Dissecting the U, M, S and C genomes of wild relatives of bread wheat (*Aegilops* spp.) into chromosomes and exploring their synteny with wheat

István Molnár[1*], Jan Vrána[2], Veronika Burešová[2], Petr Cápal[2], András Farkas[1], Éva Darkó[1], András Cseh[1†], Marie Kubaláková[2], Márta Molnár-Láng[1], Jaroslav Doležel[2]

[1] Agricultural Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, Brunszvik u. 2, H-2462 Martonvásár, Hungary

[2] Institute of Experimental Botany, Centre of the Region Haná for Biotechnological and Agricultural Research, Šlechtitelů 31, CZ-78371 Olomouc, Czech Republic

[*]For correspondence:

Tel. +36-22-569504, Fax: +36-22-460213, E-mail: molnar.istvan@agrar.mta.hu

[†]Present address: Division of Plant and Crop Sciences, School of Biosciences, The University of Nottingham, Sutton Bonington Campus, Loughborough, UK

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SUMMARY (236words)

Goat grasses(*Aegilops spp.*) contributed to the evolution of bread wheat and are importantsources of genes and alleles for modern wheat improvement. However, their use in alien introgression breeding is hindered by poor knowledge of their genome structure and a lack of molecular tools. The analysis oflarge and complex genomes may be simplified by dissecting them into single chromosomes via flow cytometric sorting. In some species this is not possible due to similarities in relative DNA content among chromosomes withina karyotype.This work describes the distribution of GAA and ACG microsatellite repeats on chromosomes of the U, M, S and C genomes of *Aegilops*, and the use of microsatellite probes to label thechromosomes in suspension by fluorescence *in situ* hybridization (FISHIS). Bivariate flow cytometric analysis of chromosome DAPI fluorescence andfluorescence of FITC-labelled microsatellites made it possible to discriminate allchromosomes andsort them with negligible contamination by other chromosomes.DNA of purified chromosomes was used as a template for PCR usingCOS markers with known positions on wheat A, B and Dgenomes. Wheat-*Aegilops* macrosyntenic comparisons using COS markers revealed significant rearrangements in the U and C genomes, while the M and S genomes exhibited structure similar to

wheat. Purified chromosome fractions provided an attractive resource to investigate the structure and evolution of the *Aegilops* genomes, and the COS markers assigned to *Aegilops* chromosomes will facilitate alien gene introgression into wheat.

SIGNIFICANCE STATEMENT (74WORDS)

Bivariate flow cytometric analysis of DNA content and FITC-labelled microsatellites enabled all the chromosomes in the U, M, S and C genomes of *Aegilops*to be discriminated and purified. Mapping COS markers with known position in the wheat genome to flow-sorted *Aegilops* chromosomes revealed significant evolutionary rearrangements in the U and C genomes, but not in the M and S genomes. COS markers assigned to *Aegilops* chromosomes will facilitate alien introgression breeding in wheat.

INTRODUCTION (1264words)

Bread wheat (*Triticum aestivum* L., 2n=6x=42, AABBDD genome) plays a fundamental role in the human diet. The pressure to produce enough food for the growing world population under achanging climate underlines urgent need for new high-yielding varieties with improved stress tolerance and quality-related traits. Breeding such varieties may be facilitated by employing new biotechnological tools and utilizing the extantgenetic diversity among the wild relatives of wheat (Feuillet et al., 2008).

The genus *Aegilops* (goatgrass) belongs to the tribe *Triticeae* and comprises eleven diploid, ten tetraploid and two hexaploid species (Van Slageren 1994). The U, M, S and C genomes were identified in nineteen (eight diploid and eleven polyploid) *Aegilops* species (Kilian et al., 2011). These species represent a rich source of genes and gene complexes that can be utilized in wheat improvement *via* chromosome-mediated gene transfer. Forexample, *Ae. umbellulata* Zhuk. (2n=2x=14, UU) and *Ae. comosa* Sm. in Sibth. & Sm. (2n=2x=14, MM) are known sources of important agronomic traits such as tolerance to biotic (BYDV, Cereal cyst nematode, Hessian fly, Leaf rust, Stripe rust, Tan spot, and Powdery mildew) and abiotic stresses (Drought, Frost, Heat, Salt, Zn-deficiency), nutritional and bread-making quality (Molnár et al., 2004;Schneider et al., 2008; Kozub et al., 2011; Dulai et al., 2014; Farkas et al., 2014).

Ae. speltoides Tausch. (2n=2x=14, SS) is the closest relative to the wheat B genome (Dvorak et al., 1998) and is an attractive source of genes providing tolerance against Leaf rust, Stem rust and Powdery mildew and for other traits, such as grain hardness protein, heat tolerance and tolerance to manganese toxicity (Schneider et al., 2008; Kilian et al., 2011). The genome of *Ae. markgrafii* (Greuter) Hammer (2n=2x=14, CC) codes for resistance genes against leaf rust and powdery mildew, genes for high protein and lysine content, and alleles affecting bread-making quality (Friebe et al., 1992;Potz et al., 1996;Liu et al., 2003;Riar et al., 2012).

Over the past decades, efforts were made to transfer *Aegilops* chromatin into wheat, resulting in addition, substitution and translocation lines containing chromosomes and chromosome segments from *Ae. umbellulata*, *Ae. comosa*, *Ae. speltoides* and *Ae. markgrafii*(Jiang et al., 1994; Friebe et al., 1996;Schneider et al., 2008, Kilian et al., 2011). Despite the valuable genetic variation within the wild relatives of wheat, andsuccessful introgression of some favorable genes, the potential of alien gene transfer has been largely underutilized in wheat breeding.

The use of wild genes and alleles in breeding programs is hampered by laborious and time-consuming development of alien introgression lines. The main tools for their selection and characterization arelow-throughput cytogenetic methods, such as C-banding (Fiebe et al., 1996), fluorescence *in situ* hybridization (FISH,Rayburn and Gill 1985;Schwarzacher and Heslop-Harrison 2000**;** Schneider et al., 2005) and genomic *in situ* hybridization (GISH, Schwarzacher et al., 1989;Le et al., 1989). However, the potential of FISH to identify alien chromosomes and their segments is limited by small number of suitable probes, low throughout and inability to detect very small introgressions.

The efficiency of introgression breeding and the development of high density genetic maps of*Aegilops* is limited by small number of molecular markers suitablefor high-throughput screening (Zhang et al., 1998). In recent decades, wheat-specific RFLP (Restriction Fragment Length Polymorphism), SSR (Simple Sequence Repeat), AFLP (Amplified Fragment Length

Polymorphism)and COS (Conserved Orthologous Set) markers weretested in *Aegilops* species (Peil et al., 1998; Schneider et al., 2010;Rey et al., 2015). Nagy et al. (2006) used S-SAP (Sequence-Specific Amplification Polymorphism) technology to produce 14 and 30 genome-specific markers for *Ae. umbellulata* and *Ae. biuncialis* (2n=4x=28, $U^bU^bM^bM^b$), respectively. More recently, Diversity Arrays Technology (DArT) markers and microarray hybridization-based sequence-independent marker systems wereused to develop a high-density genetic map of wheat × wild emmer (Peleg et al., 2008). The advent of next generation sequencing (NGS) technologies led to the development of SNP-based platforms for wheat genotyping (Rey et al., 2015).However,low representation of wild wheat relatives in the SNP design may limit the utility of these platforms in alien introgression breeding (Winfield et al., 2015) and new genomic resources need to be generated from wild relatives of wheat.

