

Original Article

**Flow sorting of C-genome chromosomes from wild relatives of wheat *Aegilops caudata*,
Ae. triuncialis and *Ae. cylindrica*, and their molecular organization**

István Molnár^{1*}, Jan Vrána², András Farkas¹, Marie Kubaláková², András Cseh¹, Márta
Molnár-Láng¹, Jaroslav Doležel²

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

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

* *For correspondence:*

Tel: +00-36-22-569504

E-mail: molnar.istvan@agrar.mta.hu

Running title: C-genome chromosome sorting in *Aegilops*

Abstract

- *Background and Aims* *Aegilops caudata* (CC) and its natural hybrids *Ae. triuncialis* (U^tU^tC^tC^t) and *Ae. cylindrica* (D^cD^cC^cC^c) represent a rich reservoir of useful genes for wheat improvement, but the limited information available on their genome structure and the shortage of molecular (cyto-) genetic tools hamper the utilization of the extant genetic diversity. This study provides the complete karyotypes obtained after FISH with repetitive DNA probes, and evaluates the potential of flow cytometric chromosome sorting in the three species.

- *Methods* The flow karyotypes obtained after the analysis of DAPI-stained chromosomes were characterized and the chromosome content of the peaks on the flow karyotypes was determined by FISH. Twenty-nine conserved orthologous set (COS) markers covering all seven wheat homoeologous chromosome groups were used for PCR with DNA amplified from flow-sorted chromosomes and genomic DNA.

- *Key Results* FISH with repetitive DNA probes revealed that chromosomes 4C, 5C, 7C^t, T6U^tS.6U^tL-5C^tL, 1C^c and 5D^c could be sorted with purities ranging from 66 to 91%, while the remaining chromosomes could be sorted in groups of two to five. This identified a partial wheat–C genome homology for group 4 and 5 chromosomes. In addition, 1C chromosomes were homologous with group 1 of wheat; a small segment from group 2 indicated 1C-2C rearrangement. The extensively rearranged structure of chromosome 7C relative to wheat was also detected.

- *Conclusions* The possibility of purifying *Aegilops* chromosomes provides an attractive opportunity to investigate the structure and evolution of the *Aegilops* C genome and to develop molecular tools to facilitate the identification of alien chromatin and support alien introgression breeding in wheat.

Keywords: *Aegilops caudata*, *Aegilops triuncialis*, *Aegilops cylindrica*; flow cytometric chromosome sorting; conserved orthologous set markers; physical mapping; Fluorescence *in situ* hybridization; Genomic *in situ* hybridization

INTRODUCTION

Wild relatives of wheat (*Triticum aestivum*) are potential sources of important genes and alleles for wheat improvement (Sears, 1956; Friebe *et al.*, 1996). Recently they have received increasing attention due to the urgent need to breed improved cultivars of wheat tolerant to various diseases and to the negative effects of the changing climate. Many wild relatives of primary interest belong to the genus *Aegilops* (goatgrass), which comprises a number of species, including the D-genome progenitor of bread wheat. All these species could be potentially used to increase the genetic variation of hexaploid wheat. However, unlike *Ae. tauschii*, for which a physical map and a draft genome sequence have become available recently (Luo *et al.*, 2013; Jia *et al.*, 2013), the genomes of the other species have not been characterized in detail, hampering their use in wheat improvement.

Ae. caudata L. (syn: *Ae. markgrafii* (Greuter) Hammer) ($2n=2x=14$; CC) is an annual diploid species growing in the Mediterranean region, with an area of distribution ranging from southern France to northern Syria and northern Iraq, and is most abundant in the Aegean region and western Turkey (Van Slageren, 1994). This species is the C genome progenitor of the annual allotetraploids *Ae. cylindrica* Host. ($2n=4x=28$; D^cD^cC^cC^c) and *Ae. triuncialis* L. ($2n=4x=28$; U^tU^tC^tC^t), which are potential sources of important traits for wheat improvement, such as resistance to barley yellow dwarf virus, rusts, powdery mildew and various pests (Hessian fly and Cyst nematode), and tolerance to abiotic stresses (cold, drought, salinity) (Friebe *et al.*, 1996; Monneveux *et al.*, 2000; Colmer *et al.*, 2006; Schneider *et al.*, 2008;

Kilian *et al.*, 2011). Moreover, these species carry genes for high grain micronutrient content (Rawat *et al.*, 2009).

The genes and alleles of interests can be introduced into wheat from wild relatives by interspecific hybridization (Friebe *et al.*, 1996; Cox, 1998). To date, the development of six wheat-*Ae. caudata* chromosome addition lines (Friebe *et al.*, 1992), two substitution lines 5C(5A) and 5C(5D) (Muramatsu, 1973; Friebe *et al.*, 1992), and two wheat-*Ae. cylindrica* addition lines (Bai *et al.*, 1995) has been reported. Some of the main tools for the selection and characterization of wheat-*Aegilops* introgression lines are cytogenetic methods such as C-banding (Fiebe *et al.*, 1996) and methods based on *in situ* hybridization: FISH (Rayburn and Gill, 1986; Mukai *et al.*, 1993) and GISH (Schwarzacher *et al.*, 1989; Le *et al.*, 1989). Genomic *in situ* hybridization (GISH) makes it possible to visualize the constituent genomes in allopolyploid species such as wheat and related *Aegilops* species (Molnár-Láng *et al.*, 2014). In the case of *Ae. cylindrica*, Linc *et al.* (1999) used fluorescein-labelled *Ae. caudata* genomic DNA as a probe, with unlabelled genomic DNA *Ae. tauschii* as a competitor to detect C^c-genome chromatin. The discrimination of closely related genomes by GISH was improved by Sánchez-Morán *et al.* (1999), who used several differentially labelled total gDNA probes simultaneously, allowing the discrimination of the A, B, D and R genomes in wheat-rye derivatives. This method involves the application of indirect labelling (labelling DNA with nucleotide-conjugated haptens such as biotin-16-dUTP or digoxigenin-11-dUTP and detecting them with hapten-specific antibodies conjugated with fluorochromes such as anti-digoxigenin-FITC or avidin-Cy3, respectively), which significantly increased the intensity of the hybridization signals, thus facilitating genome discrimination. Two-colour or multi-colour GISH has also been used for the parallel visualization of the constituent genomes in allopolyploid *Aegilops* species such as *Ae. geniculata* (U^gU^gM^gM^g) and *Ae. biuncialis* (U^bU^bM^bM^b) (Molnár *et al.*, 2011a), but no information is available on the simultaneous

discrimination of the C^c and D^c genomes of *Ae. cylindrica* and the C^t and U^t genomes of *Ae. triuncialis*.

The sequential use of two-colour GISH and FISH enables the chromosomal distribution of repetitive DNA probes in allopolyploid species to be characterized more accurately, as the chromosomes belonging to different genomes could be identified (Molnár *et al.*, 2011a). Fluorescence *in situ* hybridization (FISH) with repetitive DNA probes results in chromosome-specific hybridization patterns, allowing the chromosomes in the species of the *Triticeae* / *Aegilops* taxa to be identified (Badaeva *et al.*, 1996a; 1996b; 2004). Probably the most frequently used repetitive DNA probes for the characterization of cereal genomes have been the pAs1 repetitive sequence originating from *Aegilops squarrosa* (syn: *Ae. tauschii*) (Rayburn and Gill, 1986), the tandem organized DNA sequence pSc119.2 from rye (Bedbrook *et al.*, 1980), which was re-cloned by McIntyre *et al.* (1990), and the 18S-5.8S-26S rDNA clone, pTa71 (Gerlach and Bedbrook, 1979). The genomic distribution of the repeats was described for diploid *Aegilops* species, including the C-genome donor *Ae. caudata*, by Badaeva *et al.* (1996a;b) and was used to study the evolution of the D- and U-genome chromosomes of *Aegilops*, including *Ae. cylindrica* and *Ae. triuncialis* (Badaeva *et al.*, 2002; 2004). However, the complete karyotype has not yet been described using the probes pAs1, pSc119.2 and pTa71 in *Ae. cylindrica* or *Ae. triuncialis*, and, most importantly, the chromosomes belonging to the C^c and C^t genomes have not been identified (Badaeva *et al.*, 2002; 2004). The lack of information available for the cytomolecular identification of the C-genome chromosomes hampers the use of the genetic potential of goatgrasses in wheat pre-breeding programmes.

Another difficulty preventing the wider use of wild alleles has been the limited knowledge available on the genome structure of goatgrasses and the lack of genomic tools. The advent of next generation sequencing (NGS) platforms paved the way for cost-effective large-scale

DNA sequencing in plants, including bread wheat and related species (Margulies *et al.*, 2005; Schatz *et al.*, 2010; Edwards and Batley, 2010; You *et al.*, 2011; Brenchley *et al.*, 2012; Ling *et al.*, 2013; Marcussen *et al.*, 2014; IWGSC, 2014; Pfeifer *et al.*, 2014; Choulet *et al.*, 2014). The most efficient approach for designing markers for *Ae. caudata*, *Ae. cylindrica* and *Ae. triuncialis* would be to use their reference genome sequences. Unfortunately, this route is currently not realistic in *Aegilops* species due to their large genome size, which exceeds 4 Gbp/1C in the diploid *Ae. caudata* and is significantly larger in the polyploids (*Ae. cylindrica*: 9.379 Gbp/1C, *Ae. triuncialis*: 9.711 Gbp/1C) (Doležel *et al.*, 2003; Eilam *et al.*, 2008). The high proportion of repetitive DNA, which typically exceeds 90% of the nuclear genomes in *Triticeae* /*Aegilops* species, represents a further obstacle to the assembly of short sequence reads obtained by NGS sequencing (Dvořák, 2009).

The difficulties encountered due to genome complexity can be partially overcome by isolating and sequencing single chromosomes, which represent smaller and defined parts of the nuclear genome. During the past decades, methods for flow cytometric chromosome sorting have been developed in a variety of plant species, including wheat, barley and rye (Doležel *et al.*, 2009; 2014). The application of genomic tools to wheat chromosomes was made possible by developing procedures for the purification of specific wheat chromosomes by flow cytometric sorting (Vrána *et al.*, 2000) and protocols for the preparation of intact DNA from sorted chromosomes, suitable for cloning (Šafář *et al.*, 2004) and sequencing (Šimková *et al.*, 2008). These advances allowed the application of NGS technology to chromosomes to develop molecular markers, construct sequence-ready physical maps and produce a reference sequence for the first wheat chromosome (Paux *et al.*, 2008; IWGSC, 2014; Choulet *et al.*, 2014).

The application of high-throughput NGS technology (Edwards and Batley, 2010) and the ability to flow-sort chromosome arms (Kubaláková *et al.*, 2002) permitted the sequencing of each chromosome arm of wheat except for 3B, which was sequenced as a complete

chromosome (IWGSC, 2014). The exploitation of gene order conservation in grass genomes and the availability of high-density SNP-based genetic maps enabled the construction of virtual gene order maps (genome zippers), which inferred the positions of 21,221, 22,051 and 22,813 genes in the A, B and D genomes of bread wheat, respectively (IWGSC, 2014).

Chromosome genomics depends on the ability to isolate chromosomes via flow-cytometric sorting. The flow-cytometric analysis of mitotic chromosomes has been reported in hexaploid and tetraploid wheat, in their diploid progenitors and in other related species in the genera *Aegilops* and *Dasyphyrum* (Molnár *et al.*, 2011b; Grosso *et al.*, 2012; Doležel *et al.*, 2012; Molnár *et al.*, 2014). To date, flow cytometric chromosome analysis and sorting has not been reported in diploid *Ae. caudata* or its natural hybrids *Ae. cylindrica* and *Ae. triuncialis*. As the technology could greatly aid the transfer of genes from wild relatives to cultivated wheat, as well as the study of evolution within the *Triticeae/Aegilops* complex, we set out to explore the possibility of using flow sorting to isolate individual chromosomes from *Ae. caudata*, *Ae. cylindrica* and *Ae. triuncialis*. The complete karyotypes of these species were described by sequential FISH and two-colour GISH, and were used to identify the chromosome content of individual peaks of the flow karyotypes obtained after the analysis of DAPI-stained chromosomes. DNA amplified from isolated chromosomes was used as a template for PCR with conserved orthologous set (COS) markers with the aim of identifying their genomic location in the *Aegilops* species. The results of the present work represent an important step towards analysing the molecular organization of chromosomes in C-genome *Aegilops* species and providing molecular (cyto-)genetic tools to support alien gene transfer in wheat improvement programmes.

MATERIALS AND METHODS

Plant material

Aegilops caudata accession MvGB428, *Aegilops cylindrica* accession MvGB1719 and *Ae. triuncialis* accession MvGB585, maintained at the Martonvásár Cereal Genebank, were used for flow cytometric chromosome analysis and sorting, for *in situ* hybridization experiments and for COS marker analysis. Total genomic DNA from *Ae. tauschii* MvGB605, *Ae. umbellulata* MvGB420, *Secale cereale* cv. ‘Lovászpatonai’, *Oryza sativa* cv. ‘Bioriza’ and *Triticum turgidum subsp. durum* cv. ‘Mv Makaróni’ was also used for *in situ* hybridization experiments, while the hexaploid wheat (*T. aestivum* L.) genotype Mv9kr1 was used for COS marker analysis.

Preparation of liquid suspensions of chromosomes

Cell cycle synchronization in root tip meristem cells was carried out with hydroxyurea and their accumulation in metaphase using amiprohos-methyl, as described by Kubaláková *et al.* (2005). Suspensions of intact chromosomes were prepared from metaphase-enriched root tips according to Vrána *et al.* (2000). Briefly, 50 roots were cut 1 cm from the root tip, fixed in 2% (v/v) formaldehyde in Tris buffer 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 (Doležel *et al.*, 1989) at pH 9. Metaphase chromosomes were released after homogenization with Polytron PT1300 homogenizer (Kinematica AG, Littau, Switzerland) at 20,000 rpm for 13 sec. The homogenate 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 argon ion laser set to multiline UV and 300 mW output power. The

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 thousand chromosomes were analysed in each sample and the results were displayed as histograms of relative DAPI fluorescence intensity (flow karyotypes). In order to verify the chromosome content of individual peaks on the flow karyotypes, one thousand chromosomes were sorted from each peak at rates of approximately 5–10 per second into a 15µl drop of PRINS buffer 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), *Ae. caudata* (C genome), *Ae. umbellulata* (U genome), *Secale cereale* and *Oryza 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 genomic DNA of *Ae. tauschii*, *S. cereale* and rice as described by Nagaki *et al.* (1995), Contento *et al.* (2005) and Chang *et al.* (2010), respectively. *Afa*, pSc119.2 and the 18S rRNA unit were labelled with digoxigenin-11-dUTP (Roche), biotin-16-dUTP (Roche, Mannheim, Germany) 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. The genomic DNA from *Ae. umbellulata* and *Ae. tauschii* was labelled with digoxigenin-11-dUTP (U and D genomic probes), while genomic DNA from *Ae. caudata* was labelled with biotin-16-dUTP (C genomic probe) by random priming, and sheared by autoclaving. Unlabelled genomic DNA from durum wheat

(*T. turgidum* subsp. *durum*) was sheared by autoclaving and used as blocking DNA. Digoxigenin and biotin were detected using anti-digoxigenin-rhodamine Fab fragments (Roche) and streptavidin-FITC (Roche), respectively.

