89.3 %, respectively) from *T. urartu, Ae. speltoides* and *Ae. tauschii*. Further improvements in the protocol might lead to the increased purity of these chromosome fractions. However, as demonstrated by Mayer et al. (2011), the level of chromosome contamination observed in the present study does not compromise the bioinformatic analysis of the sequence data obtained by sequencing DNA amplified from single chromosome fractions. Other chromosomes from the diploid wheat species formed composite peaks on the flow karyotypes and could be sorted into groups of three. This limitation could be overcome in the future using the multiparametric flow cytometric analysis of chromosomes labelled by FISH with fluorescent probes for microsatellites, as shown by Giorgi et al. (2013). The FISH karyotypes of diploid wheat species developed in this study and by Megyeri et al. (2012) might serve as a guide when choosing probes for labelling chromosomes prior to flow-sorting.

Table 2 Assignment of COS markers specific for wheat homoeologous groups 1–7 to peaks I–III on flow karyotypes of *T. urartu* MvGB115, *Ae. speltoides* MvGB905 and *Ae. tauschii* MvGB605

Marker	Wheat homoeologous group	*T. urartu*			*Ae. speltoides*			*Ae. tauschii*		
		I	II	III	I	II	III	I	II	III
$X_{BE443103}$	1	X			X			X		
$X_{GPI:C:732519}$	1	X			X			X		
$X_{GPI:C:746781}$	1	X			X			X		
$X_{BE445693}$	2			X			X			X
$X_{GPI:C:719382}$	2			X			X			
X_{tr150}	2			X			X			X
X_{2N}	2			X			X			X
X_{3B}	3			X			X			X
$X_{BM134465}$	3			X			X		X	
$X_{BE404709}$	3			X						X
$X_{GPI:C:748004}$	4	X			X			X		
$X_{GPI:C:731424}$	4	X			X			X		
$X_{GPI:C:725135}$	4	X			X			X		
X_{4S}	4	X				X		X		
$X_{GPI:C:748166}$	5								X	
$X_{GPI:C:729592}$	5		X			X			X	
X_{5A}	5		X			X			X	
$X_{GPI:C:726959}$	5		X			X			X	
$X_{GPI:C:740549}$	6	X			X			X		
X_{6A}	6	X				X		X		
X_{6N}	6	X			X			X		
$X_{BE352570}$	7			X			X			X
$X_{GPI:C:771171}$	7			X			X			X
$X_{GPI:C:770073}$	7			X			X			X
$X_{GPI:C:767323}$	7		X				X			X

To conclude, this study represents an important step forward in developing chromosome genomics for the wild genetic resources of wheat. The flow karyotypes of *T. urartu, Ae. speltoides* and *Ae. tauschii* were characterized and the chromosome content of all the peaks on the karyotypes was determined for the first time. The ability to purify group 5 chromosomes in the wild diploid progenitors of wheat paves the way for the rapid re-sequencing of single chromosomes isolated from diverse populations, the physical mapping of DNA sequences to particular chromosomes using PCR, the construction of chromosome-specific BAC libraries and next generation sequencing to identify low-copy and genic sequences and to develop new markers. The COS markers assigned to the chromosomes of diploid wheats could be used in pre-breeding programmes to select chromosome segments carrying agronomically useful genes in *T. aestivum*—wild wheat recombinant lines.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The authors declare that the experiments comply with the current laws of the countries (Czech Republic and Hungary) in which they were performed.

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