Poor knowledge of syntenic relationships between wheat and *Aegilops* chromosomes is another obstacle hampering the use of wild genetic diversity in wheat breeding. Collinearity between the homoeologous wheat and alien chromosomes may be interrupted as a consequence of evolutionary chromosome rearrangements in the *Aegilops*genomes (Devos et al., 1993,Zhang et al., 1998). Thus, genes on alien chromosome segments do not compensate for the loss of wheat genes and this may have a negative effect on agricultural performance of the wheat-alien translocations. Clearly, better knowledge on the genome organization of wild crop relatives and the

development of new molecular resources and tools are needed if the extant genetic diversity of wild *Aegilops* speciesis to be better utilized.

The analysis of large *Triticeae* genomes can be simplified by dissecting them into individual chromosomes by flow cytometric sorting (Doležel et al., 2007). As demonstrated in bread wheat, barley and rye, flow-sorted chromosomes are suitable for next generation sequencing (NGS) to establish linear gene order and assess gene synteny with other species (Mayer et al., 2011;Martis et al., 2013**;** IWGSC 2014). High purity offlow-sorted chromosome fractions makes them an ideal template for PCR-based analyses and toassignmolecular markers to *Aegilops* chromosomes (Molnár et al., 2011b). Using gene-based COS markers and chromosomesflow-sorted from wheat-*Aegilops* introgression lines, Molnár et al. (2013) assigned 132 and 156 loci to the M- and U-genome chromosomes, respectively, of *Ae. comosa*, *Ae. umbellulata*, *Ae. biuncialis* and *Ae. geniculata*. The genomic position of orthologue unigene EST-contigs, which were used to design the COS markers, made it possible to investigate syntenic relationships between the U and M genomes of *Aegilops* and wheat using *Brachypodium* and rice as references. Unfortunately, in somespecies, flow cytometric chromosome analysis and sorting based on DAPI fluorescence alone fails to discriminate and sort allchromosomes. Thus, only chromosomes 1U, 3U and 6U could be purified from *Ae. umbellulata* and only $1U^b$ from *Ae. biuncialis,* while the remaining chromosomes could only be sorted in groups (Molnár et al., 2011b). This limitation prevented a detailed comparative analysis with wheat

and hampered the use of the chromosome-based approach to sequence the genomes of wild relatives of wheat chromosome by chromosome.

To overcome this problem, Giorgi et al. (2013) developed a method termed FISHIS (FISH in suspension), which fluorescently labels specific microsatellite sequences on chromosomes in suspension. Some microsatellites, such as GAA and ACG motifs, form large clusters on chromosomes of *Aegilops* species and are detectable on mitotic metaphase spreads usingFISH (Molnár et al., 2011a), providing an opportunity to employ these repeats for fluorescent labelling of chromosomes prior to flow cytometry. Encouraged by the results obtained bygenomicsanalysesof chromosomes flow-sorted from cereal crops, and motivated by theneed to support alien introgression breeding of wheat, we set out to expand chromosome genomics in *Aegilops* and develop molecular tools and resources.

Here we report on the use of two microsatellite repeats, GAA and ACG, as probes for FISH to identify mitotic chromosomes of *Ae. umbellulata*, *Ae. comosa*, *Ae. speltoides* and *Ae. markgrafii*. The same microsatellite repeat probes were used to fluorescently label chromosomes in suspension prior to flow-cytometric analysis to facilitate sorting all chromosomes fromdiploid progenitors of the U, M, S and C genomes of *Aegilops*. DNA amplified from flow-sorted chromosomes was used for PCR with COS markers to obtain insights into the macrosyntenic relationships between the genomes of *Aegilops* and bread wheatat chromosome level.

RESULTS (1829words)

Chromosomal distribution of GAA and ACG repeats

In order to investigate the potential of GAA and ACG repeatsas probes for fluorescent labelling chromosomes in suspension and to provide additional chromosomal landmarks foridentification of *Aegilops* chromosomes and chromosome segments, sequential FISH was carried out on mitotic metaphase plates of *Ae. umbellulata*, *Ae. comosa*, *Ae. speltoides* and *Ae.markgrafii*using probes for the two microsatellites and probesfor tandem repeats pSc119.2, Afa family and 18S rDNA (Fig. 1).The karyotypesobtained are shown in Fig. 2 and detailed in Table S1.Only minor differences in fluorescent labelling patterns were observed between this workand the results obtained by Badaeva et al. (1996ab) (Table S1), and we could identify allchromosomes in the diploid *Aegilops* species.The labelling efficiency (i.e. the number and intensity of hybridization signals) of the microsatellite probes(Table S2), showed significant intragenomic differences amongthe four *Aegilops* species.No differences in FISH labelling patterns were observed between thetwo accessions of*Ae. markgrafii*(MvGB428 and MvGB607).

Flow sorting of mitotic chromosomes after FISHIS

When suspensions of mitotic chromosomes from diploid*Ae. umbellulata, Ae. comosa, Ae. speltoides and Ae. markgrafii*were analyzed for the distribution of DAPI fluorescence intensity(flow karyotypes), narrow peaks were obtained, giving better chromosome resolution as compared to our previous work (Molnár

et al., 2011b, 2014, 2015). This was probably due to the fact that we used a BD FACSAria II SORP flow sorter in this study,which employs a gel-coupled flow cell instead of the classic jet-in-air system of BD FACSVantage flow sorter. The former system is more efficient in collecting fluorescence light pulses and provides better stability of the fluid stream.

Monovariate flow karyotype of *Ae. umbellulata* consisted ofpeaks I - III representing chromosomes 1U, 6U and 3U, respectively, and one composite peak IV containingthe chromosomes 2U, 4U, 5U and 7U (Fig. 3a). The bivariate flow karyotype obtained after FISHIS with a probe for GAA motif consisted of seven clearly separated populations corresponding to the seven chromosomes of *Ae. umbellulata*(Fig. 3b). The chromosomes were assigned to the chromosome populations by FISH withprobes for pSc119.2, Afa family and 18S rDNA on chromosomes flow-sorted onto microscope slides (Table S3). Better resolution of chromosome populations after bivariate flow karyotyping resulted in high purity (88-98%) of sorted chromosome fractions (Table 1).

Bivariate flow karyotyping in *Ae. comosa*after FISHIS with a probe for GAA(Fig.4a) revealed three chromosome populations (IV, VI and VII) representing chromosomes 6M, 3M and 7M, respectively (Fig. S1). The three chromosomes could be sorted with apurity of 96.7%, 94.2% and 93.3%, respectively. On the other hand, populations of 1M and 4M, and 2M and 5M overlapped, resulting in lower purities (1M: 44.8%, 4M: 53.8%, 5M: 86.5%, 2M: 62.6%). To improve chromosome discrimination, double FISHIS was employed

with probes for GAA and ACG (Fig. 4b). This resulted in better separation of the chromosome populations and allowed chromosomes 1M, 2M, 4M and 5M to be sorted at purities of 79.6%, 73.6%, 78.4% and 90.2%, respectively (Fig. S1, Table S3). Importantly, the purity of the sorted 3M, 6M and 7M fractions also improved (Table 1).

As the combined use of GAA and ACG microsatellite repeats for FISHIS had a positive effect on bivariate flow karyotyping in *Ae. comosa*, the same approach was used in *Ae. speltoides* and *Ae. markgrafii*. Differences in the abundance of GAA and ACG motifs between chromosomes were largeenough to allow separation of all S- and C-genome chromosomes (Fig. 5). FISH analysis on flow-sorted chromosomes of *Ae. speltoides* showed that the populations of chromosomes 1S, 3S and 5S, on which GAA and ACG repeats are less abundant (Fig. 2), were allocated in regions III, V and IV of the bivariate flow karyotype, characterized by lower FITC fluorescence intensity (Fig. 5a; Fig. S2; Table S3). On the other hand, chromosome 4S, which has strong and complex GAA and ACG hybridization patterns, was assigned to the population with the highest level of FITC fluorescence (Fig. 5a; region I).