Pretreatments and stringency washes (Schneider-Linc *et al.*, 2005), were applied only to slides containing root tip metaphase cells. These steps were omitted in experiments with flow-sorted chromosomes. The hybridization mix (30 μL per slide), containing 50% formamide, 2xSSC, 10% dextran sulphate, 20 ng 18S rDNA, and 70 ng each of the pSc119.2 and *Afa* family probes in the presence of salmon sperm DNA, was denatured at 80°C for 10 min and stored on ice for 5 min. Chromosomal DNA was denatured in the presence of the hybridization mix at 75°C for 6 min and allowed to hybridize overnight at 37°C. To detect the hybridization signals, 10 $\mu\text{g mL}^{-1}$ each of streptavidin-FITC and anti-digoxigenin-Rhodamin were used. Finally, the slides were counterstained with 2 $\mu\text{g mL}^{-1}$ DAPI and examined with a Zeiss Axioskop-2 fluorescence microscope using a Plan Neofluar oil objective 63x, N.A. 1.25 (Zeiss, Oberkochen, Germany) and optical filter sets for DAPI (Zeiss filter set 02) and 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 hybridization mix for GISH (30 μL per slide), containing 50% formamide, 2xSSC, 10% dextran sulphate, 50 ng C genomic probe and 35 ng D (or U) genomic probes and 2.1 μg blocking DNA, was denatured at 80°C for 10 min and stored on ice for 5 min. Chromosomal DNA was denatured in the presence of the hybridization mix at 75°C for 6 min and allowed to hybridize overnight at 42°C.

Relative chromosome lengths were determined as the ratio of chromosome length to the length of the whole chromosome complement by measuring the lengths of at least 20

chromosomes per homoeologous group on DAPI-stained images of *Ae. caudata* MvGB428, *Ae. triuncialis* MvGB585 and *Ae. cylindrica* MvGB1719.

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 with 40 µl of sterile deionized water. The chromosomes were treated with proteinase 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 COS marker analysis.

COS marker analysis

The preparation of genomic DNA from the accessions of *Ae. caudata* MvGB428, *Ae. cylindrica* MvGB1719 and *Ae. triuncialis* MvGB585, used for the flow-cytometric analysis and from the wheat (*T. aestivum* L.) genotype Mv9kr1 was carried out as described by Cseh *et al.* (2013). A total of 29 conserved orthologous set (COS) markers [**Supplementary information, Table S1**] specific for wheat homoeologous groups I-VII were chosen from publicly available COS marker collections (Quraishi *et al.*, 2009; 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/>).

PCR reactions were performed as described by Molnár *et al.* (2014) in a 12 µl reaction volume using a reaction mix consisting of 1× PerfectTaq Plus PCR Buffer (5 Prime GmbH, Hamburg, Germany) and 0.4 µM primers, while 50 ng genomic DNA or 1.5 ng of amplified chromosomal DNA were 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):
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)).
The annealing temperature and PCR reaction profiles are summarized together with the
primer sequences in Table S1. PCR amplicons were separated using a Fragment AnalyzerTM
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 showed differences between the three *Aegilops* species in the number of peaks and in the degree of resolution of individual peaks on the flow karyotypes (Fig. 1). The flow karyotype of *Ae. caudata* had four well-resolved peaks (Fig. 1A). In *Ae. triuncialis*, two peaks (peaks I and II) were well resolved, while three were only partially resolved (peaks III, IV and V) (Fig. 1B). Out of the six peaks identified on the flow karyotype of *Ae. cylindrica*, four were well-resolved (peaks I, II, V and VI), while peaks III and IV were incompletely discriminated (Fig. 1C). The chromosome peaks on the flow karyotype of diploid *Ae. caudata* were observed at lower fluorescence intensity channels (380-500) as compared to those of the tetraploid species *Ae. triuncialis* (channels 390-610) and *Ae. cylindrica* (channels 390-590) (Fig. 1).

In situ hybridization on mitotic metaphase spreads

In order to develop a reference karyotype for the identification of flow-sorted chromosomes, the accessions *Ae. caudata* MvGB428, *Ae. triuncialis* MvGB585 and *Ae. cylindrica* MvGB1719 were investigated with fluorescence *in situ* hybridization (FISH) and genomic *in situ* hybridization (GISH). FISH using repetitive DNA probes (*Afa* family, pSc119.2, 18S rDNA) on root tip mitotic metaphase spreads allowed the identification of the whole set of chromosomes in *Ae. caudata*, *Ae. triuncialis* and *Ae. cylindrica* (Fig. 2). The accessions of *Ae. cylindrica* and *Ae. triuncialis* were also investigated using two-colour GISH in order to discriminate the constituent genomes. The C^c- and D^c-genome chromosomes were clearly discriminated in *Ae. cylindrica* using differentially labelled total genomic DNA from *Ae. caudata* and *Ae. tauschii* (Fig. 2C). Similarly, the use of labelled genomic DNA from *Ae. caudata* and *Ae. umbellulata* allowed the parallel visualisation of the C^t- and U^t-genome chromosomes in *Ae. triuncialis* (Fig.2E).

The application of two-colour GISH revealed the presence of a disomic reciprocal U^t/C^t translocation in *Ae. triuncialis* with pericentromeric breakpoints. The rearranged C^t chromosome was clearly identified as 5C^t on the basis of diagnostic *Afa* and 18S rDNA signals on the short arm and of the telomeric pSc119.2 signal on the long arm. The acrocentric U^t chromosome involved in the reciprocal translocation had *Afa* signals in the terminal region of the short arm and in an intercalary position on the long arm. This chromosome structure and hybridization pattern corresponded to chromosome 6U, as observed earlier in *Ae. umbellulata* (Molnár *et al.*, 2011a) and the reciprocal translocations were thus identified as T1: 6U^tS.6U^tL-5C^tL and T2: 5C^tS.5C^tL-6U^tL.

Differences in the FISH patterns were observed between the *Ae. caudata* genotype used in this study and those used previously (Badaeva *et al.*, 1996a; b). A strong pSc119.2 signal was observed on the long arm of 3C, which was lacking from the same position on 4C. When the allotetraploid species *Ae. cylindrica* and *Ae. triuncialis* were compared with their

diploid C genome ancestor *Ae. caudata*, differences were detected in the FISH hybridisation patterns in some of the C chromosomes. For instance, the 18S rDNA signal on 1C was weaker in *Ae. cylindrica* and stronger in *Ae. triuncialis* in comparison with their diploid progenitor. Similarly, the strong telomeric pSc119.2 signal on 4CL of *Ae. cylindrica* was missing in *Ae. caudata* and *Ae. triuncialis*, while the telomeric pSc112.2 signal on 6CL was not observed in *Ae. cylindrica*, and an additional subtelomeric pSc119.2 band was observed in *Ae. triuncialis*.

The D^c genome of *Ae. cylindrica* showed FISH hybridization patterns similar to those of diploid *Ae. tauschii* (Molnár *et al.*, 2014), with minor differences on chromosome 2D^c (strong Afa signals at intercalary and telomeric positions on the short and long arms, respectively), 3D^c (lack of a telomeric pSc119.2 signal on the short arm) and 4D^c (the presence of a strong telomeric Afa signal on the long arm). Only minor differences were observed between the U genome of *Ae. triuncialis* and those of the diploid progenitor *Ae. umbellulata* (Molnár *et al.*, 2011a), involving the hybridization pattern of chromosomes 4U^t (lack of a telomeric pSc119.2 signal on the long arm) and 6U^t (lack of a centromeric Afa signal).

These differences were not great enough to hinder chromosome identification and all the chromosomes in the diploid and polyploid *Aegilops* species could be distinguished according to their fluorescence labelling patterns. Moreover, the differences in the FISH patterns will facilitate the identification of each of the *Aegilops* chromosomes in the wheat background. The idiogram representing the genomic distribution of the Afa family, pSc119.2 and 18S rDNA sequences in *Ae. caudata* MvGB428, *Ae. cylindrica* MvGB1719 and *Ae. triuncialis* MvGB585 is shown in Figure 3.

Description of flow karyotypes

The chromosome content of individual peaks on the flow karyotypes was determined on flow-sorted chromosomes after FISH with probes for *Afa* family, pSc119.2 and 18S rDNA repeats (Fig. 4, Table 1). The strong 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 *Ae. caudata*, peaks I and II contained chromosomes 1C, 6C and 7C and 2C and 3C, respectively. Peaks III and IV corresponded to chromosomes 5C and 4C, representing 15.15% and 14.72% of the *Ae. caudata* genome, which could be sorted at a purity of 66.2% and 91.3%, respectively (Fig. 4, Table 1). In *Ae. cylindrica* Peaks II and V contained chromosomes 1C^c and 5D^c representing 6.32% and 7.61% of the genome, which could be sorted at a purity of 67.5% and 84.9%, respectively (Fig. 4, Table 1). The composite peaks I and III comprised groups of chromosomes (6C^c and 7C^c, and 3C^c, 1D^c and 6D^c, respectively), while peaks IV and VI contained chromosomes 2C^c, 4C^c, 5C^c and 4D^c, and 2D^c, 3D^c and 7D^c, respectively. In *Ae. triuncialis*, peaks I and II corresponded to the rearranged chromosome T1 (6U^tS.6U^tL-5C^tL) and 7C^t (representing 5.70% of the genome), which could be sorted at a purity of 90.1% and 70.0%, respectively. The composite peaks III and IV contained groups of chromosomes: 3C^t, 6C^t, 1U^t and 3U^t, and 1C^t, 4C^t and 4U^t, respectively, while peak V contained the chromosomes 2C^t, 2U^t, 5U^t and 7U^t, and the translocation chromosome T2 (5C^tS.5C^tL-6U^tL) (Fig. 4, Table 1).

Assignment of COS markers to peaks on flow karyotypes

A set of conserved orthologous set (COS) markers specific for seven wheat homoeologous chromosome groups were mapped to the subgenomic DNA samples obtained after sorting chromosomes from individual peaks on the flow karyotypes. Out of the 29 COS markers investigated, 28 gave PCR products in *Ae. caudata* MvGB428, and all 29 in the *Ae. cylindrica* MvGB1719 and *Ae. triuncialis* MvGB585 accessions [**Supplementary Information, Table**

S2]. The 29 markers resulted in a total of 55 PCR products (range: 1-6 PCR products/marker/genotype, mean: 1.89 PCR products) in the diploid *Ae. caudata* MvGB428, while 125 products were amplified in the tetraploid *Aegilops* species (71 and 54 products in *Ae. cylindrica* MvGB1719 and *Ae. triuncialis* MvGB585, respectively). Out of the 55 PCR products detected in the diploid *Ae. caudata*, 38 (69.09%) and 17 (30.9%) were polymorphic and non-polymorphic, respectively, relative to the tetraploid *Ae. cylindrica*, while 31 (56.36%) and 24 (43.63%) were polymorphic and non-polymorphic, respectively, relative to the tetraploid *Ae. triuncialis*. Out of the 180 PCR products amplified by the 29 COS markers in the three *Aegilops* species, 144 (80.0%) were polymorphic relative to the wheat genotype Mv9kr1, and can thus be used for the marker-assisted selection of wheat-*Aegilops* introgression lines in pre-breeding programmes.

Because each chromosome of the investigated *Aegilops* species has a major location in one of the peaks on a 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 (Figure 5). For example, the markers $X_{GPI:C:731424}$ and $X_{GPI:C:725135}$, which are specific for group 4 chromosomes of wheat, produced a 260 bp and a 319 bp PCR amplicon, respectively, with continuously increasing yield in the *Ae. caudata* flow karyotype peaks I, II, III and IV, with no amplicon in peak I for $X_{GPI:C:731424}$, and no amplicon in peaks I-II for $X_{GPI:C:725135}$ (Figure 5), [Supplementary Information, Table S2]. The 4C chromosome content was 0%, 0%, 16.20% and 91.37%, respectively (Table 1). With these markers a similar relationship could be observed for the yield of PCR amplicons specific to group 4 chromosome as for the relative content of group 4 chromosomes determined by cytomolecular methods for the flow karyotype peaks in *Ae. triuncialis* and *Ae. cylindrica*.

Based on differences in the yield of PCR amplicons between the flow karyotype peaks, all the PCR amplicons could be assigned to peaks on the flow karyotypes of *Ae. caudata*, *Ae. triuncialis* and *Ae. cylindrica* (Table 2) , [Supplementary Information, Table S2]. The putative chromosomal location of the markers is shown in Table 2. The group I-specific markers were assigned to the flow karyotype peaks containing group 1 chromosomes (peak I of *Ae. caudata*, peak IV of *Ae. triuncialis*, specific for 1C^t and peaks II and III of *Ae. cylindrica*, containing chromosomes 1C^c and 1D^c, respectively). The markers specific for the wheat homoeologous group IV were assigned mainly to flow karyotype peaks representing chromosomes 4C, 4C^t/4U^t and 4C^c/4D^c (i.e. peaks IV of *Ae. caudata*, *Ae. triuncialis* and *Ae. cylindrica*). Most of the group 5-specific markers produced PCR amplicons at the highest intensity in flow karyotype peaks containing group 5 chromosomes (i.e. peak III in *Ae. caudata*, peaks I and V in *Ae. triuncialis* and peaks IV and V in *Ae. cylindrica*). Markers specific for the wheat homoeologous group 7 were observed mainly in flow karyotype peak II in *Ae. caudata* (containing chromosomes 2C and 3C), in peaks III (containing 3C^t, 6C^t, 1U^t and 3U^t) and V (containing 7U^t) in *Ae. triuncialis* and in peaks III (where chromosome 3C^c was identified) and VI (where chromosome 7D^c was located) in *Ae. cylindrica*. Interestingly, only two of the five group 7-specific markers gave intense PCR amplicons in flow karyotype peak I of *Ae. caudata*, which contains chromosome 7C, and none of the group 7-specific markers produced intense PCR amplicons in peaks containing chromosome 7C in *Ae. triuncialis* (i.e. in peak II) and *Ae. cylindrica* (i.e. in peak I).

The markers specific for the wheat homoeologous group 3 gave the highest yield of PCR amplicons in flow karyotype peak I of *Ae. caudata*, to which chromosomes 1C, 6C and 7C were assigned. In the case of *Ae. triuncialis*, the highest amount of PCR products was observed in peak III, consistent with the cytological results assigning chromosomes 3C^t and 3U^t to this peak. In *Ae. cylindrica*, a specific pattern for the peak location of the PCR

amplicons was not observed with the group 3-specific markers. Moreover, no typical peak location was observed for the PCR products with markers specific for wheat homoeologous groups 2 and 6. For example, no PCR products were located with group 2-specific markers in Peak II of *Ae. caudata*, where chromosome 2C was located.

