Analysis of the B chromosomes undergoing root-specific elimination during the embryogenesis of *Aegilops speltoides*

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1. Introduction

1.1. General features of B chromosomes

The basic diploid chromosome set is essential for normal growth and development of an organism. The loss or gain of even one chromosome can cause changes of the normal developmental course. However, some extra chromosomes exist, which may occur as an optional addition to the standard chromosome set in all eukaryotic phyla (Houben et al., 2013a). These supernumerary chromosomes do not affect significantly the development and fitness of an organism, when present in a low number. In general, chromosomes, which are dispensable and occur in addition to the basic chromosome complement in some individuals, may be referred to as B chromosomes, accessory or supernumerary chromosomes. Here we consider these designations as equal. The term "B chromosome" (Bs) was proposed by Randolph (1928) to distinguish between supernumerary chromosomes and the chromosomes of the basic complement. The standard chromosomes are called "A chromosomes" (As). B chromosomes are often considered as selfish autonomous elements, so-called genetic parasites, bearing no advantages to the host and exploiting its cellular machinery to successfully propagate themselves (Burt and Trivers, 2008). Wilson (1907) discovered the first B chromosomes in an insect of the genus *Metapodius*. In plants, the first Bs were found in *Secale cereale* L. (Nakao, 1911).

The three major criteria to distinguish Bs from chromosome fragments and other types of extra chromosomes are as follows (Jones and Houben, 2003; Jones et al., 2008):

(i) Bs are an optional addition to the host genome and may be present or absent from individuals in the same population;

(ii) Bs never pair and recombine with A chromosomes during meiosis;

(iii) the inheritance of Bs is non-Mendelian and irregular.

In plants, B chromosomes exist mainly in wild or semi-wild populations and were rarely found in cultivated forms (Jones et al., 2008). Their occurrence is more common in outbreeders than in inbreeding species (Burt and Trivers, 1998). The preferential occurrence of Bs in outbreeding plant species was previously discussed in the context of balancing detrimental effects of B chromosomes in high numbers by crosses with individuals devoid of Bs (Palestis et al., 2004). A relationship between

genome size and the existence of Bs was noticed, too. Most species with Bs were discovered among organisms with a large genome compared to those with a small genome. A possible explanation of this phenomenon is that organisms with larger genomes could better tolerate B chromosome associated effects (Palestis et al., 2004; Trivers et al., 2004).

Recently a database summarizing all the information available on the B chromosome carrying species was generated (D'Ambrosio et al., 2017). It includes more than five thousand entries and the total number of species known to carry B chromosomes is estimated to be 2828 (D'Ambrosio et al., 2017). Despite that Bs are widespread in all eukaryotic taxa, including animals, fungi and plants, little is understood about the origin, maintenance and accumulation mechanisms of this enigmatic constituent of the genome. In plants, B chromosomes may have potential applications for crop improvement and genetic studies, and intense research were devoted to this subject. However, a general overview of this topic is not available yet.

1.2. B chromosome carrying species in the genus *Aegilops*

Aegilops is evolutionary the closest relative to one of the most economically important cereal species – wheat (*Triticum*). *Triticum* and *Aegilops* are considered to be among the youngest genera in the tribe Triticeae (Bernhardt et al., 2017). The taxonomy of *Aegilops* is complicated and different taxonomic classifications exist. The currently most comprehensive and commonly accepted one is the system revised by Van Slageren (1994). *Aegilops* species contributed two of the three subgenomes of hexaploid wheat. Although, there are still disputes about the identity of those particular species. The most supported theory is that *Aegilops speltoides* TAUSCH. and *Aegilops tauschii* COSS. possess the genomes closest to the B- and D-subgenomes of wheat, and may be considered as their donors (Miki et al., 2019; Petersen et al., 2006).

Ae. speltoides (2n=2x=14, genome type: S) belongs to the section Sitopsis which includes also four other diploid S-genome carrying species, namely *Aegilops bicornis* (FORSSK.) JAUB. & SPACH, *Aegilops searsii* FELDMAN & KISLEV EX HAMMER, *Aegilops sharonensis* EIG and *Aegilops longissima* SCHWEINF. & MUSCHL. These putative donors of the wheat B-subgenome were extensively studied at the chromosomal and molecular level (Haider, 2013). However, a comparative study of

the S-genome karyotype evolution based on a combination of Giemsa C-banding and fluorescent *in situ* hybridization (FISH) for all diploid and polyploid species is missing.

The genus *Aegilops* is a part of the wheat secondary gene pool and gene transfer from *Aegilops* into wheat is possible once the interspecific barriers are overcome (Harlan and de Wet, 1971). Due to the adaptation of *Aegilops* species to different environmental conditions and resistance to biotic and abiotic factors, they are often used in wheat pre-breeding (Schneider et al., 2008). Another interesting feature is that some diploid *Aegilops* species are known to possess B chromosomes.

B chromosomes in the genus *Aegilops* were discovered relatively late, 1957 in *Aegilops mutica* BOISS. (syn. *Triticum tripsacoides* (JAUB. & SPACH) BOWDEN and *Amblyopyrum muticum* (BOISS.) EIG) (Mochizuki, 1957). In 1971, Bs were reported in *Ae. speltoides* (Simchen et al., 1971). Except for the general characteristics of Bs, such as their supernumerary nature, complete loss of recombination with A chromosomes and non-Mendelian inheritance, *Aegilops* Bs demonstrate another peculiar feature. While their number is constant in the aerial organs of B-carrying individuals, Bs are completely absent from the roots (Mendelson and Zohary, 1972; Ohta, 1995a).

Beside in *Ae. speltoides* and *Ae. mutica*, B chromosome candidates were also reported in self-pollinating species of the genus *Aegilops*, such as the diploid species *Ae. tauschii* (Mehra and Sharma, 1977; Sheidai, 2008) and *Aegilops umbellulata* ZHUK. (Sheidai et al., 2002), and the tetraploid species *Aegilops cylindrica* HOST (Chennaveeraiah and Löve, 1959; Ghorbani et al., 2015; Sheidai, 2008), *Aegilops columnaris* ZHUK. (Chennaveeraiah and Löve, 1959) and *Aegilops triuncialis* L. (Sheidai, 2008; Sheidai et al., 2002). However, it is not clear whether the reported 'supernumerary' chromosomes are truly B chromosomes or represent fragmented or rearranged A chromosomes.

1.3. Characteristics of *Ae. speltoides* B chromosomes

Ae. speltoides may carry up to eight (Raskina et al., 2004), submetacentric B chromosomes characterized by prominent telomeric and interstitial Giemsa C-bands (Friebe et al., 1995). The length of a B chromosome is about 2/3 of the average A chromosome length (Simchen et al., 1971). Friebe et al. (1995) identified the first B-located repeats (pSc119.2 repeat and 5S rDNA) of *Ae. speltoides* in both arms of the

Bs (Friebe et al., 1995). Depending on the genotype, a 5S rDNA locus can be observed only in one B chromosome arm (Raskina et al., 2011). Intercalary clusters of the Spelt1 tandem repeat and the Ty3-gypsy retroelement were detected in the long arm of *Ae. speltoides* Bs (Belyayev and Raskina, 2013; Hosid et al., 2012). Aside from these previously known repeats usually abundant in Triticeae species, there are no other data available on the molecular composition of the *Ae. speltoides* B chromosome. Interestingly, the B of rye possesses a high amount of plastid- and mitochondrion-derived sequences (Martis et al., 2012). Whether the B of *Ae. speltoides* shows a comparable accumulation of organellar DNA was hitherto an open question.

The evolutionary relationship of *Ae. speltoides*, *Ae. mutica* and rye B chromosomes is not yet understood. Giemsa C-banding analysis has shown that the Bs of *Ae. speltoides* and *Ae. mutica* differ from each other by the amount and distribution of constitutive, Giemsa C-banding positive heterochromatin (Friebe et al., 1995). There is also no meiotic pairing observed between *Ae. speltoides* and *Ae. mutica* Bs in hybrids of these two species (Vardi and Dover, 1972). This may be considered as an indication that Bs of these two *Aegilops* species are rather distant from each other. Until now, there are no data available on the interaction between *Aegilops* and rye Bs in the same genetic background.

It is tempting to ask whether a proto-B arose in a common ancestor of *Aegilops* and *Secale*. After divergence of both genera, have the B chromosomes evolved further in their respective species, or did the Bs originate independently? Based on the latest data on Triticeae phylogeny, *Secale* and *Taeniatherum caput-medusae* (L.) Nevski are closest relatives of the *Aegilops*/*Triticum* complex, as shown in Figure 1 (Bernhardt et al., 2017). So far, there is no information available on the age, sequence content and possible origin of *Ae. speltoides* and *Ae. mutica* Bs. Such data further combined with what is known about the B chromosomes of the closest B-carrying genus *Secale*, would help to shed light on the evolutionary pathways of Triticeae B chromosomes.

The physiological effects of the presence of B chromosomes on the host organism are difficult to study. This is because Bs are mainly present in outbreeding species and it is not always possible to differentiate between effects caused by the Bs from those arising due to natural variation of A chromosome-located genes.

Nevertheless, some authors report that the presence of one to three Bs has an effect on *Ae. speltoides* plants, such as an earlier emergence of spikes, which at certain conditions may appear to be beneficial. In contrast, a higher number of Bs has a negative effect on plant fitness and increases the number of aborted pollen (Belyayev et al., 2010; Mendelson and Zohary, 1972). It has also been reported that the plant vigour is not much affected by Bs, but the reproductive fitness may depend on their number (Cebria et al., 1995).
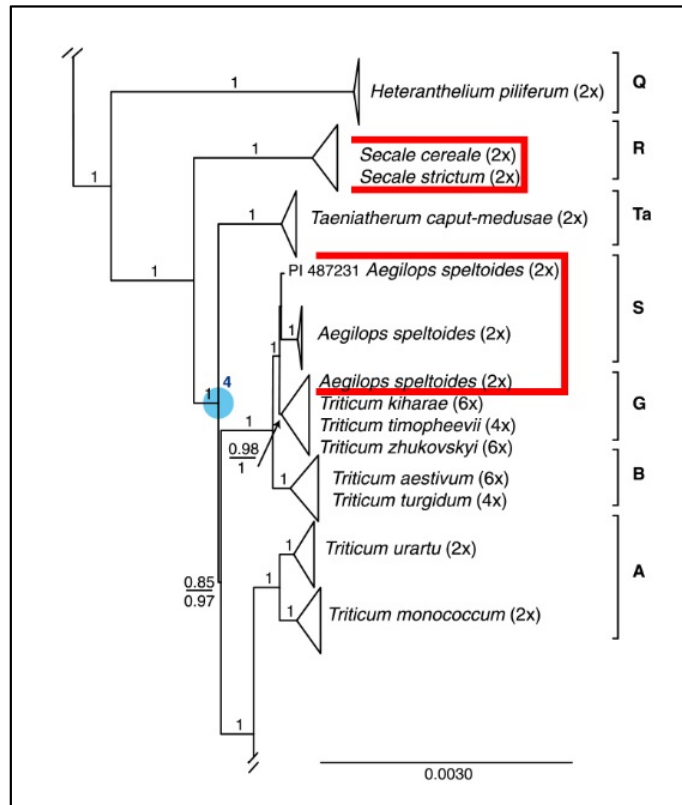
Figure 1. Dated phylogenetic tree derived from the sequences of entire chloroplast genomes showing the relationships of *Secale* and *Aegilops*/*Triticum* (modified from Bernhardt et al., 2017). The age of the node indicated with a blue circle is 4.1-3.4 M years. The letters on the right indicate genomic groups.

The behaviour of Bs during meiosis and pollen grain mitosis in *Ae. speltoides* was described first by Mendelson and Zohary (1972). In case of one B, two scenarios are possible. A single B is lagging at anaphase I and telophase I and, as a result, it is not included into daughter nuclei and forms micronuclei at the end of meiosis. Alternatively, the B can migrate towards one pole and gets included in a daughter nucleus in 15 - 20% of the cells. Meiosis of plants with two Bs appears regular if they form a bivalent, and their segregation proceeds normally, as was observed in 50% of

cases. Otherwise, two Bs do not pair and appear as univalents in meiosis I. If the number of Bs is higher than two, different chromosome associations from univalents to multivalents are possible (Figure 2).
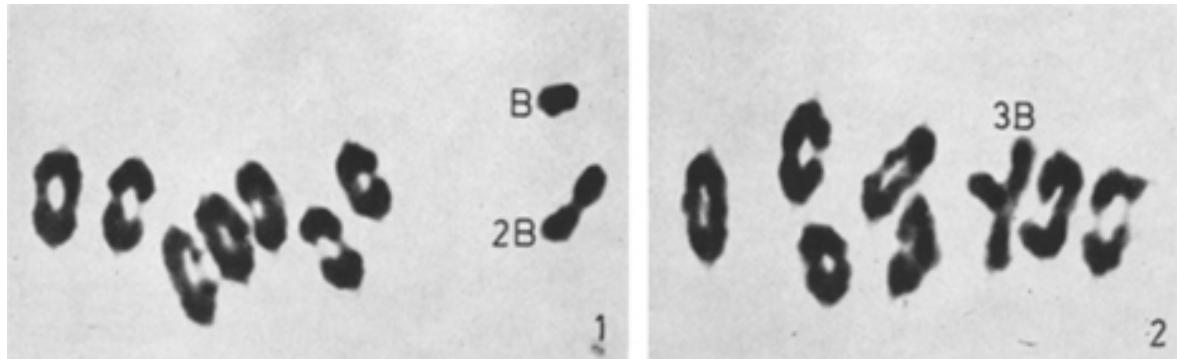
Figure 2. Meiotic metaphase I pairing of *Ae. speltoides* B chromosomes in a plant with 3 Bs (adopted from Simchen et al., 1971). (1) Formation of one univalent and one bivalent B. (2) All three B chromosome pair and form a trivalent.

A specific accumulation mechanism, also known as B chromosome drive, is essential for the successful maintenance of a selfish chromosome. Drive increases the number of Bs in the progeny and balances their meiotic loss. The way in which drive may occur is species- and B chromosome-type specific. It may be directed nondisjunction during first or second pollen grain mitosis, preferential fertilization of the egg cells by B carrying male gametes, preferential meiotic segregation in embryo sac mother cells, or somatic nondisjunction (Jones and Rees, 1982). Accumulation of Bs in *Ae. speltoides* occurs presumably via directed nondisjunction during first pollen grain mitosis as the analysis of pollen grains revealed their accumulation in the generative nucleus (Mendelson and Zohary, 1972). However, it is unknown how the drive of Bs is controlled. In *Ae. mutica* (Ohta, 1995b) and rye (Banaei-Moghaddam et al., 2012; Müntzing, 1945) B chromosomes also accumulate via directed nondisjunction, with the only difference that in rye drive occurs during the female gametophyte development as well.

The control of the B-specific accumulation process is well studied in rye (Figure 3). The B of rye carries a nondisjunction control region at the end of the long chromosome arm. This was demonstrated by using lines carrying different types of Bs: normal, short arm iso-B, long arm iso-B and deficient B, lacking the terminal part of the long arm. These types of Bs show different segregation behaviours during the first pollen mitosis. Bs lacking the long arm or the terminal part of the long arm divide

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regularly (Lima-de-Faria, 1962). Later using the gametocidal system in wheat lines with additional rye Bs, Endo and co-authors demonstrated that besides the trans-acting element at the distal part of long B arm also the B pericentromeric sticking sites are responsible for nondisjunction (Endo et al., 2008). The behaviour of different rye B chromosome types in different genetic backgrounds was summarized and shown as schemata by Houben et al. (2013b) (Figure 3). The B-specific repeat families E3900 and D1100 are the main components of the nondisjunction control region. They are highly transcribed in anthers, where the nondisjunction process occurs. However, their exact role in the nondisjunction process, as well as the sequence composition of pericentromeric sticking sites, is not yet known (Banaei-Moghaddam et al., 2012; Carchilan et al., 2007).
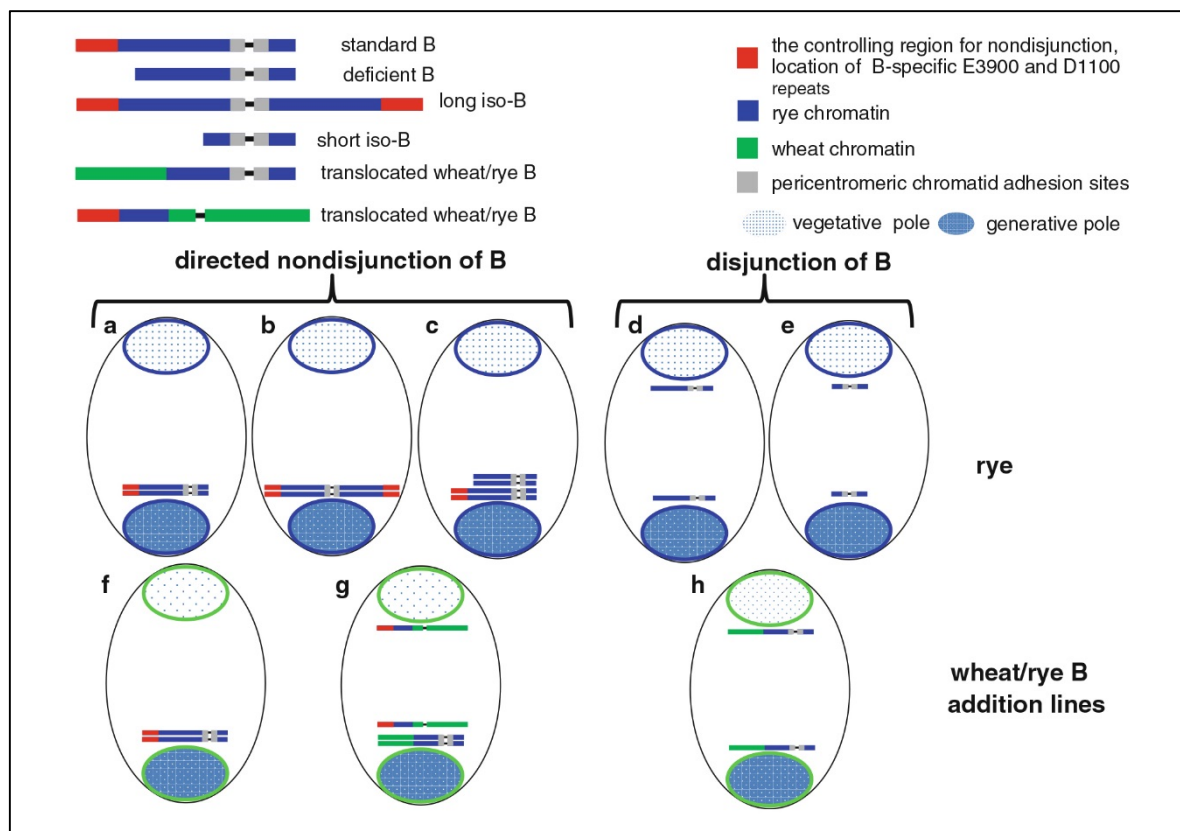
Figure 3. Schemata summarizing the function of the distal region of the long B chromosome arm and the pericentromeric sites in the process of directed nondisjunction of the rye B during first pollen mitosis (adopted from Houben et al., 2013b). (a-c) Directed nondisjunction of standard B (a), B long arm isochromosome (b), and deficient B together with standard B (c). (d-e) Disjunction of B chromosomes lacking terminal part of the long arm: deficient B (d) and B short arm isochromosome (e). (f) Nondisjunction of a standard rye B chromosome in the wheat background. (g) Reciprocal A/B translocation and nondisjunction of the translocated chromosome possessing the B centromere. (h) Translocated A/B chromosome lacking the terminal region shows normal disjunction.

Despite many potential applications, the B chromosome of *Aegilops* was never introduced into hexaploid wheat as a stable additional chromosome. Possibly, due to the negative impact of the Bs presence on the hybrid fertility (Friebe et al., 2000). Whether Bs affect the meiotic chromosome pairing in hybrids between hexaploid wheat and *Ae. speltoides* remains an open question (Vardi and Dover, 1972). Homoeologous pairing is controlled by the *Ph1* locus, which is localized at chromosome arm 5BL of wheat. If chromosome 5B is lacking in F1 *Triticum aestivum* L. × *Ae. speltoides* hybrids with Bs, the B chromosome can compensate for the missing 5B and prevents homoeologous pairing of the standard A chromosomes (Dover, 1975; Riley, 1974). In F1 hybrids of *Ae. speltoides* with tetraploid wheat possessing AABB or AAGG genomes, the presence of Bs was shown to decrease the homoeologous pairing in a dosage-dependent manner (Sano and Tanaka, 1980).

1.4 Organ-specific distribution of B chromosomes

The absence in roots and stable presence in all other organs is a specific feature of the B chromosome in *Aegilops* (Mendelson and Zohary, 1972; Ohta, 1995a). In contrast, in rye Bs are present in all organs in a constant number. The regular loss of extra chromosomes in a particular plant organ was reported for the first time in *Sorghum purpureo-sericeum* (A. Rich.) Aschers. & Schweinf. (Janaki-Ammal, 1940). In the genus *Sorghum*, B chromosomes are characterized by high instability in somatic tissues but maintained regularly in pollen mother cells (Darlington and Thomas, 1941). It was reported that the Bs of *S. purpureo-sericeum* undergo elimination during almost the entire life cycle of B-carrying plant. In primary roots Bs are lost during the seed development, shoots and inflorescences are losing Bs in the course of plant growth. Only in pollen mother cells, Bs are present in invariable number, while in ovaries micronuclei as an indication of elimination were found (Darlington and Thomas, 1941). In *Sorghum stipoideum* (Ewart & Jean White) C.A.Gardner & C.E.Hubb. numerical variations were observed between panicles and even between spikelets of the same panicle. The Bs also vary in number in tapetal cells, while in stem and leaf meristems they are completely lost (Wu, 1992).

In Poaceae, somatic instability of Bs was found also in several other species. The Bs of *Agropyron cristatum* (L.) Gaertn. were shown to be absent only in adventitious roots, while present in primary root, stem meristem and pollen mother

cells (Baenziger, 1962). In diploid varieties of *Poa alpina* L., the Bs are usually absent in roots (Müntzing and Nygren, 1955) and leaves (Müntzing, 1949) but present in pollen mother cells in fairly constant number. However, the occasional presence of Bs in primary, but not in adventive, roots was also described (Müntzing, 1948; Håkansson, 1948; Milinkovic, 1957). In *Dactylis glomerata*, numerical variations of Bs were found between tillers of the same plant, while the B number is constant within one tiller and in spikelets of the same panicle. The complete absence of Bs in some tillers of B-carrying individuals and constant presence in others might suggest that loss of Bs occurs during tiller differentiation (Puteyevsky and Zohary, 1971).

Many other examples of numerical instability of Bs in plants and animals were reviewed by Jones and Rees (1982). It is likely that the somatic variation of B chromosome number is a more common phenomenon, as often only one type of tissue is used for chromosome counting. Thus, organ- or tissue-specific chromosome number variation can remain unnoticed.

The causes of B chromosome numerical variation between organs of the same individual and the mechanisms responsible for their elimination are still unknown. A possible explanation of B chromosome instability in somatic tissues and their constant presence in germline cells was proposed for *S. purpureo-sericeum*. Presumably, Bs have defective centromeres, which are together with defective mitotic spindles leading to the elimination of Bs in somatic tissues. The mitotic spindles of dividing germline cells are supposed to be normal, which prevents the loss of Bs (Darlington and Thomas, 1941). Håkansson speculated that in *P. alpina* B centromeres are weaker in comparison with A centromeres, which results in B chromosome instability (Håkansson, 1948). In *Xanthisma texanum* DC. Bs are absent in roots and present in constant number in shoots and in germline cells (Semple, 1976). Observation of cell divisions during embryo development revealed lagging of Bs during anaphases. The elimination of Bs was explained by the inability of the spindle to interact with the B centromeres because of some unknown factors (Berger et al., 1955).

Summarizing all above mentioned studies and taking into account that in general the behaviour of Bs during somatic cell division is regular, one may think that there should be a particular reason for their elimination in some organs. However, till now no explanations for that were proposed and very few recent studies on this

subject were conducted. Beside the preferential elimination of Bs in roots, in some species Bs may be absent in leaves and tillers and also vary in number between cells of the same organ.