Two accessions of *Ae. markgrafii* (MvGB428 and MvGB607) were used to secure enough seed to allowreplications of the experiments. FISH on flow-sorted chromosome fractions showed that chromosomes 4C, 6C and 7C,which hadcomplex, strong microsatellite hybridization patterns (Fig. 2), were represented by populations VII, III and I, respectively, on bivariate flow

karyotype(Fig. 5b), while chromosomes 1C, 2C, 3C and 5C, which had lower GAA and ACG content, were assigned to populations with lower FITC fluorescence intensity (Fig. 5b; Fig. S3). With the exception of chromosomes 2S and 7C, which could be sorted at purities of 84.4% and 80.9%, respectively, bivariate flow cytometry after FISHIS with probes for GAA and ACG permitted complete sets of chromosomes from *Ae. speltoides* and *Ae. markgrafii*to be sorted at purities exceeding 93% and 90%, respectively (Table 1, Table S3).

Sorting chromosome arms after FISHIS

Stimulated by the positive results, we checked the utility of bivariate flowcytometry to purify chromosome arms of *Aegilops* from wheat-*Ae. umbellulata* ditelosomic addition lines. Chromosome suspensions of wheat (*T. aestivum* cv. Chinese Spring)-*Ae. umbellulata* double ditelosomic addition lines CSDtA2US (Figure 6a), CSDtA2UL (Figure 6b) and CSDtA7UL (Figure 6c) were labelled by FISHIS with a probe for GAA. Chromosome arms 2US, 2UL and 7UL of *Ae. umbellulata*could be easily discriminated from wheat chromosomes on bivariate flow karyotypes (Figure 6a-c; Fig. S4), allowing these arms to be sortedat high purities ranging from 88 to 94%.

Assignment of COS markers to U, M, S and C chromosomes

COS markers designed from wheat ESTs for which chromosome deletion bin map positions are known were assigned to *Aegilops* U-, M-, S- and C-genome chromosomes using PCR, with DNA amplified from flow-sorted chromosomes as a template (Table S4). Of the 123 COS markers, 100 amplified PCR

products from genomic DNA of at least one of the four *Aegilops* species (Supplementary Data S1). The 100 markers resulted in a total of 544PCR products in the four *Aegilops* species (137, 131, 127 and 142 amplicons in *Ae. umbellulata*, *Ae. comosa*, *Ae. speltoides* and in the two accessions of *Ae. markgrafii*, respectively).

Because each of the *Aegilops* chromosomes has a major location in one of the populations on bivariate flowkaryotype (Table 1; Table S3), the highest amount of PCR product obtained with a COS marker identified the population with the locus-carrying chromosome (Supplementary Data S1; Table S3). However, if the amounts of PCR product were similar in two different chromosome populations, it was not possible to discriminate between the intragenomic duplication anda false positive chromosomal assignment. Thus, COS markers which gave differences of less than 10% between the PCR product amountsof two different chromosome populations were excluded from further analysis. In total,466PCR products (225polymorphic and 241non-polymorphic with respectto wheat) were assigned to *Aegilops* chromosomes (Supplementary Data S1).

Out of 118 loci assigned to U-genome chromosomes of diploid *Ae. umbellulata*(Table S5), 63 loci (53.38%) were polymorphic relative to wheat cv. GK Öthalom. In*Ae. comosa*, where 114loci were mapped to M-genome chromosomes, 53loci (46.49%) were polymorphic. Of the 120loci assigned to S-genome chromosomes of *Ae. speltoides*,56loci (46.66%) showed size

polymorphism. Finally, 53(46.49%) of the 114 loci mappedto C-genome chromosomes of *Ae. markgrafii*were polymorphic. Chromosome-specific COS markers with significant (≥5bp) length polymorphism between wheat cv. GK Öthalom and *Aegilops*species (Table 2) will be suitable formarker-assisted selection of wheat-*Aegilops* introgression lines.

Wheat-*Aegilops* homology at chromosome level

Using the genetic map data and the deletion bin positions of the source ESTs (Supplementary Data S2), the 100 COS markers assigned to *Aegilops* chromosomes were physically mapped on wheat B, A and D genomes (Figure 7, Figure S5-6). This providedan overviewof the genome relationships between wheat and *Aegilops* species (Figure 7, Figure S5-6).

The coverage of wheat B-genome chromosomes 3B, 5B, 6B and 7B with COS markers (16, 15, 15 and 20 markers / chromosome, respectively) was better as compared to the remaining chromosomes (1B, 2B and 4B with 12, 11 and 10 markers, respectively). Similar results were obtained for the A-genome chromosomes and to some extent for the D-genome chromosomes, where 17, 15 and 20 markers were specific for chromosomes 3D, 6D and 7D, respectively (Figure S5 and S6). Based on the presence or absence of COS markers on the same homoeologous group chromosomes in wheat and *Aegilops*, genetic relationships were quantified usingthe Jaccard similarity coefficients (Table S6) (Kosman and Leonard 2005).

At the whole genome level, the structures of the S-genome chromosomes of *Ae. speltoides*and the M genome of *Ae. comosa*were the most similar to wheat, followed by the U genome of *Ae. umbellulata*, while the structure of the C genome in *Ae. markgrafii* differed considerably. At chromosome level, the group 1 and group 5 chromosomes of *Aegilops* species generally showed greatermacrosynteny with wheat than the remaining chromosome groups (Table S6).

The chromosomal locations of orthologous genes revealed structural relationships between the U-genome chromosomes of *Ae. umbellulata* and the A, B and D genomes of wheat. For example, COS marker *c746642,* specific for wheat (W) chromosome group 2 (W2), was located on chromosome 6U, COS marker *c755442* specific for W3 was located on 7U, four markers indicated homology between the short arms of W4 and 6U, while twomarkers indicatethat intercalary part of the long arm of W6 is related to 4U. Another part of the W6 long arm, represented by fivemarkers,was found to behomologous to 2U (Figure 7, Figure S5-6).

Chromosomes of *Ae. comosa*exhibited greatersyntenywith wheat than those of*Ae. umbellulata*. However, some rearrangements were observed relative to wheat. One COS marker indicated presence of a W5 fragment on 2M and four markers suggesteda homology between W7 and 3M (Figure 7, Figure S5-6). As expected, the S genome of *Ae. speltoides* was closely related to wheat. However, two COS markers indicated genome rearrangements between W2

and 3S, whiletwo markers specific for W4 were found on 6S. Homology between the long arm of W6 and 4S was indicated by three markers and between W6 and 3S by two markers (Figure 7, Figure S5-6).

In *Ae. markgrafii*, chromosomes 1C and 5C exhibited the greatest synteny withwheat homoeologous groups, although three markers indicated the presence of a W5-specific region on chromosome 2C. It seems that the long arms of 2B and 3B, and the short arm of 4B are related to 7C. Five markers located on the long arm of 4B and four markers specific for different parts of 2B were detected on chromosome 4C, indicating their homology. Twelve markers specific for 6B were located on chromosome 2C, while elevenmarkers indicated homology between 7B and 3C.