DISCUSSION

During the last decade, the application of genomic tools to flow-sorted chromosomes (Doležel *et al.*, 2007) has facilitated the structural and functional analysis of chromosomes, including the high-throughput development of markers, the construction of ready-to-sequence physical maps, and positional gene cloning, mainly in species with high socio-economic importance such as bread wheat, durum wheat, barley and rye (Lysák *et al.*, 1999; Vrána *et al.*, 2000; Kubaláková *et al.*, 2003; Kubaláková *et al.*, 2005; IWGSC, 2014). As the allele diversity in wild related species has great potential for wheat breeding, the utilization of chromosome-based approaches could facilitate the transfer of alien genes to wheat (Tiwari *et al.*, 2014). To date, chromosomes have only been purified by flow cytometry from a limited number of wild species, including *Aegilops* species with U, M, S and D genomes, *T. urartu* and *Dasypyrum villosum* (Molnár *et al.*, 2011b; Grosso *et al.*, 2012; Giorgi *et al.*, 2013; Molnár *et al.*, 2014). The present work extends the potential of chromosome genomics to C-genome *Aegilops* species: diploid *Ae. caudata* (CC) and its tetraploid relatives *Ae. triuncialis* (U^tU^tC^tC^t) and *Ae. cylindrica* (C^cC^cD^cD^c).

The determination of the chromosome content of peaks on flow karyotypes revealed that the C-genome chromosomes of tetraploid *Ae. triuncialis* and *Ae. cylindrica* were represented by

peaks at lower fluorescence channels relative to the U^t- and D^c-genome chromosomes, suggesting that the C-genome chromosomes are smaller than the U^t- and D^c-genome chromosomes. This is in agreement with the nuclear DNA amounts published for *Ae. caudata* (4.63-4.84 pg/1C DNA), *Ae. umbellulata* (5.05-5.38 pg/1C DNA) and *Ae. tauschii* (5.08-5.17 pg/1C DNA) (Furuta 1970; Eilam et al. 2007; 2008), and indicates that their genomes have undergone different evolutionary changes. As the number of genes per monoploid genome is similar in plants, differences in genome size are mainly due to repetitive DNA sequences, of which retroelements are considered to play a dominant role in genome expansion (Feuillet and Keller, 2002; Bennetzen, 2007; Lisch, 2009). Senerchia *et al.* (2014) investigated the nucleotide diversity of 16 long terminal repeat (LTR) retrotransposon families in diploid *Aegilops* species, including *Ae. caudata*, *Ae. tauschii* and *Ae. umbellulata*. It was shown that the genetic diversity of transposable elements, such as *Fatima* and *Sabine*, was greater in *Ae. caudata*, while other retrotransposon families such as *BARE1*, *Maximus* or *WHAM* exhibited greater diversity in *Ae. tauschii* or *Ae. umbellulata*, indicating that specific types of transposable elements had undergone differential proliferation in various *Aegilops* genomes during their evolution.

In the present work two-colour GISH and FISH were employed with a set of probes suitable for describing the karyotypes of the accessions used for flow cytometric chromosome sorting. The genomic distribution of these probes was previously described in *Ae. caudata*, and in the tetraploid species *Ae. triuncialis* and *Ae. cylindrica* (Badaeva *et al.*, 1996a; b; 2002; 2004). The chromosomes of *Ae. triuncialis* and *Ae. cylindrica* were similar to those of the ancestral species *Ae. caudata*, *Ae. umbellulata* and *Ae. tauschii* (Molnár *et al.*, 2011b; 2014). The number of variable FISH bands compared with the diploid ancestors was lower in *Ae. triuncialis* than in *Ae. cylindrica*, indicating that *Ae. triuncialis* had greater homology with the progenitor genomes than *Ae. cylindrica*. This was supported by the frequency of non-

polymorphic COS marker loci, which was higher (43.63%) between *Ae. caudata* and *Ae. triuncialis* than between *Ae. caudata* and *Ae. cylindrica* (30.9%). The greater homology of *Ae. triuncialis* with the progenitor genomes may reflect the more recent origin of this species compared with *Ae. cylindrica*.

GISH revealed a disomic 5C^t/6U^t reciprocal translocation with pericentromeric breakpoints in the *Ae. triuncialis* accession MvGB585. Intra- and intergenomic rearrangements were also identified by Badaeva *et al.* (2004) in *Ae. triuncialis*, *Ae. kotschyi* and *Ae. columnaris*, but the translocation breakpoints could not be accurately mapped due to the limitations of the C banding technique. The use of two-colour GISH permitted the identification of U/M intergenomic translocations in four accessions of *Ae. biuncialis* and in two accessions of *Ae. geniculata* (Molnár *et al.*, 2011a) with clearly determined pericentromeric breakpoints. These results are consistent with previous observations in other *Triticeae* species indicating that pericentromeric regions are prone to rearrangements, including duplications, inversions and deletions (Eichler and Sankoff, 2003; Qi *et al.*, 2006). The preferential location of translocation breakpoints in pericentromeric regions suggests that pericentromeric heterochromatin clusters may play a role in the formation of intergenomic exchanges in the allopolyploid *Triticeae/Aegilops* species (Raskina *et al.*, 2008; Molnár *et al.*, 2011a). The present study also indicates that intergenomic translocations may promote the dissection of the genome into chromosomes in wild species, and that flow-sorted translocation chromosomes can be used for the physical mapping of markers.

It has been well documented that C-genome *Aegilops* species are attractive sources of numerous agronomic traits. Riar *et al.* (2012) described the wheat-*Ae. caudata* introgression line T291-2, which carries a homoeoallele of the leaf rust resistance gene orthologue *Lr57*. Romero *et al.* (1998) produced a wheat-*Ae. triuncialis* translocation, TR-3531, carrying the Hessian fly resistance gene *H30* and a resistance gene, *Cre7*, against *Heterodera avenae*

(Martin-Sanchez *et al.*, 2003). The present study demonstrates that chromosomes 4C and 5C of *Ae. caudata*, chromosome 7C^t and the translocation chromosome 6U^tS.6U^tL-5C^tL of *Ae. triuncialis*, and chromosomes 1C^c and 5D^c of *Ae. cylindrica* can be purified from wild relatives of bread wheat. Chromosomes of *Triticeae/Aegilops* species that are homoeologous with the chromosomes flow-sorted in this study have been reported to carry important genes and alleles ensuring biotic and abiotic stress tolerance and adaptation to various agro-ecological conditions. For example, chromosome 6U of *Ae. umbellulata* contains a leaf rust resistance gene, *Lr9*, that is one of the most effective in providing resistance to leaf rust pathogen *P. triticina* (Friebe *et al.*, 1996; Vida *et al.*, 2011). The *VRN1* gene, which is critical for the adaptation of polyploid wheats to autumn sowing, has been mapped to collinear regions of the long arms of chromosomes 5A, 5B and 5D (Galiba *et al.*, 1995; Snape *et al.*, 1997). Chromosome group 5 of hexaploid wheat also carries major loci affecting winter hardiness and freezing tolerance (*FR-1* (Sutka and Snape, 1989), and *FR-2* (Vágújfalvi *et al.*, 2003; Dhillon *et al.*, 2010)). Other loci affecting drought and salt tolerance and the crossability of wheat have also been mapped on the group 5 chromosomes (Quarrie *et al.*, 1994; Koebner *et al.*, 1996; Riley and Chapman, 1967; Sitch *et al.*, 1985; Krolow, 1970). The chromosomes flow-sorted from *Ae. caudata* and its related species may facilitate the dissection of useful alleles of the above-mentioned genes and their use in wheat improvement to cope with the pleiotropic effects of climate change (Feuillet *et al.*, 2008).

The present results confirm and expand the results of previous studies on 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 80% of the products obtained with chromosomes isolated from wild relatives were polymorphic relative to those obtained in hexaploid wheat suggested that a substantial part of the genetic diversity of wild progenitors is due to the variability of intron regions (Yu *et al.*, 2005). Thus, polymorphic COS markers

will be suitable for identifying the chromatin of wild *Aegilops* species introduced into wheat and for marker-assisted selection to facilitate the transfer of useful agronomic traits (Quraishi *et al.*, 2009).

Twenty-five of the 29 COS markers used in the present study were also used in a previous study (Molnár *et al.*, 2014), when twenty of them were assigned to the same homoeologous group chromosomes of diploid wheats, *T. urartu*, *Ae. speltoides* and *Ae. tauschii*, as in hexaploid wheat. In the present study, the chromosomal location of the same set of markers was not so consistent in the C-genome *Aegilops* species as in the diploid progenitors of wheat, indicating the presence of genome rearrangements in the C genomes relative to wheat.

These results indicate that the 1C chromosomes (1C, 1C^t and 1C^c) are mainly homologous to group 1 chromosomes of wheat (W), but some markers indicate the presence of conserved orthologous regions specific for W2 (in the case of 1C and 1C^c) and W6 (in 1C^c). Homology between the 4C chromosomes (4C, 4C^t and 4C^c) and W4, and partial homology between the 5C chromosomes (5C, 5C^t and 5C^c) and W5 was also observed. On the other hand, more markers proved homology between the W3 and 7C chromosomes (7C, 7C^t and 7C^c), suggesting the presence of a group3/group7 rearrangement in the C genomes relative to hexaploid wheat.

The macrosynthetic relationship between the U genome, another widely distributed genome in the genus *Aegilops*, and wheat was investigated by mapping RFLP markers on an *Ae. umbellulata* segregating mapping population and by mapping COS markers on wheat-*Aegilops* addition lines and flow-sorted chromosomes (Zhang *et al.*, 1998; Molnár *et al.*, 2013). These studies indicated the presence of a small fragment related to W2 on 1U and the presence of a region syntenic with W3 on chromosome 7U (Zhang *et al.*, 1998; Molnár *et al.*, 2013). It can be assumed that similar group1/group2 and group3/group7 rearrangements are present in the C genomes of *Ae. caudata* and related polyploids. However, a larger number of

COS markers assigned to the chromosomes of *Aegilops* species and the preparation of isolated chromosome fractions with higher purity will be needed if the genome rearrangements and synteny perturbations occurring in the C genome and other *Aegilops* genomes relative to bread wheat are to be effectively studied.

The present study showed that chromosomes 4C and 5C of *Ae. caudata*, chromosome 7C^t and translocation chromosome 6U^tS.6U^tL-5C^tL of *Ae. triuncialis*, and chromosomes 1C^c and 5D^c of *Ae. cylindrica* can be purified from wild relatives of bread wheat. FISH analysis on flow-sorted chromosome fractions showed that these chromosomes can be isolated at high purity. Further improvements in the chromosome sorting 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 sequence data obtained by sequencing DNA amplified from single chromosome fractions. Other chromosomes of the diploid and tetraploid C-genome *Aegilops* species formed composite peaks on the flow karyotypes and could be sorted as groups of two, three, four or five. If microsatellite probes can be applied for the fluorescent labelling of the chromosomes in these species, multiparametric flow cytometric analysis may facilitate the isolation of the remaining chromosomes, as shown by Giorgi *et al.* (2013). The FISH karyotypes of *Ae. caudata*, *Ae. triuncialis* and *Ae. cylindrica* developed in this study might serve as a guide when choosing probes for labelling chromosomes prior to flow-sorting and may support the introgression of *Aegilops* chromosomes into wheat.

To conclude, this study represents an important step forward in developing chromosome genomics for wild genetic resources of wheat. The flow karyotypes of *Ae. caudata*, *Ae. triuncialis* and *Ae. cylindrica* were characterized, and the chromosome content of all the peaks was determined for the first time. The ability to purify chromosomes 4C, 5C, 7C^t, T6U^tS.6U^tL-5C^tL, 1C^c and 5D^c in wild genetic resources of wheat paves the way for their

next-generation sequencing to facilitate comprehensive gene content analysis and allele discovery as well as the development of gene-based markers for specific genomic regions. COS markers assigned to the chromosomes of C-genome *Aegilops* species could be used in pre-breeding programmes to select chromosome segments carrying agronomically useful genes in *T. aestivum* – *Aegilops* recombinant lines.

Supplementary Information

Table S1 provide information about the COS markers used in the present study, their primer sequences and the annealing temperature used to amplify products by the specific PCR reaction profiles.

Table S2 provide information about Size and concentration of PCR products of COS markers amplified from total genomic DNA of wheat (Mv9kr1), *Ae. caudata*, *Ae. cylindrica* and *Ae. triuncialis* and from subgenomic DNA samples derived from chromosomes sorted from individual peaks on the flow karyotypes of *Ae. caudata*, *Ae. cylindrica* and *Ae. triuncialis*.

Funding

This work was supported by the Hungarian National Research Fund (K112226, PD105594), by TÁMOP-4.2.2.A-11-1-KONV-2012-0008, by a János Bolyai Research Scholarship from the Hungarian Academy of Sciences (for MI), by an OECD fellowship (TAD/CRP JA00079297), and by the Ministry of Education, Youth and Sports of the Czech Republic (grant LO1204 from the National Program of Sustainability I). AC gratefully acknowledges support from a Marie Curie Fellowship Grant (FP7-PEOPLE-2013-IEF-625671) under the seventh framework program of the European Union.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Jarmila Číhalíková, Bc. Romana Šperková, and Zdenka Dubská for their assistance with chromosome sorting and DNA amplification.

LITERATURE CITED

- Badaeva ED, Friebe B, Gill BS. 1996a.** Genome differentiation in *Aegilops*. 1. Distribution of highly repetitive DNA sequences on chromosomes of diploid species. *Genome* **39**:293-306.
- Badaeva ED, Friebe B, Gill BS. 1996b.** Genome differentiation in *Aegilops*. 2. Physical mapping of 5S and 18S-26S ribosomal RNA gene families in diploid species. *Genome* **39**:1150-1158.
- Badaeva ED, Amosova AV, Muravenko OV, et al. 2002.** Genome differentiation in *Aegilops*. 3. Evolution of the D-genome cluster. *Plant Systematics and Evolution* **231**:163-190.
- Badaeva ED, Amosova AV, Samatadze TE, Zoshchuk SA, Shostak NG, et al. 2004.** Genome differentiation in *Aegilops*. 4. Evolution of the U-genome cluster. *Plant Systematics and Evolution* **246**:45-76.
- Bai D, Scoles GJ, Knott DR. 1995.** Rust resistance in *Triticum cylindricum* Ces. (4x, CCDD) and its transfer into durum and hexaploid wheats. *Genome* **38**:8–16.