Impaired centromere function and noninteraction with the mitotic spindle are proposed reasons for the somatic instability of Bs. However, no direct evidence exists for this assumption. On the other hand, if we consider Bs as dispensable and selfish elements of the genome, their elimination from somatic tissues could be a way to restore an initial state of the genome without genetic parasites, while retention of Bs in the germline is caused by some B-specific mechanism allowing to maintain themselves in the progeny of host organism.

1.5 Programmed DNA/chromosome elimination in eukaryotes

Does the organ-specific elimination of Bs in plants occur in a programmed way or is it just a matter of genome imbalance, due to the presence of a significant amount of extra DNA? This is still an intriguing question to be answered. The only report where the absence of B chromosomes in roots of *Sorghum purpureo-sericeum* was assumed to be a result of a strictly controlled and genetically programmed mechanism, similar to that known in animals, was published in 1940 (Janaki-Ammal, 1940). Otherwise, chromosome elimination in plants is usually referred to as genome instability resulting from wide hybridization and incompatibility of parental genomes (Gernand et al., 2005; Ishii et al., 2010).

In animals, programmed DNA elimination was first described in roundworms by Boveri (1887). Since then there were many other organisms discovered in which this phenomenon seems to be an essential part of the developmental program (Figure 4). Programmed DNA elimination may proceed either as so-called chromatin diminution, when chromosomes break into pieces and some of their fragments are lost (e.g., in such species as *Parascaris univalens*, *Cyclops kolensis* and *Petromyzon marinus*), or as the loss of entire chromosomes (e.g., in *Sciara coprophyla*, *Taeniopygia guttata* and *Isoodon macrourus*) (Wang and Davis, 2014).

Currently, the roundworms *Ascaris suum* and *Parascaris univalens*, the zebra finch *Taeniopygia guttata*, and the lamprey *Pteromyzon marinus* are the species in which programmed DNA elimination is best studied at the molecular level. In all these species, germline-expressed genes are eliminated from somatic cells together with a

significant amount of repetitive DNA (Biederman et al., 2018; Bryant et al., 2016; Wang et al., 2017). It is proposed that the elimination of these genes prevents their expression in somatic tissues, which may have otherwise deleterious effects (Smith, 2018; Streit, 2012). Alternatively, it may be a way to reduce the costs of maintaining high amounts of unneeded DNA in somatic cells by simply removing it (Streit, 2012).
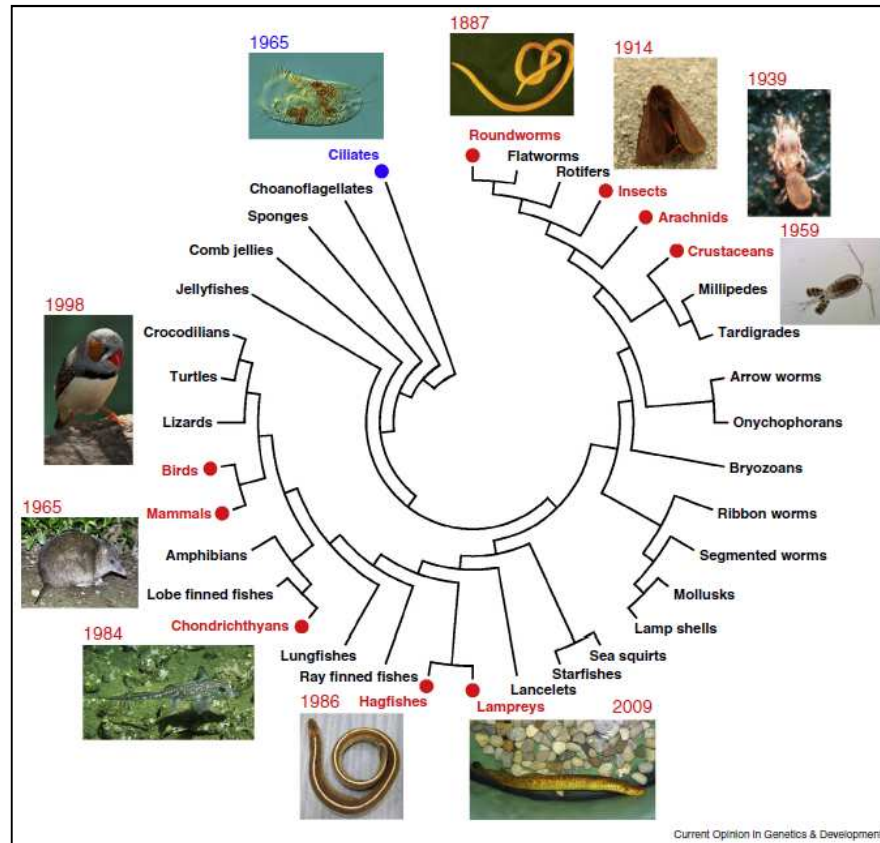
Figure 4. Programmed DNA elimination in animals (adopted from Wang and Davis, 2014). Organisms known to undergo programmed DNA elimination are depicted in the phylogenetic tree (marked by red dots). The years of programmed DNA elimination discovery in particular organisms is noted.

Altogether, programmed DNA elimination is thought to be involved in differentiation of germline and somatic cells and may act as a mechanism for irreversible gene silencing, dosage compensation and sex determination (Wang and Davis, 2014). For example, in *Sciara coprophila* sex determination involves a complex and stepwise process of programmed chromosome elimination. Interestingly, it affects not only sex chromosomes but also so-called germline limited or L chromosomes. These L chromosomes are supernumerary but considered to be indispensable unlike most of the supernumerary chromosomes in other species

(Singh and Belyakin, 2018). The dynamics of organ-specific elimination of B chromosomes in plants is poorly studied, and it is impossible to discuss whether similarity exists between this process and the programmed chromosome elimination in animals.

2. Aims of the study

The aim of the current study was, first, to investigate the molecular structure of the *Ae. speltoides* B chromosome. The understanding of the origin and the way of functioning of such enigmatic part of the genome is not possible without deep knowledge of its DNA composition. Are there any B-specific sequences present and did the Bs of *Ae. speltoides* accumulate organellar-derived DNA? These are the first questions addressed in the study.

Second, the *Ae. speltoides* B chromosome is interesting from the point of view of its organ-specific distribution. Is this a result of genome instability or a strictly controlled process? Understanding of when and how Bs are selectively lost during plant development would allow answering this question. If the elimination of Bs occurs in a programmed manner, it could make *Ae. speltoides* a possible model for studying programmed DNA elimination in plants. In animals, programmed DNA elimination seems to play an important role in the development of some species. If the mechanism of programmed DNA elimination is conserved among eukaryotes, the dispensable nature of B chromosomes may become a good background for studying the basics of chromosome elimination in *Ae. speltoides.*

3. Publications prepared in the frame of the PhD thesis

3.1. Evolution of the S-Genomes in *Triticum-Aegilops* alliance: evidences from chromosome analysis

Ruban A. S. and Badaeva E. D. (2018) Evolution of the S-Genomes in *Triticum-Aegilops* alliance: evidences from chromosome analysis. Frontiers in Plant Science 9: 1756. DOI: 10.3389/fpls.2018.01756.

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Evolution of the S-Genomes in *Triticum-Aegilops* Alliance: Evidences From Chromosome Analysis

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Five diploid *Aegilops* species of the *Sitopsis* section: *Ae. speltoides, Ae. longissima, Ae. sharonensis, Ae. searsii*, and *Ae. bicornis*, two tetraploid species *Ae. peregrina* (=*Ae. variabilis*) and *Ae. kotschyi* (*Aegilops* section) and hexaploid *Ae. vavilovii* (*Vertebrata* section) carry the S-genomes. The B- and G-genomes of polyploid wheat are also the derivatives of the S-genome. Evolution of the S-genome species was studied using Giemsa C-banding and fluorescence *in situ* hybridization (FISH) with DNA probes representing 5S (pTa794) and 18S-5.8S-26S (pTa71) rDNAs as well as nine tandem repeats: pSc119.2, pAesp_SAT86, Spelt-1, Spelt-52, pAs1, pTa-535, and pTa-s53. To correlate the C-banding and FISH patterns we used the microsatellites $(CTT)_{10}$ and $(GTT)_9$, which are major components of the C-banding positive heterochromatin in wheat. According to the results obtained, diploid species split into two groups corresponding to *Emarginata* and *Truncata* sub-sections, which differ in the C-banding patterns, distribution of rDNA and other repeats. The B- and G-genomes of polyploid wheat are most closely related to the S-genome of *Ae. speltoides*. The genomes of allopolyploid wheat have been evolved as a result of different species-specific chromosome translocations, sequence amplification, elimination and re-patterning of repetitive DNA sequences. These events occurred independently in different wheat species and in *Ae. speltoides*. The 5S rDNA locus of chromosome 1S was probably lost in ancient *Ae. speltoides* prior to formation of Timopheevii wheat, but after the emergence of ancient emmer. Evolution of *Emarginata* species was associated with an increase of C-banding and $(CTT)_{10}$-positive heterochromatin, amplification of Spelt-52, re-pattering of the pAesp_SAT86, and a gradual decrease in the amount of the D-genome-specific repeats pAs1, pTa-535, and pTa-s53. The emergence of *Ae. peregrina* and *Ae. kotschyi* did not lead to significant changes of the S*-genomes. However, partial elimination of 45S rDNA repeats from 5S* and 6S* chromosomes and alterations of C-banding and FISH-patterns have been detected. Similarity of the S^v-genome of *Ae. vavilovii* with the S^s genome of diploid *Ae. searsii* confirmed the origin of this hexaploid. A model of the S-genome evolution is suggested.

Keywords: wheat, *Aegilops*, S-genome of *Ae. speltoides*, S*-genome of other *Aegilops* species, chromosome, karyotype evolution, C-banding, FISH

INTRODUCTION

Evolutionary goat grasses, or *Aegilops* are closely related to wheat and contributed two of the three subgenomes of hexaploid bread wheat (Sears, 1969; Kihara, 1975; Feldman, 2001). The natural distribution area of the genus *Aegilops* L. covers the Mediterranean basin, southwestern and central Asia (Witcombe, 1983; Kimber and Feldman, 1987; Van Slageren, 1994; Kilian et al., 2011). Their center of origin is thought to be located in Transcaucasia (Hammer, 1980; Van Slageren, 1994), or in the Fertile Crescent (Kimber and Feldman, 1987). These regions contain the highest concentration of *Aegilops* species. Goat grasses inhabit a broad range of environments and are characterized by very wide adaptation. Owing to this, many goat grasses exhibit good resistance to fungal diseases and pests (Hammer, 1980; Gill et al., 1985; Makkouk et al., 1994; El Bouhssini et al., 1998; Monneveux et al., 2000; Schneider et al., 2008; Zhao et al., 2016), heat, drought or frost tolerance and cold hardiness (Limin and Fowler, 1985; Damania et al., 1992; Monneveux et al., 2000; Pradhan et al., 2012). Some *Aegilops* accessions are characterized by high grain quality and increased micronutrient content (Rawat et al., 2011; Farkas et al., 2014; Rakszegi et al., 2017) that can be used for wheat improvement. Although many agronomically useful genes have already been transferred from *Aegilops* to common wheat varieties or breeding lines (Knott and Dvorák, 1976; Schneider et al., 2008; Rawat et al., 2011; McIntosh et al., 2013; Zhang et al., 2015), their genetic potential in broadening genetic diversity of wheat is not fully exploited. Utilization of gene pool of *Aegilops* requires good knowledge of genetics and genomics of these species, including their karyotypes and chromosomal structures.

In addition to the great potential for wheat breeding, goat grasses can also be an attractive model for studying mechanisms of reticulate evolution. Depending on taxonomical system, the genus *Aegilops* is classified into 20 (Kihara, 1954), 22 (Zhukovsky, 1928; Eig, 1929; Van Slageren, 1994), 24 (Kimber and Feldman, 1987), 25 (Chennaveeraiah, 1960), or 26 species (Witcombe, 1983). These species are split into sections based on morphological criteria or genome composition. At present, the system suggested by Van Slageren (1994) is commonly accepted; therefore, we will follow this nomenclature. According to it, 10 *Aegilops* species are diploid and 12 – polyploid, that were formed as a result of hybridization of different diploid progenitors.

Based on a series of pioneering works of the famous Japanese geneticist (Kihara, 1937, 1949, 1954, 1957, 1963; Lilienfeld, 1951; Kihara et al., 1959), diploid *Aegilops* were divided into three major genomic groups, C, D, and S. The C-genome group included two species; the D-genome group included four species; and the S-genome group consisted of three species of the *Sitopis* (Jaub. & Spach) Zhuk. section: *Ae. longissima* Schweinf. & Muschl. (including *Ae. sharonensis* Eig), *Ae. bicornis* (Forssk.) Jaub. & Spach, and *Ae. speltoides* Tausch (Kihara, 1937, 1949; Lilienfeld, 1951). A new diploid species of the *Sitopsis* section— *Ae. searsii* Feldman and Kislev ex Hammer, has been discovered later by Feldman and Kislev (1977). Analysis of the karyotype, meiotic chromosome pairing, pollen fertility and seed set in *Ae. longissima x Ae. searsii* hybrids showed that *Ae. searsii*

possesses the S*-genome (Feldman et al., 1979; Yen and Kimber, 1990a).

Thus, current taxonomy recognizes five diploid species carrying the S-genome: *Ae. speltoides* including ssp. *ligustica* (Savign.) Fiori (SS) and ssp. *speltoides* Boiss., *Ae. bicornis* (S^bS^b), *Ae. searsii* (S^sS^s), *Ae. sharonensis* ($S^{sh}S^{sh}$), and *Ae. longissima* (S^lS^l) (Van Slageren, 1994; Kilian et al., 2011; Feldman and Levy, 2015). These species are morphologically similar, but can be easily distinguished by their habitat, climatic adaptation, and distribution areas. Based on differences in spike morphology, Eig (1929) divided the *Sitopsis* group into two sub-sections, *Truncata* and *Emarginata*. Subsection *Truncata* includes only one species–*Ae. speltoides* (SS), which grows in central, eastern, and northern part of the *Sitopsis* area. This species consists of two forms, *ligustica* and *auscheri,* which differ in their fruiting spike and the mode of seed dispersal (Eig, 1929; Zohary and Imber, 1963), but are similar in karyotype structure (Chennaveeraiah, 1960). Their hybrids are fully fertile and show complete meiotic chromosome pairing (Zohary and Imber, 1963). *Ae. speltoides* has the lowest nuclear DNA content (1C = 5.81 ± 0.123 pg) within the *Sitosis* group (Eilam et al., 2007) and differs significantly from *Emarginata* species in its chromosome morphology (Chennaveeraiah, 1960), Giemsa C-banding (Teoh and Hutchinson, 1983; Friebe and Gill, 1996; Friebe et al., 2000) and FISH patterns (Yamamoto, 1992a,b; Jiang and Gill, 1994b; Badaeva et al., 1996a,b; Salina et al., 2006b; Raskina et al., 2011; Belyayev and Raskina, 2013).

The subsection *Emarginata* includes four species: *Ae. bicornis, Ae. searsii, Ae. sharonensis,* and *Ae. longissima,* which grow in the central and southern part of the *Sitopsis* section habitat (Feldman and Kislev, 1977). Study of the chromosome pairing of intraspecific hybrids (Kihara, 1954, 1963; Feldman et al., 1979; Yen and Kimber, 1989, 1990a,b,c), similarity of karyotype structure (Riley et al., 1958; Chennaveeraiah, 1960), the number and distribution of 5S and 45S rDNA loci (Yamamoto, 1992a,b; Badaeva et al., 1996b), and the distribution of pSc119.2 sequence (Badaeva et al., 1996a) suggest a close relationship of *Emarginata* species, although they differ from each other in genome size (Eilam et al., 2007) and C-banding patterns (Friebe and Gill, 1996).

Morphologically, *Ae. bicornis* is the most primitive species in this group (Eig, 1929). It is more difficult to produce hybrids with *Ae. bicornis* than with other *Aegilops* of the S-genome group (Kimber and Feldman, 1987). Genome size of *Ae. bicornis* (1C = 6.84 ± 0.097 pg) is only little larger than that of *Ae. searsii* (1C = 6.65 ± 0.091 pg), and is lower than of *Ae. longissima* (1C = 7.48 ± 0.082 pg) or *Ae. sharonensis* (1C = 7.52 ± 1.000 pg) (Eilam et al., 2007). Morphologically *Ae. searsii* resembles *Ae. longissima,* but differs from it in a number of morphological traits which are considered as evolutionary advanced (Feldman and Kislev, 1977). *Ae. longissima* x *Ae. searsii* hybrids exhibit meiotic irregularities and are highly sterile (Feldman et al., 1979). By contrast, the F_1 hybrids *Ae. longissima* x *Ae. sharonensis* are fertile and show complete chromosome pairing in meiosis. Isolation of these species is caused by different ecological requirements (Feldman and Levy, 2015). According to other hypothesis (Waines and Johnson, 1972), *Ae. sharonensis* could be a hybrid between

Ae. longissima and *Ae. bicornis*. *Ae. longissima* carries a species-specific 4S*/7S* translocation (Tanaka, 1955; Yen and Kimber, 1990b; Friebe et al., 1993; Naranjo, 1995), while no structural rearrangements have been identified in other species of this group (Yen and Kimber, 1989, 1990a,b,c; Maestra and Naranjo, 1997, 1998; Luo et al., 2005; Dobrovolskaya et al., 2011).

The similarity of *Emarginata* species and separate position of *Ae. speltoides* within the *Sitopsis* section was confirmed by molecular analyses of nuclear and cytoplasmic DNA. Based on the variation of repeated nucleotide sequences (RNS) Dvořák and Zhang (1992) showed that the *Sitopsis* species are phylogenetically similar, but *Ae. speltoides* is clearly separated from species of the *Emarginata* group. RAPD- and AFLP analyses revealed that *Ae. speltoides* forms a cluster with polyploid wheats, which is separated from other *Sitopsis* species (Kilian et al., 2007, 2011; Goryunova et al., 2008). Study of organellar DNAs by PCR-single-strand conformational polymorphism (PCR-SSCP) revealed high similarity of *Ae. bicornis* - *Ae. sharonensis* - *Ae. longissima* plasmons and their distinctness from plasmon of *Ae. speltoides* (Wang et al., 1997).

Comparative sequence analysis provided further insights into the evolution of *Triticum* and *Aegilops* and allowed the estimation of divergence time of different genomic groups. Comparison of chloroplast (Yamane and Kawahara, 2005; Golovnina et al., 2007; Gornicki et al., 2014; Middleton et al., 2014; Bernhardt et al., 2017) and nuclear DNA sequences (Petersen et al., 2006; Salse et al., 2008; Marcussen et al., 2014) strongly suggest that *Ae. speltoides* occupies a basal position on the phylogenetic tree of *Aegilops/Triticum* (Petersen et al., 2006; Kawahara, 2009). Probably *Ae. speltoides* diverged from the progenitor of the Triticeae much earlier than diploid wheat and *Aegilops* species (Yamane and Kawahara, 2005; Salse et al., 2008; Gornicki et al., 2014; Middleton et al., 2014; Bernhardt et al., 2017). Estimates obtained from the analyses of nuclear DNA sequences placed the possible divergence time within the period from ∼7 MYA (Marcussen et al., 2014) to 3.5–2.7 MYA (Dvořák and Akhunov, 2005; Salse et al., 2008). Estimates obtained from chloroplast DNA favored a more recent origin of *Ae. speltoides* – 4.1–3.6 MYA (Bernhardt et al., 2017) to 2.67 ± 1.1. MYA (Middleton et al., 2014). Marcussen et al. (2014) supposed that the D-genome lineage (which indeed included D, M, and S* genome species, Sandve et al., 2015) emerged through ancient homoploid hybridization between A and S genomes. The members of *Emarginata* group are thought to radiate from common ancestor approximately 1.0–2.0 MYA (*Ae. searsii*) – 1.4 MYA (*Ae. bicornis*) – to 0.4 MYA (*Ae. sharonensis*) (Marcussen et al., 2014; Feldman and Levy, 2015).

Hypothesis that the B and G genomes of polyploid wheats originated from a diploid S-genome *Aegilops* species was put forward in the middle XXth (Sears, 1956; Riley et al., 1958). Different taxa were suggested as potential progenitors of polyploid wheat (Haider, 2013). All species of the *Sitopsis* section have been considered as the B-genome donors: *Ae. speltoides* (Sarkar and Stebbins, 1956; Tanaka et al., 1979; Bahrman et al., 1988; Kerby et al., 1990; Daud and Gustafson, 1996; Maestra and Naranjo, 1998; Yan et al., 1998; Blake et al., 1999; Rodríguez et al., 2000a; Haider, 2013), *Ae. bicornis* (Sears, 1956),

Ae. longissima (Tanaka, 1956; Konarev et al., 1976; Konarev, 1980; Peacock et al., 1981), *Ae. searsii* (Feldman and Kislev, 1977; Nath et al., 1983, 1984; Kerby et al., 1990; Liu et al., 2003), *Ae. sharonensis* (Kushnir and Halloran, 1981) or yet unknown species of the *Emarginata* group (Kerby et al., 1990). Molecular analyses of common wheat genome and genomes of related species confirmed the ancestry of wheat B- genome from *Ae. speltoides* or the species close to it (Talbert et al., 1991; Petersen et al., 2006; Goryunova et al., 2008; Salse et al., 2008; Marcussen et al., 2014). Based on the analysis of nuclear or plastid DNA, ancient tetraploid emmer could emerge 0.4–0.8 MYA (Huang et al., 2002; Dvořák and Akhunov, 2005; Yamane and Kawahara, 2005; Golovnina et al., 2007; Gornicki et al., 2014; Marcussen et al., 2014; Middleton et al., 2014; Bernhardt et al., 2017).

The origin of the G-genome of *Triticum timopheevii* Zhuk. from the S-genome of *Ae. speltoides* was first hypothesized by Giorgi and Bozzini (1969) based on comparison of chromosome morphologies and was later confirmed by numerous studies including chromosome pairing analysis of intraspecific hybrids (Shands and Kimber, 1973; Tanaka et al., 1979; Maestra and Naranjo, 1999; Rodríguez et al., 2000a), comparison of C-banding (Badaeva et al., 1996a) and ISH patterns (Jiang and Gill, 1994a,b; Salina et al., 2006b), isozyme profiles (Konarev et al., 1976; Nakai, 1978; Jaaska, 1980), AFLP- (Kilian et al., 2007, 2011) and RFLP-analyses (Dvořák and Zhang, 1990; Talbert et al., 1991; Dvořák, 1998), sequencing of nuclear (Huang et al., 2002) and cytoplasmic DNA (Sasanuma et al., 1996; Yamane and Kawahara, 2005; Golovnina et al., 2007; Gornicki et al., 2014). These studies revealed that *Ae. speltoides* is more closely related to the G genome of *T. timopheevii* than to the B-genome of common wheat and suggested that ancient *T. timopheevii* could emerge approximately 0.4 MYA (Huang et al., 2002; Gornicki et al., 2014).

The S*-genome is identified in two tetraploid *Aegilops* species belonging to the section *Aegilops* L.: *Ae. peregrina* (Hach. in Fraser) Maire & Weiller (=*Ae. variabilis* Eig, U^PU^PS^PS^P) and *Ae. kotschyi* Boiss. (U^kU^kS^kS^k). Based on the "analyzer" method H. Kihara (1954) proposed that *Ae. peregrina* is a hybrid between *Ae. umbellulata* Zhuk. and a diploid species of the *Sitopsis* group (Lilienfeld, 1951), although conventional chromosome staining did not reveal the S*-genome in these species (Chennaveeraiah, 1960). Cytoplasmic genomes of *Ae. peregrina* and *Ae. kotschyi* are most closely related to the cytoplasmic genome of *Ae. searsii* (Ogihara and Tsunewaki, 1988; Siregar et al., 1988). However, meiotic analysis of the F$_1$ hybrids between *Ae. kotschyi* and induced autotetraploid of three *Sitopsis* species showed that *Ae. kotschyi* shared the S* genome with *Ae. longissima* (Yen and Kimber, 1990d). Yu and Jahier (1992) come to the same conclusion based on chromosome pairing analysis in hybrids of *Ae. variabilis* (=*Ae. peregrina*) with different *Sitopsis* species. RFLP profiles of RNS suggested that the S* genome of *Ae. peregrina* and *Ae. kotschyi* could have originated from *Ae. longissima* or *Ae. sharonensis* or the species immediately preceding the divergence of these diploids (Zhang et al., 1992). C-banding and FISH analyses confirmed highest similarity of the S*-genome of these tetraploids with *Ae. longissima* or *Ae.*

sharonensis (Jewell, 1979; Jewell and Driscoll, 1983; Friebe et al., 1996; Badaeva et al., 2004; Zhao et al., 2016).