Table 3 provides a complete list ofconserved genomic regions between hexaploid wheat genomes and chromosomes from theU, M, S and C genomes of diploid *Aegilops* species as identified in the present work.

DISCUSSION (2459words)

The exploitation of *Aegilops* species for wheat improvement has been the subject of research for more than a century. Yet,with a few exceptions, thelarge genetic diversity of *Aegilops*remainsuntapped(Schneider et al., 2008; Kilian et al., 2011). The present work aims to contribute to the efforts to change this by developing approaches to simplify the analysis of *Aegilops* genomes, describing

relationships between (sub)genomes of bread wheat and genomes of four *Aegilops* species, and developing markers to facilitate exploitation of important traits in wheat breeding programs.

We demonstrate that it is possible to dissect the large U, M, S and C genomes of *Aegilops* into individual chromosomes representing 12.0% - 15.8% of the whole genome. This should facilitate the analysis and mapping these complex genomes whose 1C values exceed 4Gbp (U: ~4,938 Mbp, M: ~6,044 Mbp, S: ~5,036 Mbp, C: ~4,528 Mbp), and which comprisehigh proportion of repetitive DNA (57% and 61% for*Ae. speltoides* and *Ae. tauschii,* respectively) (Kilian et al., 2011;Shangguan et al., 2013). Slicing the genomes into single chromosomes provides a powerful approach to perform structural and functional genome analysis(Doležel et al., 2014;Rey et al., 2015).

Chromosome samples are traditionallystained by DAPI and classified according to their relative DNA content using flow cytometry. Only chromosomes whose DAPI fluorescence intensity differsfrom other chromosomes in a karyotype can be discriminated and purified (Doležel et al., 1992). As many species have chromosomes of similar size, individual chromosomescannot be easily discriminated based on DAPI staining alone. Thus, only group 5 chromosomes could be sorted from *Ae. tauschii* and *Ae. speltoides*(Molnár et al., 2014), chromosome 4C from *Ae. markgrafii* (Molnár et al., 2015) and chromosomes 1U, 3U and 6U from *Ae. umbelulata* (Molnár et al., 2011b).

To overcome the difficultyto sort particular chromosomes, Vrána et al. (2015) suggested dissectingcomposite chromosome peaks representing several chromosomes into smaller sections enriched for the chromosomes of interest,whileCápal et al. (2015) developed a protocol for sequencing single flow-sorted chromosomes. While useful forcertain applications, these approaches do not allow particular chromosomes to be sortedat high purity and/orin large numbers. On the other hand, labellingspecific DNA sequences by FISH should facilitate discrimination of otherwise indistinguishable chromosomesand their sorting in large numbers (Lucretti et al., 2014).The present results show that the distribution of GAA and ACG hybridization signalsdiffers within the U, M, S and C genomes.Theseresults are on line with previous observations that microsatellite trinucleotide repeats (GAA, AAC, ACG) provide diagnostic landmarks to identify chromosomesin cereals such as wheat, barley and rye (Kubaláková et al., 2005; Cuadrado et al., 2008)and in *Aegilops*species withthe U and M genomes (Molnár et al., 2011a). The GAA and ACG karyotypes obtainedin the present study show that the microsatellitesprovide useful chromosomal landmarks also in*Ae. speltoides* and *Ae. markgrafii*.

Motivated by the results of FISH on mitotic metaphase chromosomes,we used FISHIS (Giorgi et al., 2013) tolabel the microsatellite repeats on chromosomes in suspension to improve chromosome discrimination and facilitate chromosome sorting in *Ae. umbellulata*, *Ae. comosa*, *Ae. speltoides* and *Ae. markgrafii.*Relative positions of populations representing individual

chromosomes on bivariate flow karyotypes DAPI vs. microsatellite-FITC agreed well with the number and intensity of GAA or ACG bands observedon mitotic metaphases.

In *Ae. comosa*, *Ae. speltoides* and *Ae. markgrafii*, FISHIS with the GAA probe alone did not discriminate the complete chromosome complements. This was achieved by dual FISHIS with probes for GAA and ACG, which increased the FITC signal diversity and improved discrimination of individual chromosomes. These results indicatethat FISHIS with an appropriate mix of probes for microsatellite repeatsmay improvediscrimination of individual chromosomes, even if the probes arelabeled with the same fluorochrome. This approach could increase the potential of chromosome genomicsin Triticeae and perhaps also in other species.

Contamination of sorted chromosome fractions by other chromosomes orchromosome fragments is common in flow cytometric chromosome sorting (Lysák et al., 1999;Vitulo et al., 2011;Doležel et al., 2012). The presentresults demonstrated that bivariate flow karyotyping after FISHIS not only increased the number of *Aegilops* chromosomes that could be discriminated and sorted, but also increased the purity inflow-sorted fractions. This is in line with the observations of Giorgi et al. (2013).

The range of applications of flow-sorted chromosomes keeps expanding (Doležel et al., 2012), and includesphysical mapping using FISH (Valárik et al.,

2004), construction of large-insert DNA libraries (Šafář et al., 2004), optical mapping (Staňková et al., 2015), development of DNA markers (Bartoš et al., 2008), and physical mapping on DNA arrays (Mayer et al., 2011). Shot-gun NGS represents a particularly important application of flow-sorted chromosomes and has been the foundation of many international genome sequencing projects, including barley, rye and bread wheat (Mayer et al., 2011; Martis et al.,2013; IWGSC 2014).

The ability to purify chromosomes from the U, M, S and C genomes of *Aegilops* and production of microgram DNA amounts from them opens avenues for the application of chromosome genomics in *Aegilops* to support alien introgression breeding. For example, Tiwari et al. (2014) flow-sorted short arm of chromosome $5M^g$ from a wheat-*Ae. geniculata* ditelosomic addition line and sequenced it by Illumina technology. Out of the 2,178 $5M^gS$-specific SNPs identified, forty-four were validated by KASP assay and used to identify $5M^gS$-specific chromosome segments in released wheat germplasm lines. These results highlighted the importance of DNA samples derived from wild wheat relatives and their suitability forNGS and development of high-throughput genotyping assays to identify alien introgressions.

Alien gene transfer induced by homoeologous recombination (Riley and Chapman 1958; Sears 1977) depends on chromosome collinearity and may be hampered by irregularities inmeiotic pairing of alien chromosomes with their wheat homoeologues due to structural rearrangements (Ceoloni et al.,

1988;Devos et al., 1993; Cuadrado et al., 1997; Lukaszewski et al., 2004). The lack of knowledge on the evolutionary relationshipsbetweenwheat and *Aegilops* hampers alien gene transfer, for example due to non-compensating translocations, (Friebe et al., 1996;Ceoloni and Jauhar 2006). The knowledge of wheat-*Aegilops* macrosyntenic relationships is also important to supporttargeted development of molecular markers specific for *Aegilops* chromosome regions potentially responsible for agronomic traits of interest (Burt and Nicholson 2011) and to minimize the amount of undesirable alien chromatin.

Wheat-*Ae. umbellulata* macrosynteny was investigated using RFLP-based genetic map of *Ae. umbellulata* (Zhang et al., 1998; Devos and Gale 2000) and at least eleven rearrangements were found that differentiated U-genome chromosomes from the D genome of wheat. Later, Molnár et al. (2013) used wheat-specific COS markers on wheat-*Aegilops* addition lines and flow-sorted chromosomes to describe relationships between wheat genome and the U and M genomes of diploid and polyploid *Aegilops*. The present work extends the comparative analysis of wheat and *Aegilops*to the S and C genomes of *Ae. speltoides* and *Ae. markgrafii*. We used complete sets of chromosome-derived DNA samples to assign COS markers to *Aegilops* chromosomes and compare the structure of the *Aegilops*U, M, S and C genomes with the A, B and D genomes of hexaploid wheat. Polymorphic markers assigned to U-, M-, S- or C-genome chromosomes will be useful to support the transfer of alien chromosomes or chromosome arms into wheat.