- Bedbrook J, Jones J, O'Dell M, Thompson RD, Flavell RB. 1980.** A molecular description of telomeric heterochromatin in *Secale* species. *Cell* **19**:545–560.
- Bennetzen JL. 2007.** Patterns in grass genome evolution. *Current Opinion in Plant Biology* **10**:176–181.
- Brenchley R, Spannag M, Pfeifer M, et al. 2012.** Analysis of the bread wheat genome using whole-genome shotgun sequencing. *Nature* **491**:705-710.
- Burt C, Nicholson P. 2011.** Exploiting co-linearity among grass species to map the *Aegilops ventricosa*-derived *Pchl* eyespot resistance in wheat and establish its relationship to *Pch2*. *Theoretical and Applied Genetics* **123**:1387–1400.
- Chang KD, Fang SA, Chang FC, Chung MC. 2010.** Chromosomal conservation and sequence diversity of ribosomal RNA genes of two distant *Oryza* species. *Genomics* **96**:181–190.
- Choulet F, Alberti A, Theil S, et al. 2014.** Structural and functional partitioning of bread wheat chromosome 3B. *Science* **345**:1249721.
- Colmer TD, Flowers TJ, Munns R. 2006.** Use of wild relatives to improve salt tolerance in wheat. *Journal of Experimental Botany* **57**:1059–1078.
- Contento A, Heslop-Harrison JS, Schwarzacher T. 2005.** Diversity of a major repetitive DNA sequence in diploid and polyploid *Triticeae*. *Cytogenetic and Genome Research* **109**:34–42.
- Cox TS. 1998.** Deepening the wheat gene pool. *Journal of Crop Production* **1**:1–25.
- Cseh A, Soós V, Rakszegi M, Türkösi E, Balázs E, Molnár-Láng M. 2013.** Expression of HvCslF9 and HvCslF6 barley genes in the genetic background of wheat and their influence on the wheat β -glucan content. *Annals of Applied Biology* **163**:142-150.

- Dhillon T, Pearce SP, Stockinger EJ, Distelfeld A, Li C, et al. 2010.** Regulation of freezing tolerance and flowering in temperate cereals: the VRN-1 connection. *Plant Physiology* **153**:1846-1858.
- Doležel J, Binarová P, Lucretti S. 1989.** Analysis of nuclear DNA content in plant cells by flow cytometry. *Biologia Plantarum* **31**:113-120.
- Doležel J, Bartoš J, Voglmayr H, Greilhuber J. 2003.** Nuclear DNA content and genome size of trout and human. *Cytometry* **51**:127-128.
- Doležel J, Kubaláková M, Paux E, Bartoš J, Feuillet C. 2007.** Chromosome based genomics in the cereals. *Chromosome Research* **15**:51–66.
- Doležel J, Šimková H, Kubaláková M, et al. 2009.** Chromosome genomics in the *Triticeae*. In: Muehlbauer GJ, Feuillet C, eds. *Genetics and genomics of the Triticeae*. Plant genetics and genomics: crops and models, Vol. 7. Springer, US, 285-316.
- Doležel J, Vrána J, Safář J, Bartoš J, Kubaláková M, Šimková H. 2012.** Chromosomes in the flow to simplify genome analysis. *Functional and Integrative Genomics* **12**:397-416.
- Doležel J, Vrána J, Cápál P, Kubaláková M, Burešová V, Šimková H. 2014.** Advances in plant chromosome genomics. *Biotechnology Advances* **32**:122-136.
- Dvořák J. 2009.** *Triticeae* genome structure and evolution. In: Muehlbauer GJ, Feuillet C, eds. *Genetics and genomics of the Triticeae*. Plant genetics and genomics: crops and models, Vol. 7. Springer, US, 685-711.
- Edwards D, Batley J. 2010.** Plant genome sequencing: applications for crop improvement. *Plant Biotechnology Journal* **8**:2-9.
- Eichler EE, Sankoff D. 2003.** Structural dynamics of eukaryotic chromosome evolution. *Science* **301**:793-797.
- Eilam T, Anikster Y, Millet E, Manisterski J, Sagi-Assif O, Feldman M. 2007.** Genome size and genome evolution in diploid *Triticeae* species. *Genome* **50**:1029-1037.

- Eilam T, Anikster Y, Millet E, Manisterski J, Feldman M. 2008.** Nuclear DNA amount and genome downsizing in natural and synthetic allopolyploids of the genera *Aegilops* and *Triticum*. *Genome* **51**:616–627.
- Feuillet C, Keller B. 2002.** Comparative genomics in the grass family: Molecular characterization of grass genome structure and evolution. *Annals of Botany* **89**:3-10.
- Feuillet C, Langridge P, Waugh R. 2008.** Cereal breeding takes a walk on the wild side. *Trends in Genetics* **24**:24-32.
- Friebe B, Schubert V, Blüthner WD, Hammer K. 1992.** C-banding pattern and polymorphism of *Aegilops caudata* and chromosomal constitutions of the amphiploid *T. aestivum* – *Ae. caudata* and six derived chromosome addition lines. *Theoretical and Applied Genetics* **83**:589–596.
- Friebe B, Jiang J, Raupp WJ, McIntosh RA, Gill BS. 1996.** Characterization of wheat-alien translocations conferring resistance to diseases and pests: Current status. *Euphytica* **91**:59–87.
- Furuta Y. 1970.** DNA content per nucleus in *Aegilops* species. *Wheat Information Service* **30**:20-22. doi:
- Galiba G, Quarrie SA, Sutka J, Morgounov A, Snape JW. 1995.** RFLP mapping of the vernalization (*Vrn1*) and frost resistance (*Fr1*) genes on chromosome 5A of wheat. *Theor Appl Gene Theoretical and Applied Genetics* **90**:1174–1179.
- Gerlach WL, Bedbrook JR. 1979.** Cloning and characterization of ribosomal RNA genes from wheat and barley. *Nucleic Acids Research* **7**:1869–1885.
- Giorgi D, Farina A, Grosso V, Gennaro A, Ceoloni C, Lucretti S. 2013.** FISHIS: fluorescence in situ hybridization in suspension and chromosome flow sorting made easy. *PLoS ONE* **8**:e57994. doi:10.1371/journal.pone.0057994

- Grosso V, Farina A, Gennaro A, Giorgi D, Lucretti S. 2012.** Flow sorting and molecular cytogenetic identification of individual chromosomes of *Dasyphyrum villosum* L. (*H. villosa*) by a single DNA probe. *PLoS ONE* 7:e50151. doi:10.1371/journal.pone.0050151
- Howard T, Rejab NA, Griffiths S, et al. 2011.** Identification of a major QTL controlling the content of B-type starch granules in *Aegilops*. *Journal of Experimental Botany* 62:2217-2228.
- Jia J, Zhao S, Kong X, et al. 2013.** *Aegilops tauschii* draft genome sequence reveals a gene repertoire for wheat adaptation. *Nature* 496:91–95.
- Kilian B, Mammen K, Millet E, et al. 2011.** *Aegilops*. In: Kole C, ed. *Wild crop relatives: genomic and breeding resources, Cereals*. Springer-Verlag, Berlin, Heidelberg, 1-76.
- Koebner RMD, Martin PK, Orford SM, Miller TE. 1996.** Responses to salt stress controlled by the homeologous group 5 chromosomes of hexaploid wheat. *Plant Breeding* 115:81–84.
- Krolow KD. 1970.** Untersuchungen über die kreuzbarkeit zwischen weizen und roggen. *Zeitschrift Für Pflanzenzüchtung* 64:44-72.
- Kubaláková M, Macas J, Doležel J. 1997.** Mapping of repeated DNA sequences in plant chromosomes by PRINS and C-PRINS. *Theoretical and Applied Genetics* 94:758–763.
- Kubaláková M, Vrána J, Číhalíková J, Šimková H, Doležel J. 2002.** Flow karyotyping and chromosome sorting in bread wheat (*Triticum aestivum* L.). *Theoretical and Applied Genetics* 104:1362-1372.
- Kubaláková M, Valárik M, Bartoš J, et al. 2003.** Analysis and sorting of rye (*Secale cereale* L.) chromosomes using flow cytometry. *Genome* 46:893–905.
- Kubaláková M, Kovářová P, Suchánková P, et al. 2005.** Chromosome sorting in tetraploid wheat and its potential for genome analysis. *Genetics* 170:823-829.

- Le HT, Armstrong KC, Miki B. 1989.** Detection of rye DNA in wheat-rye hybrids and wheat translocation stocks using total genomic DNA as a probe. *Plant Molecular Biology Reporter* 7:150-158.
- Linc G, Friebe B, Kynast RG, et al. 1999.** Molecular cytogenetic analysis of *Aegilops cylindrica* Host. *Genome* 42:497–503.
- Ling H-Q, Zhao S, Liu D, et al. 2013.** Draft genome of the wheat A-genome progenitor *Triticum urartu*. *Nature* 496:87-90.
- Lisch D. 2009.** Epigenetic regulation of transposable elements in plants. *Annual Review of Plant Biology* 60:43–66.
- Luo M-C, Gu YQ, You FM, et al. 2013.** A 4-gigabase physical map unlocks the structure and evolution of the complex genome of *Aegilops tauschii*, the wheat D-genome progenitor. *Proceedings of the National Academy of Sciences of the United States of America* 110:7940-7945.
- Lysák MA, Čihalíková J, Kubaláková M, Šimková H, Künzel G, Doležel J. 1999.** Flow karyotyping and sorting of mitotic chromosomes of barley (*Hordeum vulgare* L.). *Chromosome Research* 7:431–444.
- Marcussen T, Sandve SR, Heier L, et al. 2014.** Ancient hybridizations among the ancestral genomes of bread wheat. *Science* 345:1250092 doi:10.1126/science.1250092
- Margulies M, Egholm M, Altman WE, et al. 2005.** Genome sequencing in microfabricated high-density picolitre reactors. *Nature* 437:376-380.
- Martin-Sanchez JA, Gomez-Colmenarejo M, Del Moral J, et al. 2003.** A new Hessian fly resistance gene (*H30*) transferred from the wild grass *Aegilops triuncialis* to hexaploid wheat. *Theoretical and Applied Genetics* 106:1248–1255.
- Mayer KFX, Martis M, Hedley PE, et al. 2011.** Unlocking the barley genome by chromosomal and comparative genomics. *Plant Cell* 23:1249-1263.

- McIntyre CL, Pereira S, Moran LB, Appels R. 1990.** New *Secale cereale* (rye) DNA derivatives for the detection of rye chromosome segments in wheat. *Genome* **33**:635-640.
- Molnár I, Cifuentes M, Schneider A, Benavente E, Molnár-Láng M. 2011a.** Association between simple sequence repeat-rich chromosome regions and intergenomic translocation breakpoints in natural populations of allopolyploid wild wheats. *Annals of Botany* **107**:65–76.
- Molnár I, Kubaláková M, Šimková H, Cseh A, Molnár-Láng M, Doležel J. 2011b.** Chromosome isolation by flow sorting in *Aegilops umbellulata* and *Ae. comosa* and their allotetraploid hybrids *Ae. biuncialis* and *Ae. geniculata*. *PLoS ONE* **6**:e27708. doi:10.1371/journal.pone.0027708
- Molnár I, Šimková H, Leverington-Waite M, et al. 2013.** Syntenic relationships between the U and M genomes of *Aegilops*, wheat and the model species *Brachypodium* and rice as revealed by COS markers. *PLoS ONE* **8**:e70844. doi:10.1371/journal.pone.0070844
- Molnár I, Kubaláková M, Šimková H, et al. 2014.** Flow cytometric chromosome sorting from diploid progenitors of bread wheat, *T. urartu*, *Ae. speltooides* and *Ae. tauschii*. *Theoretical and Applied Genetics* **127**:1091-1104.
- Molnár-Láng M, Molnár I, Szakács É, Linc G, Bedő Z. 2014.** Production and molecular cytogenetic identification of wheat-alien hybrids and introgression lines. In: Tuberosa R, Graner A, Frison E, eds, *Genomics of plant genetic resources, Vol. 1, Managing, sequencing and mining genetic resources*. Springer, Netherlands, 255-283.
- Monneveux P, Zaharieva M, Rekika D. 2000.** The utilisation of *Triticum* and *Aegilops* species for the improvement of durum wheat. In: Royo C, Nachit M, Di Fonzo N, Araus JL, eds, *Durum wheat improvement in the Mediterranean region: New challenges*. CIHEAM, Zaragoza, 71-81.

- Mukai Y, Nakahara Y, Yamamoto M. 1993.** Simultaneous discrimination of the three genomes in hexaploid wheat by multicolour fluorescence in situ hybridization using total genomic and highly repeated DNA probes. *Genome* **36**:489-494.
- Muramatsu M. 1973.** Genetic homology and cytological differentiation of the homoeologous-group-5 chromosomes of wheat and related species. In: Sears ER, Sears LMS, eds, *Proceedings of the 4th international wheat genetics symposium*. Agriculture Experiment Station, College of Agriculture, University of Missouri, Columbia, USA, 719–724.
- Nagaki K, Tsujimoto H, Isono K, Sasakuma T. 1995.** Molecular characterization of a tandem repeat, Afa family, and its distribution among *Triticeae*. *Genome* **38**:479–486.
- Parida SK, Kumar ARK, Dalal V, Singh NK, Mohapatra T. 2006.** Unigene derived microsatellite markers for the cereal genomes. *Theoretical and Applied Genetics* **112**:808-817.
- Paux E, Sourdille P, Salse J, et al. 2008.** A physical map of the 1-gigabase bread wheat chromosome 3B. *Science* **322**:101-104.
- Pfeifer M, Kugler KG, Sandve SR, et al. 2014.** Genome interplay in the grain transcriptome of hexaploid bread wheat. *Science* **345**:1250091. doi: 10.1126/science.1250091
- Qi L, Friebe B, Gill BS. 2006.** Complex genome rearrangements reveal evolutionary dynamics of pericentromeric regions in the *Triticeae*. *Genome* **49**:1628-1639.
- Quarrie SA, Gulli M, Calestani C, Steed A, Marmioli N. 1994.** Location of a gene regulation drought-induced abscisic acid production on the long arm of chromosome 5A of wheat. *Theoretical and Applied Genetics* **89**:794–800.
- Quraishi UM, Abrouk M, Bolot S, et al. 2009.** Genomics in cereals: from genome-wide conserved orthologous set (COS) sequences to candidate genes for trait dissection. *Functional & Integrative Genomics* **9**:473-484.

- Raskina O, Barber JC, Nevo E, Belyayev A. 2008.** Repetitive DNA and chromosomal rearrangements: speciation-related events in plant genomes. *Cytogenetic and Genome Research* **120**:351–357.
- Rayburn AL, Gill BS. 1986.** Isolation of a D genome specific repeated DNA sequence from *Aegilops squarrosa*. *Plant Molecular Biology Reporter* **4**:102-109.
- Rawat N, Tiwari VK, Singh N, et al. 2009.** Evaluation and utilization of *Aegilops* and wild *Triticum* species for enhancing iron and zinc content in wheat. *Genetic Resources and Crop Evolution* **56**:53-64.
- Riar AK, Kaur S, Dhaliwal HS, Singh K, Chhuneja P. 2012.** Introgression of a leaf rust resistance gene from *Aegilops caudata* to bread wheat. *Journal of Genetics* **91**:155-161.
- Riley R, Chapman V. 1967.** The inheritance in wheat of crossability with rye. *Genetics Research* **9**:259-267.
- Romero MD, Montes MJ, Sin E, et al. 1998.** A cereal cyst nematode (*Heterodera avenae* Woll.) resistance gene transferred from *Aegilops triuncialis* to hexaploid wheat. *Theoretical and Applied Genetics* **96**:1135–1140.
- Šafář J, Bartoš J, Janda J, et al. 2004.** Dissecting large and complex genomes: Flow sorting and BAC cloning of individual chromosomes from bread wheat. *Plant Journal* **39**:960-968.
- Sánchez-Morán E, Benavente E, Orellana J. 1999.** Simultaneous identification of A, B, D and R genomes by genomic in situ hybridization in wheat-rye derivatives. *Heredity* **83**:249-252.
- Schatz M, Langmead B, Salzberg S. 2010.** Cloud computing and the DNA data race. *Nature Biotechnology* **28**:691-693.
- Schneider A, Molnár I, Molnár-Láng M. 2008.** Utilisation of *Aegilops* (goatgrass) species to widen the genetic diversity of cultivated wheat. *Euphytica* **163**:1-19.