Ae. vavilovii (Zhuk.) Chennav. ($D^1D^1X^{cr}X^{cr}S^vS^v$) is a hexaploid taxa belonging to section *Vertebrata* Zhuk. emend Kihara, complex Crassa. *Ae. vavilovii* originated from hybridization of tetraploid *Ae. crassa* Boiss. with a species of *Emarginata* group, possibly *Ae. longissima* (Kihara, 1963; Kihara and Tanaka, 1970). Originally *Ae. vavilovii* was treated as a subspecies of hexaploid *Ae. crassa,* and its taxonomic rank was raised to independent biological species by Chennaveeraiah (1960). Although this author was unable to determine genome constitution of *Ae. vavilovii,* he noticed a pairwise similarity of the satellite chromosomes in karyotype of this species.

Yen and Kimber (1992) failed to identify the exact donor of the S^v-genome of *Ae. vavilovii* based on analysis of chromosome pairing in the F_1 hybrids of *Ae. vavilovii* with induced autotetraploids of the *Sitopsis* species and proposed that the S^v-genome is substantially modified. By using molecular markers (Talbert et al., 1991) showed that the S^v-genome of *Ae. vavilovii* is related to the S^*-genome of *Emarginata* group. Data collected by molecular methods (Zhang and Dvořák, 1992), C-banding and FISH analyses (Badaeva et al., 2002; Zhang et al., 2002) confirmed, that *Ae. vavilovii* contains the S^v-genome that could probably derive from *Ae. searsii* (Badaeva et al., 2002).

Because of the genetic relatedness of the S-genome *Aegilops* species and polyploid wheats as well as of their potential for wheat improvement, they have been attracting the attention of researchers over the past century. Numerous intraspecific hybrids have been created to transfer desired genes from *Aegilops* to wheat (Schneider et al., 2008). Sets of addition, substitution or translocation wheat-*Aegilops* lines, including *Ae. speltoides* (Friebe et al., 2000; Liu et al., 2016), *Ae. searsii* (Pietro et al., 1988; Friebe et al., 1995), *Ae. sharonensis* (Olivera et al., 2013), *Ae. longissima* (Friebe et al., 1993), and polyploid *Ae. peregrina,* (Jewell and Driscoll, 1983; Friebe et al., 1996; Yang et al., 1996) and *Ae. kotschyi* (Rawat et al., 2011) were obtained and characterized using a combination of C-banding and analyses with the group-specific molecular or isozyme markers. As a result of these studies, the genetic classifications were developed for C-banded chromosomes of several S-genome species (Friebe and Gill, 1996).

From another side, the S-genomes were extensively examined by FISH with various DNA probes (Yamamoto, 1992a; Badaeva et al., 1996a,b, 2002, 2004; Belyayev et al., 2001; Zhang et al., 2002; Giorgi et al., 2003; Salina et al., 2006b, 2009; Raskina et al., 2011; Ruban et al., 2014; Molnár et al., 2016; Zhao et al., 2016). Probe pSc119.2 was used most frequently (Badaeva et al., 1996a, 2002, 2004; Molnár et al., 2016; Zhao et al., 2016), however, the pSc119.2 signals are located predominantly in subtelomeric chromosome regions, thus hindering unequivocal chromosome identification. Probe pAs1, which proves to be highly informative for many *Aegilops* species, is not very useful for the S-genome analysis owing to a small number of detected sites (Badaeva et al., 1996a). In most papers FISH-labeled *Aegilops* chromosomes were classified based on their morphology, which is not sufficient to determine their correspondence to the genetic

nomenclature of C-banded chromosomes. Owing to this, it was necessary to find FISH markers for the precise identification of all S-genome chromosomes and coordination of classification systems.

Recently, Komuro et al. (2013) isolated and characterized a number of repetitive DNAs from the wheat genome, which can potentially be used for molecular-cytogenetic analysis of wheat and *Aegilops* species. Several new sequences have been described in other papers (Salina et al., 1998, 2009; Adonina et al., 2015; Badaeva et al., 2015; Zhao et al., 2016). In this study we characterized the S genomes of diploid and polyploid *Triticum* and *Aegilops* species using C-banding and FISH with a set of "classical" [pSc119.2, pAs1, pTa71, pTa794, Spelt-1, Spelt-52] and novel [pAesp_SAT86, $(CTT)_n$, $(GTT)_n$, pTa-535, pTa-s53] probes in order to assess evolutionary changes in the *Triticum-Aegilops* alliance.

MATERIALS AND METHODS

Plant Material

Five diploid (*Aegilops speltoides, Ae. longissima, Ae. sharonensis, Ae. searsii, Ae. bicornis*), two tetraploid (*Ae. peregrina* and *Ae. kotschyi*) and one hexaploid (*Ae. vavilovii*) *Aegilops* species carrying the S-genome have been examined in comparison with two tetraploid wheats, *T. timopheevii* and *T. dicoccoides*. The list of accessions, their ploidy level, genome constitution and the origin are given in **Table S1**.

DNA Probes

Following probes were used for FISH:

Plasmid clones pTa71 - a 9 kb long sequence of common wheat encoding 18S, 5.8S and 26S rRNA genes including spacers (Gerlach and Bedbrook, 1979), pTa794 – a 420 bp long sequence of wheat containing the 5S rRNA gene and intergenic spacer (Gerlach and Dyer, 1980), pAs1 - a 1 kb fragment derived from *Ae. tauschii* and belonging to *Afa* family (Rayburn and Gill, 1986), pSc119.2 – a 120 bp long sequence isolated from rye (Bedbrook et al., 1980), pTa-s53 – a 587 bp DNA fragment isolated from common wheat (Komuro et al., 2013), Spelt-1 – a 150 bp fragment isolated from *Ae. speltoides* (Salina et al., 1997), Spelt-52 (homolog of pAesKB52) – a 276 bp long DNA fragment isolated from *Ae. speltoides* (Salina et al., 2004a), and pAesp_SAT86 - a new satellite family with a monomer length of 86 bp isolated from Ae. *speltoides* genomic DNA (Badaeva et al., 2015) and showing 91-94% similarity to wheat repeat pTa-713 described in Komuro et al. (2013) were labeled with dUTP-ATTO-488, dUTP-ATTO-550, dUTP-ATTO-647N by nick-translation using an Atto NT Labeling Kit (Jena Bioscience, Germany) or with FITC (fluorescein-12-dUTP, Roche, Germany) or biotin (biotin-16-dUTP, Roche, Germany) by nick-translation using the Nick Translation Mix (Roche, Germany) according to manufacturers' instruction.

Probe pTa535-1 was used as 5′ 6-carboxyfluorescein (6-FAM) or 6-carboxytetramethylrhodamine (TAMRA) end-labeled (MWG, Germany) oligo probe (5′-AAA AAC TTG ACG CAC GTC ACG TAC AAA TTG GAC AAA CTC TTT CGG AGT ATC AGG GTT TC-3′) (Komuro et al., 2013; Tang et al., 2014).

The oligo-$(CTT)_{10}$ or complementary oligo-$(GAA)_{10}$ probes [thereafter $(CTT)_n$] were labeled with 5/6-Sulforhodamine 101-PEG3-Azide or 6-Carboxyfluorescein Azide by click chemistry (Baseclick, Germany).

The oligo-$(GTT)_9$ probe labeled at the $3'$-end with fluorescein-12-dUTP was synthesized in the Laboratory of Biological Microchips at the Engelhardt Institute of Molecular Biology, Moscow, Russia.

Giemsa C-Banding Method

The Giemsa C-banding method described in Badaeva et al. (1994) was used for analysis. Seeds were soaked in water for 24 h at room temperature and then kept at 4°C overnight on wet filter paper in Petri dishes. For the next 24 h Petri dishes were placed at 24°C. Roots were cut and treated with 0.05% colchicine for 3 h. Further, roots were fixed in 45% acetic acid for 4 h, washed with distilled water and treated with 0.2 N HCl for 15 min at 4°C and for 5 min at 60°C. After overnight treatment with a 4 mg/ml Cellulysine (Fluka, Switzerland) solution at 24°C root meristems were squashed in drop of 45% acetic acid. Slides were frozen in liquid nitrogen and coverslips were removed. After that slides were placed into 96% ethanol at room temperature. Chromosomes of wheat were classified according to nomenclature suggested in Gill et al. (1991), Badaeva et al. (2016); chromosomes of *Aegilops* species were classified according to the nomenclature of Friebe et al. (1993, 1995, 1996, 2000), Friebe and Gill (1996). Karyotype of one typical accession per each species was taken as standard for alignment of C-banding and FISH patterns.

Fluorescence *in situ* Hybridization

Detailed protocols of the pretreatment of the materials, fixation and chromosomal preparation are given in Badaeva et al. (2017). Briefly, seeds were germinated in Petri dishes on wet filter paper at 24°C in dark. Roots were excised when 2 cm long, treated with ice-cold water for 24 h, and fixed with ethanol:acetic acid (3:1) fixative for at least 4 days at room temperature. Before slide preparation roots were stained in 2% acetocarmine for 15 min. Meristems were cut off and squashed in a drop of 45% acetic acid. Slides were frozen in liquid nitrogen and coverslips were removed with a razor blade. The slides were kept in 96% ethanol in a freezer.

Hybridization mixture contained 1 g dextran sulfate dissolved in 1 ml of distilled water, 5 ml deionized formamide, 1 ml of 20x SSC, 1 ml Herring sperm DNA (10 mg/ml, Promega, USA). Per slide 40–60 ng of each labeled probe were added to 18 μl hybridization mixture. Post hybridization washes were carried out as follows: for probes labeled with biotin or fluorescein the slides were washed in 0.1x SSC 2 × 10 min, then in 2x SSC 2 × 10 min at 42°C. Slides hybridized with directly labeled probes were washed at 58°C in 2x SSC for 20 min. The probes labeled with fluorescein were detected using anti-fluorescein/Oregon green®, rabbit IgG fraction, Alexa Fluor® 488 conjugated antibody (Molecular Probes, USA). Biotin was detected with sptreptavidin-Cy3 (Amersham Pharmacia Biotech, USA). The slides were counter-stained with DAPI ($4'$,6-diamidino-2-phenylindole) in Vectashield mounting media

(Vector laboratories, Peterborough, UK) and examined with a Zeiss Imager D-1 microscope. Selected metaphase cells were captured with an AxioCam HRm digital camera using software AxioVision, version 4.6. Images were processed in Adobe Photoshop[R], version CS5 (Adobe Systems, Edinburgh, UK). For classification, chromosomes were aligned with the C-banding patterns based on the hybridization patterns of labeled CTT- and GTT-satellite sequences.

RESULTS

Analysis of Diploid Species

According to the C-banding and FISH patterns of nine probes, five diploid species of the *Sitopsis* section split into two groups corresponding to taxonomically recognized sub-sections *Truncata* (*Ae. speltoides*) and *Emarginata* (*Ae. longissima, Ae. sharonensis, Ae. searsii, Ae. bicornis*).

Sub-section *Truncata: Ae. speltoides*

The karyotype of *Ae. speltoides* consists of metacentric or submetacentric chromosomes; the chromosome pairs 1S and 6S carry large satellites in their short arms (**Figure S1**). All chromosomes contain large Giemsa-positive pericentromic heterochromatin, prominent subtelomeric C-bands, and some small or medium sized interstitial bands. Giemsa-patterns allowed the identification of all *Ae. speltoides* chromosomes. We observed significant variations of Giemsa bands between plants within and between accessions. Heteromorphism of homologous chromosome has been recorded in all studied genotypes (**Figure S1**).

The $(CTT)_{10}$ clusters (**Figure 1**, CTT) are located in proximal and interstitial chromosome regions, overlapping with Giemsa N-bands (Jiang and Gill, 1994b). No $(CTT)_{10}$ signals were found in the sub-telomeric parts of the chromosomes possessing C-bands. The $(GTT)_9$ probe forms prominent proximal clusters (**Figure 1**, GTT, **Figures 2**, **3f**), often exceeding the size of $(CTT)_n$-signals. The abundance of the GTT-microsatellite is an important diagnostic feature of *Ae. speltoides* chromosomes.

The pSc119.2 labeling patterns are represented by subtelomeric and interstitial signals allowing the discrimination of all *Ae. speltoides* chromosomes. Some hybridization sites are found in all genotypes, whereas other vary in the presence and signal size (**Figure 1**). Based on dual-color FISH with $(CTT)_{10}$ and pSc119.2 probes we corrected previously published classification of pSc119.2-labeled chromosomes (Badaeva et al., 1996a) according to the genetic nomenclature (Friebe et al., 2000). In particular, the chromosomes 2S and 3S have been renamed.

Major NORs are detected on chromosomes 1S and 6S, and one pair of 5S rDNA loci are mapped on the chromosome 5S (**Figures 1**, **2**). In addition, accession TA1873 shows one minor site on the long arm of one 5S chromosome.

Repeat pAesp_SAT86 exhibits significant variation of labeling patterns between *Ae. speltoides* genotypes (**Figure 1**). Two sites located in the short arm of 4S and pericentromeric region of 7SL are permanent (**Figure 1**, yellow arrows). In genotypeTS89 this repeat is transferred to the long arm of 4S, probably due to a
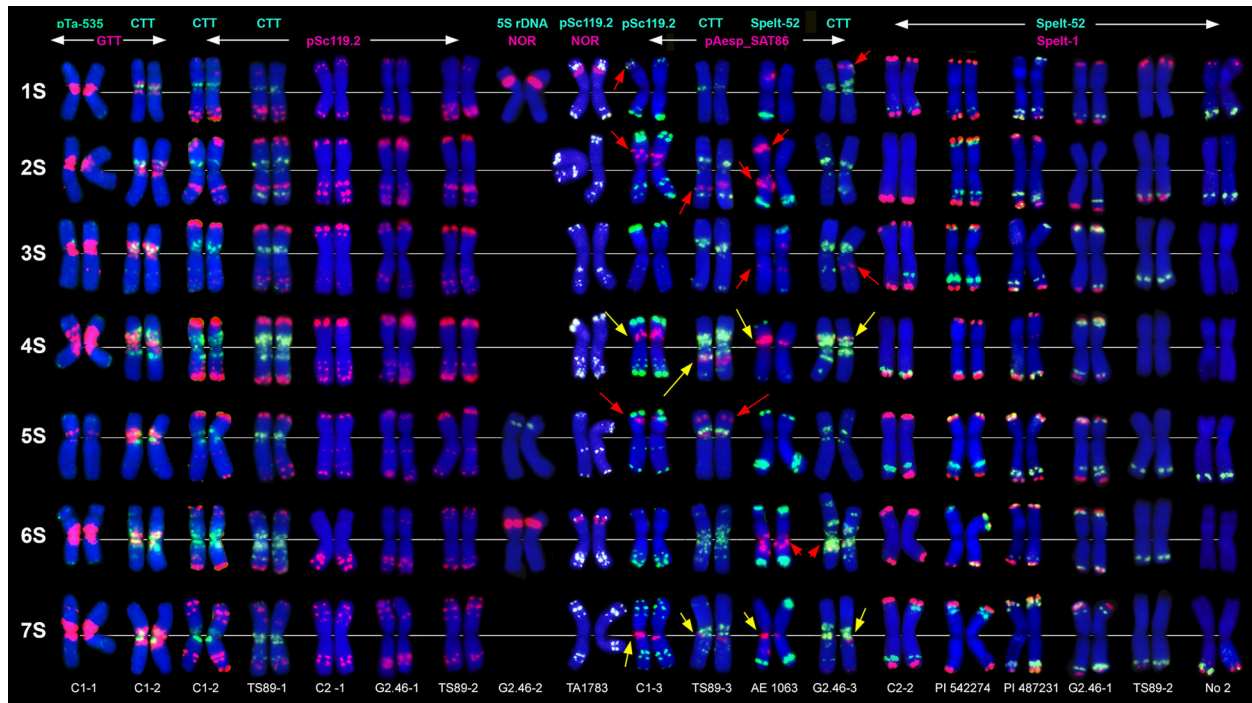
FIGURE 1 | Localization of different DNA sequences on chromosomes of *Ae. speltoides*. Probe combinations are shown on the top; signal color corresponds to probe name. Accessions numbers are indicated in the bottom: C1-1–C1-3 genotypes from Technion park, Haifa, Israel; TS89-1–TS89-3–genotypes from Katzir, Israel; C2-1–C2-2–genotypes from Nahal Mearot, Israel; G2.46-1–G2.46-3–genotypes from Ramat haNadiv, Israel. Permanent pAesp_SAT86 loci are indicated with yellow arrows; polymorphic sites are shown with red arrows.

pericentric inversion. Several facultative pAesp_SAT86 sites were found in more than one genotype (**Figure 1**, red arrows), while some signals were detected in single genotypes on either one or both homologous chromosomes.

The labeling patterns of Spelt-1 and Spelt-52 probes are highly polymorphic (**Figure 1**). The Spelt-1 sequence is located in subtelomeric regions of either one or two chromosome arms. The number of loci per diploid genome varied from six (TS89 Katzir and No2 from Turkey) to 27 (PI 542274 from Turkey). Genotypes differ from each other in the size and chromosome location of the Spelt-1 clusters. The Spelt-52 signals of variable size are located in distal chromosome regions, proximally to Spelt-1. The number of Spelt-52 clusters per diploid genome varied from eight to 22 (**Figure 1**), the size and chromosomal distribution are highly polymorphic. Genotypes differ from each other in a ratio of Spelt-1/Spelt-52 repeats. Thus, the Spelt-1 could significantly prevail over Spelt-52, or the Spelt-52 could be more abundant (**Figure 1**).

Only few inconsistent, dot-like pTa-535 signals have been detected in *Ae. speltoides* (**Figure 1**). No hybridization was found with pAs1 and pTa-s53 probes.
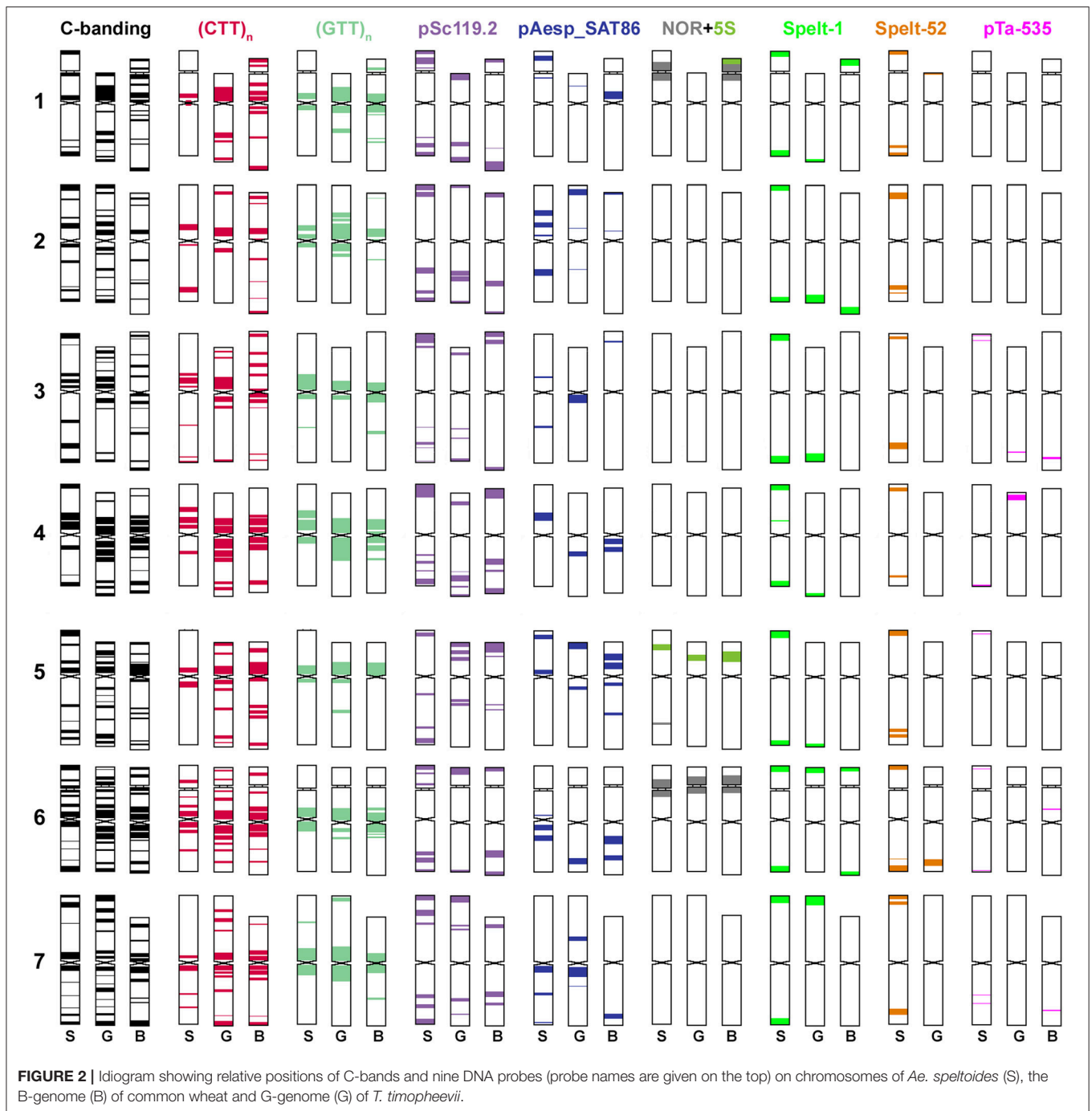
Sub-section Emarginata

Four species of the *Emarginata* sub-section have a similar karyotype, which is distinct from that of *Ae. speltoides* (**Figures S1**, **S2**). Chromosome pairs 5S* and 6S* carry unequal satellites: large on 6S* and small on 5S* chromosomes (**Figure S2**). Most *Ae. sharonensis* genotypes collected in Keshon

(Israel) are heterozygotes (**Figures S2c1,c2**) indicating that open pollination is common in this population.

The karyotypes of *Emarginata* species differ in heterochromatin content detected by Giemsa staining. *Ae. bicornis* and *Ae. searsii* showed small-to-medium C-bands located in interstitial chromosome regions (**Figures S2a1–b4**). *Ae. sharonensis* and *Ae. longissima* exhibit prominent pericentromeric and subtelomeric and many interstitial C-bands (**Figures S2c1–d10**). C-banding patterns allowed the chromosome identification in all *Emarginata* species. A species-specific translocation between 4S* and 7S* is found in all *Ae. longissima* accessions.

The (CTT)$_{10}$-hybridization pattern (**Figures 3d,e,i,j**, **4**, **5**) corresponds to the C-banding pattern. As expected, *Ae. bicornis* and *Ae. searsii* carry predominantly small CTT-signals (**Figures 3e,j**), while *Ae. sharonensis* and *Ae. longissima* possess prominent pericentromeric and distinct interstitial CTT-clusters. In contrast to *Ae. speltoides*, the (GTT)$_9$ probe hybridizes poorly on the chromosomes of *Emarginata* species. Probably, accumulation of heterochromatin in this evolutionary lineage was mainly due to amplification of CTT-repeat, contributing to an increase of nuclear DNA content in *Ae. sharonensis/Ae. longissima* genomes as compared to *Ae. bicornis/Ae. searsii* (Eilam et al., 2007).

Emarginata species display similar pSc119.2 hybridization patterns consisting of subtelomeric signals of variable size in one or both chromosome arms. Interstitial loci were rarely found

FIGURE 2 | Idiogram showing relative positions of C-bands and nine DNA probes (probe names are given on the top) on chromosomes of *Ae. speltoides* (S), the B-genome (B) of common wheat and G-genome (G) of *T. timopheevii*.