The U genome-wheat homoeologouos relationships observedin this work were similar to those reported by Zhang et al. (1998) and Gale and Devos (1998). We found that 1U was related mainly to W1 which was also true for *Aegilops*group 1 chromosomes 1M, 1S and 1C. Danilova et al. (2014) used FISH to map full-length cDNA clones to wheat chromosomes. With 2-6 probes per chromosome arm, the authors observed close relationship between chromosomes 1U, 1C and W1. According to Zhang et al. (1998), the distal part of the long arm ofW1 (represented by 3 RFLP markers) was related to chromosome 6U. In our work, relatively large distal bins on the long arm of W1 were represented by 1, 0 and 3 COS markers in 1A, 1B and 1D, respectively. Presumably these COS markers were located more proximally on the long arm of W1 than the RFLP markers used by Zhang et al (1998) and thus failed to detect the 6U-specific region.

In the present work, all group 2 COS markers were located on 2U,except for marker *c746642*in the terminal bin of W2L, which was located on 6U in agreement with Zhang et al. (1998). According to Gale and Devos (1998), W3 was homoeologous to 3U (represented by 8 RFLP markers) and 7U (based on two RFLP markers). We also detected most of the W3 markers (10 COS markers) on 3U. However, one marker specific for the terminal part of the short arm of W3 was located on 7U. According to Zhang et al. (1998) and Gale and Devos 1998), the short arm of W4 was related to 6U, whilethe long arm to 4U and 5U. In this work, COSmarkers specific for the short arm of W4 werealso located on6U, while those specific for the intercalary bin of thelong arm were

assigned to 4U.However, in contrast toZhang et al. (1998),we did not detect anyW4 COS markers on chromosome 5U.

We detectedCOS markers from W5on 5U, but unlike Gale and Devos (1998),we did not observehomoeology with 4U asthe most distal part of the long armof W5 was not represented by COS markers. W5 was also found to be closely related to chromosome 5M of *Ae. comosa*,while one marker suggesteda relationship with 2M. A homoeology between W5 and $5M^g$ of *Ae. geniculata*was also observed by Tiwari et al. (2015) who showed that approximately 72% of the annotated $5M^g$ genes had sequence identity to wheat genes on chromosomes 5A, 5B and 5D. Chromosomes 5S and 5C were also found to be homoeologous with W5 in the present work, whilethree markers on the long arm of W5, were detected on 2C.

Homoeologous chromosome group 6, andchromosomes 6A and 6D in particular,have segmental homoeology to the short arm of *Ae. umbellulata* chromosome 6U,and long arms of 4U and 6U (Zhang et al. 1998; Gale and Devos 1998).In general, the present work confirmed the previous observations (three W6 COS markers were detected on each of 6U and 4U), but unlike the earlier results, five W6 markers suggested a relationship with 2U. Mapping the group 6 COS markers revealedsignificant homoeology ofW6 chromosomesto chromosome 6M of *Ae. comosa*,and less pronounced homoeology tochromosome 6Sof *Ae. speltoides*. On the other hand, W6 was related to 2C in *Ae. markgrafii.*

Gale and Devos (1998) noted that the short arm and a significant part of the long arm of W7 was homoeologous to 7U,the distal part of W7 long arm was related to 6U,while the terminal part was homoeologous to chromosome 4U of *Ae. umbellulata*. On line with these observationswe detected three of the five W7 short arm markers, and nine of the thirteenW7 long arm markers on 7U,whilethreemarkers from the distal bins of W7 long arm were found on 6U. For the group 7 chromosomes, the wheat-*Aegilops* macrosynteny was highest in *Ae. speltoides*, and lower in *Ae. comosa,*while no synteny was found between W7 and the chromosome7Cof *Ae. markgrafii*.

We have detectedpreviously unknown wheat-*Ae. umbellulata* genome relationships. For example, COS marker *c755444* specific for the proximal bin of the W3 long arm was assigned to 6U and W6 marker *c750237*was assigned to 5U. We detected such local breaks in the wheat-*Aegilops* genome relationshipsalso in *Ae. comosa*, *Ae. speltoides* and *Ae. markgrafii.* These resultsare consistentwith the observationsof Dobrovolskaya et al. (2011) who observed local synteny perturbations between *Ae. speltoides* and wheat. However, 76 out of 90 markers mapped in *Ae. speltoides*were assigned to chromosomes homoeologous with wheat, confirmingthat the species is highly syntenic with wheat (IWGSC 2014).

According to Jaccard similarity coefficients estimated in this work,the S genome of *Ae. speltoides*and the M genome of *Ae. comosa*are structurally similar to the wheatgenomes, while the U genome of *Ae. umbellulata* and the C genome of

*Ae. markgrafii*in particular,are significantly different. These results are on line with previous phylogenetic studiesin which*Ae. umbellulata* and *Ae. markgrafii* formed a closer sub-cluster on the *Aegilops-Triticum* clade,indicating greatergenetic similarity, relative to *Ae. comosa* and *Ae. speltoides* (Petersen et al., 2006; Mahelka et al., 2011).

Evolutionary genome rearrangements in *Ae. markgrafii* relative to wheat as describedin the present studyindicate a need to rename fourC-genome chromosomes. As twelveout of nineteenW6 COS markers identified homology betweenchromosomes 2C andW6($J_{W6,2C}$: 0.800), we suggest renaming chromosome 2C to 6C. Elevenout of fifteen markers indicated homology between3C and W7($J_{W7,3C}$: 0.611),and thus we suggest renaming 3C to 7C. Similarly, five markers mapped to chromosome 7C were specific to W2($J_{W2,7C}$: 0.454), and five to W4, so chromosome 7C could be renamed 2C. Finally, out of three markers identified on chromosome 6C, two were related to W7 and one to W3 indicating a need to rename 6C to 7C or 3C.However, we note that the low number of markers per chromosome allowed only macro-level comparisons and a more detailed comparative analysis is needed before changing the chromosome nomenclature of *Ae. markgrafii*.Sequencing DNA from flow-sorted U, M, S andC genome chromosomes and comparison of their gene content with thatof wheat chromosomes (IWGSC 2014) couldprovide detailed information about the synteny between*Aegilops* genomes and wheat.

This workrepresents an important step forward in developing chromosome genomics for wild relatives of wheat. FISH karyotypes will facilitateidentification of*Aegilops* chromatin transferred to wheat. Bivariate flow karyotyping after FISHIS makes it possible to dissect the genomes of four important gene sources for cultivated wheat, *Ae. umbellulata*, *Ae. comosa*, *Ae. speltoides* and *Ae. markgrafii*into single chromosomes. This provides an opportunityfor detailedcharacterization of theirgenomes, including gene content, allele discovery and targeted development of gene-based markers from specific genomic regions. The knowledge of homoeologous relationships between wheat and *Aegilops* species atchromosome-level will be an important guide fortargeted development of markers and for planning introgression breeding programs. COS markers assigned to chromosomes of the *Aegilops* species will be useful in pre-breeding programs to select chromosome segments carrying agronomically useful genes in *T. aestivum – Aegilops* recombinant lines. Altogether, these results promise to accelerate genomic studies onwild relatives of bread wheat and support pre-breeding studies that arerequired to meet the future challenges of food security and sustainable agriculture.