- Schwarzacher T, Leitch AR, Bennett MD, Heslop-Harrison JS. 1989.** In situ localization of parental genomes in a wild hybrid. *Annals of Botany* **64**:315-324.
- Sears ER. 1956.** The transfer of leaf-rust resistance from *Aegilops umbellulata* to wheat. *Brookhaven Symposia in Biology* **9**:1-22.
- Senerchia N, Felber F, Parisod C. 2014.** Contrasting evolutionary trajectories of multiple retrotransposons following independent allopolyploidy in wild wheats. *New Phytologist* **202**:975-985.
- Šimková H, Svensson JT, Condamine P, et al. 2008.** Coupling amplified DNA from flow-sorted chromosomes to high-density SNP mapping in barley. *BMC Genomics* **9**:294.
- Sitch LA, Snape JW, Firman SJ. 1985.** Intrachromosomal mapping of crossability genes in wheat (*Triticum aestivum*). *Theoretical and Applied Genetics* **70**:309-314.
- Snape JW, Semikhodskii A, Fish L, et al. 1997.** Mapping frost resistance loci in wheat and comparative mapping with other cereals. *Acta Agronomica Hungarica* **45**:265–270.
- Sutka J, Snape JW. 1989.** Location of a gene for frost resistance on chromosome 5A of wheat. *Euphytica* **42**:41–44.
- The International Wheat Genome Sequencing Consortium (IWGSC). 2014.** A chromosome-based draft sequence of the hexaploid bread wheat (*Triticum aestivum*) genome. *Science* **345**:1251788. doi:10.1126/science.1251788
- Tiwari VK, Wang SC, Sehgal S, et al. 2014.** SNP Discovery for mapping alien introgressions in wheat. *BMC Genomics* **15**:273. doi:10.1186/1471-2164-15-273.
- Vágújfalvi A, Galiba G, Cattivelli L, Dubcovsky J. 2003.** The cold regulated transcriptional activator *Cbf3* is linked to the frost-tolerance gene *Fr-A2* on wheat chromosome 5A. *Molecular Genetics and Genomics* **269**:60–67.

- Van Slageren MW. 1994.** *Wild wheats: a monograph of Aegilops L. and Amblyopyrum (Jaub. & Spach) Eig (Poaceae)*, Wageningen, the Netherlands: Agricultural University, Aleppo, Syria: International Center for Agricultural Research in Dry Areas.
- Vida Gy, Cséplő M, Gulyás G, Karsai I, et al. 2011.** Effectiveness of major resistance genes and identification of new sources for disease resistance in wheat. *Acta Agronomica Hungarica* 59:241-248. doi:10.1556/AAgr.59.2011.3.7
- Vrána J, Kubaláková M, Šimková H, Číhalíková J, Lysák MA, Doležel J. 2000.** Flow-sorting of mitotic chromosomes in common wheat (*Triticum aestivum* L.). *Genetics* 156:2033–2041.
- You FM, Huo N, Deal KR, et al. 2011.** Annotation-based genome-wide SNP discovery in the large and complex *Aegilops tauschii* genome using next-generation sequencing without a reference genome sequence. *BMC Genomics* 12:59.
- Yu J, Wang J, Lin W, et al. 2005.** The genomes of *Oryza sativa*: A history of duplications. *PLoS Biology* 3:e38. doi:10.1371/journal.pbio.0030038
- Zhang H, Jia J, Gale MD, Devos KM. 1998.** Relationships between the chromosomes of *Aegilops umbellulata* and wheat. *Theoretical and Applied Genetics* 96:69–75.

FIGURE LEGENDS

Fig. 1 Flow karyotypes obtained after the analysis of DAPI-stained chromosome suspensions prepared from *Ae. caudata* MvGB428 ($2n=2x=14$, CC) (A), *Ae. triuncialis* MvGB585 ($2n=4x=28$, $U^tU^tC^tC^t$) (B) and *Ae. cylindrica* MvGB1719 ($2n=2x=14$, $D^cD^cC^cC^c$) (C). The flow karyotype of *Ae. caudata* consists of two composite peaks, I and II, representing groups of three and two chromosomes, respectively, while peaks III and IV are specific for chromosomes 5C and 4C, respectively. The flow karyotype of *Ae. triuncialis* comprises three composite peaks, III, IV and V, representing chromosome groups of three to five chromosomes, while peaks I and III are specific for chromosomes T1 and $7C^t$, respectively. The flow karyotype of *Ae. cylindrica* consists of four composite peaks, I, III, IV and VI, representing chromosome groups of two to four chromosomes, while peaks II and V are specific for chromosomes $1C^c$ and $5D^c$, respectively.

Fig. 2 Molecular cytogenetic identification of mitotic chromosomes in *Ae. caudata* MvGB428 (A), *Ae. cylindrica* MvGB1719 (B, C) and *Ae. triuncialis* MvGB585 (D, E) by fluorescence *in situ* hybridization (FISH) (A, B, D) using Afa family, pSc119.2 and pTa71 repetitive DNA probes and by two-colour genomic *in situ* hybridization (GISH) using D- and C- (C), or U- and C-genome probes (E). On the FISH images (A, B, D) pSc119.2 sites are green, Afa family signals are red and 18S rDNA signals are yellow. On the GISH images the C genome is visualized in green (C, E), while the D (C) and U genomes (E) are in brown. Disomic U/C reciprocal translocations (T1: $6U^tS.6U^tL-5C^tL$ and T2: $5C^tS.5C^tL-6U^tL$) in *Ae. triuncialis* are indicated by arrows. Scale bar = 10 μ m.

Fig. 3 Idiogram and karyotype of the chromosomes of *Ae. caudata* MvGB428 (C), *Ae. cylindrica* MvG1719 (C^c, D^c) and *Ae. triuncialis* MvGB585 (C^t, U^t) showing the genomic distribution of repetitive DNA sequences pSc119.2 (green), Afa family (red) and 18S rDNA (yellow). Disomic U/C reciprocal translocations T16U^tS.6U^tL-5C^tL and T25C^tS.5C^tL-6U^tL in *Ae. triuncialis* are indicated by * and **, respectively. The positions for hybridization signals and translocation breakpoints on the chromosome arms are given in fraction lengths determined as means of 15-20 measurements.

Fig. 4 Mitotic metaphase chromosomes sorted from individual peaks of flow karyotypes of *Ae. caudata* MvGB428, *Ae. triuncialis* MvGB585 and *Ae. cylindrica* MvGB1719. The sorted chromosomes were identified after FISH with probes for repetitive DNA sequences pSc119.2 (green), Afa family (red) and 18S rDNA (yellow). The chromosomes were assigned to the peaks in which their frequencies were the highest. Four representative examples are given for each chromosome.

Fig. 5 PCR products of conserved orthologous set (COS) markers amplified from bread wheat genotype Mv9kr1 (Mv), from total genomic DNA (gDNA) and from subgenomic DNA samples derived from chromosomes of specific peaks on flow karyotypes (I-VI) of *Ae. caudata* MvGB428, *Ae. triuncialis* MvGB585 and *Ae. cylindrica* MvGB1719. The representative pictures shows markers $X_{GPI:C:731424}$ (A) and $X_{GPI:C:725135}$ (B), specific for group 4 chromosomes of wheat.

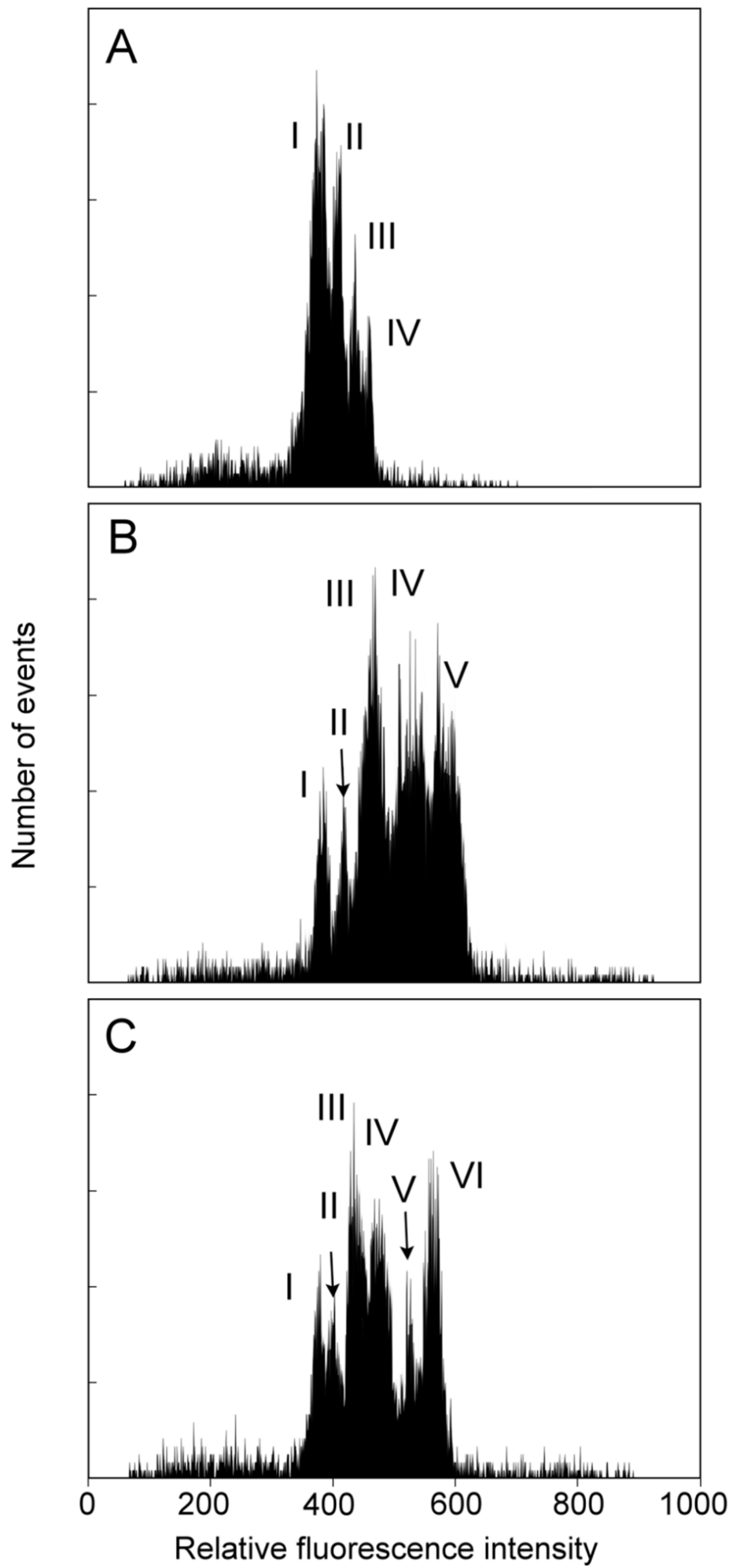
Table 1. Assignment of chromosomes to peaks on flow karyotypes of *Aegilops caudata*, *Ae. cylindrica* and *Ae. triuncialis*. The numbers give the relative proportion (as a percentage) of specific chromosome types in individual peaks. The highest chromosome concentrations are highlighted in bold.

Species	Genome	Chromosome peaks						
		I	II	III	IV	V	VI	
<i>Ae. caudata</i>	C	1	38.03	3.54	-	0.50		
		2	3.52	45.02	11.10	-		
		3	6.86	28.20	6.48	-		
		4	-	-	16.20	91.37		
		5	0.74	7.44	66.20	8.12		
		6	32.09	4.78	-	-		
		7	18.73	0.79	-	-		
No. of chromosomes analysed		539	376	216	197			
<i>Ae. cylindrica</i>	C ^c	1	13.46	67.50	-	-	-	-
		2	1.90	-	0.90	24.50	1.2	0.5
		3	-	-	21.60	-	-	-
		4	-	-	3.10	27.0	0.8	-
		5	-	-	13.40	17.3	-	-
		6	44.80	4.20	-	-	-	-
		7	36.50	9.10	-	-	-	-
	D ^c	1	3.20	16.20	26.80	1.6	-	-
		2	-	-	-	-	2.0	34.5
		3	-	-	-	4.4	8.94	45.6
		4	-	-	-	20.9	-	-
		5	-	-	-	0.8	84.95	0.5
		6	-	2.80	33.90	3.2	-	-
		7	-	-	-	-	0.8	18.7
No. of chromosomes analysed		156	142	439	248	246	197	
<i>Ae. triuncialis</i>	C ^t	1	-	14.43	14.14	31.22	-	
		2	-	-	-	6.69	8.78	
		3	-	-	21.21	0.74	-	
		4	-	0.53	0.67	29.36	-	
		6	-	7.48	17.84	0.74	-	
		7	9.85	70.05	2.02	0.37	-	
		U ^t	1	-	4.27	16.49	-	-
	2		-	-	-	1.85	16.21	
	3		-	-	28.28	-	-	
	4		-	-	-	28.62	-	
	5		-	-	-	-	22.29	
	7		-	-	-	0.37	19.59	
	T1		90.15	3.20	-	-	-	
	T2	-	-	-	-	33.1		
No. of chromosomes analysed		193	187	297	269	148		

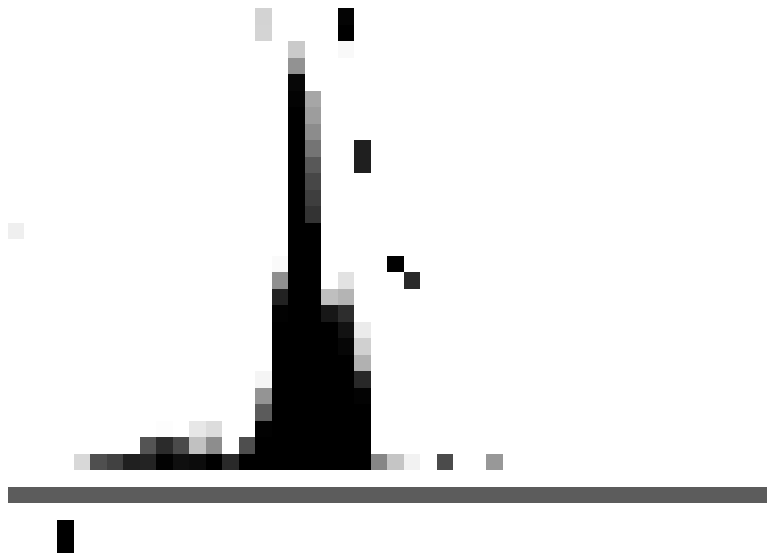
Table 2. Assignment of COS markers specific for wheat homoeologous groups 1-7 to peaks I-VI on flow karyotypes of *Ae. caudata* MvGB428, *Ae. triuncialis* MvGB585 and *Ae. cylindrica* MvGB1719. The putative chromosomal location of the markers is given in brackets.