(**Figures 4**, **5**). Permanent interstitial sites are found on 2S*S (*Ae. sharonensis* and *Ae. bicornis*), 4S^{l}L (*Ae. longissima*), and 7S^s (*Ae. searsii*) only. The pSc119.2 cluster in the middle of 5S*S is present in all *Ae. searsii* accessions and some *Ae. longissima* and *Ae. sharonensis* lines (**Figure 4**). One or two polymorphic pSc119.2 sites were rarely observed on 1S^{b}L and 4S^{b}L of *Ae. bicornis*.

The number and location of 5S and 45S rDNA loci in *Emarginata* species is similar and differ from that in *Ae. speltoides*

(**Figures 1**, **4**). Major NORs are located on 5S*S and 6S*S and permanent minor NORs are found on 1S*S (**Figure 4**). Additional minor sites were detected in the terminal region of 6S*L of all *Ae. searsii* accessions and some lines of *Ae. bicornis* and *Ae. longissima*. All species possess two 5S rDNA loci located in the short arms of chromosome 1S* and 5S*, distally (1S*) or proximally (5S*) to the 45S rDNA loci.

The distribution of pAesp_SAT86 signals is species- and chromosome-specific. An intraspecific polymorphism was
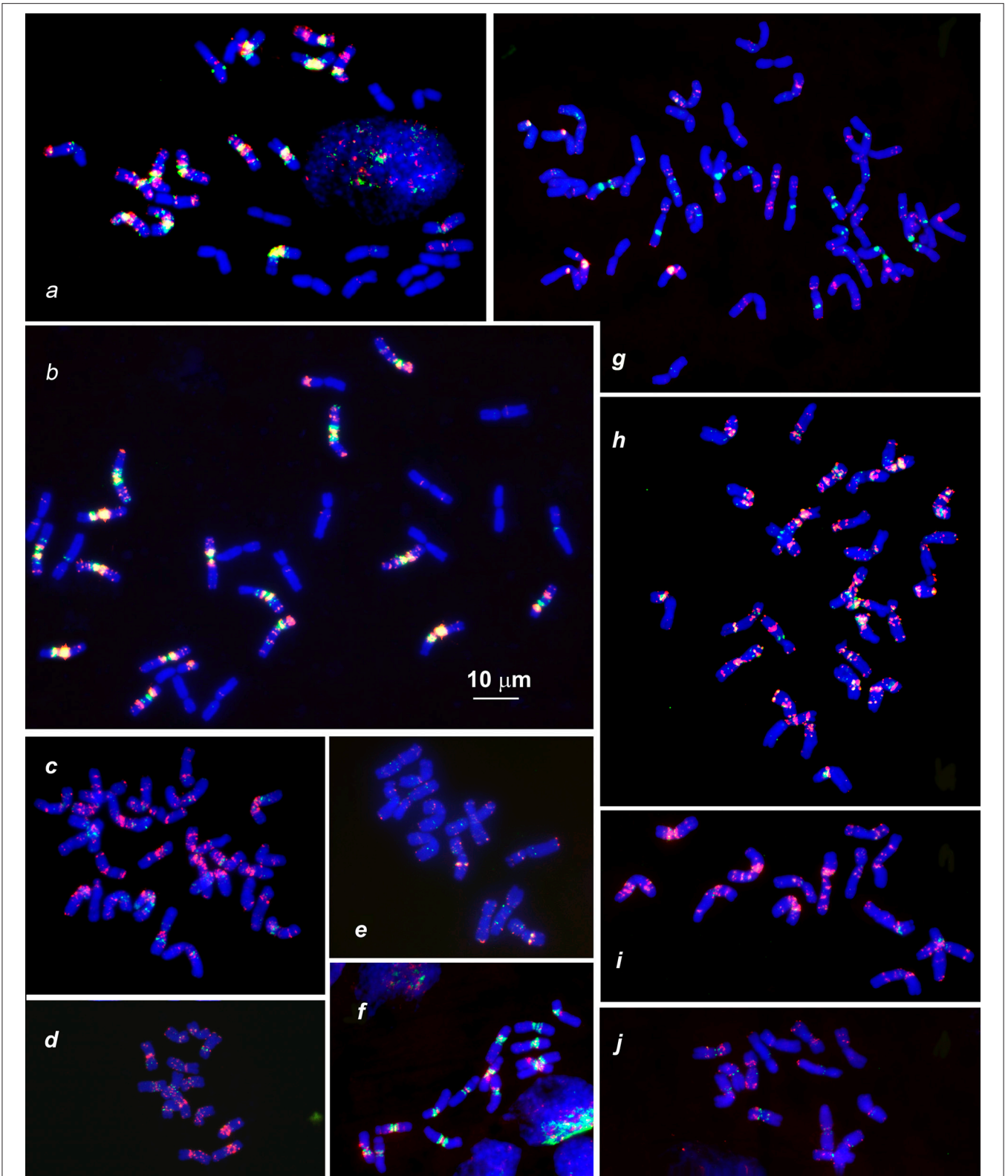
FIGURE 3 | Distribution of $(CTT)_{10}$ and $(GTT)_9$ probes (red and green colors respectively) on chromosomes of wheat and *Aegilops* species: **(a)**, *Triticum dicoccoides* (IG 46396); **(b)**, *T. araraticum* (IG 116164); **(c)**, *Ae. kotschyi* (TA2206); **(d)**, *Ae. longissmia* (AE 904); **(e)**, *Ae. searsii* (AE 1071); **(f)**, *Ae. speltoides* (C1, Technion park, Israel); **(g)**, *Ae. vavilovii* (K-3637); **(h)**, *Ae. peregrina* (C11, Nahal Mearot, Israel); **(i)**, *Ae. sharonensis* (C6, Keshon, Israel); **(j)**, *Ae. bicornis* (K-666). Scale bar, 10 μm.
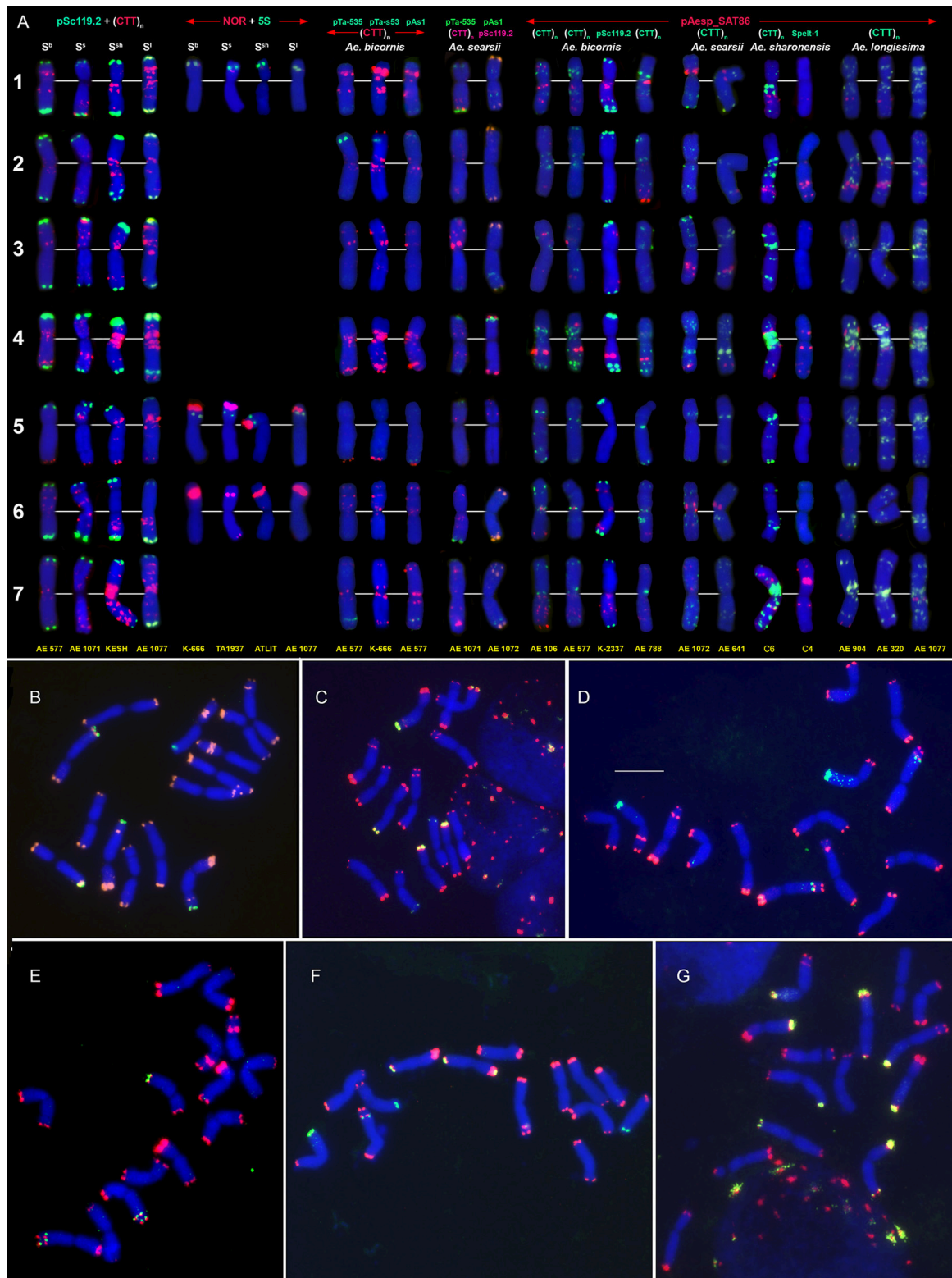
FIGURE 4 | Distribution of repeated DNA sequences on chromosomes of four species of *Emarginata* group **(A)**, *Ae. bicornis* (S^b^), *Ae. searsii* (S^s^), *Ae. sharonensis* (S^sh^), and *Ae. longissima* (S^l^). **(A)** Probe combinations are given on the top, accession names are shown below karyograms. Signal color corresponds to probe name. 1–7 – homoeologous groups. Polymorphisms of Spelt-52 patterns on *Ae. longissima* **(B–D)** and *Ae. sharonensis* **(E–G)** chromosomes: **(B)**, K-905; **(C)**, K-907; **(D)**, C3 (HaBonim); **(E)**, C6 (Keshon); **(F)**, C7 (HaBonim); **(G)**, i-570030. The pSc119.2 signals are shown in red, Spelt-52–in green. Scale bar, 10 μm.

FIGURE 5 | Idiogram showing relative positions of C-bands and nine DNA probes (probe names are given on the top) on the S*genome chromosomes of *Ae. bicornis* (b), *Ae. searsii* (s), *Ae. sharonensis* (sh), *Ae. longissima* (l), *Ae. peregrina* (p), *Ae. kotschyi* (k), and *Ae. vavilovii* (v).
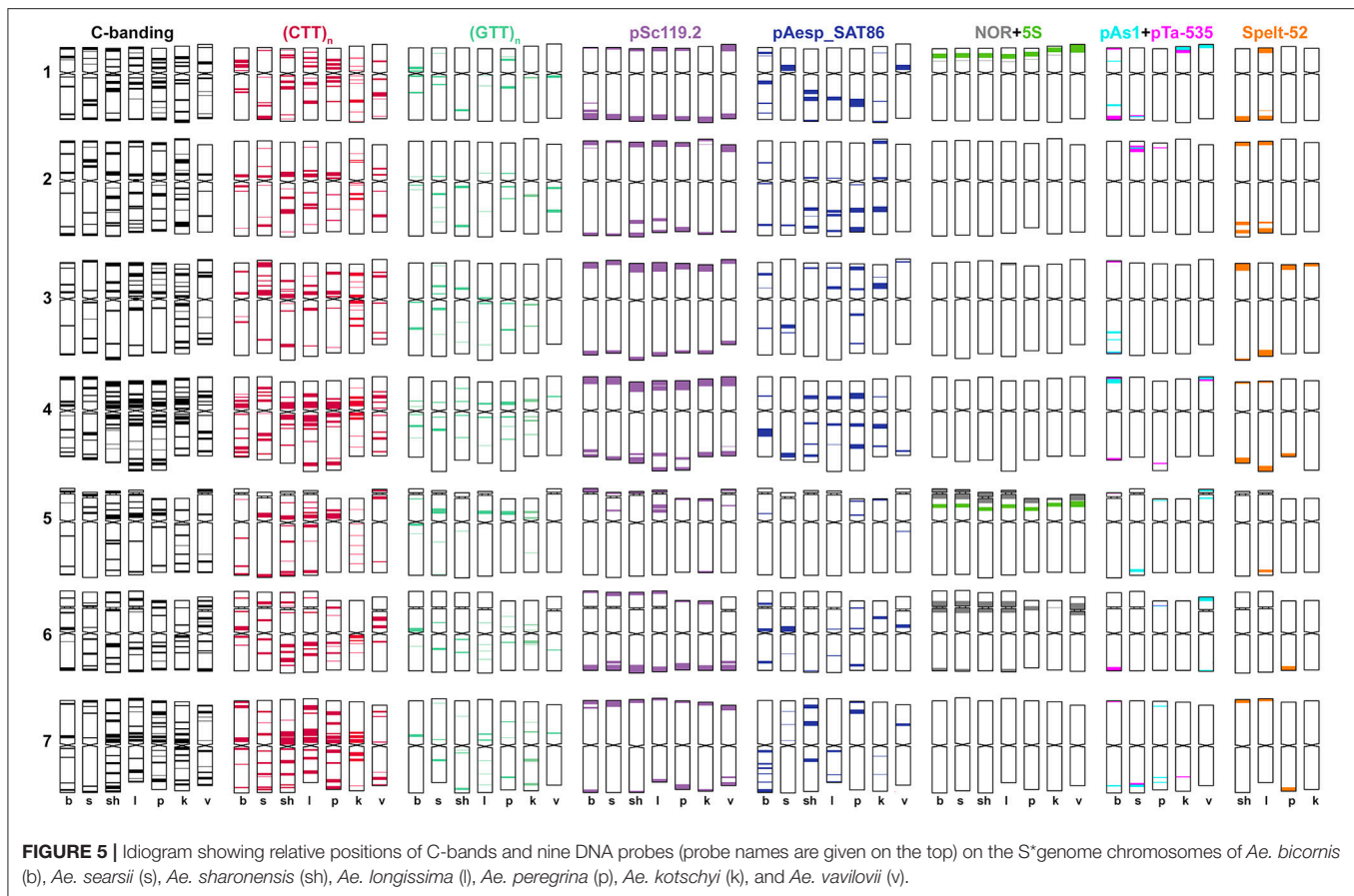
detected in *Ae. bicornis* and *Ae. longissima,* labeling patterns are virtually invariable in *Ae. searsii* (**Figures 4**, **5**). Distribution of pAesp_SAT86 clusters on *Ae. sharonensis* and *Ae. longissima* chromosomes is similar and differs from *Ae. bicornis* and *Ae. searsii,* which, in turn, are clearly distinct from each other. No similarity between homoeologous chromosomes of different species has been observed, though the chromosome 3S^s (*Ae. searsii*) shows almost the same distribution of pAesp_SAT86 sequence as the chromosomes 2S* of *Ae. sharonensis* and *Ae. longissima.*

The Spelt-1 repeat was not found in any *Emarginata* species, and Spelt-52 is detected in *Ae. sharonensis* and *Ae. longissima* only (**Figures 4B–G**). Signals of variable size are located in terminal regions of either one or both arms of all chromosomes except 6S*. Only two interstitial loci are found in the long arms of 2S* and 4S*. Distribution of Spelt-52 is highly diverse and polymorphisms are often observed even between homologous chromosomes. Depending on genotype, the number of signals ranges from 0 to 14. Most *Ae. longissima* accessions carry a Spelt-52 site in the long arm of 5S*, while it is absent in six out of 8 *Ae. sharonensis* accessions (**Table S2**). No other differences in labeling patterns were found between these species.

Distinct signals of the D-genome specific probes pAs1, pTa-535 or pTa-s53 are revealed in *Ae. bicornis* and *Ae. searsii* only (**Figures 4A**, **5**). Two small pTa-535 sites are found in the distal

parts of 2S^{b}S and 7S^{b}L chromosomes of *Ae. bicornis;* the first one overlaps with pTa-s53, and the second—with pAs1 sites. The pTa-535 probe hybridizes to subterminal regions of five pairs of *Ae. searsii* chromosomes, 1S^{s}L and 6S^{s}L exhibiting the largest signals. A relatively intense pAs1 signal is detected in a terminus of 4S^{s}S and few very weak interstitial signals are observed on 1S^{s}L, 3S^{s}L, and 7S^{s}L. Faint, dispersed, non-specific pAs1 signals are distributed in distal halves of *Ae. sharonensis* and *Ae. longissima* chromosomes, while pTa-s53 and pTa-535 did not hybridize to the chromosomes of these species.

Analysis of Polyploid Species: Wheats

Differences between emmer and Timopheevii wheat are mainly due to species-specific translocations identified in both evolutionary lineages (Naranjo et al., 1987; Liu et al., 1992; Jiang and Gill, 1994a; Maestra and Naranjo, 1999; Salina et al., 2006a). The (CTT)$_{10}$ signals on *T. araraticum* and *T. dicoccoides* chromosomes (**Figures 3a,b**, **6a,c,h**) mainly correspond to the C-bands, whereas (GTT)$_9$ forms large clusters in proximal regions of all B- and G-genome chromosomes (**Figure 2**, G,B; **Figures 3a,b**); their positions mainly overlapped with the location of (CTT)$_{10}$ clusters. A similar pattern is also observed in *Ae. speltoides* (**Figure 3f**).

Although the pSc119.2 hybridization patterns in these two wheat species are distinct and species-specific, they share some
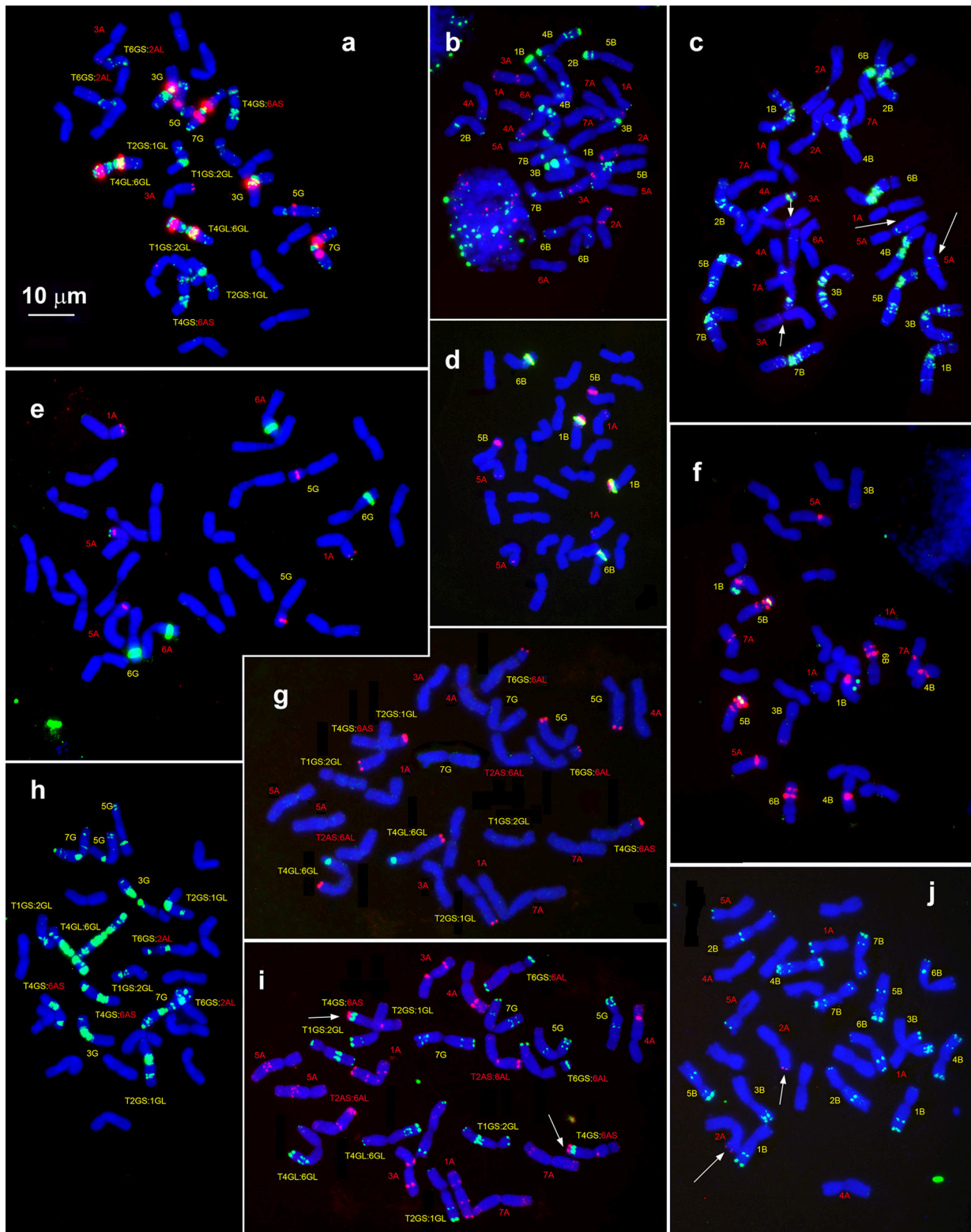
FIGURE 6 | Hybridization patterns of (CTT)$_{10}$ (**a,c,h**, green), pSc119.2 (**b,i,j**, green), pTa-535 (**c,i**, red), pTa-s53 (**b,h**, red), pAesp_SAT86 (**f,h**, red), NORs (**d,e**, green), and 5S rDNA (**d,e**, red), 5S rDNA (**f**, green); Spelt-1 (**j,g**, red) and Spelt-52 (**g**, green) on metaphase chromosomes of *T. dicoccoides,* IG 46396 (**b–d,f,j**) and *T. araraticum,* K-59940 (**a,e,h,g,i**). Position of pTa-s53 hybridization sites on *T. dicoccoides* chromosomes (**c**), huge cluster of pTa-535 sequence on the chromosome 4GS (**i**) and Spelt-1 site on *T. dicoccoides* chromosome 2AL (**j**) are indicated with arrows. Scale bar, 10 μm.
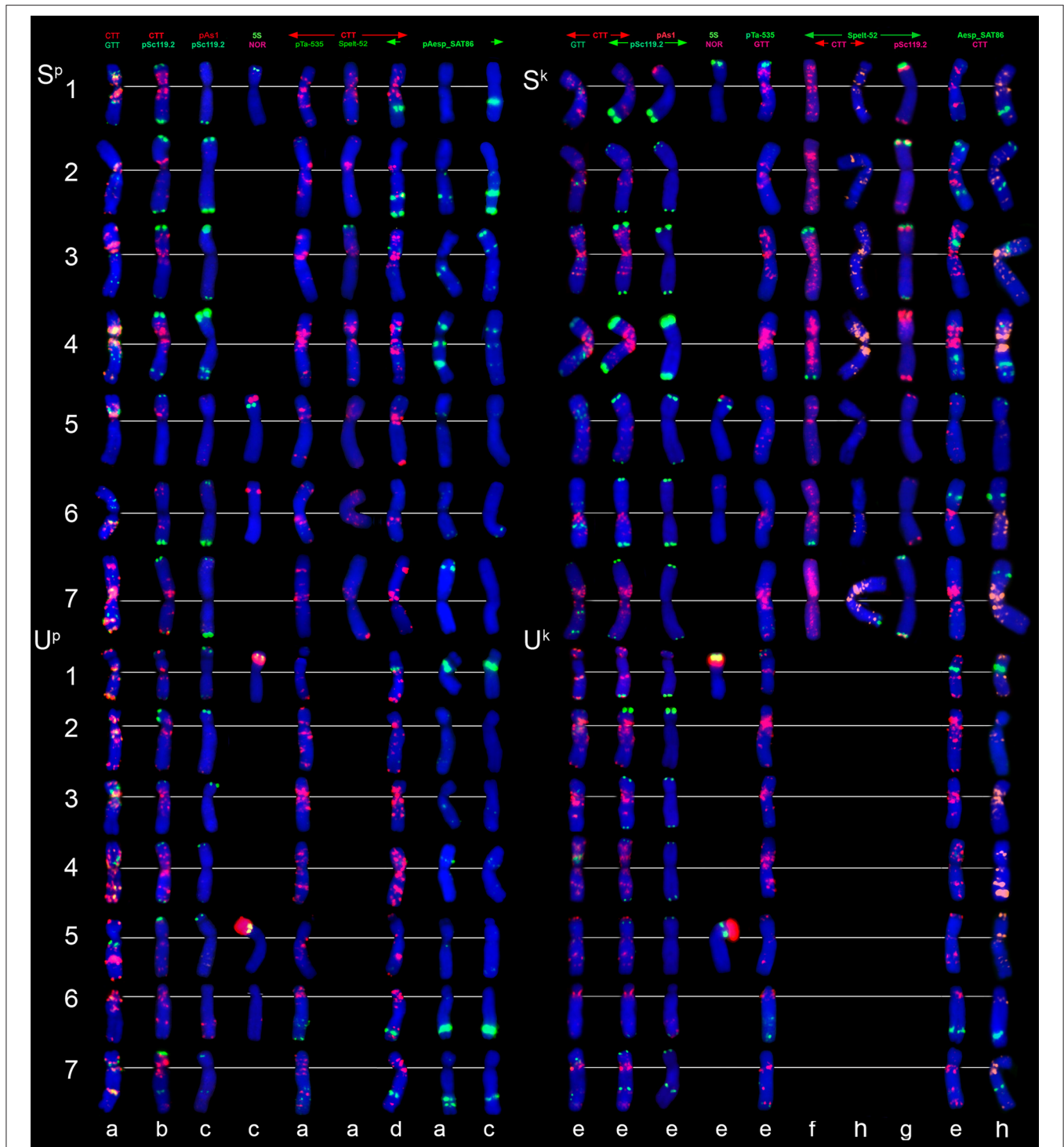
FIGURE 7 | Distribution of repetitive DNA families on chromosomes of *Ae. peregrina* left side and *Ae. kotschyi* (right side of the figure): **(a)**, C11 (Nahal Mearot, Israel); **(b)**, C8 (Haifa, Carmel, Israel); **(c)**, K-61; **(d)**, C9 (Keshon, Israel); **(e)**, TA2206; **(f)**, K-91; **(g)**, hybrid *Ae. umbellulata* TU04 × *Ae. sharonensis* TH02; **(h)**, K-2905. Probe combinations are given on the top; signal color corresponds to probe name. The S-genome chromosomes are shown on the top, the U-genome–on the bottom part of the figure.

similar features. As in *Ae. speltoides,* pSc119.2 signals are located in interstitial and subtelomeric regions of orthologous chromosome allowing a complete chromosome identification.