EXPERIMENTAL PROCEDURES (1250words)

Plant material

Seeds of *Aegilops umbellulata*Zhuk. accessionAE740/03 (2n=2x=14; UU) were kindly provided by the Institute of Plant Genetics and Crop Plant Research

(Gatersleben, Germany). The accessions of *Ae. comosa*Sm. in Sibth. & Sm.MvGB1039 (2n=2x=14, MM), *Ae. speltoides*Tausch.MvGB905 (2n=2x=14, SS) and *Ae. markgrafii*(Greuter) Hammer MvGB428 and MvGB607 (2n=2x=14, CC) are maintained atthe Martonvásár Cereal Genebank (Hungary). Wheat (*Triticum aestivum*L.) cv. Chinese Spring-*Ae. umbellulata* ditelosomic addition lines 2US, 2UL and 7UL (Friebe et al., 1995) were kindly provided by Dr. Bernd Friebe(Wheat Genetics Resource Center, Kansas State University, USA).Accessions of *Secale cereale*L. cv. *'Petkus',Ae.tauschii*Coss. MvGB605, *Oryza sativa* L. cv. 'Bioryza' and *T. aestivum* L. cv. 'GK Öthalom' were also used in the present study and were obtained from the Cereal Research Non-Profit Company, Szeged, Hungary

Flow cytometric chromosome analysis and sorting

Suspensions of intact mitotic metaphase chromosomes were prepared from synchronized root tips of young seedlings following Vrána et al. (2000) and Kubaláková et al. (2005). The chromosome samples were fluorescently labelled by FISHIS using oligonucleotides 5'-FITC-GAA_7-FITC-3' and/or 5'-FITC-ACG_7-FITC-3' (Sigma) and counterstained by DAPI (4´,6-diamidino 2-phenylindole) as described by Giorgi et al. (2013). Bivariate flow karyotyping and chromosome sorting weredone on a FACSAria II SORP flow cytometer and sorter (Becton Dickinson Immunocytometry Systems, San José, USA). Chromosome samples were analyzed at rates of 1500–2000 particles per second, and bivariate flow karyotypes FITC vs. DAPI fluorescence were acquired. Sort windows were set on dotplotsFITC vs. DAPI, and chromosomes were sorted at rates of 15-20 /

sec. Flow-sorted chromosomes were identified and the purity in sorted chromosome fractions was determined according to Molnár et al. (2011b). Briefly, approximately one thousand chromosomes were sorted from each chromosome populationidentified on bivariateflow karyotype into a 15 µl drop of PRINS buffer supplemented with 5% (w/v) sucrose on a microscope slide (Kubaláková et al. 1997). The slides were air-dried and used for FISH with probes for pSc119.2, pTa71 and Af*a* family repetitive DNA sequences.

Amplification of chromosomal DNA

Three batches of 30,000 chromosomes each were sorted from each chromosome population identified onbivariate flow karyotypes. The chromosomes were treated with proteinase K, after which their DNA was purified and 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). Three independent MDA products from each sorted chromosome fraction were pooled into one sample to reduce amplification bias (Table S1) and used as template for PCR reaction with primers for COS markers.

Fluorescence *in situ* hybridization (FISH)

pSc119.2 and Afa-family repeats were amplified from genomic DNA of *S. cereale* and *Ae. tauschii*and labelled with biotin-16-dUTP (Roche, Mannheim, Germany) and digoxigenin-11-dUTP (Roche), respectively, using PCR (Nagaki et al., 1995;Contento et al., 2005).18S unit of 45S ribosomal RNA gene was

amplified using PCR from genomic DNA of rice (Chang et al., 2010) and labelled with 50% biotin-16-dUTP and 50% digoxigenin-11-dUTP. GAA and ACG microsatellites were amplified from genomic DNA of *T. aestivum* and labelled with digoxigenin-11-dUTP (Roche) and biotin-16-dUTP (Roche), respectively, using PCR. Digoxigenin and biotin were detected using anti-digoxigenin-rhodamine Fab fragments (Roche) and streptavidin-FITC (Roche), respectively.

FISH was performedon chromosomes flow-sorted onto microscopic slides and on slides prepared by squashing meristem root tips (Molnár et al., 2011a). The pretreatment and stringent washing steps were omitted in experiments onflow-sorted chromosomes. Chromosome preparations were examined under a Zeiss AxioImager M2 fluorescence microscope system equipped with an AxioCam MRm CCD camera (Zeiss, Oberkochen, Germany), and the images were compiled with AxioVision v4.8 software (Zeiss) as described by Mikó et al. (2015). After capturing FISH signals on metaphase plates, the slides were washed and re-hybridized with GAA and ACG microsatellite probes at 42°C using the protocoldescribed above.

COS marker analysis

Genomic DNA was prepared according to Cseh et al. (2013) from *Ae. umbellulata*, *Ae. comosa*, *Ae. speltoides* and *Ae. markgrafii* MvGB428 and MvGB607, which were also used for flow cytometric analyses, and from wheat cv. ‘GK Öthalom’. PCRwith primers for 123 COS markers (Quraishi et al., 2009;

Supplementary Data S1) specific for wheat homoeologous groups I - VII, was performed in12µL reaction volumes as described by Molnár et al. (2014) using a touchdown reaction profile: 94°C (2 min);10 cycles of 94^{o}C (0.5 min), Ta+5^{o}C (0.5 min) decreased in 0.5^{o}C increments for every subsequent set of cycles, 72^{o}C (1 min);30 cycles of 94^{o}C (0.5 min), Ta^{o}C (0.5 min), 72^{o}C (1 min);hold at 72^{o}C (2 min). PCR products were separated usinga Fragment Analyzer Automated CE System equipped with a 96-Capillary Array Cartridge (effective length 33 cm) (Advanced Analytical Technologies, Ames, USA) and analyzed withPROsize v2.0 software.The annealing temperature (Ta) for each COS marker, together with data on the PCR amplicons, are included in Supplementary Data S1.

DNA sequence analysis

A deletion bin map was constructed for each wheat chromosome showing positions of the COS markers (Quraishi et al., 2009). To order the markers along the chromosomes, EST sequences of the COS markers (Quraishi et al., 2009, Supplementary Data S2) were used as queries in BLASTn searches to identify the scaffold containing the EST in the assembled chromosome survey sequences of hexaploid wheat (https://urgi.versailles.inra.fr/blast/blast.php; IWGSC 2014) Throughout the study, BLAST hits with E-values smaller than 2.8e^{-08}, identity % > 58.44 and alignment length > 100bp were considered significant (Supplementary Data S2). The relative order and genetic distance (in cM) of the EST-specific scaffolds were obtained by searching the scaffold IDs in the GenomeZipper (v.5) of wheat chromosome arms

(https://urgi.versailles.inra.fr/download/iwgsc/zipper/; IWGSC 2014) (Supplementary Data S2).

Visualization of wheat–*Aegilops* orthologous relationships

In order to visualize wheat-*Aegilops* homoeologous relationships,a genetic map and physical deletion bin map of wheat were constructed showing positions of the mapped COS markers. Separate maps were drawn for the B, A, and D genomes of wheat (Figs. 7, S5, S6). The deletion bins were divided into as many parts as the number of COS markers located in the bins. The marker-specific bin parts were color-coded to show the homoeologous group location of the markers. For each homoeologous group (1-7), five wheat chromosome bin maps were displayed, one for awheat genome (B or A and D) and one each for the *Aegilops* genomes U, M, S and C. This allowed to visualize the homoeologous group positions of the relevant wheat chromosome segments in the genomes of wheat and *Aegilops.*Moreover, a table was assembledshowing the number of wheat homoeologousgroup-specific COS markers located on each of the*Aegilops* chromosome (Table 3). This highlighted wheat genomic regions related to a given chromosome in *Aegilops*.