Marker	Hom. Group in wheat	<i>Ae. caudata</i>				<i>Ae. cylindrica</i>						<i>Ae. triuncialis</i>					
		I	II	III	IV	I	II	III	IV	V	VI	I	II	III	IV	V	
X _{BE443103}	1	X (1C)					X (1C)	X (1D)							X (1U)	X (1C)	
X _{GPI:C:732519}	1	X (1C)					X (1C)	X (1D)								X (1C)	
X _{GPI:C:746781}	1	X (1C)					X(1C)	X (1D)								X (1C)	X
X _{BE445693}	2	X				X							X				
X _{tr150}	2			X (5C)					X (5C)	X (5D)							X (5C,5U)
X _{GPI:C:746665}	2	X(1C)					X (1C)	X									X (2U)
X _{2N}	2	X(1C)					X (1C)					X (2D)	X				X (2U)
X _{3B}	3	X						X	X			X (3D)			X (3U)		
X _{BM134465}	3	X							X						X (3U)		
X _{BE404709}	3	X				X				X	X (3D)				X (3U)	X	
X _{GPI:C:748004}	4				X (4C)				X (4C,4D)							X (4C,4U)	
X _{BE496216}	4	X				X			X (4C,4D)							X (4C,4U)	
X _{GPI:C:731424}	4				X (4C)				X (4C,4D)							X (4C,4U)	
X _{GPI:C:725135}	4				X (4C)				X (4C,4D)							X (4C,4U)	
X _{4S}	4				X (4C)				X (4C,4D)							X (4C,4U)	
X _{GPI:C:729592}	5			X (5C)					X (5C)	X (5D)							X
X _{GPI:C:748166}	5								X (5C)	X (5D)		X(5C)					
X _{5A}	5	X (7C)		X (5C)		X			X (5C)	X (5D)	X		X (7C)				X (5C)
X _{tr77}	5	X							X (5C)		X	X (7C)					
X _{GPI:C:717465}	5			X (5C)						X (5D)		X (5C)					
X _{GPI:C:726959}	5			X (5C)					X (5C)	X (5D)							X (5U,5C)
X _{6A}	6		X			X	X (1C)							X			X

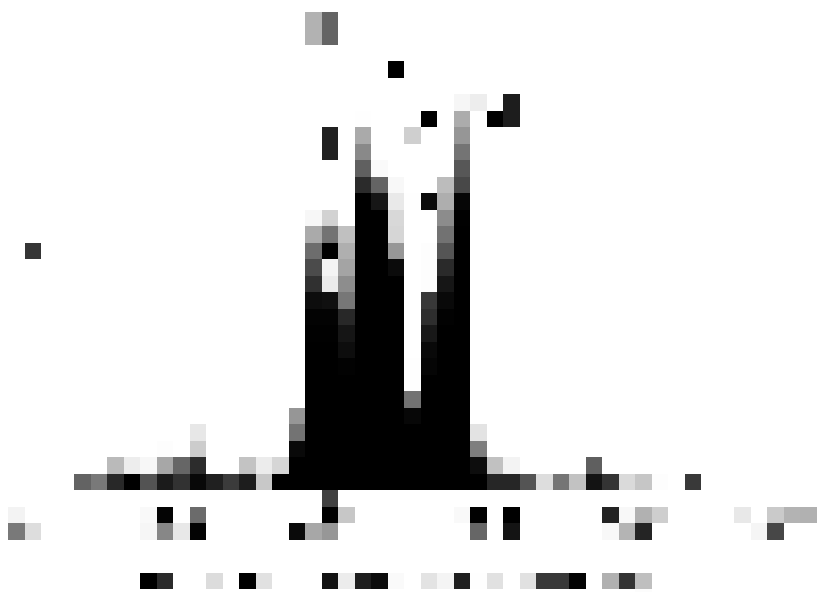
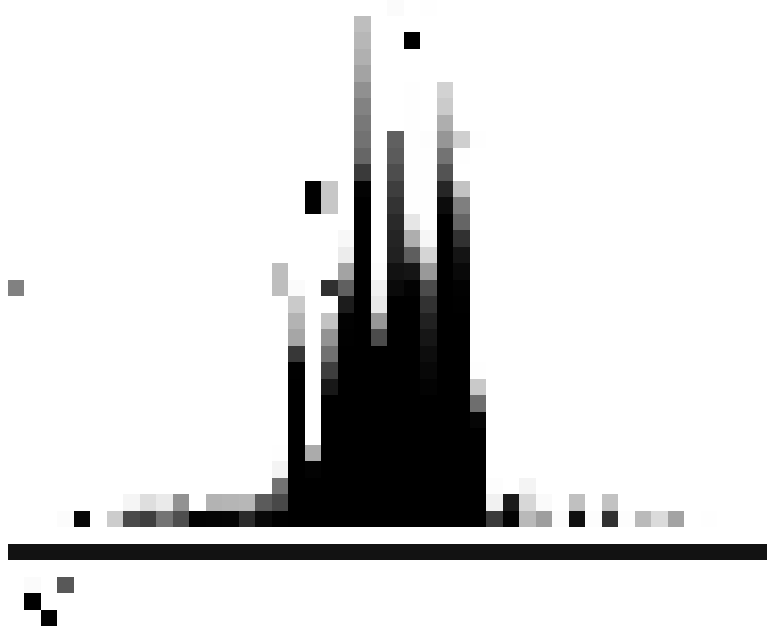
X _{6N}	6			X (5C)			X (1C)								X
X _{GPI:C:740549}	6	X						X							X
X _{BG264012}	7	X	X (3C)							X (7D)			X (3C)		
X _{BE352570}	7			X (5C)			X	X (3C)		X (7D)			X (3C)		X (7U)
X _{GPI:C:771171}	7		X (3C)					X (3C)		X (7D)					X (7U)
X _{GPI:C:770073}	7		X (3C)					X (3C)		X (7D)			X (3C)		X (7U)
X _{GPI:C:767323}	7	X	X (3C)					X (3C)		X (7D)			X (3C)		X (7U)

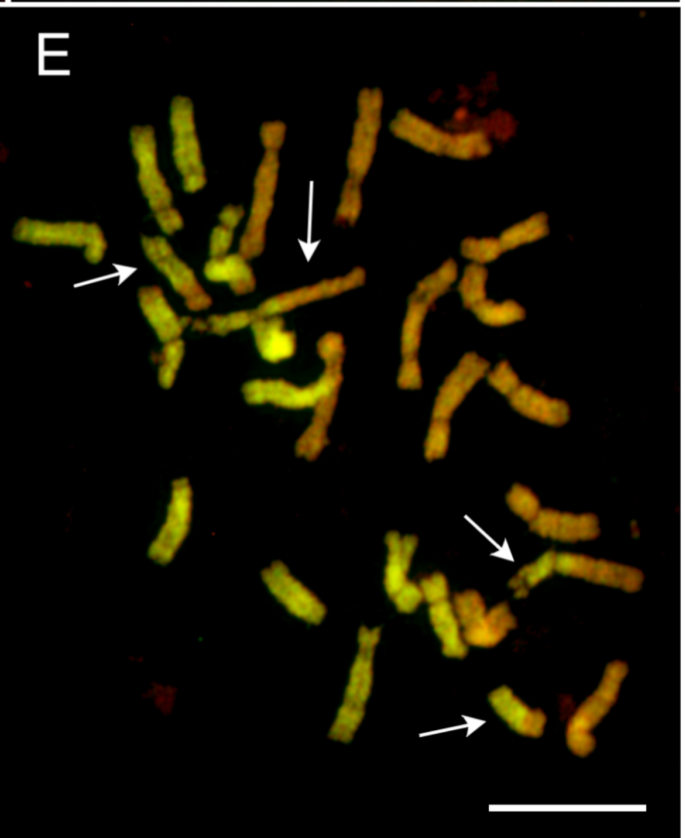
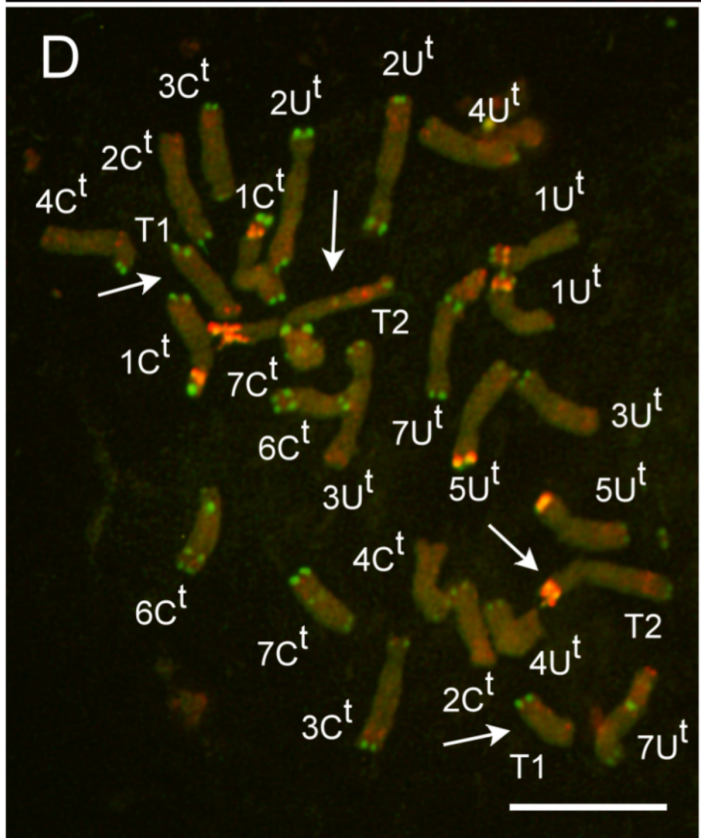
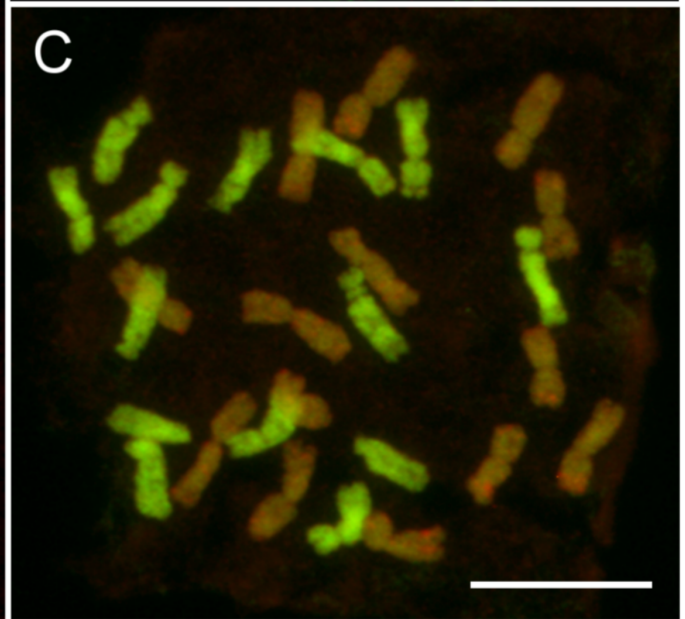
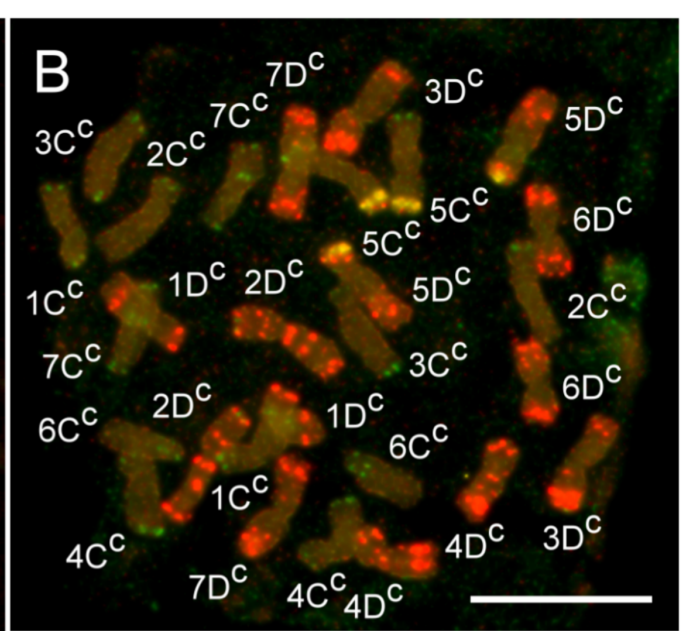
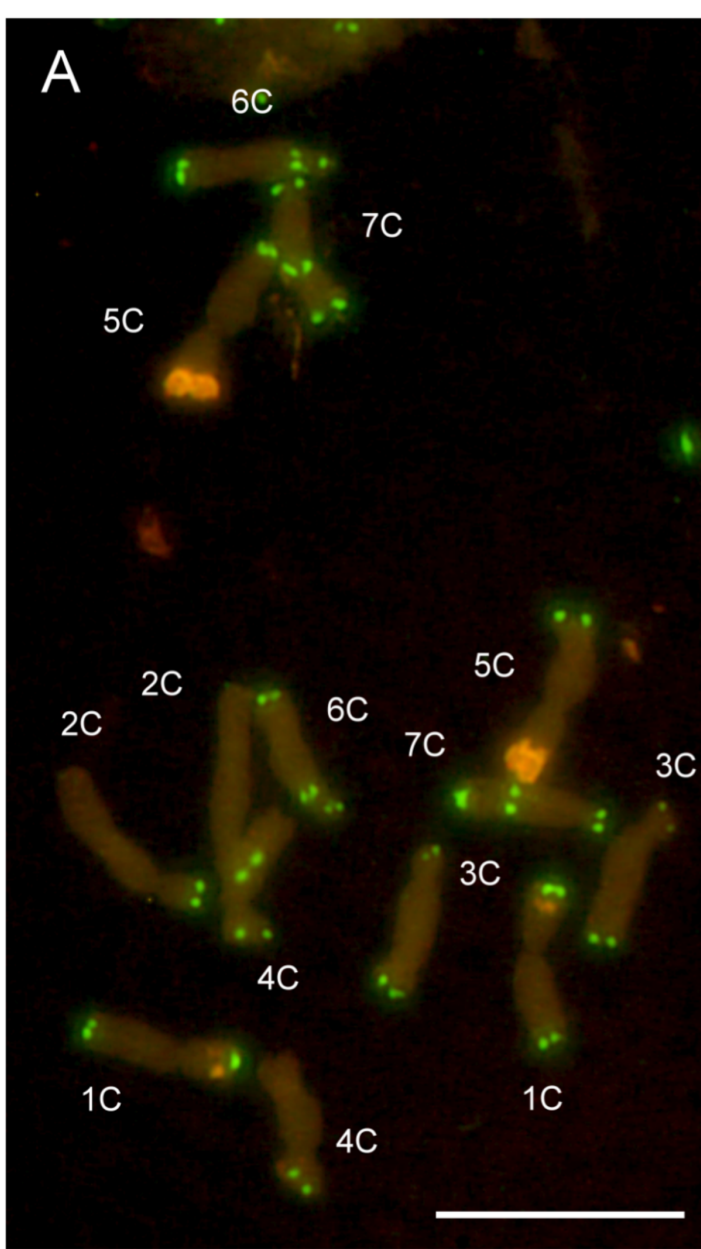