Two chromosome pairs of *T. araraticum* and *T. dicoccoides* carry major NORs (**Figures 6d,e**). These are 1B and 6B in *T. dicoccoides* and 6G and 6A^t in *T. araraticum* (transfer of NORs

from 1G to 6A[t] is due to species-specific translocation 1G-4G-6A[t] in Timopheevii lineage, Jiang and Gill, 1993). Group 1 and 5 chromosomes of *T. dicoccoides* display eight 5S rDNA signals (**Figure 6d**), but only six - in *T. araraticum* (chromosomes 1A[t], 5A[t], and 5G, see **Figure 6e**). Chromosome 5S of *Ae. speltoides* shows one 5S rDNA locus, therefore 1S likely lost the 5S rDNA locus in the progenitor of *Ae. speltoides* after the formation of ancient emmer, but prior to the divergence of Timopheevii wheat.

The pAesp_SAT86 clusters are found on both A and B/G genome chromosomes (**Figures 6a,f**), *T. dicoccoides* and *T. araraticum* show different labeling patterns and both exhibit broad intraspecific polymorphisms (Badaeva, unpublished). A large pAesp_SAT86 signal is found on 1BS of all emmer (**Figure 6f**) and common wheat (Komuro et al., 2013), but it is absent on 1G of *T. timopheevii* (**Figure 6a**). By contrast, huge 3GL- and 7GL-located pericentromeric pAesp_SAT86 clusters are missing on the homoeologous chromosomes of emmer wheat. At the same time, similar labeling patterns were observed on 4B/4G, 5B/5G, and 6B/6G of these species.

Very weak Spelt-52 signals were seen on 1GS and large on 6GL of *T. araraticum*. The same sequence was not detectable in wild emmer. Two faint Spelt-1 signals were revealed on the chromosome pair 2A of *T. dicoccoides* (**Figure 6j**), whereas ten clear signals were observed on chromosomes 6A[t]S, 1GL, 4GL, 5GL, and 6GS of *T. araraticum* (**Figure 6g**).

Probe pTa-535 hybridized predominantly on the A-genome chromosomes of both wheat species (**Figures 6b,i**, red color). A large pTa-535 cluster was found on the short arm of 4G of *T. araraticum* (**Figure 6i**, indicated with arrows). Overlapping, small pAs1/ pTa-535 signals are detected in distal halves of 3GL and 3BL. In addition, faint pAs1 signals were found in the satellite of 1B, in the middle of 6BS and 7BL of wild emmer. *T. araraticum* carries small pAs1 loci on 5GL and 7GL and in the satellite of 6A[t] (data not shown). Only weak pTa-s53 signals were observed on chromosomes 3AS and 5AL of *T. dicoccoides* (**Figure 6c**), and no hybridization was found on *T. araraticum* (**Figure 6h**).

Polyploid *Aegilops*: Ae. peregrina and Ae. kotschyi

Ae. peregrina and *Ae. kotschyi* are both tetraploids with the same genome constitutions UUS*S*. Their C-banding patterns are generally similar, however, some differences in morphology and heterochromatin distribution on chromosomes 2S*, 4S*, and 7S* are observed (**Figure S3**). According to C-banding patterns, *Ae. peregrina* carries 4S-7S* translocation and therefore the S[p]-genome is originated from *Ae. longissima*. The S[k] genome of *Ae. kotschyi* is more diverged from the S*-genomes of *Emarginata* species, but shares similar structure and C-banding pattern of chromosome 4S* with *Ae. sharonensis* (**Figures S2, S3**).

FISH with (CTT)$_{10}$ and (GTT)$_9$ probes reveals large CTT-clusters on all chromosomes, but only few weak GTT-signals on some U and S*-genome chromosomes of both species (**Figures 3c,h**). Distribution of (CTT)$_{10}$ probe corresponds to the C-banding patterns (**Figure 7**, **Figure S3**), and dual-color FISH allows aligning of the CTT/C-banding and

pSc119.2-FISH patterns (**Figure 5**). Positions of pSc119.2 clusters on chromosomes of the two species are similar except for 4S*, which carries two prominent subtelomeric signals in *Ae. kotschyi*, but one huge cluster in the short and two smaller sites in the long arm in *Ae. peregrina* (**Figure 7**). Labeling patterns varies between the accessions. Owing to subterminal location of pSc119.2 sites and polymorphism of labeling patterns, applicability of the pSc119.2 probe for chromosome identification of *Ae. peregrina* and *Ae. kotschyi* is limited.

Distribution of 45S and 5S rDNA loci on *Ae. peregrina* and *Ae. kotschyi* chromosomes is similar **Figure 7**, (NOR+5S) and corresponds to that in the parental species. Signal size of pTa71 probe (45S rDNA) on 5S[k] and especially 6S[k] chromosomes of *Ae. kotschyi* is significantly smaller than on the orthologous chromosomes of *Ae. peregrina*, which can be an indicative of more extensive gene loss at the respective loci.

FISH reveals similar hybridization patterns of pAesp_SAT86 probe on chromosomes of *Ae. peregrina* and *Ae. kotschyi*. According to dual-color FISH, the largest pAesp_SAT86 signals are located on chromosomes 1S*L (polymorphic), 2S*L, 1US, 6UL, and 7UL. Chromosomes 3S* and 4S* carry medium and 5S*S, 6S*L, and 4US – faint signals (**Figure 7**). Labeling patterns of chromosomes 3S*, 4S*, 6S*, and 7S* are polymorphic. In contrast to *Ae. peregrina* and diploid *Emarginata* species, the chromosome 6S[k] of *Ae. kotschyi* carries large pAesp_SAT86 cluster in the short arm.

The Spelt-1 repeat is not found in these tetraploid species, while Spelt-52 is revealed only in few accessions of *Ae. kotschyi* and *Ae. peregrina*. Small Spelt-52 clusters are observed on four out of seven S*-genome chromosome: 3S*S, 4S*L, 6S*L, and 7S*L. Number of signals varies from two to six (**Figure 7**), nearly half of genotypes we examined do not exhibit any hybridization. This is strictly different from what is observed in a newly synthesized hybrid *Ae. umbellulata x Ae. sharonensis*, in which 12 distinct Spelt-52 signals are observed in either one or both arms of chromosome pairs 1S*, 2S*, 3S*, and 7S* (**Figure 7g**).

Very few weak pAs1 and pTa-535 signals are located predominantly on the U-genome chromosomes of *Ae. kotschyi* and *Ae. peregrina* (**Figure 7**), while the pTa-s53 sequence is totally absent.

Polyploid *Aegilops*: Ae. vavilovii

The hexaploid species *Ae. vavilovii* with the genome constitution D[1]D[1]X[cr]X[cr]S[v]S[v] is characterized by a medium amount of Giemsa bands. Small and medium sized bands are distributed predominantly in interstitial chromosome regions (**Figure 8**). Two chromosome pairs are submetacentrics with small satellites, which morphologically correspond to 5S*. Two other pairs are metacentrics with large satellites, which is typical for chromosome 6S*. The C-banding pattern of *Ae. vavilovii* is similar to the parental species: *Ae. crassa* (Badaeva et al., 1998, 2002) and *Ae. searsii* (Friebe et al., 1995; Friebe and Gill, 1996). Intraspecific variations due to chromosomal rearrangements were identified in two of the three accessions of *Ae. vavilovii*.

The distribution of (CTT)$_{10}$ signals is generally similar to the observed C-banding patterns (**Figure 8**). The (GTT)$_9$ probe results in distinct signals on five pairs of the S[v] genome

FIGURE 8 | C-banding (left) and FISH patterns of *Ae. vavilovii* chromosomes: a – K-3635; b – K-3637. Chromosomes are assigned to genome D[1] (left), X[cr] (middle), and to the S[v] genome (right) according to similarity with chromosomes of *Ae. crassa* (Badaeva et al., 1998) and *Ae. searsii* (Friebe et al., 1995). Probe combinations are shown on the top, signal color corresponds to probe name. Chromosomes derived from *Ae. crassa* are designated with letters (A–N); the S[v] genome chromosomes are numbered 1–7 according to genetic nomenclature (Friebe et al., 1995).

chromosomes and small to medium clusters on seven pairs of the D[1] and X[cr] genome chromosome. (GTT)$_9$ signals only partially overlap with (CTT)$_{10}$ loci (**Figure 3g**).

Probe pSc119.2 hybridized with all S[v] and some X[cr] genome chromosomes. Signals are located in subterminal chromosome regions; interstitial sites were found in the middle of 5S[v]S and in the distal region of 4S[v]L and 7S[v]L (**Figure 8**).

Probe pTa71 revealed eight major and eight minor NOR sites in *Ae. vavilovii*. The major NORs are located on group 5 and 6 chromosomes belonging to S[v] and X[cr] genomes. Minor NORs mapped on all three pairs of group 1 chromosomes and, surprisingly on 6D[1]S. Six of 5S rDNA sites are located on group 1 and 5 chromosomes. An additional, minor 5S rDNA locus is detected in the proximal region of an unknown small metacentric chromosome (**Figure 8**, shown with arrow).

The pAesp_SAT86 signals of different sizes were detected on many *Ae. vavilovii* chromosomes; the number of loci varies from one to three per chromosome (**Figure 8**). Distribution of pAesp_SAT86 sites on 2S[v], 3S[v], and 7S[v] is different from

Ae. searsii, while the remaining chromosomes of these genomes show similar labeling patters.

The A/D-genome-specific probes pAs1, pTa-535, and pTa-s53 hybridize mainly to the D[1]-genome and partially to X[cr] genome chromosomes of *Ae. vavilovii* (**Figure 8**). The S[v] genome possesses the lowest amount of these sequences. Small pAs1 signals were observed only in terminal regions of 4S[v]S and 6S[v]S. Neither Spelt-1, nor Spelt-52 hybridization sites were detected in *Ae. vavilovii*.

DISCUSSION

Karyotype analysis as a tool for studying evolutionary processes must be based on an unified chromosome nomenclature. The first classification of chromosomes according to their homoeologous relationships and genome affinities was developed for common wheat by Sears (1954), and since then it is used as standard in genetic and cytogenetic studies of the *Triticeae*. Although the nomenclature of Giemsa C-banded chromosomes is now

available for many *Aegilops* species, including *Ae. speltoides, Ae. searsii, Ae. longissima,* and *Ae. peregrina* (Friebe and Gill, 1996), their correspondence to the distribution of FISH probes is not known.

In order to link C-banding and FISH patterns (Jiang and Gill, 1993) developed a method of sequential C-banding and *in situ* hybridization analysis. An alternative approach was suggested by Pedersen and Langridge (1997), who used the barley probe pHvG38 containing a GAA-satellite sequence for the identification of wheat chromosomes. Later this sequence was successfully used for the FISH analyses of wheat, barley, rye and some other cereal chromosomes (Pedersen et al., 1996; Cuadrado et al., 2000; Vrána et al., 2000; Cuadrado and Jouve, 2002; Kubaláková et al., 2005; Kato, 2011; Komuro et al., 2013; Adonina et al., 2015; Badaeva et al., 2016), however it was rarely applied for *Aegilops* species (Molnár et al., 2005, 2016; Mirzaghaderi et al., 2014).

The CTT–labeling patterns of *Aegilops* chromosomes obtained in our study basically correspond to their C-banding patterns. Therefore, we used the CTT-signals as landmark to identify chromosomes according to the genetic nomenclature. This allowed us to compare karyotypes based on chromosome homoeology and to trace chromosomal changes that could have occurred over the course of species evolution.

Ae. speltoides and Polyploid Wheats Are Cytogenetically Distinct From the S*-Genome of Other Diploid and Polyploid *Aegilops* Species

Based on C-banding and FISH patterns it is possible to divide the S-genome chromosomes of diploid and polyploid wheat and *Aegilops* species into two distinct groups. The first one includes *Ae. speltoides* and polyploid wheat. The second contains four diploid species of the *Emarginata* sub-section and three polyploid *Aegilops,* in agreement with molecular phylogenetic analyses (Yamane and Kawahara, 2005; Golovnina et al., 2007; Goryunova et al., 2008; Salse et al., 2008; Gornicki et al., 2014; Marcussen et al., 2014; Middleton et al., 2014; Feldman and Levy, 2015). The main diagnostic features of these groups can be described as follows.

1. The satellite chromosomes of the S-genome of *Ae. speltoides* and B/G-genomes of polyploid wheats belong to homoeologous groups 1 and 6 (Dvorák et al., 1984; Friebe et al., 2000). The satellites are large and nearly equal in size (Chennaveeraiah, 1960). The satellite of *T. timopheevii* chromosome 1G is transferred to 6A[t] as a result of a species-specific translocation (Jiang and Gill, 1994a; Rodríguez et al., 2000b; Dobrovolskaya et al., 2011). Major 45S rDNA sites are located on the short arms of group 1 and 6 chromosomes (**Figure 2**) (Yamamoto, 1992a,b; Jiang and Gill, 1994b; Badaeva et al., 1996b; Raskina et al., 2011; Belyayev and Raskina, 2013; Molnár et al., 2016). In addition to major NORs, Jiang and Gill (1994b) revealed minor 45S rDNA loci in the long arm of chromosome 1B of common and durum wheat, 1G of *T. timopheevii* and 1S of *Ae. speltoides,* which were never observed in other S*-genome *Aegilops*

species. Diploid *Emarginata* species possess two pairs of satellite chromosomes assigned to genetic groups 5 and 6 (Friebe et al., 1993, 1995; Friebe and Gill, 1996); satellites significantly differ in size (Chennaveeraiah, 1960). The secondary constrictions of 5S* and 6S* are suppressed in polyploid *Ae. peregrina* and *Ae. kotschyi,* but are extended in hexaploid *Ae. vavilovii.* FISH with the probe pTa71 revealed major 45S rDNA sites on 5S* and 6S* chromosomes of diploid and polyploid *Aegilops* species, but signal sizes were significantly reduced in tetraploid *Ae. peregrina* and *Ae. kotschyi* (**Figures 4**, **7**). Permanent minor 45S rDNA loci were present on chromosome 1S*, and additional minor site was detected in the terminus of 6S*L of all *Ae. searsii* and some *Ae. bicornis* and *Ae. longissima* accessions (**Figure 5**). Earlier we also found minor 45S rDNA locus in a terminus of the short arm of an unknown chromosome, probably 3S[l], of *Ae. longissima,* accession TA1912 (Badaeva et al., 1996b). These observations are in agreement with previously published results (Yamamoto, 1992a,b; Friebe et al., 1993; Badaeva et al., 1996b, 2002, 2004).

2. The S, B, and G genomes are enriched in GTT-repeats (**Figures 1**–**3**). This microsatellite is especially abundant in proximal chromosome regions, but rarely appears in interstitial locations. The GTT-sites do not always overlap with the CTT-clusters, and proximal GTT-signals could be observed in chromosome regions lacking Giemsa C-bands. By contrast, the S*-genome chromosomes of *Aegilops* species show poor labeling with the $(GTT)_9$ probe (**Figures 3**, **5**). The GTT-interstitial signals mainly overlap with the $(CTT)_n$ clusters (**Figures 3c–e**, **h–j**).

3. The distribution of pSc119.2 repeat in *Ae. speltoides* and the B/G genomes of wheat observed in our study (**Figures 1**, **2**) is similar to what was reported before (Jiang and Gill, 1994a; Badaeva et al., 1996a; Schneider et al., 2003; Kubaláková et al., 2005; Salina et al., 2006b; Komuro et al., 2013) and is distinct from the S*-genome chromosomes of other *Aegilops* species in preferentially interstitial signal location.

4. The Spelt-1 sequence is present in the S-genome of *Ae. speltoides* (Salina et al., 1997, 2006b; Raskina et al., 2011; Belyayev and Raskina, 2013) and the B/G genomes of polyploid wheats (Salina, 2006; Salina et al., 2006b; Zoshchuk et al., 2007, 2009), but it is absent from the S*-genome of other diploid and polyploid *Aegilops* species.

Different Families of Repetitive DNA Show Different Evolutionary Rates

Our data and previous findings imply that the evolutionary rate varies between different families of repetitive DNAs. Despite distinct differences between *Ae. speltoides*/polyploid wheats and other S*-genome *Aegilops* species in the distribution of rDNA probes, the patterns of 45S and 5S rDNA loci was highly conserved within each group. Only minor intra-and inter-specific variations were observed,

(1) Regarding the appearance of minor NORs, which occur at similar positions on the orthologous chromosomes (Yamamoto, 1992a,b; Badaeva et al., 1996b), and

(2) The decrease of signal size on the S*-genome chromosomes of tetraploid *Aegilops* species (Yamamoto, 1992a,b; Badaeva et al., 2004). Such signal reduction could be explained by uniparental elimination of genes (Shcherban et al., 2008).

The distribution of the rye-derived pSc119.2 repeat is also found to be relatively conserved within each of the two S-genome groups. This sequence with a 120 bp-long repeat unit is broadly distributed in the *Triticaea* and some *Aveneae* species and constitutes large and evolutionary old component of their genomes (Contento et al., 2005). The repeat units isolated from wheat, rye, barley and *Aegilops* species showed 70-100% similarity to each other. Nucleotide sequences of pSc119.2 repeat units are not species-specific, and one site may contain diverse members of the family (Contento et al., 2005). The authors proposed that these individual pSc119.2 sites are transferred as blocks and can be translocated within the genome resulting in position variation and site numbers. Similar was observed in our material. Most cereals, including barley (Taketa et al., 2000; Zhao et al., 2018), *Aegilops* (Badaeva et al., 1996a, 2002, 2004; Linc et al., 1999; Molnár et al., 2005, 2016), *Agropyron* (Brasileiro-Vidal et al., 2003; Li et al., 2018; Said et al., 2018), *Elytrigia* (Linc et al., 2012), *Haynaldia* (Zhang et al., 2013), possess predominantly subtelomeric pSc119.2 clusters. Therefore, a terminal location of pSc119.2 satellite family is probably a more primitive character compared to interstitial locations. Intercalary pSc119.2 sites are typical for *Ae. speltoides* (Badaeva et al., 1996a; Molnár et al., 2016), B- and G-genomes of polyploid wheats (Jiang and Gill, 1994a; Schneider et al., 2003), and rye (Cuadrado and Jouve, 2002); the rye genome being highly rearranged relative to wheat (Liu et al., 1992). Strong differences in the distribution of pSc119.2 sites in the R and S genome chromosomes suggest that transposition of this repeat proceeded in genomes of rye and *Ae. speltoides* independently, likely, after their radiation from the ancestral form.

Comparison of C-banding patterns with the distribution CTT+ GTT-microsatellite sequences shows that heterochromatin blocks detected by Giemsa staining in different *Triticum* and *Aegilops* species could have different sequence composition. Thus, *Ae. speltoides* chromosomes carry prominent proximal and telomeric C-bands and only few intercalary bands, which is considered as primitive karyotype structure (Stebbins, 1971). Only proximal bands overlap with both $(CTT)_{10}$ and $(GTT)_9$ clusters. The GTT-repeat is more abundant in these chromosomal regions. Intercalary C-bands correspond to CTT-signals, and probably they are composed by this microsatellite mainly. Neither $(CTT)_{10}$, nor $(GTT)_9$ signals were detected in telomeric heterochromatin, which is enriched in Spelt-1 and Spelt-2 repeats.

The C-banding patterns of the S*-genome *Emarginata* species and their polyploid derivatives are very similar to their CTT-hybridization patterns indicating that this sequence is a major component of Giemsa-positive heterochromatin. The GTT-microsatellite is present in much lower quantities, and only few C-bands contain this sequence solely. Species of this genomic group exhibit drastic differences in the content of C-positive heterochromatin. Diploid *Ae. bicornis, Ae. searsii,*

and hexaploid *Ae. vavilovii* are low heterochromatic; the $(CTT)_{10}$-signals are small and located mainly in the intercalary chromosome regions. Karyotypically *Ae. searsii* is distinct from other diploid species and its divergence was accompanied mainly by heterochromatin re-pattering visualized by Giemsa-staining and FISH with the CTT-microsatellite probe. The genomes of *Ae. sharonensis, Ae. longissima, Ae. kotschyi,* and *Ae. peregrina* are highly heterochromatic; prominent C-bands and CTT-signals are distributed in proximal and intercalary chromosome regions (**Figure 5**). Thus, massive amplification of the CTT-repeat occurred at the stage of radiation of *Ae. sharonensis* and *Ae. longissima*, resulting in an increase of nuclear DNA (Eilam et al., 2007) and the amount of heterochomatin.

Three tandemly repeated DNA families, pAesp_SAT86, Spelt-1, and Spelt-52 show the highest rate of evolution in the *Triticum-Aegilops* group. pAesp_SAT86 sequence is detected in all S-genome species (**Figures 1**, **3**, **7**, **8**) and the B/G genomes of polyploid wheat (**Figure 6**). The labeling patterns are extremely variable in *Ae. speltoides* (**Figure 1**) and differ from polyploid wheat species which, in turn, are distinct from each other (Komuro et al., 2013; Badaeva et al., 2016). Diploid *Emarginata* species and their polyploid derivatives display species-specific patterns of pAesp_SAT86 probe (**Figures 4–8**). *Ae. bicornis* shows the highest degree of intraspecific pAesp_SAT86-polymorphism, while little variation has been observed in *Ae. searsii, Ae. sharonensis* (**Figure 4**), *Ae. kotschyi* (**Figure 7**), and *Ae. vavilovii* (data not shown). *Ae. bicornis* and *Ae. searsii* differ from each other and from other species of this group (**Figure 5**). *Ae. sharonensis* is more similar with *Ae. longissima* and *Ae. peregrina* in the distribution of pAesp_SAT86 clusters and only slightly different from *Ae. kotschyi*. The pTa-713 (homolog of pAesp_SAT86) hybridization patterns of *Ae. peregrina* reported by Zhao et al. (2016) is consistent with our results, though there are some discrepancies in chromosome designations.