Calculation of Jaccard similarity coefficients

Pairwise similarity between the structure of chromosomes within the same homoeologous groups of wheat and *Aegilops* species was determined using Jaccard's coefficient $J_{(i1,i2)}$ =a/(a+b+c) (Kosman and Leonard 2005). For a given

homoeologous group A, a = the number of markers present on group A chromosomes for both wheat and a corresponding *Aegilops* species; b = the number of markers where species i_1 (i.e. wheat) has a band on the group A chromosome, but i_2 (i.e.*Aegilops*) does not; c = the number of markers where the *Aegilops* species i_2 has a band on the group A chromosome, but i_1 (wheat) does not. Jaccard's coefficients were calculated for each homoeologous group I - VII between wheat and each *Aegilops* species, and the similarity values are givenin Table S6.

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The authors declare that there is no conflict of interest.

SUPPORTING INFORMATION (213words)

Figure S1. Identification of chromosomes flow-sorted from*Ae. comosa* using FISH.

Figure S2. Identification of chromosomes flow-sorted from *Ae. speltoides*using FISH.

Figure S3. Identification of chromosomes flow-sorted from *Ae. markgrafii* using FISH.

Figure S4. Identification of chromosome arms 2US, 2UL and 7UL flow-sorted from wheat-*Ae. umbellulata* ditelosomic addition lines using FISH.

Figure S5. Wheat–*Aegilops* orthologous relationships from the genomic perspective of A-genome chromosomes.

Figure S6. Wheat–*Aegilops* orthologous relationships from the genomic perspective of D-genome chromosomes.

Table S1. Karyotypic description of *Aegilops* chromosomes with probes pSc119.2, Afa family and 18S rDNA.

Table S2. Labelling efficiency of GAA and ACG repeats for*in situ* hybridisation on the chromosomes of *Aegilops*

Table S3. Chromosome assignment to populations on bivariate flow karyotypes of *Aegilops umbellulata*, *Ae. comosa*, *Ae. speltoides* and *Ae. markgrafii*.

Table S4. DNA yields after the multiple displacement amplification of DNA fromflow-sorted chromosome fractions.

Table S5. The number of COS marker loci assigned to U, M, S and C genome-chromosomes of *Aegilops* species.

Table S6. Jaccard similarity coefficients (J) calculated between the same homoeologous group chromosomes in wheat and *Aegilops* species.

Data S1. PCR products of COS markers amplified from wheat and *Aegilops* species.

Data S2.BLASTn search results and Genome Zipper data used for ordering COS markers on wheat chromosomes.

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Tables

Table 1: The effect offlow cytometric chromosome analysismethod on the number of discriminated and sorted chromosomes and purity inflow-sorted chromosome fractions.

Method	*Ae. umbellulata*			*Ae. comosa*			*Ae. speltoides*			*Ae. markgrafii*		
	Chr	% of the genome	Purity (%)	Chr.	% of the genome	Purity (%)	Chr.	% of the genome	Purity (%)	Chr.	% of the genome	Purity (%)
Monoparametric (DAPI)	1U	12.9	98.9[#]	-[#]			5S	13.8	89.8[##]	4C	12.4	91.3[###]
	3U	13.3	86.4[#]	-			-			-		
	6U	13.4	74.1[#]	-			-			-		
Biparametric (DAPI + FITC)	1U	12.9	98.9	1M	14.7	79.6	1S	13.8	98.8	1C	13.1	91.8
	2U	14.3	88.7	2M	13.1	73.6	2S	15.2	84.4	2C	15.8	94.4
	3U	13.3	96.4	3M	15.6	96.7	3S	15.5	95.7	3C	15.1	89.6
	4U	15.5	90.1	4M	12.6	78.0	4S	13.1	93.0	4C	12.4	97.9
	5U	15.1	93.2	5M	14.2	90.2	5S	13.8	99.2	5C	15.5	90.7
	6U	13.4	94.2	6M	13.6	99.6	6S	13.4	97.1	6C	12.0	91.9
	7U	15.2	98.0	7M	15.8	98.4	7S	14.9	99.0	7C	15.7	80.1

[#], [##], [###]: data from Molnár et al. 2011b, 2014 and 2015, respectively.

Table 2. COS markers showing polymorphic (≥5bp) PCR amplicons between wheat and *Aegilops* species, which are considered suitable for identification of introgressions of the U-, M-, S- and C-genome chromosomes from *Ae. umbellulata,Ae. comosa, Ae. speltoides* and *Ae. markgrafii* into hexaploid wheat. The size (in bp) of the chromosome-specific loci is shown in brackets.

Homoeologous group in *Aegilops*	*Ae. umbellulata* (UU)	*Ae. comosa* (MM)	*Ae. speltoides* (SS)	*Ae. markgrafii* (CC)
1	*c757212 (244), c735941 (238), c743018 (298, 310), c726029 (418), c743346 (275), c737520 (327), c744747 (320), c758392 (379, 390),*	*c757212 (285), c735941 (238), c743346 (277), c737520 (327), c744747 (317),*	*c757212 (280), c735941 (227, 239), c743018 (305, 317), c743346 (278), c737520 (330), c744747 (317),*	*c757212 (285), c735941 (237), c743018 (298, 310), c743346 (274), c737520 (327), c744747 (320), c751053 (498), c765452 (357),*
2	*c740970 (207), c757237 (190, 194), c767104 (443), c741435 (201), c760549 (430), c742110 (194, 198), c742079 (374),*	*c740970 (207), c757237 (230, 233), c762599 (267, 269),*	*c720763 (323, 326),*	*c756721 (307), c765220 (298, 302, 310), c744766 (239), c747871 (655), c724406 (628), c741435 (588), c760549 (428), c753637 (442), be496986 (629), c771657 (888), c748987 (260), c754211 (288, 291),*
3	*c752137 (399, 410), c805553 (442, 451), c772427 (371), c757460 (633), c756279 (308), c755305 (263)*	*c805553 (450), c772427 (371), c751053 (502), c752685 (597), c771860 (374), c740781 (413), c756279 (285), c761505 (1374), c750237 (517), c732202 (232), c740257 (280), c748987 (260),*	*c757237 (228), c746642 (654), c805553 (450), c751053 (595), c739776 (323), c741435 (468),*	*c767104 (422), c805553 (442, 451), 760830 (300, 305), bf484254 (556), c747342 (655), c745166 (243), c740257 (280),*
4	*c759427 (557, 552), c765452 (310, 322), c724406 (633), be496986 (716),*	*c743018 (298, 310), c733078 (458), c765452 (310, 322), c760004 (697), bf484254 (536)*	*c770094 (432), c742110 (561),*	*c740970 (207), c757237 (225, 228), c757460 (654),*
5	*c762599 (269), c743567 (588), c758334 (630), c728956 (340), c756721 (308), c771643 (370), c748436 (873), c749645 (354, 362), c765220 (300, 304, 313), c732202 (322),*	*c743567 (585), c756721 (295), c748436 (745), c749645 (316, 326), c765220 (297, 301, 309), c732202 (254),*	*c762599 (267, 269), c743567 (585), c758334 (630), c756721 (311), c744654 (328), c748436 (810), c724685 (674), c749645 (348, 356), c765220 (299, 304, 312),*	*c762599 (264, 269), c743567 (585), c758334 (622), c748436 (795), c749645 (339, 348),*
6	*c746642 (673), c771614 (286), c760004 (690), c744766 (238), c747871 (657), c753637 (424), c760754 (430), c771657 (836),*	*c744766 (254), c747871 (660), c724406 (700), c760549 (430), c753637 (424), be496986 (647),*	*c740781 (412), c765452 (304, 308), c760004 (177), c737067 (470), c744766 (251), c747871 (660), c724406 (694), c760549 (428),*	*c743137 (514),*