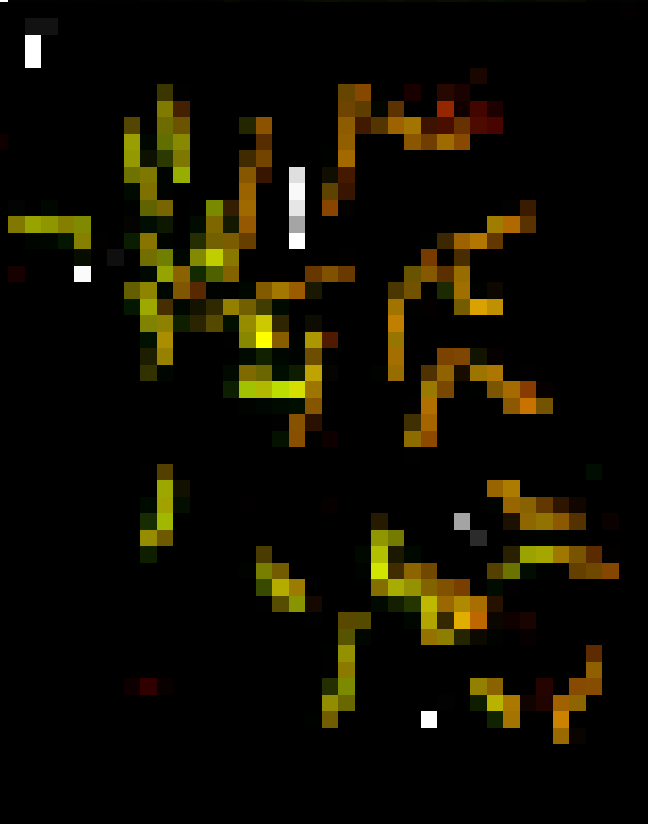
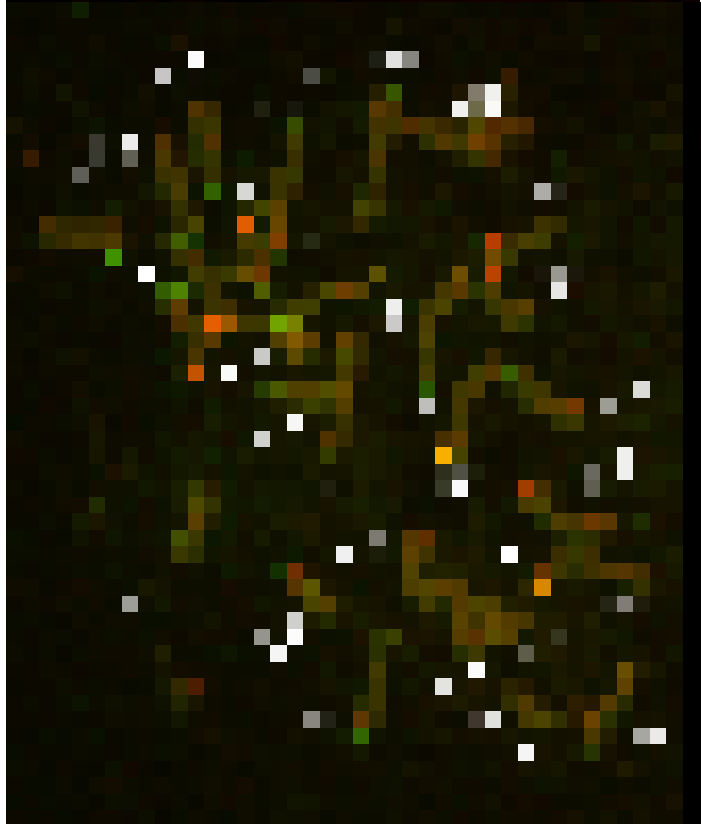
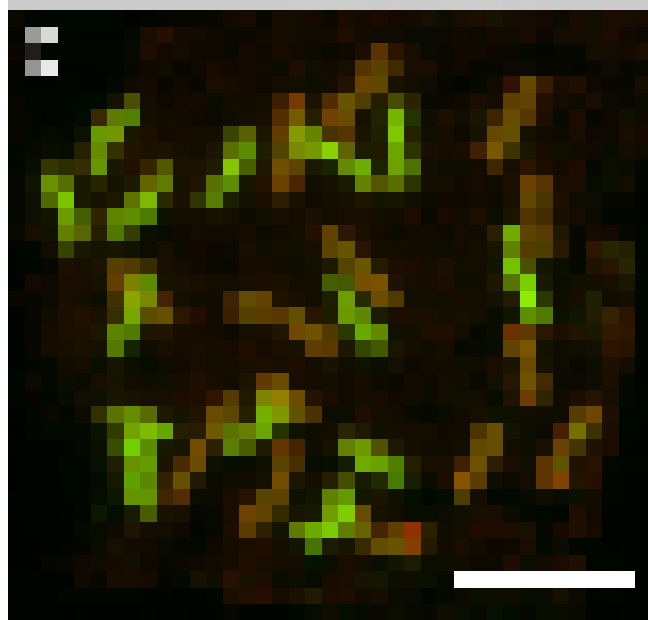
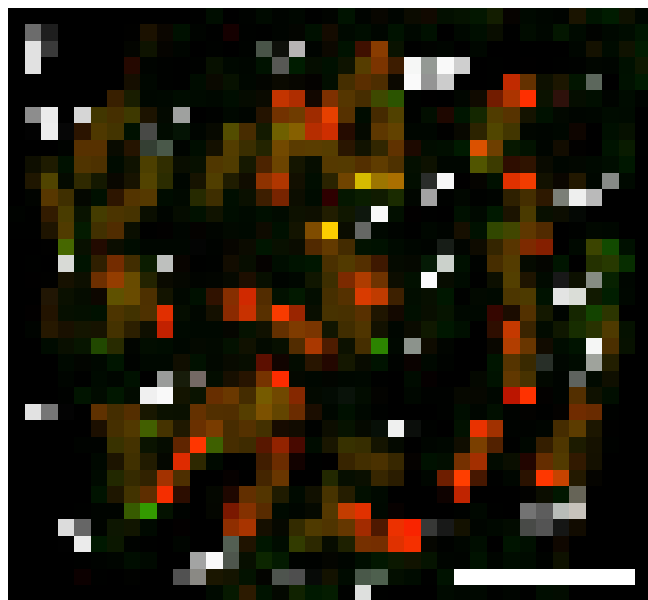
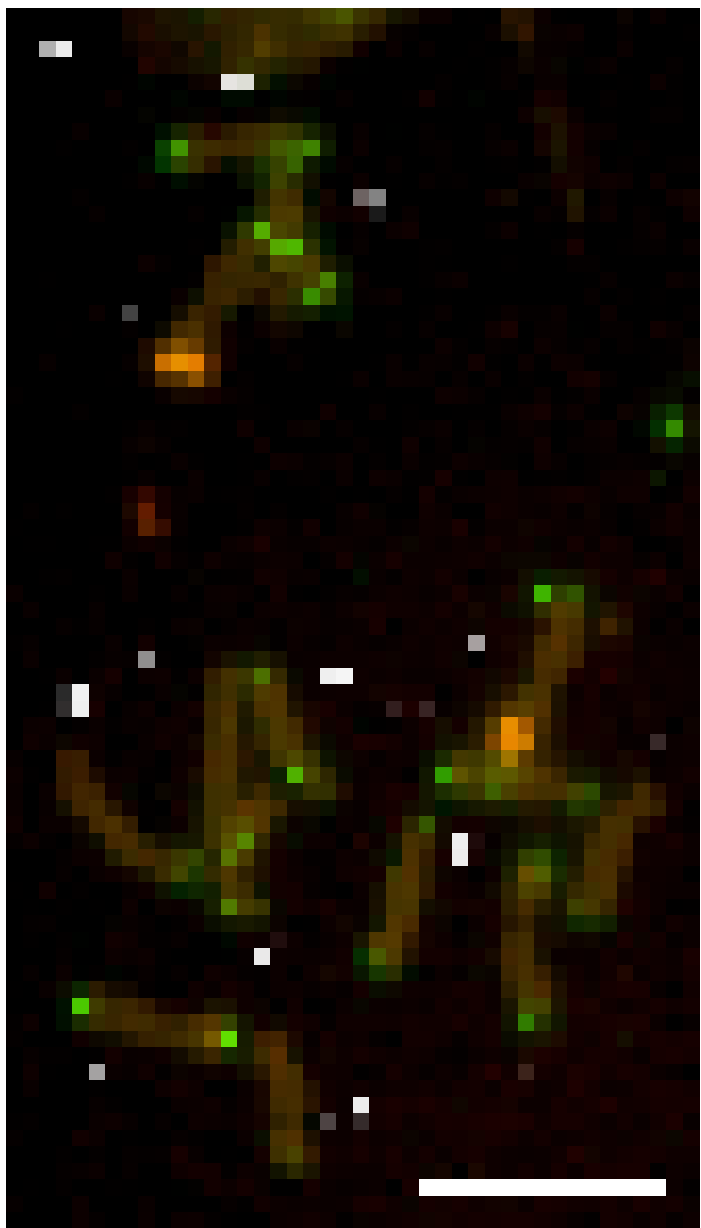
■
■ ■