The Spelt-1 repeat is found in *Ae. speltoides* and the B/G genomes of polyploid wheats. In *Ae. speltoides* it comprises nearly 2% of the nuclear genome ($10^5–10^6$ copies). The copy number of constituent sequence related to Spelt-1 is ∼40–60 reduced in genomes of tetraploid wheats, and ∼1200–2400 times reduced in genomes of other *Triticeae* (Pestsova et al., 1998; Salina et al., 1998; Salina, 2006). Minor amounts of Spelt-1 exist in genomes of rye, cultivated barley, most diploid and polyploid wheat as well as *Aegilops* species indicates that this sequence was already present in minor quantities in the common ancestor of the *Triticeae* (Salina et al., 1998). High homology (97–100%) of individual repetitive units implies that massive amplification of Spelt-1 repeat occurred in ancient *Ae. speltoides* after radiation from the common ancestor of the *Triticeae* (Salina et al., 1998; Salina, 2006). Spelt52 is homologous to the pAesKB52 repeat isolated earlier from *Ae. speltoides* by Anamthawat-Jonsson and Heslop-Harrison (1993). This repeat consists of monomers of two types, Spelt52.1 and Spelt52.2, which share a homologous stretch of 280 bp and have two regions without sequence similarity of 96 and 110 bp, respectively. *Ae. speltoides* displays intraspecific variation in the occurrence of Spelt52 monomer types, whereas *Ae. longissima, Ae. sharonensis,* and *Ae. bicornis* showed no

interspecific variation (Salina et al., 2004a). The Spelt-52 is abundant in *Ae. speltoides* accounting for approximately 1% of nuclear genome (Anamthawat-Jonsson and Heslop-Harrison, 1993; Salina, 2006) and is also highly represented (1.0×10^4 – 2.5 $\times 10^5$ copies) in *Ae. longissima* and *Ae. sharonensis*, but it present in minor quantities in *Ae. bicornis* and *Ae. searsii* (Salina, 2006).

Coincidently with previous findings (Salina et al., 2006b; Raskina et al., 2011; Belyayev and Raskina, 2013), we observed significant intraspecific variation of Spelt-1 and Spelt-52 labeling patterns (**Figure 2**). Strict differences in a ratio of Spelt-1/ Spelt-52 repeats detected between genotypes can be due to geographical origin of the material. Earlier, Raskina et al. (2011) found that the amount of Spelt-1 and, in lower extent, the Spelt-52 repeat decreases in marginal populations of *Ae. speltoides*.

Although pAs1 and pTa-535 repeats are abundant in some cereal genomes (Rayburn and Gill, 1986; Badaeva et al., 1996a; Taketa et al., 2000; Komuro et al., 2013), they are poorly represented in the S genomes of *Triticum* and *Aegilops*. Thus, we failed to detect any pAs1signals in *Ae. speltoides*, but Molnár et al. (2016) revealed small pAs1 signals on the chromosome 3S. Wheat chromosomes 3BL - 3GL and 7BL - 7GL possess pAs1 and pTa-535 clusters in similar positions (Schneider et al., 2003; Badaeva et al., 2016), although they are not detected in *Ae. speltoides*. Probably, these loci were present in the genome of ancient *Ae. speltoides*, but they were eliminated after radiation of polyploid wheats. The pAs1 and pTa-535 repeats are also poorly represented in genomes of *Emarginata* species. Two distinct interstitial pAs1 sites overlapping with either pTa-535, or with pTa-s53 loci are found in *Ae. bicornis*. pAs1 and pTa-535 are less abundant in *Ae. searsii*, *Ae. sharonensis* and *Ae. longissima*. Only chromosome 1S^k of tetraploid *Ae. kotschyi* contains a distinct pAs1/pTa-535 cluster, and these sequences are absent from the S* genomes of *Ae. peregrina* and *Ae. vavilovii*.

Evolution of the S-Genome

Summarizing our data and the results of other authors (Kihara, 1954; Chennaveeraiah, 1960; Kihara and Tanaka, 1970; Yen and Kimber, 1990b; Zhang and Dvořák, 1992; Zhang et al., 1992; Dvořák, 1998; Feldman and Levy, 2015), the following scenario of the S-genome evolution can be suggested (**Figure 9**).
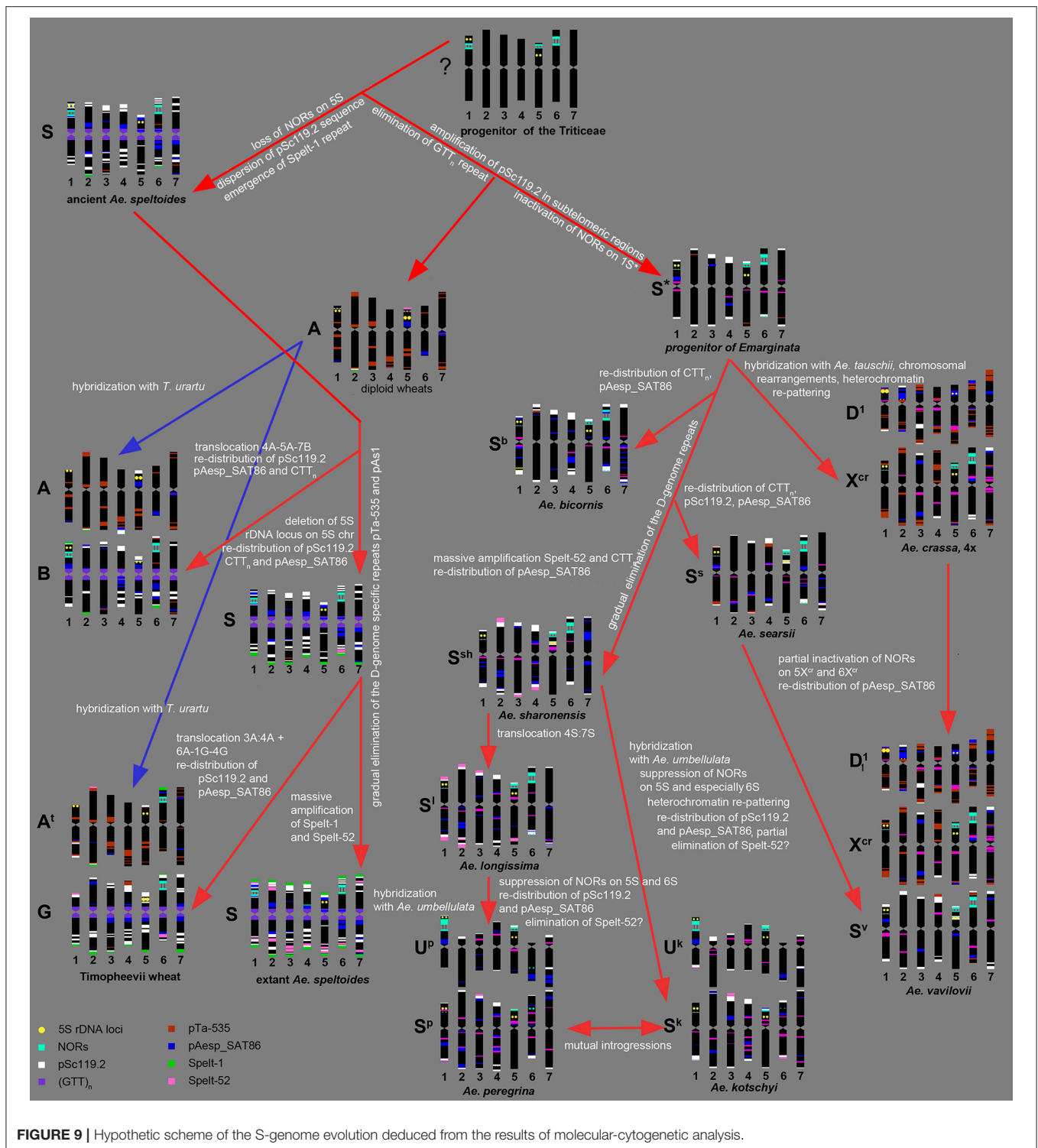
According to molecular phylogeny, *Ae. speltoides* is the most distinct diploid *Aegilops*, which diverged from the common ancestor very early, prior to the split of diploid wheat and *Aegilops* species (Salse et al., 2008; Gornicki et al., 2014; Marcussen et al., 2014; Feldman and Levy, 2015). Divergence of *Ae. speltoides* from an ancestral form was not associated with major translocations, because neither meiotic analysis (Rodríguez et al., 2000a), nor microsatellite mapping (Dobrovolskaya et al., 2011) detected structural chromosmal rearrangements in the S-genome. However some genomic changes not causing linkage group perturbations did probably occur at the early stages of *Ae. speltoides* speciation. As was shown earlier, major NORs in *Triticum* and *Aegilops* species are located on group 1, 5, and 6 chromosomes (Appels et al., 1980; Appels and Honeucutt, 1986), while 5S rDNA loci are located separately from NORs in the short arms of group 1 and 5 chromosomes (Appels et al., 1980; Dvořák et al., 1989). The chromosome 5S of *Ae. speltoides* and

B/G genome of polyploid wheats does not contain 45S rDNA loci, therefore the loss of respective NOR probably occurred prior to formation of ancient emmer. Other early genomic changes of ancient *Ae. speltoides* included the transposition of the pSc119.2 repeat from subtelomeric to interstitial chromosome regions and also the amplification of Spelt-1 repeat.

As mentioned above, *Ae. speltoides* and the B/G genomes of polyploid wheats are characterized by the abundance of GTT-microsatellite (Cuadrado et al., 2000; Badaeva et al., 2016), which is poorly represented in diploid wheats (Badaeva et al., 2015) and most *Aegilops* species (**Figure 3**). This difference can be caused by massive amplification of GTT-repeat in the ancient *Ae. speltoides*. Alternatively, this repeat could be eliminated from the progenitor of wheat and *Aegilops* species. Taking into consideration the abundance of GTT-repeat in rye and *Hordeum* (Cuadrado and Jouve, 2002, 2007; Dou et al., 2016), the second scenario seems to be more likely. The progenitor of *Ae. speltoides* probably possessed minor amounts of Spelt-52 and the D-genome specific repeats pTa-535, or pAs1, as they are still present in *Ae. speltoides* and the B/G genomes of polyploid wheats (Schneider et al., 2003; Badaeva et al., 2016; Molnár et al., 2016). However, these sequences could be of the A-genome origin, which spread to the S-genome following allopolyploidization.

The emergence of tetraploid emmer was accompanied by the species-specific translocation involving the chromosomes 4A-5A-7B (Naranjo et al., 1987; Liu et al., 1992; Maestra and Naranjo, 1999). In addition to structural chromosome rearrangements, other genetic and epigenetic changes occurred in a newly formed polyploid, including inactivation of the 45S rDNA loci on the A-genome chromosomes, re-distribution of Giemsa C-bands and repetitive DNA families on both A and B-genome chromosomes. Evolution of polyploid wheat resulted in polymorphisms of various DNA sequences and heterochromatin patterns that were described in many publications (Friebe and Gill, 1996; Schneider et al., 2003; Badaeva et al., 2016).

Subsequent evolution of *Ae. speltoides* occurred independently of polyploid emmer and was accompanied by several transposon insertions (Salse et al., 2008) and the loss of the 5S rDNA locus on the chromosome 1S, which is present in emmer and common wheat (Mukai et al., 1990), but absent in *T. timopheevii* (Badaeva et al., 2016) and modern *Ae. speltoides*. Although *T. timopheevii* derived from the same parental species as emmer, different parental genotypes were involved in the origin of these two lineages (Golovnina et al., 2007). Timopheevii wheat emerged much later, than ancient emmer - nearly 0.4 MYA (Gornicki et al., 2014) and its formation was accompanied by different species-specific translocation involving the chromosomes 1G-4G-6A^t + 3A^t-4A^t (Jiang and Gill, 1994a; Maestra and Naranjo, 1999; Rodríguez et al., 2000b; Dobrovolskaya et al., 2011). As a result, a major NOR was translocated from chromosome 1G to 6A^t, and a massive cluster of the A/ D-genome specific repeat pTa-535 appeared on the short arm of chromosome 4G (**Figure 6g**). Existence of Spelt-52 sites and a spread of Spelt-1 to most *T. timopheevii* chromosomes (Salina et al., 2006b; Zoshchuk et al., 2007; Badaeva et al., 2016) suggests a massive amplification of these sequences in *Ae. speltoides* prior to emergence of ancient *T. timopheevii*.

FIGURE 9 | Hypothetic scheme of the S-genome evolution deduced from the results of molecular-cytogenetic analysis.

Results of molecular cytogenetic analysis suggest that genome re-structuring process is still ongoing in natural populations of *Ae. speltoides.* This is exemplified by intraspecific C-banding polymorphisms and diversity of labeling patterns of pAesp_SAT86, Spelt-1 and Spelt-52 probes observed in this

and other studies (Belyayev and Raskina, 2011, 2013; Raskina et al., 2011), fluctuation of copy number of retrotransposons and tandem repeats, and high number of chromosomal rearrangements (Belyayev and Raskina, 2013; Shams and Raskina, 2018).

The species of the *Emarginata* group are closely related to each other (Eig, 1929; Kihara, 1954; Friebe and Gill, 1996; Kilian et al., 2011; Gornicki et al., 2014; Feldman and Levy, 2015), which is supported by their similar karyotypes (Chennaveeraiah, 1960), C-banding and pSc119.2-labeling patterns (Badaeva et al., 1996a), distribution of rDNA probes (Yamamoto, 1992a,b; Badaeva et al., 1996b). Separation of *Emarginata* species from a common ancestor was associated with inactivation of major NORs on chromosome 1S* accompanied with the significant loss of 45S rDNA repeat copies. Despite similarity of pSc119.2 labeling patterns, there are obvious, but discontinuous changes in the patterns of other sequences. Our data show that most drastic changes occurred probably at the stage of radiation of *Ae. sharonensis-Ae. longissima*. These are massive amplification of Spelt-52 and CTT-repeats resulting in the gain of heterochromatin in *Ae. sharonensis* and *Ae. longissima*, leading to an approximately 12% increase of nuclear DNA content in *Ae. sharonensis/ Ae. longissima* as compared to *Ae. searsii/ Ae. bicornis* (Eilam et al., 2007). By contrast, the amount of the D-genome repeats pTa-535, pAs1 and especially pTa-s53 gradually decreased, and these sequences nearly disappeared in genomes of *Ae. sharonensis* and *Ae. longissima*. Spelt-52 patterns of *Ae. sharonensis* and *Ae. longissima* chromosomes are highly polymorphic. The similar distribution of all analyzed DNA sequences on chromosomes of *Ae. sharonensis* and *Ae. longissima* (**Figures 3**, **4**) point to a rather recent divergence of these species, which was accompanied by the species-specific translocation 4S*-7S* in *Ae. longissima*.

Formation of tetraploid *Ae. peregrina* and *Ae. kotschyi* did not cause significant alterations of the parental genomes. Considering the structure of chromosome 4S*, the S^p-genome of *Ae. peregrina* was donated by *Ae. longissima,* while *Ae. sharonensis* or the form preceding the split of these diploids could be the source of the S*-genome of *Ae. kotschyi*. These data are consistent with observations of other authors (Yu and Jahier, 1992; Zhang et al., 1992; Friebe et al., 1996), however they contradict the hypothesis about the possible ancestry of *Ae. searsii* in the origin of *Ae. peregrina* (Siregar et al., 1988). Merging of U and S* genomes in the tetraploid *Ae. peregrina* and *Ae. kotschyi* led to inactivation of 45S rDNA loci on the S*-genome chromosomes (**Figure 7**). Similar was also recorded in the artificial allopolyploid *Ae. umbellulata* × *Ae. sharonensis* (Shcherban et al., 2008). Significantly smaller 45S rDNA sites on *Ae. kotschyi* chromosome 6S^k compared to the 6S^p of *Ae. peregrina* evidences in favor of a higher extent of gene loss at the respective locus, which can be due to earlier origin of *Ae. kotschyi*. The assumption that *Ae. kotschyi* is an older species is also supported by higher divergence of C-banding patterns relative to the parental species.

Interestingly, *Ae. peregrina* and *Ae. kotschyi* both possess only minor quantities of the Spelt-52 repeat, which is abundant in their diploid parents. According to the analyses of artificial wheat-*Aegilops* or *Aegilops-Aegilops* hybrids, the Spelt-52 was either amplified or retained at the same level upon polyploidization (Salina et al., 2004b). Considering these results we can expect massive amplification of the Spelt-52 sequence in *Ae. peregrina* and *Ae. kotschyi* genomes. However, this is not the

case. Low amount of Spelt-52 in these species can be caused by the so-called "originator effect," if they obtained their S* genomes from genotype depleted with this repeat, or it can be caused by sequence elimination after formation of tetraploids.

The S^v-genome chromosomes of *Ae. vavilovii* are very similar to the S^s-genome chromosomes of *Ae. searsii,* which further supports their close relationships (Zhang and Dvorák, 1992; Dubkovsky and Dvorák, 1995). Our results strongly suggest that the X^{cr} genome of *Ae. vavilovii* is also the derivative of the S* genome of an unknown *Emarginata* species, but not of *Ae. speltoides* as proposed by Dubkovsky and Dvorák (1995); Edet et al. (2018). Significant differences between the X^{cr} and S^s genomes, as well between X^{cr} and S*-genomes of all diploid *Emarginata* species in the C-banding and labeling patterns demonstrate that the X^{cr} genome was significantly modified during speciation.

CONCLUSIONS

Analysis of the S-genomes of diploid and polyploid *Triticum* and *Aegilops* species using FISH with nine DNA probes, including 5S and 45S rDNA, two microsatellites and five tandem repeats showed an isolated position of *Ae. speltoides* among other *Aegilops* species. In addition, close relationships with the B and G genomes of polyploid wheats were observed, thus confirming previous molecular-phylogenetic data (Yamane and Kawahara, 2005; Petersen et al., 2006; Golovnina et al., 2007; Salse et al., 2008; Gornicki et al., 2014; Marcussen et al., 2014; Middleton et al., 2014; Bernhardt et al., 2017). The evolution of polyploid wheats was associated with different species-specific chromosome translocations and the amplification/ elimination of repeats, re-pattering or, possibly with an exchange of repetitive DNA families with the A-genome chromosomes. Evolutionary changes in the *Ae. speltoides* genome occurred independently from polyploid wheats.

Diploid *Aegilops* species of *Emarginata* group are similar, but are substantially different from *Ae. speltoides* based on C-banding and FISH patterns. The genome evolution in this group was mainly associated with an increase of high copy DNA fraction due to amplification of CTT-repeat, re-distribution of C-bands, (CTT)$_n$-, (GTT)$_n$-, and pAesp_SAT86-clusters, massive amplification of Spelt-52 and gradual elimination of the D-genome-specific sequences pAs1, pTa-535 and pTa-s53. These changes were more profound at the stage of divergence of *Ae. sharonensis/Ae. longissima*. Tetraploid *Ae. peregrina* and *Ae. kotschyi* originated independently from hybridization of *Ae. umbellulata* with *Ae. longissima* (*Ae. peregrina*) or *Ae. sharonensis* or its immediate precursor (*Ae. kotschyi*). The S*-genomes of both tetraploids show little differences to the parental species. The S^k-genome is characterized by more modifications than the S^p-genome, suggesting that *Ae. kotschyi* is older than *Ae. peregrina*. Chromosome introgressions recorded in some accessions of both species (Badaeva et al., 2004) can be explained by gene flow between *Ae. peregrina* and *Ae. kotschyi*.

Our study confirmed that *Ae. vavilovii* is a natural hybrid between tetraploid *Ae. crassa* and *Ae. searsii*. The similarity of C-banding and FISH patterns of *Ae. vavilovii* and corresponding parental species points to rather recent origin of this hexaploid. The assumption that the X^{cr} genome is an additional derivative of the S*genome obtained from an unknown or extinct species of the *Emarginata* group, which was substantially modified over the course of evolution is supported.

AUTHOR CONTRIBUTIONS

EB planned the research, performed and coordinate the analysis. EB and AR performed the research and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01756/full#supplementary-material

Figure S1 | Diversity of the C-banding patterns in *Aegilops speltoides* accessions: **(a)**, No1, from Turkey (provided by Dr. N. Aminov); **(b–f)**, genotypes collected from Israeli populations; **(b,c)**, C1, Technion park, Haifa; **(d)**, C2, Nahal Mearot; **(e)**, G2.46, Ramat haNadiv; **(f)**, C14, Keshon; **(g)**, no 2734 (unknown provided by Dr. B. Kilian); **(h)**, No 2 from Iran (provided by Dr. N. Aminov); **(i)**, i-570060; **(j)**, TS89, Katzir, Israel; **(k)**, PI 487233, and **(l)**, PI 487231 (from Syria); **(m)**, PI 542269 (Turkey). Arrows show unbalanced chromosome modifications.

Figure S2 | Interspecific and intraspecific variation of the C-banding patterns in *Ae. bicornis* **(a1–a3)**, *Ae. searsii* **(b1–b4)**, *Ae. sharonensis* **(c1–c8)**, and *Ae. longissima* **(d1–d10)**. Accession codes: **(a1)**, TA1942; **(a2)**, TB04-3; **(a3)**, TB10-2; **(b1)**, G.7.15; **(b2)**, TE01-1; **(b3)**, G7.12; **(b4)**, IG 47619; **(c1,c2)**, C6, Keshon; **(c3)**, C5, Caesaria; **(c4)**, TH04; **(c5)**, TH01; **(c6)**, TH02; **(c7)**, C4, Atlit; **(c8)**, C7, HaBonim; **(d1)**, TL06; **(d2)**, TL01; **(d3)**, G6.77 (Sa'ad); **(d4)**, G6.58 (Tel Akko); **(d5)**, TL03; **(d6)**, C3 (HaBonim); **(d7)**, G6.32 (Nizzanim); **(d8)**, G6.55 (Zomet Shoked); **(d9)**, G17-3; **(d10)**, TL05.

Figure S3 | C-banding polymorphism of *Ae. peregrina* **(a–f)** and *Ae. kotschyi* **(g–j)** chromosomes: **(a)**, TA1888; **(b)**, C11 (Nahal Mearot, Israel); **(c)**, K-61; **(d)**, C12 (Caesaria, Israel); **(e)**, C13 (Natufia, Israel); **(f)**, i-570632; **(g)**, PI 487279; **(h)**, K-91; **(i)**, TA2206; **(j)**, K-201; **(k)** K-2905. 1–7, homoeologous groups.

Table S1 | List of material and their origins.

Table S2 | Distribution of Spelt-52 probe on chromosomes of *Ae. longissima* and *Ae. sharonensis*.

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3.2. How next-generation sequencing has aided our understanding of the sequence composition and origin of B chromosomes

How Next-Generation Sequencing Has Aided Our Understanding of the Sequence Composition and Origin of B Chromosomes

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Abstract: Accessory, supernumerary, or—most simply—B chromosomes, are found in many eukaryotic karyotypes. These small chromosomes do not follow the usual pattern of segregation, but rather are transmitted in a higher than expected frequency. As increasingly being demonstrated by next-generation sequencing (NGS), their structure comprises fragments of standard (A) chromosomes, although in some plant species, their sequence also includes contributions from organellar genomes. Transcriptomic analyses of various animal and plant species have revealed that, contrary to what used to be the common belief, some of the B chromosome DNA is protein-encoding. This review summarizes the progress in understanding B chromosome biology enabled by the application of next-generation sequencing technology and state-of-the-art bioinformatics. In particular, a contrast is drawn between a direct sequencing approach and a strategy based on a comparative genomics as alternative routes that can be taken towards the identification of B chromosome sequences.