	c754211 (281, 287), *c743137 (478),*		*c753637 (514),* *be496986 (633),*	
7	*c760830 (300, 305),* *bf484254 (568),* *c759439 (849),* *c747342 (663),* *c745166 (243),*	*c760830 (300, 305),* *be494425 (531),* *c759439 (851),* *c747342 (668),* *c754211 (281, 287, 290),* *c743137 (514),*	*c760830 (300, 305),* *bf484254 (568),* *c732202 (644),* *c771657 (819),* *c741119 (760),* *c747342 (696),* *c745166 (243),* *c740257 (280),* *c769080 (349),* *c753911 (165),* *c754211 (289, 292),* *c743137 (515),*	*c720763 (308, 311),* *c746642 (694),* *c744070 (215),* *c765452 (309, 313, 321),* *c760004 (685),*

Table 3. Genomic regions conserved between hexaploid wheat and U, M, S and C genome-chromosomes of *Ae. umbellulata*, *Ae. comosa*, *Ae. speltoides* and *Ae. Markgrafii*, respectively. The number of COS markers representing wheat homoeologous regions is shown in parentheses.

Homoeologousgroup in *Aegilops* chromosomes		*Ae. umbellulata*	*Ae. comosa*	*Ae. speltoides*	*Ae. markgrafii*
1		W1 (12) W3 (1)	W1 (10)	W1 (12)	W1 (10) W3 (4)
2	2US	W2 (2) W6 (5)	W2 (7) W5 (1)	W2 (4) W3 (2)	W5 (3) W6 (12) W7 (4)
	2UL	W2 (5)			
3		W3 (10) W7 (1)	W3 (15) W4 (1) W6 (1) W7 (4)	W2 (2) W3 (13) W6 (2)	W2 (1) W3 (2) W6 (1) W7 (11)
4		W4 (5) W6 (3)	W1 (2) W4 (7) W7 (1)	W4 (7) W6 (3) W7 (1)	W2 (4) W3 (1) W4 (4)
5		W5 (14) W6 (1) W7 (1)	W5 (12) W7 (2)	W5 (14)	W5 (10) W7 (1)
6		W2 (1) W3 (1) W4 (3) W6 (3) W7 (4)	W6 (10)	W2 (1) W3 (1) W4 (2) W6 (8)	W3 (1) W7 (2)
7	7US	W7 (1)	W7 (12)	W7 (18)	W1 (1) W2 (5) W3 (2) W4 (5)
	7UL	W3 (1) W7 (11)			

Figure legends (654words)

Figure 1.FISH on mitotic metaphase plates of *Aegilops markgrafii* with probes for GAA (green) and ACG (red) microsatellites(a - c), and with probes for 18S rDNA (yellow) and pSc119.2 repeat (green) (d).Chromosomes were counterstained by DAPI (grey). Bar = 10 µm.

Figure 2. Representative karyotypes of *Aegilops umbellulata* (AE740/03), *Ae. comosa* (MvGB1039), *Ae. speltoides* (MvGB905) and *Ae. markgrafii* (MvGB428) after FISH with repetitive DNA probes. The signals of GAA and ACG probes were visualized as green and red, respectively, while the probes for 18S rDNA (yellow), Afa family repeat (red) and pSc119.2 repeat (green) were detected simultaneously. Chromosomes were counterstained by DAPI (grey).

Figure3. Flow cytometric analysis and sorting of *Ae. umbellulata* chromosomes. (a) Distribution of fluorescence intensity (flow karyotype) obtained after the analysis of DAPI-stained suspensions of mitotic chromosomes. Monovariate flow karyotype comprises peaks I – III representing chromosomes 1U, 6U and 3U, respectively, and a composite peak of the remaining four chromosomes. (b) Bivariate (DAPI vs. GAA-FITC) flow karyotyping and sorting in*Ae. umbellulata*. FISHIS with probes for GAAresolvedseven chromosome groups (I-VII colored regions). (c - i) Chromosomes were flow-sorted from the colored regions I - VII onto microscope slides and identified by FISH with probes for DNA repeats

pSc119.2 (red), Afa family (green) and 18S rDNA (yellow). All seven chromosomes of *Ae. umbellulata* could be sorted at purities 88%-98%.Bar = 20 µm.

Figure 4. Bivariate flow karyotyping and flow sorting of *Ae. comosa* chromosomes. (a) FISHIS with probes for GAA resolvedonly three chromosome groups (IV, VI and VII colored regions) specific for chromosomes 3M, 6M and 7M. (b) Dual FISHIS with probes for GAAand ACG resolved all seven M-genome chromosomes of *Ae. comosa,* which could be flow sorted at purities of 73%-99%.Chromosomes were assigned to the colored regions by FISH using probes for 18S rDNA (yellow), Afa family (red) and pSc119.2 (green). Chromosomes were counterstained by DAPI (grey).

Figure 5. Bivariate flow karyotyping and flow sorting of chromosomes from (a) *Ae. speltoides* and (b) *Ae. markgrafii*. Dual FISHIS with probes for GAAand ACG resolved all S-genome and C-genome chromosomes,whichcould be flow-sorted at purities of 84%-99% and 80%-97%, respectively.Chromosomes were assigned to the colored regions by FISH using probes for 18S rDNA (yellow), Afa family (red) and pSc119.2 (green). Chromosomes were counterstained by DAPI (grey).

Figure 6. Bivariate flow karyotyping after FISHIS with a probe for GAAand flow sorting *Ae. umbellulata* chromosome arms from wheat (*T. aestivum* cv. Chinese Spring)-*Ae. umbellulata* double ditelosomic addition lines CSDtA2US,

CSDtA2ULand CSDtA7UL. (a) FISHIS allowed discrimination ofthe homoeologous genomes A, D and B of hexaploid wheat (blue and green boxes, respectively) and populations representing 2US (a), 2UL (b) and 7UL (c). Chromosome arms 2US, 2UL and 7ULwere identified using FISH with probes for Afa family (green) and pSc119.2 (red) and could be sorted at purities of 94.9%, 90.3% and 88.3%, respectively.Chromosomes were counterstained by DAPI (grey).

Figure 7. Visualization of wheat–*Aegilops* orthologous relationships from the perspective of wheat B-genome chromosomes.Genetic map positions of the source ESTs of the COS markers are indicated on the left, while the physical positions on the deletion bin map are indicated on the right. Each marker assigned to chromosomes of *Ae. umbellulata*(U), *Ae. comosa*(M), *Ae. speltoides*(S) and *Ae. markgrafii*(C) is positioned to its known bin position and ordered within eachchromosomal bin by the cM value of the marker-containing scaffold obtained from the Genome Zipper of the corresponding wheat chromosome arm.The wheat deletion bins were divided into windows according to the number of markers and each window was color-coded to visualize the marker position on the homoeologous groups of *Triticum*/*Aegilops* chromosomes. When a marker mapped to two chromosomes within a genome, the marker-window was double color-coded. Marker windows and chromosome bins without markers were colored white.