■
■
■
■
■
■
■
■







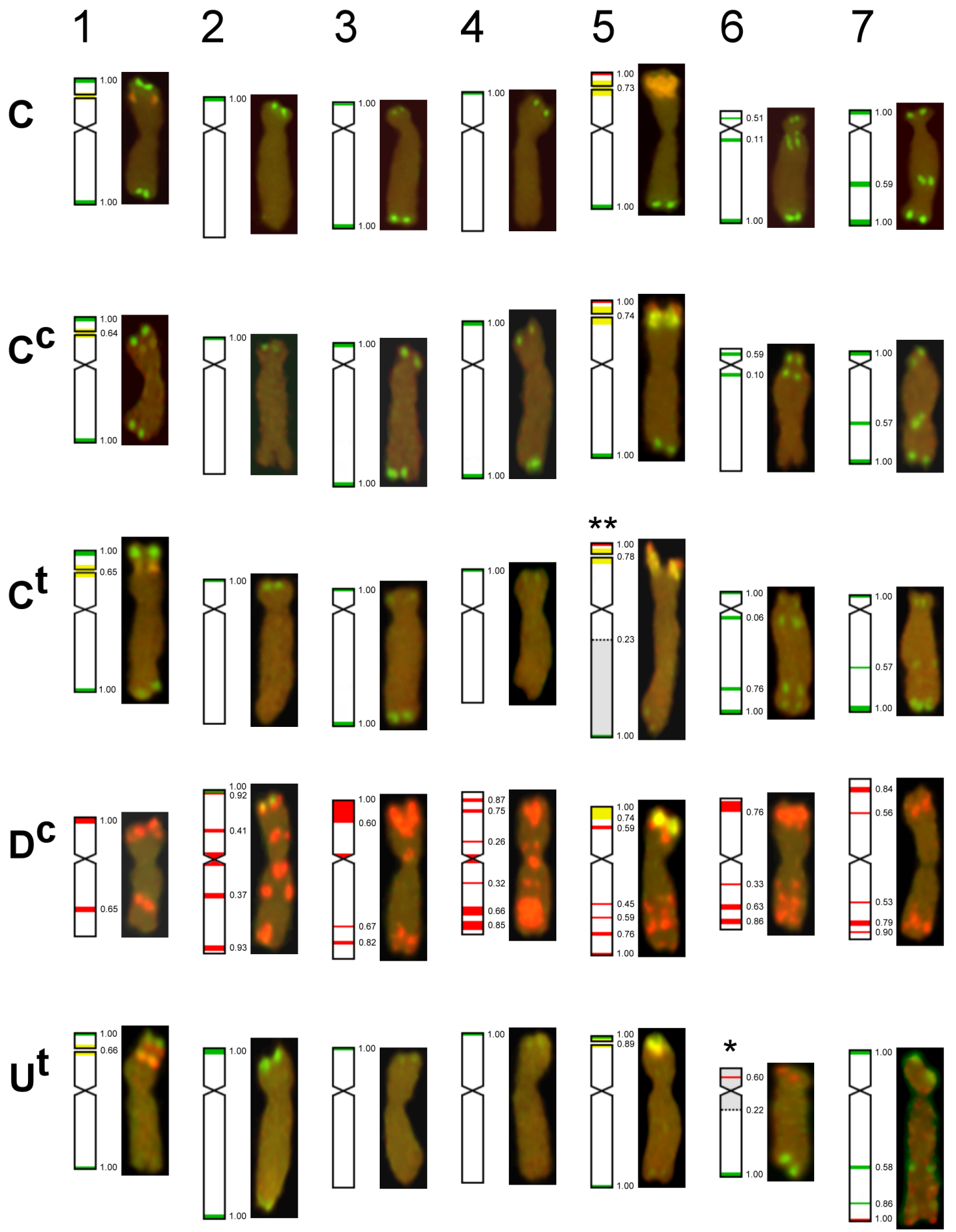

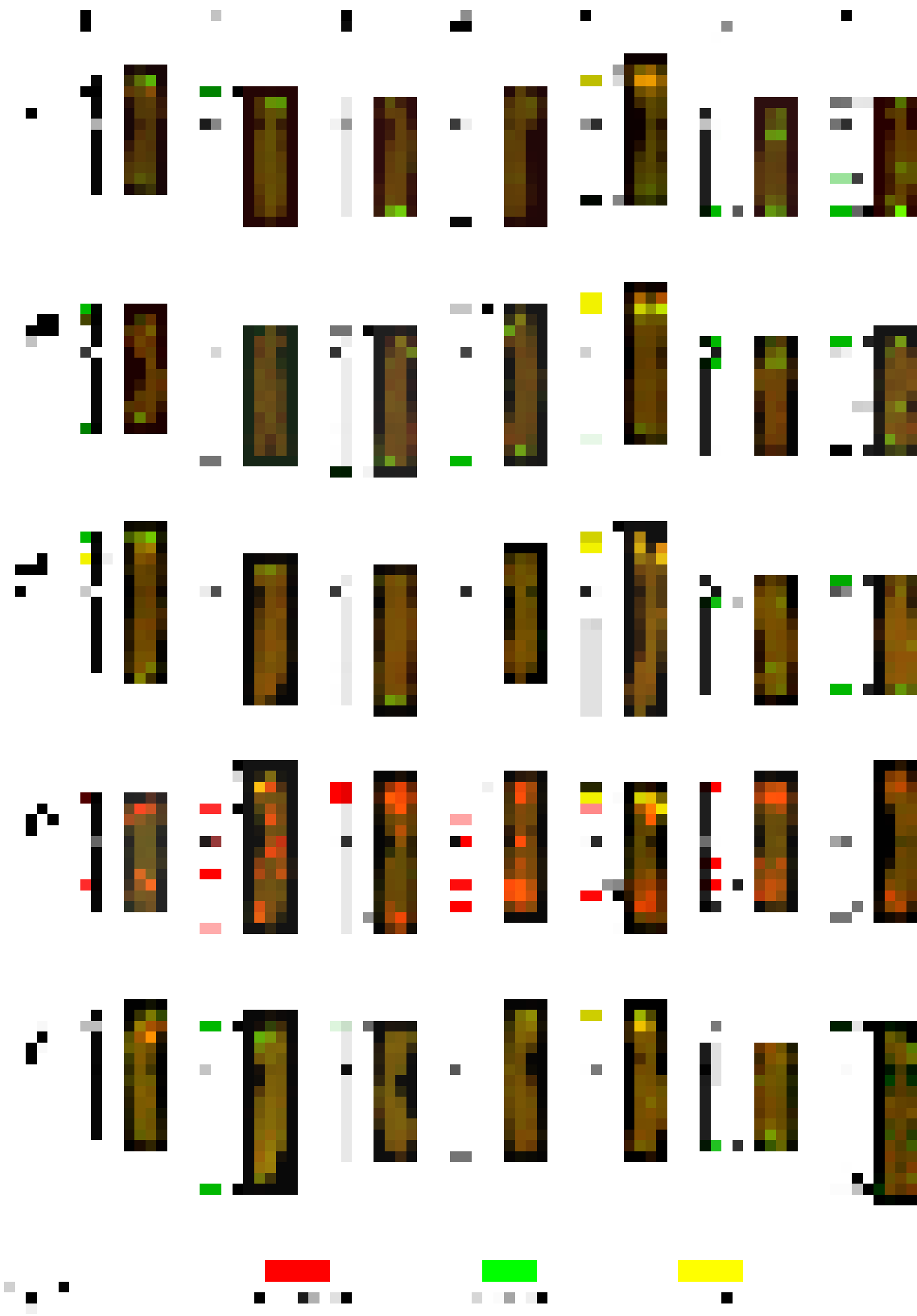


Fig.3

 Afa family

 pSc119.2

 18S



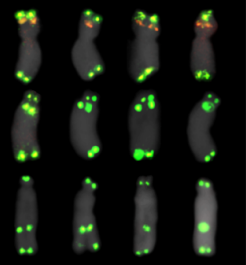
Ae. caudata

Ae. triuncialis

Ae. cylindrica

Peak I.

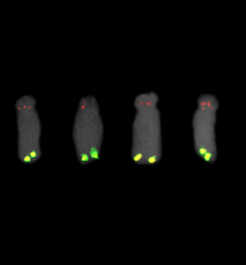
1C



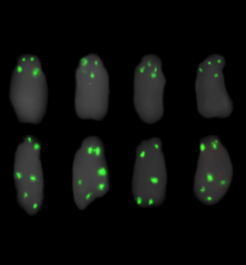
6C

7C

T1



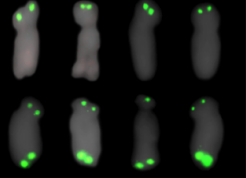
6C^c



7C^c

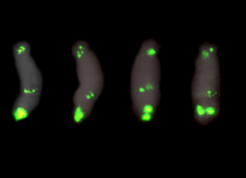
Peak II.

2C

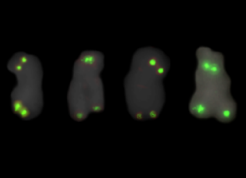


3C

7C^t

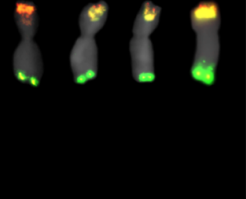


1C^c



Peak III.

5C

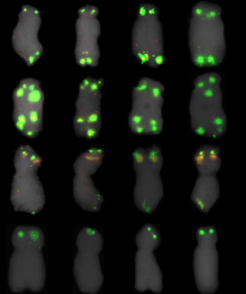


3C^t

6C^t

1U^t

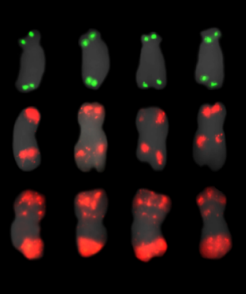
3U^t



3C^c

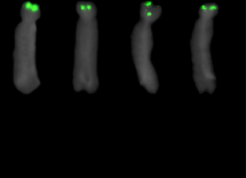
1D^c

6D^c



Peak IV.

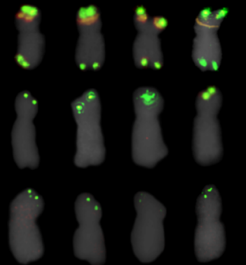
4C



1C^t

4C^t

4U^t

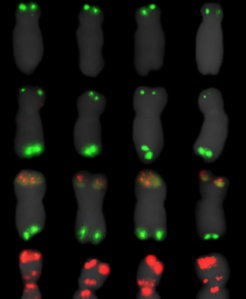


2C^c

4C^c

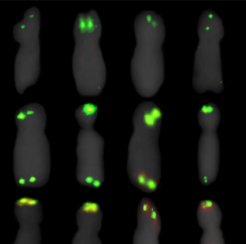
5C^c

4D^c



Peak V.

2C^t

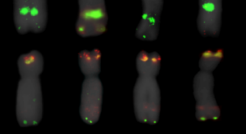


2U^t

5U^t

7U^t

T2

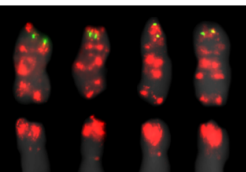


5D^c



Peak VI.

2D^c



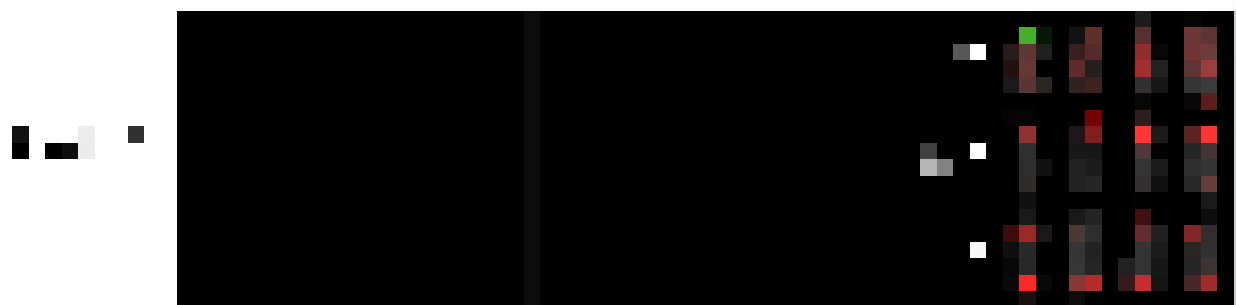
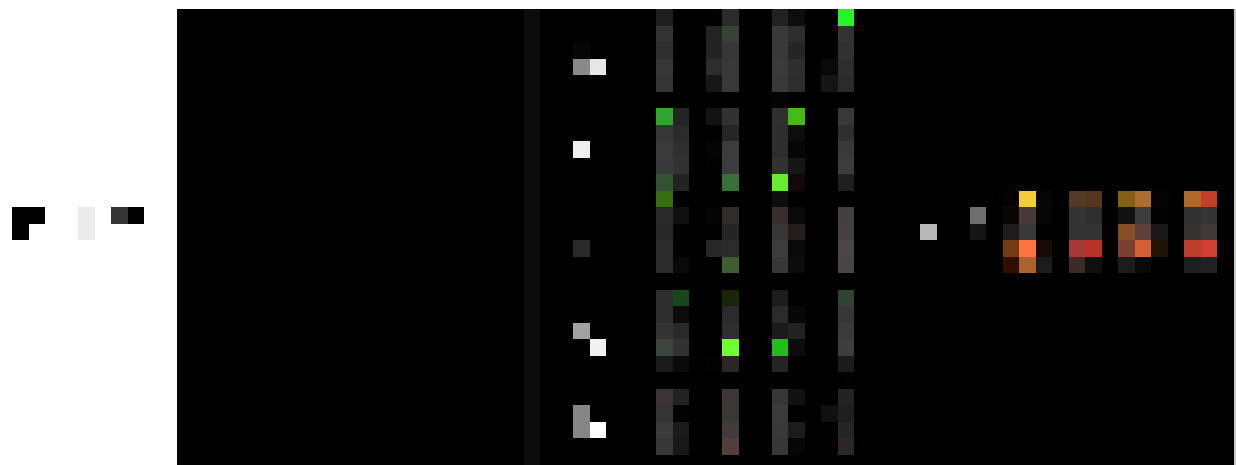
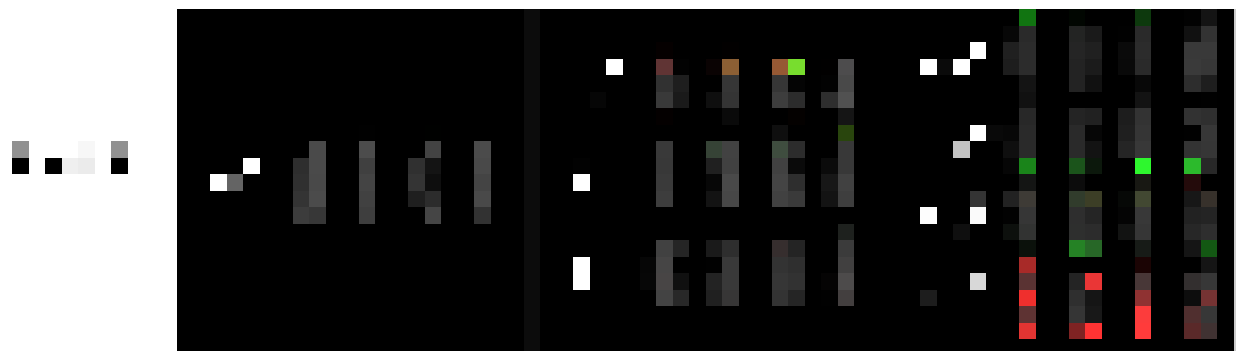
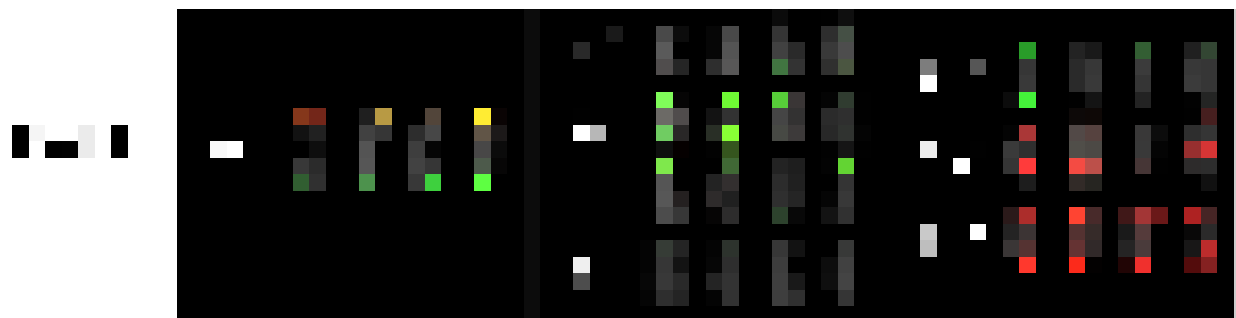
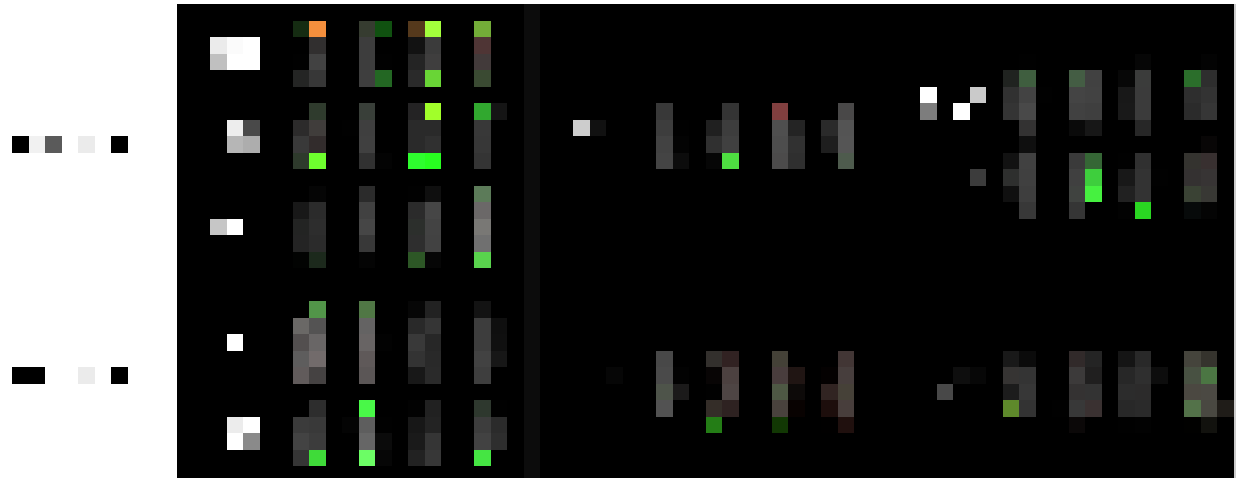
3D^c

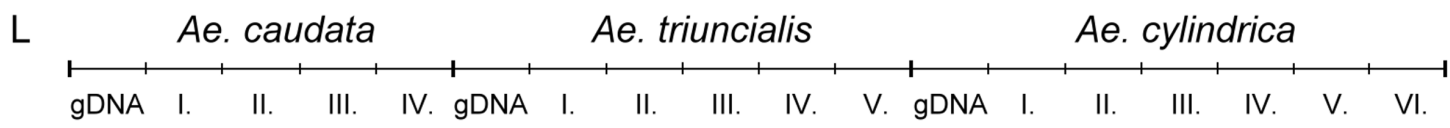
7D^c

0 1 2 3 4 5 6 7 8 9

0 1 2 3 4 5 6 7 8 9

0 1 2 3 4 5 6 7 8 9





A



B