1. Recent Discoveries Related to the Origin and Evolution of B Chromosomes

The origin and evolution of the B chromosomes, which appear to make a non-essential contribution to the overall genome, have puzzled cytogeneticists for over a century. These generally smaller than standard (A) chromosomes are transmitted in a higher than expected frequency, leading to a rise in their number from one generation to the next, in a process termed "drive" [1,2]. The quantum improvement in DNA sequencing power achieved by so-called next-generation sequencing (NGS), along with associated analytical methodologies, now allows for a rigorous investigation of the nucleotide composition of the B chromosomes. The result will finally provide an unequivocal answer to whether or not they harbor genes, whether they affect the function of the genome and how they originated. A number of B-chromosome-carrying species, representing a broad range of taxa, have been targeted in recent years to address these issues. The outcome of applying NGS and extensive cytogenetic analyses has been that the B chromosomes, despite their being non-essential, have been shown to share much in common with A chromosomes, and that they evolved in the various taxa in comparable ways.

Among the plant B chromosome carriers, the major focus on the sequence composition of B chromosomes has been on rye (*Secale cereale*). Based on molecular clock calculations, it has been estimated that rye B chromosomes originated approximately 1.1–1.3 million years ago, 0.4–0.6 million years after the formation of the *Secale* genus [3]. Analysis of flow-sorted B chromosomes has shown that they harbor a substantial amount of A-chromosome-derived DNA sequences. On the basis of these sequences, the B chromosomes represent a multichromosomal mosaic, with the two A chromosomes 3R and 7R making the largest contribution. The distribution of repetitive DNA

along the B chromosome is largely similar to that found in the A chromosomes, although certain transposable elements are either noticeably rarer or noticeably more abundant in the B chromosomes than in the A chromosomes. Two repetitive sequences, arranged as tandem repeats, were shown some time ago to be B-chromosome-specific, but NGS has now uncovered a further nine sequences that appear to be strongly enriched in the B chromosomes—these are most likely tandemly arranged, and are concentrated either in the non-disjunction control region or in the pericentromere. Some of B-specific repeats are transcribed in a tissue-type specific manner [4]. Other sequences have clearly been derived from organellar (plastids and mitochondria) genomes, which is similarly the case for the B chromosomes of the grass species (and wheat progenitor) *Aegilops speltoides* [5]. As a result, it has been proposed that the rye B chromosomes arose in a stepwise manner, possibly as a by-product of evolutionary rearrangements of the A chromosome complement. The suggestion is that the progenitor of the B chromosome arose in conjunction with a segmental or whole genome duplication event, during which segments of several A chromosomes coalesced. An independent mode of evolution of the B chromosomes requires that they no longer are able to associate meiotically with their A chromosome progenitor(s). The prediction flowing from this scenario is that B chromosomes are more likely to have arisen in taxa which have experienced major karyotypic rearrangements [3]. The B chromosomes found in wild and cultivated rye populations of diverse geographical origin are structurally highly conserved, an observation which has been taken to suggest that, despite their rapid initial evolution, once they had become established, their rate of further structural change and their accumulation of repetitive sequence became greatly attenuated. Intriguingly, however, the level of nucleotide polymorphism appears to be much higher in B chromosome genic sequences than in their A chromosome homologs [6]. Some of these genic sequences are actively transcribed and their transcripts may well be functional [7]: for example, a copy of *AGO4B* residing on a B chromosome is transcribed and has been shown to possess RNA slicer activity, at least in vitro [8].

Uniquely among the many taxa that carry B chromosomes, only fungal species harbor definitively functional B chromosomes; in some cases, they even endow a selective advantage on the host. Some are known to harbor genes encoding the virulence function, which allows for host colonization, and others genes underlying potentially adaptive traits [9,10]. The single-molecule real-time (SMRT) sequencing of a strain of the fungal pathogen *Fusarium poae* carrying at least one B chromosome has established that the A and B chromosomes differ in their content—specifically, the former harbor few transposable elements and no gene duplications, while up to 25% of the latters' sequence is composed of transposable elements, and gene duplications are frequent [11]. Similarly, the B chromosome sequence of *Nectria haematococca*, a fungal pathogen belonging to the *Fusarium solani* species complex, comprises a higher proportion of repetitive DNA than does that of the A chromosome complement; its GC content is lower, and it includes both single copy and duplicated genes [12]. The understanding is that fungal B chromosomes represent a part of the genome able to evolve faster than the standard chromosome complement, thereby permitting the rapid development of pathogenicity without disturbing the core genome (see review by Croll and McDonald [13]).

An NGS-enabled comparison of the genomic sequences of 0B and 4B males of the grasshopper species *Eyprepocnemis plorans*, along with the assembled transcriptomes of 0B and 1B females, has revealed ten B chromosome protein-encoding genes, four of which are complete and six truncated [14]. The abundance of transcript derived from half of these genes was significantly higher in the B chromosome carriers, and in some cases, the increase in abundance could be correlated with the number of B chromosome copies present. A gene ontology analysis has suggested that these B-chromosome-encoded genes are predominantly involved in the regulation of cell division, but it has not been established as yet whether the transcripts generated from the B chromosome gene copies are functional.

A sequence analysis of the satellite repeat fraction present in the grasshopper *Eumigus monticola* genome has suggested that one of the autosomes contributed the most for B chromosome formation [15]. Selected satellite repeats and 5S rDNA showed a similar distribution in the proximal third of autosome

S8 and the B chromosome. Two repetitive families, which were considered, on the basis of in situ hybridization, to be B-chromosome-specific, were represented by a high copy number. A bioinformatic analysis concluded, however, that both were in fact also to be found in the A genome complement, although at a density which was too low to detect cytogenetically. The observation was taken as supportive of the intraspecific origin of the B chromosomes. Such conclusions remain provisional however, given the dynamic behavior of satellite repeats. Ruiz-Ruano et al. [16] have also analyzed the repetitive DNA content of micro-dissected B chromosomes carried by the migratory locust *Locusta migratoria*, and demonstrated a substantial difference between the proportions of the B and A chromosome sequence represented by repetitive DNA—respectively, 94.9% and 64.1%. Six different satellite repeats were located on the B chromosome, whereas only one member of the A chromosome complement harbored all of these satellite sequences. On this basis, this chromosome, along with a second autosome, which shares histone gene sequences with the B chromosome, have been proposed as the putative donors of the B chromosome sequences. A further feature of this B chromosome is a 17 Kbp segment composed of 29 distinct transposable elements, indicative of the occurrence of multiple insertion events within this region.

The most drastic impact of B chromosomes documented in the literature relates to the jewel wasp (*Nasonia vitripennis*), in which the B chromosomes (also referred to as the "paternal sex ratio" (PSR) chromosomes) are transmitted exclusively via the sperm, and act to eliminate one set of A chromosomes during the zygote's first mitosis [17,18]. As a result, a female zygote is converted into a male embryo (see review by Aldrich and Ferree [19]). The PSR-induced elimination of the paternal A chromosomes is regulated by post-translational modifications to the histones associated with the sperm's chromatin [20]. A transcriptomic analysis of the *N. vitripennis* testis has identified a number of PSR-specific transcripts, which may either encode a functional protein or may represent long non-coding RNA [17]. As yet it remains unclear both how these transcripts relate to the key chromatin modifications and what the nature of the controlling mechanism may be.

A plausible example of the de novo formation of a B chromosome has recently come to light in *Drosophila melanogaster*. Although the presence of B chromosomes has been documented in the *Drosophila* genus since at least 1980, they were first noted in *D. melanogaster* karyotype as recently as 2014 in an established stock containing the *mtrm*[126] allele of the *matrimony* (*mtrm*) gene. Importantly, no B chromosomes had been identified either in the stock from which the *mtrm*[126] mutant line was created, or in stocks bearing different *mtrm* alleles [21]. The implication was that a B chromosome had formed over the course of the ten-year period of the stock's maintenance. These particular B chromosomes were highly heterochromatic and resembled chromosome 4 with respect to the arrangement of certain heterochromatin-related satellite repeats. Their presence has been associated with the meiotic non-disjunction of achiasmate copies of chromosome 4 in females. The B chromosomes do not apparently possess a strong drive mechanism and are thought to be mitotically unstable [21]. While these de novo formed B chromosomes appear to offer an appropriate model for revealing the origin and evolution of B chromosomes, as yet little information has been gathered concerning their sequence composition.

The acquisition of genomic sequence obtained from individual cichlid fish (*Astatotilapia latifasciata*) either carrying or not carrying B chromosomes, as well as that of micro-dissected B chromosome sequence has permitted some clarification regarding the origin and evolution of the B chromosomes present in this species [22]. The sequence data have suggested that a proto B chromosome formed before the diversification of the main lineages of the Lake Victoria population, induced by segmental duplications occurring within the autosomes. Three different A chromosomes appear to have provided most of the material making up the B chromosome, but there remains a level of similarity to most of the A chromosomes. The development of a proto B chromosome appears to reflect an accumulation of A chromosome-derived fragments, followed by a burst of sequence amplification and the establishment of a drive mechanism. Besides the large proportion of repetitive DNA present on the B chromosomes (larger than that on the A chromosomes), a number of genic sequences are also present, although most

of these are gene fragments. The few genes remaining intact present are likely either to have been transferred rather recently to the B chromosomes, or to possess functions important for the maintenance and transmission of the B chromosomes. Among the genic sequences detected which have retained a similar structure to their A chromosome homologs, several are associated with the process of cell division, namely the structure of the kinetochore, recombination, cell cycle progression and microtubule organization. Valente et al. [22] have suggested that those that appear to be transcribed are likely involved in the transmission of the B chromosomes. The PCR amplification of some B chromosome sequences present in the cichlid fish *Metriaclima zebra* and six other Lake Malawi cichlids has revealed a link between the B chromosomes and the sex of the zygote [23], which has been reported to be the case as well for a number of species [24]. The underlying mechanism of this association is either an effect of drive, which results in one of the sexes carrying a higher number of B chromosomes, or represents the outcome of a mechanism that ensures that B chromosomes are more frequent in males or females in which it drives [23].

A high-quality assembly of the domestic dog genome sequence was combined with a comparative cytogenetics approach by Becker et al. [25] to address aspects of genome architecture in a selection of canid species. As part of the study, the sequence composition of the B chromosomes of the common fox (*Vulpes vulpes*) and two subspecies of raccoon dog (*Nyctereutes procyonoides*) was revealed in some detail. In addition to the identification of additional copies of the proto-oncogene *cKIT* [26], the presence of other genomic regions shared by A and B chromosomes was successfully demonstrated. The inference was that the canid B chromosomes likely arose in a single ancestral species as a byproduct of a genome rearrangement event(s), which led to speciation in the Canidae. The canid B chromosomes represent a pool of duplicated sequences, including cancer-associated genes, many of which are associated with chromosome breakpoints [25,27].

The Siberian roe deer (*Capreolus pygargus*) and grey brocket (*Mazama gouazoubira*) are both B-chromosome-carrying members of the Cetartiodactyla [28]. In contrast to the evolution of the canid B chromosomes, in this case, the B chromosomes seem likely to have originated independently. The acquisition of sequence from flow-sorted B chromosomes has enabled a demonstration that both harbor mainly repetitive DNA, with some representation of autosomal sequences undergoing pseudogenization and of amplified non-repetitive sequences. However, both the composition of the repetitive DNA and the spectrum of A-chromosome-derived sequences present differed greatly between the two species. Those in *C. pygargus* harbored at least two duplicated A chromosome regions containing three genes, and the level of heterozygosity and the number of haplotypes was high. In contrast, the *M. gouazoubira* B chromosomes were relatively homogeneous. There were 26 duplicated regions, harboring 34 intact and 21 partial gene sequences. The presence of both the proto-oncogenes *cKIT* and *RET* [25] in the *M. gouazoubira* B chromosomes suggests that the A chromosome genomic regions that become involved in B formation are not random [28].

Chromosome-specific probes generated from flow-sorted B chromosomes of the rodent *Holochilus brasiliensis* have been exploited for in situ hybridization in *Oryzomyini* spp. [29]. These experiments have revealed some common sequences, referred to as the "anonymous *Oryzomyini* shared heterochromatic region" (OSHR), which are found in 12 of the 15 species analyzed. The OSHR is thought to have arisen 3.0–7.8 million years ago on the sex chromosomes of an ancestral species, spreading later to some autosomes as well as to established B chromosomes through the action of transposable elements. An independent evolution of B chromosomes in the genus *Oryzomyini* has been proposed by Ventura et al. [29]. As yet, the origin of the heterogeneous B chromosomes remains unclear. The proposition is that, in rodents, both the autosomes and the sex chromosomes have contributed to the evolution of B chromosomes [30]. The involvement of sex chromosomes has also been suggested in the frog species *Leiopelma hochstetteri* [31].

2. The Acquisition of Sequences Enriched in B Chromosome

Differences between the A and B chromosomes with respect to their size, structure, and pattern of meiotic pairing behavior offer the opportunity to isolate the B chromosomes via micro-dissection. In plant species where it is difficult to synchronize mitotic division across many cells, advantage can be taken of the natural synchrony associated with meiosis, particularly in the anthers, where large numbers of pollen mother cells passage through meiosis simultaneously. In the earliest reported use of micro-dissection to obtain B chromosome-specific sequences, Sandery et al. [32] attempted to clone into lambda phage DNA obtained from a very large number of rye B chromosomes; although the approach was rather inefficient. The introduction of PCR was responsible for a quantum leap in efficiency, and this technology lies behind most current protocols for chromosome micro-dissection and the subsequent handling of the DNA [33–36]. Successful in situ painting of B chromosomes (e.g., rye [37], *Brachycome dichromosomatica* [38]) with labelled DNA generated after microdissection was possible because of the enrichment of chromosome-specific repetitive sequences, rather than the chromosome specific low- and single-copy sequences. A list of B chromosomes successfully isolated via micro-dissection is given in Table 1. An alternative route to acquiring chromosome-specific DNA takes advantage of the power of flow-sorting to separate chromosomes on the basis of their size [39]. A major advantage of this approach is that it isolates orders of magnitude higher numbers of chromosomes than is feasible using micro-dissection. The resulting DNA can be amplified using either degenerate oligonucleotide primed PCR [40] or Phi29 multiple displacement amplification [41]. The latter technique is more effective where longer amplicons (5–30 Kbp) are preferred [42]. Species for which flow-sorting has been successfully used to purify B chromosome DNA are listed in Table 2.

Table 1. Isolation of B chromosomes by microdissection.

Species	Reference
Rye (*Secale cereale*)	[32,37,43]
Hawks beard (*Crepis capillaris*)	[44]
Brachycome dichromosomatica	[38]
Maize (*Zea mays*)	[45]
Dung beetle (*Dichotomius sericeus*)	[46]
Locust (*Locusta migratoria*)	[16,47]
Grasshopper (*Podisma kanoi*)	[48]
Grasshopper (*Podisma sapporensis*)	[49]
Grasshopper (*Abracris flavolineata*)	[50]
Hylid frog (*Hypsiboas albopunctatus*)	[51]
Fish (*Moenkhausia sanctaefilomenae*)	[52]
Fish (*Astyanax scabripinnis*)	[53]
Cichlid fish (*Astatotilapia latifasciata*)	[22]
Fishes genus *Astyanax*: *A. paranae*, *A. fasciatus*, *A. bockmanni*	[54]
Fish (*Prochilodus lineatus*)	[55]
Pacific giant salamander (*Dicamptodon tenebrosus*)	[56]
Korean field mouse (*Apodemus peninsulae*)	[57]

Table 2. Isolation of B chromosomes by flow sorting.

Species	Reference
Rye (*Secale cereale*)	[3,4]
Red fox (*Vulpes vulpes*)	[26]
Oryzomyines (*Holochilus brasiliensis*)	[29]
Siberian roe deer (*Capreolus pygargus*)	[28]
Grey brocket deer (*Mazama gouazoubira*)	[28]

3. The In Silico-Based Identification of B Chromosome-Enriched Sequences

Various strategies have been elaborated to identify B chromosome sequences from NGS-acquired data. This section summarizes the differences between the direct and indirect (comparative) methods (Figure 1).
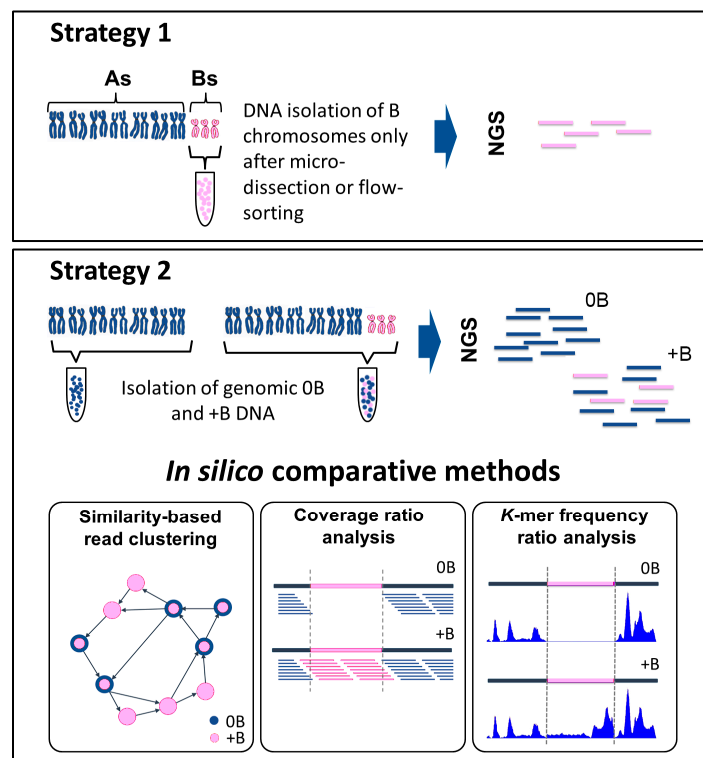
Figure 1. Direct and indirect methods used to identify B chromosome sequences using next generation sequencing (NGS). Strategy 1: the direct method. This approach requires a prior step, in which the B chromosomes are isolated either by micro-dissection or by flow-sorting. Strategy 2: the indirect method. This method requires the acquisition of sequence data from both an individual carrying a B chromosome(s) (+B dataset) and a related individual lacking any B chromosome(s) (0B dataset). The two datasets are compared using three alternative methods. In "similarity-based read clustering", a graphically based analysis is performed using, for example, the RepeatExplorer pipeline. Sequence information is transformed into graphical structures (vertices correspond to sequence reads and edges characterize the overlap between reads). Differences (presence/absence of sequence reads) in the 0B and +B datasets affect the clusters, and are used to distinguish B chromosome sequences. The two-colored circles indicate reads containing sequences from 0B and +B probes. The "coverage ratio analysis" requires an initial alignment of reads, using an alignment pipeline such as Burrows-Wheeler Alignment tool (BWA). Differences in the read coverage ratio indicate B chromosome-derived candidate regions. The pink section illustrates an example of a putative candidate region, which features the absence of reads in the 0B dataset and their presence in the +B dataset. In the "*k*-mer frequency ratio analysis" approach, a program such as the Kmasker pipeline identifies differences in the *k*-mer frequency ratio. The illustration shows an example of a B chromosome segment (shown in pink) in which the *k*-mer frequency is low or zero in the 0B dataset, but high in the +B dataset. Both the coverage ratio and *k*-mer frequency ratio analyses, but not the similarity-based read clustering approach, require a reference sequence.

3.1. Strategy 1—The Direct Route: Isolating, then Sequencing Micro-Dissected or Flow-Sorted B Chromosomes

Once B chromosomes have been isolated by either micro-dissection (Table 1) or flow-sorting (Table 2), it is possible to derive their nucleotide content by standard DNA sequencing approaches.

The benefit of this direct method is that there is an a priori assurance that most of the sequences generated are harbored by a B chromosome complement. Employing sufficient sequencing depth, in conjunction with the deployment of advanced bioinformatic tools such as the "targeted chromosome-based cloning via long-range assembly" method [58] can generate sequence assemblies of high quality. Data acquired from a low sequencing depth experiment cannot produce sufficient sequence coverage to allow for a reliable assembly. The major problem encountered with sequencing DNA from micro-dissected material is the noise generated by contamination from non-target chromosomes, from non-target species and from PCR amplification bias. Thus, sequence reads should always be tested (where possible) against reference genome sequences. Here, high specificity and sequence uniqueness is required to identify B chromosome-specific fragments.

Similar to the micro-dissection approach, flow-sorted chromosomes offer a significant reduction in sample complexity, since a specific chromosome can be purified for sequencing. An effective method of sequencing flow-sorted material platform is the so-called "Chicago Hi-C scaffolding" approach, since it requires only small amounts of template DNA [59]. The ability to assemble long sequence scaffolds aids in assessing co-linearity and synteny between B and A chromosomes, and in addressing the origin of B chromosomes sequences. The major limitation encountered with flow-sorting is the difficulty of discriminating between B and fragmented A chromosomes. Measurable progress has been made in recent years towards minimizing this source of contamination [60].

3.2. Strategy 2—The Indirect Route: Comparing Whole Genome Sequence Acquired from Individuals Carrying and Not Carrying B Chromosomes

Inferring a B chromosome location for a given sequence from whole genome sequence data requires a comparison between datasets from a pair of (preferably related) accessions, one of which carries one or more B chromosomes (+B) and the other does not (0B). In principle, the approach identifies peaks where the ratio of aligned sequences is significantly higher in the +B dataset than in the 0B dataset. These regions are identified as putative candidates that are enriched in B chromosome sequences. Here, three different methods have been suggested to identify B chromosome-enriched sequences. The use of several independent +B and 0B identification methods helps to reduce the number of false positives.

3.2.1. Similarity-Based Read Clustering

B chromosome-enriched sequences, such as satellite DNA, retrotransposons, and organelle-derived sequences, can be identified by the similarity-based clustering of NGS reads, as attempted by the RepeatExplorer pipeline, which identifies clusters of frequently overlapping reads, and interprets these as parts of repetitive elements [61]. In addition, the pipeline estimates copy numbers, based on the frequency of duplicate reads. It is able to connect adjacent sequence clusters via the use of paired-end sequence reads. Furthermore, it performs BLAST nucleotide and protein sequence (BLASTN and BLASTX) similarity searches [62] against specialized databases of repetitive elements and repeat-encoded conserved protein domains, which supports the annotation of repetitive elements. To reveal the presence of repetitive elements on a B chromosome, the analysis can be run in a comparative mode, performing a simultaneous clustering of reads from the +B and 0B samples. The structure of the clusters can be investigated using the SeqGrapheR program [61]. The approach has been applied with some success in both rye [3] and *Plantago lagopus* [63].

3.2.2. Coverage Ratio Analysis

The "coverage ratio analysis" can be performed by mapping genomic reads against a reference genome [22], as is cited in the manuscript. However, it could be also performed by mapping genomic reads against a reference transcriptome as performed by Navarro-Dominguez et al. [14]. The method works by aligning the +B and 0B dataset, looking for differences in the sequence read coverage ratio (Figure 1). Alignment software such as Burrows-Wheeler Alignment tool (BWA) [64] and Bowtie [65]

can be used to construct sequence alignment/maps [66]. Subsequently, the constructed SAM/BAM files are investigated for regions with different numbers of aligned reads. The B chromosome sequence content of the cichlid fish *A. latifasciata* was determined from high coverage whole genome sequence (acquired with an Illmuina HiSeq platform, San Diego, CA, USA) of individuals with and without the B chromosomes, and the reads were mapped onto a reference genome—in this case, that of the related cichlid species *M. zebra* [22]. The coverage ratio analysis revealed that the B chromosomes contain thousands of sequences which have copies on almost every A chromosome. Although most of the genic sequences on the B chromosomes have been fragmented, a few do appear to be intact. Subsequent sequence analysis of micro-dissected *A. latifasciata* B chromosomes has confirmed this conclusion [22].

3.2.3. *k*-mer Frequency Ratio Analysis

A third possible approach is referred to "*k*-mer frequency ratio analysis." Here, the critical variable is the *k*-mer frequency ratio (Figure 1). A *k*-mer is defined as a sequence fragment of length *k*. The method relies on the construction of a set of such *k*-mer indices covering all sequence motifs occurring in the dataset. Two programs designed to perform this task are Tallymer [67] and Jellyfish [68]. The Kmasker tool [69] can be applied to run the *k*-mer frequency ratio analysis. In addition to its core functionality of masking repetitive elements and identifying low copy sequences, Kmasker can also be used to design both probes for in situ hybridization [70] and single nucleotide polymorphism markers.

The approach was applied in the carnivorous plant species *Genlisea* to study its divergent genome size evolution [71]. In this regard, when comparing the two-sister species *Genlisea nigrocaulis* and *Genlisea hispidula* in their repeat composition, the approach revealed sequence candidates that were involved in the genome size expansion, which is a similar experiment as comparing 0B and +B datasets.

3.3. Benefits and Merits of Indirect and Direct Strategies

The major advantage of the indirect over the direct strategy lies in its not requiring a technical intervention (micro-dissection or flow-sorting), which not only incurs cost, but also introduces an unavoidable degree of contamination by off-target material. While most of the unwanted sequence can be excluded using bioinformatics approaches, this further intervention adds yet another intermediate step. Nevertheless, the direct approach gains from the fact that the bulk of the sequence acquired is relevant, while in the indirect approach, the opposite is the case, since most of the sequence acquired originates from the A chromosome complement or from the organellar genomes. Contamination in the template acquired by micro-dissection is likely to derive from off-target species (microorganisms, human) rather than from the host, whereas for the flow-sorted template, the major source of contaminating DNA is likely to be the host's A chromosome complement and/or organellar DNA. Where a reference genome sequence has been established, much of the contamination should be identifiable using homology searches, except for sequences that are shared between the B and A chromosomes. This is less obviously the case for a template acquired from micro-dissected chromosomes, as in this case, the source of the contamination is unknown. The challenge for the indirect method is to set an appropriate threshold that minimizes type I error, while still retaining a sufficient number of sequences. Defining this threshold depends on the sequencing depth, the sequence diversity to reference genome sequence and the probability of assembly error. Thus, all sequences identified via the indirect route are associated with a level of uncertainty. In general, the indirect approach is most effective for the discovery of sequences that are abundant on the B chromosomes. In some situations, technical considerations can suggest one method as more suitable than the alternative. For instance, where it is not possible to boost the number of somatic cells undergoing mitosis, flow-sorting becomes inefficient. Similarly, micro-dissection is difficult to carry out where the target chromosome cannot be readily identified on the basis of its morphology. Combining direct and indirect approaches can be an effective strategy, since the outcome of one can be used to validate the outcome of the other.

3.4. An In Silico Method Used to Identify B Chromosome Sequences

One way of assigning the origin of specific sequences to the B chromosome is to make use of synteny between closely related species, a phenomenon whereby interspecific gene order is maintained, at least within relatively short genomic segments. The "genome zipper approach" [72], which exploits this conservation of gene order, has been used in a number of plant species to order and structure NGS sequences [3,72]. As demonstrated in rye [3], the "genome zipper approach" can be extended to B chromosome sequences, once candidate sequences have been identified by a BLASTN analysis against an appropriate reference genome sequence.

4. Conclusions

Combining NGS with state-of-the-art bioinformatics is providing new ways of identifying sequences specific to B chromosomes, revealing a wealth of molecular data relevant for the study of their origin and evolution. Based on sequence data obtained from animal, plant and fungal B chromosomes, the present consensus is that the B chromosomes are composed of duplicated segments derived from potentially multiple A chromosomes, with the addition of some organellar DNA (see review by Houben et al. [73]). Some B chromosomes contain paralogs of A-chromosome-located genes, either as intact or as degenerate sequences. Genic sequences on the B chromosomes do make some contribution to the host transcriptome [74,75]. B-chromosome-specific repeats tend to be derived from the amplification of A chromosome coding and non-coding sequences [76]. The similarities between the B chromosomes and both the so-called "double minute" chromosomes and homogeneously staining regions has suggested that these structures were formed in a comparable manner [76]. There may be parallels between B chromosomes and marker chromosomes in tumor tissue formed by chromothripsis, a process in which several distinct chromosomal regions simultaneously fragment and subsequently are imperfectly reassembled [77]. Human small supernumerary marker chromosomes may serve as an appropriate model for the early evolution of the B chromosomes [78], although these do not share the drive mechanism characteristic of the B chromosomes. Taking into account the growing number of species for which B chromosome-located genic sequences with possible functions have been reported, B chromosomes cannot be considered as "genetically inert" any more. However, their physiological importance still remains at best sketchily understood.

Modern sequencing and bioinformatics methods can be expected to shed new light on the B chromosomes and thereby improve our knowledge of their genomic dynamics. A detailed understanding of the workings of the (peri)centromere will be needed before the mechanistic basis of their characteristic drive can be unraveled. Further progress in RNA sequencing technology will allow for a more rounded picture of the effect on the transcriptome of the B chromosomes to be generated. Additional analysis of the B chromosomes can be expected to provide exciting information relevant to the rapid genome changes that can occur in higher eukaryotes.

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3.3. Are B chromosomes useful for crop improvement?

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Are B chromosomes useful for crop improvement?

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Societal Impact Statement

Ensuring the sustainable production of food within the context of an increasing world population is a huge challenge facing humankind. Here we review the existence of additional, non-essential chromosomes, known as B chromosomes, and consider their potential usefulness for crop improvement. These enigmatic chromosomes have long fascinated scientists and although their origin and function in plants is somewhat obscure, new research is beginning to shed light on these mysterious chromosomes. This work suggests that B chromosomes may be useful for crop improvement, particularly in grasses, where they show promise as a mechanism to introduce new genes, which could potentially help us to produce more efficient crops.

Summary

The question of the usefulness of supernumerary B chromosomes (Bs) has long fascinated cytogeneticists. Since their discovery in 1907 thousands of species with Bs have been discovered, but their function in the genetics system remains enigmatic, from their origin, evolution, and adaptive significance, as well as their molecular structure and organization. New research is beginning to answer some of these tantalizing questions. Here, we summarize the known data and conclude that Bs are potentially useful, but only in a few known cases, especially in the grasses, and notably in terms of their engineering as plant artificial chromosomes (mini-chromosomes) to carry whole suites of transgenes outside of, and free from interference with, the normal genome.

KEYWORDS
A/B interchanges, artificial chromosomes, B-chromosomes (Bs), Bs in hybrids, chiasmata, gene mapping, mini-chromosomes, nondisjunction

1 | INTRODUCTION—WHAT ARE B CHROMOSOMES?

The continuity of chromosomes between one division of the nucleus and the next was a major topic of discussion in the early days of the science of cytogenetics. Wilson (1907, 1909) was studying this question in the insect *Metapodius terminalis,* the leaf-footed plant bug, when he made a remarkable discovery: he found B chromosomes,

which he called supernumeraries. In *Metapodius* the standard chromosome complement consists of 2n = 20 autosomes, plus a pair of sex chromosomes, and in addition to that, certain individuals also carried from 0 to 6 of what we now call supernumerary B chromosomes (Bs). The properties described by Wilson for these supernumeraries were as follows: the number varies between individuals, with some having none; there is no pairing between these supernumeraries and the standard A chromosome set; no obvious phenotypic effects; no

interaction with the environment and an irregular mode of inheritance. These properties are essentially the way that we define B chromosomes today, with the additional facts that they are usually smaller than the basic A chromosome set (As) and often heterochromatic. They can be detected at mitosis and meiosis (Figure 1), and are now known in close to two thousand species of plants and animals (Jones, 2017; Jones & Rees, 1982). A number of review papers deal with the story of B chromosomes (Table 1), including the most recent discoveries that they may contain transcribed genes. The idea that they are selfish genetic elements is still current, based on the fact that many of them have mechanisms of nondisjunction, or other modes of accumulation, which boosts their number over generations until modulated by their harmful effects and elimination due to pairing failure at meiosis.

There are many questions on the properties of these enigmatic Bs, concerning their origin, their behavior as selfish DNA, their genetic status in terms of any transcribed genes, their function in the genetics systems in natural populations of their hosts and whether they have any useful properties or applications. In some cases, we are tantalizingly near to answering these questions.

2 | ROMAN'S A-B INTERCHANGE DEMONSTRATES NONDISJUNCTION OF THE MAIZE B

Maize (*Zea mays*) is the main species where we can confidently say that Bs have potentially useful applications. The presence of B chromosomes in maize was first reported by Kuwada (1915), working with sweet corn. They were named as B chromosomes (Bs) by Randolph (1928), to distinguish them from chromosomes of the basic complement referred to as A chromosomes (As). The maize B is smaller than any of the As, is rich in heterochromatin and has a near terminal centromere (Figure 2). It is genetically relatively inert, and has no effect on plant morphology or vigor when present in low numbers, and is only detrimental when 10 to 20 are present (Jones & Rees, 1982). The genetic characteristics of the maize Bs have been described in detail by Randolph (1941). In crosses made where only one of the parents carries Bs, such as ♀0B × ♂1B or ♂0B × ♂2B the progeny had more than the number of Bs expected

by Mendelian segregation. This outcome could not be explained by the behavior of the Bs at meiosis, but suggested a mitotic drive (*mitotic nondisjunction*) taking place during the divisions in the pollen grains which gave rise to the male gametes (sperm nuclei). Cytological observations were ruled out for technical reasons and genetic analysis could not be performed since the Bs carried no known genes or obvious effects on the phenotype. Roman (1947) resolved this problem of their transmission using an interchange, generated by X-rays, between A chromosome 4 and the B (A-B interchange, designated as TB-4a, Figure 3). The system works on the basis that the behavior of the B is independent of it remaining intact. The break point, in chromosome 4 is in the short arm, while for the B it is close to the junction of euchromatin heterochromatic regions (Figure 3.) The details of the behavior of the interchanges at meiosis, and their transmission through gametogenesis, are described in detail by Roman (1947) and are given in diagrammatic form by Jones and Rees (1982).

To carry out the analysis a normal female parent carrying the recessive *su* marker gene for sugary endosperm on chromosome four was crossed reciprocally with a male heterozygous for the interchange (Figure 3). The chromosome 4 segment on the B carried the dominant *Su* allele for non-sugary endosperm, and the inheritance of the marker was followed from parents to offspring. From crosses with $B^{4Su} B^{4Su}$ as the female parent all of the kernels had non-sugary endosperm—clearly B^{4Su} was transmitted to the triploid endosperm. When $B^{4Su} B^{4Su}$ was used as the male however, about half the grains were non-sugary and about half were sugary. In this case the endosperm nuclei in the sugary grains were lacking B^{4Su}. How could this be? It seemed to suggest there are two kinds of sperm nuclei, and that nondisjunction had taken place at the second pollen grain mitosis. The sperm nucleus that fertilized the egg carried two B^{4Su} chromatids and the sperm nucleus which fertilized the polar nuclei to produce the endosperm carried none, and this was confirmed.

It was also noticed that the ratio of non-sugary to sugary endosperm varied, and that the sperm nuclei carrying the B chromatids had a preference for fertilizing the egg nucleus. Roman confirmed that the preferential fertilization by the B-carrying sperm worked for normal Bs, as well as for the B/A interchange. Investigations into the mechanism of preferential fertilization, which happens about
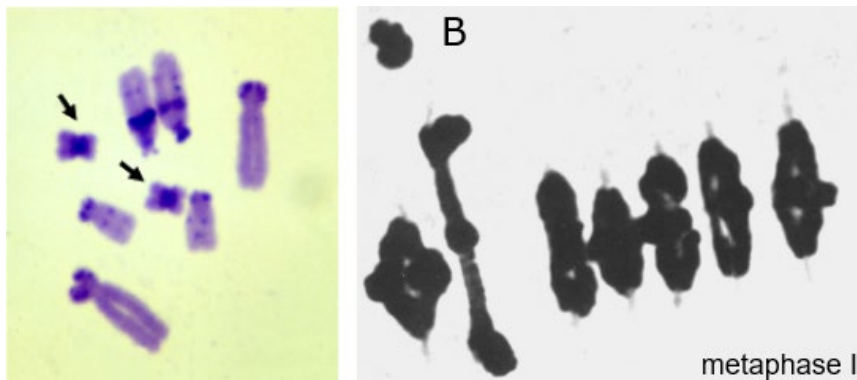
FIGURE 1 Right panel, B chromosomes at c-metaphase in *Crepis capillaris* with 2 Bs; left panel, meiosis in rye, *Secale cereale*, with 1 B

TABLE 1 Selected reviews on B chromosomes

1982	Jones RN, Rees H. *B chromosomes*. New York: Academic Press.
1995	Jones RN. B-chromosomes in plants. *New Phytologist* 131(4): 411–434.
2005	Camacho JPM. B chromosomes. In: Gregory TR ed. *The evolution of the genome*. Amsterdam: Elsevier.
2006	Burt A, Trivers R. B Chromosomes. *Genes in Conflict: The Biology of Selfish Genetic Elements*. Cambridge: Harvard University Press, 325–380.
2013	Houben A, Banaei-Moghaddam AM, Klemme S. Biology and Evolution of B Chromosomes. In: Greilhuber J, Dolezel J, Wendel JF eds. *Plant Genome Diversity Volume 2*: Springer Vienna, 149–165.
2014	Houben A, Banaei-Moghaddam AM, Klemme S, Timmis JN. Evolution and biology of supernumerary B chromosomes. *Cell Mol Life Sci* 71(3): 467–478.
2015	Banaei-Moghaddam AM, Martis MM, Macas J, Gundlach H, Himmelbach A, Altschmied L, Mayer KF, Houben A. Genes on B chromosomes: Old questions revisited with new tools. *Biochim Biophys Acta* 1849(1): 64–70.
2017	Valente GT, Nakajima RT, Fantinatti BE, Marques DF, Almeida RO, Simoes RP, Martins C. B chromosomes: from cytogenetics to systems biology. *Chromosoma* 126(1): 73–81.
2017	Houben A. B Chromosomes – A Matter of Chromosome Drive. *Frontiers in Plant Science* 8(210).
2017	Jones N. New species with B chromosomes discovered since 1980. *The Nucleus* 60(3): 263–281.
2017	Ruban A, Schmutzer T, Scholz U, Houben A. How Next-Generation Sequencing Has Aided Our Understanding of the Sequence Composition and Origin of B Chromosomes. *Genes* 8(11): 294.

two thirds of the time, and provides the mitotic drive of the maize B, have so far failed to provide a complete understanding of the process (Carlson, 1986).

## 3 | B CHROMOSOMES HAVE APPLICATIONS IN GENE MAPPING IN MAIZE

Roman's system of using X-rays to produce B-A translocations has proved to be useful for gene mapping. A large number of such translocations have been produced, and the list is summarized by Beckett (1978). Nondisjunction of the B[4] chromosome at second pollen grain mitosis is the basis of the system. Crosses are made between homozygous recessive females and a set of dominant tester translocation males. The F1 progeny will have the dominant trait in the endosperm (or the plant) when the dominant allele in the male is carried on the B[A] chromosome of the translocation, and the recessive phenotype will be "uncovered" in some of the F1 through nondisjunction, when the sperm nucleus lacking the B[A] chromosome fertilizes the egg. When a recessive gene of unknown locus is crossed as female to each member of the series, it can be uncovered by one of the translocations of the series and mapped to the arm concerned (Beckett, 1978, 1991; Birchler, 1991; Birchler & Alfenito, 1993; Carlson, 1986). More than 100 B-A translocations have now been listed and described, one for each arm (Beckett, 1991). B-A lines can

also be developed with points of breakage in different places to give some information on linkage relationships of genes within an arm. Maize B-A Translocations have also been used for mapping RFLPs (Weber & Helentjaris, 1989).

4 | B CHROMOSOME EFFECTS ON A CHROMOSOME CHIASMATA IN DIPLOIDS

There have been a number of studies of the behavior of Bs at meiosis, not only in terms of the inheritance pattern of the Bs themselves, but also on the effects they may have at meiosis on the A chromosome set (Table 2). The principle question in relation to the As is to what extent, and how, the Bs influence the pattern of chiasma frequency and distribution. The analysis shows that the effect may lead to an increase or decrease in the mean number of chiasmata in meiocytes, and that these changes may or may not be associated with changes in the variation between cells, namely the between cell variance. The *raison d'être* for this analysis being that Bs may influence patterns of genetic recombination which could have significance in modulating the release of new genetic variation, or adaptive features, in natural populations, as well as usefulness in crop improvement. The weakness in terms of this reasoning, however, is that we lack evidence relating variation in patterns of chiasma distribution to outcomes of variation in phenotypes, other than in a theoretical sense.
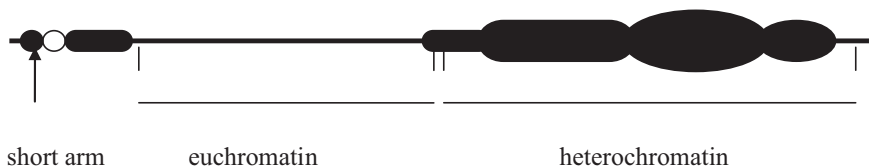
FIGURE 2 The maize B chromosome

short arm   euchromatin   heterochromatin
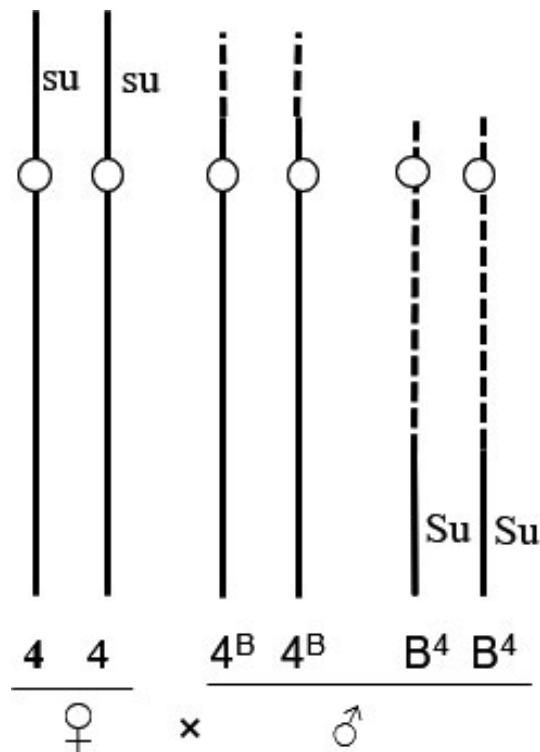
FIGURE 3 The structure of Roman's TB-4a interchange

As far as plants are concerned there are only four species which can be described as crop plants, the principle ones being *Triticum* species, *Lolium perenne*, *Secale cereale*, and *Zea mays*. The maize story is the one where the increase in mean pollen mother cell chiasma frequency is supported by an increased genetic recombination between marker genes on the long arm of chromosome 3 (Hanson, 1969). There is clearly more research needed here to provide the cytogenetic basis for these effects of Bs before we can say that they are useful.

The latest information is that B chromosomes are associated with the redistribution of genetic recombination toward lower-recombination chromosomal regions in perennial ryegrass (Harper et al., 2018). This has potential applications in plant breeding and crop improvement. In this study, the effects of the presence/absence of B chromosomes on genetic recombination was investigated through generating DArT marker genetic maps for 6 perennial ryegrass diploid populations, the pollen parents of which contained either two B or zero B chromosomes. While overall cytological estimates of chiasma frequencies were significantly lower in pollen mother cells with two B chromosomes as compared to zero Bs, the recombination frequencies within some marker intervals were actually increased, particularly for marker intervals in lower recombination regions of chromosomes, i.e., pericentromeric regions. Thus, in perennial ryegrass, the presence of two B chromosomes redistributed patterns of meiotic recombination in pollen mother cells in ways which could increase the range of allelic variation available to plant breeders.

5 | EFFECTS OF B CHROMOSOMES ON CHROMOSOME PAIRING IN HYBRID PLANTS

Mochizuki (1964) was the first to report that the B chromosomes of *Aegilops mutica* could suppress homoeologous pairing in the F1 hybrid with bread wheat (*Triticum aestivum*) × *Ae. mutica.* This work started a flurry of excitement in the wheat cytogenetics community to exploit the potential of this finding for practical applications in both the cereals and the grasses. The reasons for excitement are clear enough, namely that the Pooideae subfamily family of the Poaceae contains many of the most agronomically important temperate cereals and grasses, such as wheat, barley, oats and ryegrass. The high status of the cereals and grasses for use as food for human consumption or as forage for farm animals has led to extensive crop improvement programmes, often involving wide crosses and selection of useful hybrids. However, these wide interspecific or intergeneric crosses are often bedevilled by reduced levels of fertility, or failure of recombination or successful segregation at meiosis; Mochizuki's work offered a possible way to ameliorate the frustrations of chromosome engineers. Some intergeneric crosses between wheat and rye have been used successfully for crop improvement, and the effects upon meiotic pairing of the particular rye cultivars used have been evaluated (Fedak & Gupta, 1991). In all of the wheat × rye hybrids involving B chromosomes, the wheat variety "Chinese Spring" has always been the female parent, and the pollen parent has always been either Transbaikal or Japanese JNK strain of rye. The results of these investigations are summarized and referenced in detail in the table found in Jenkins and Jones (2004). The results are contradictory, and there is no consistent pattern for the way in which the Bs of rye interact with the pairing control genes of wheat. The rye Bs do appear to carry genetic elements that interact with the pairing control genes of wheat under some circumstances, depending on the source of the B and which wheat chromosomes are missing in the various nullisomic hybrids used.

The most hopeful approach we have so far for the usefulness of B chromosomes in plants comes from the work of Evans and Macefield (1973, 1974), who made a remarkable discovery on the effect of Bs on chromosome pairing in hybrids between the ryegrasses *L. temulentum* × *L. perenne*. The genome size, and therefore the chromosomes, of *L. temulentum* are 33% larger than those of *L. perenne*, and it comes as some surprise to learn that in the hybrid there is a high degree of pairing and chiasma formation between the homoeologous asymmetrical bivalents (Figure 4).

When 2Bs are present however, contributed by the *L. perenne* parent, there are only half the average number of bivalents, and those that are formed are mainly rods with a single chiasma. At the tetraploid level there is another remarkable event (Figure 4): namely the contrast in chromosome pairing in the hybrids with and without Bs. When Bs are lacking there is pairing and chiasma formation between homoeologous and homologous bivalents, giving rise to multivalents as well as bivalents and univalents, but the presence of Bs diploidises meiosis and only bivalents are formed involving

TABLE 2 Effects of Bs on chiasma frequency and distribution in A chromosomes

Plants	
Chiasma frequency increased	
Acanthophyllum laxiusculum (2n = 2x = 30 + 0–3Bs)	Ghaffari and Bidmeshkipoor (2002)
Allium flavum (2n = 2x = 16 + 0–2Bs)	Loidl (1982)
Centaurea kandavanensis (2n = 2s = 20 + 0–3Bs)	Ghaffari (1998)
Crepis capillaris (2n = 2x = 6 + 0–3Bs) Cell variance also increased	Parker, Jones, Edgar, and Whitehouse (1990)
Listera ovata. (2n = 2x = 34 + 0–8Bs)	Vosa and Barlow (1972)
Puschkinia libanotica (2n = 2x = 10 + 0–7Bs) Between cell variance also reduced	Barlow and Vosa (1970)
Secale cereal wild rye (2n = 2x = 14 + 0–4Bs)	Zečević and Paunović (1969)
Silene maritima (2n = 2x = 24 + 0–15Bs)	Cobon and Murray (1983)
Zea mays (2n = 2x = 20 + 0–10 Bs) Bs also increased genetic recombination between marker genes on the long arm of chromosome 3	Ayonoadu and Rees (1968); Hanson (1969)
Chiasma frequency reduced	
Aegilops speltoides, Ae. mutica (2n = 2x = 14 + 0–3Bs)	Simchen, Zarchi, and Hillel (1971)
Agropyron mongolicum, A. cristatum (2n = 2x = 14 + 0–6 Bs)	Chen, Jahier, and Cauderon (1993)
Artemisia frigida (2n = 2x = 18 + 0–2Bs)	Bakshi, Kaul, and George (1987)
Briza humilis (2n = 2x = 14 + 0 = 4Bs)	Murray (1984)
Lolium perenne (2n = 2x = 14 + 0–3Bs)	Cameron and Rees (1967)
Najas marina (2n = 2x = 12 + 1B)	Viinikka (1973)
No effect	
Allium sphaerocephalon (2n = 2x = 16 + 0–2Bs) but the between cell variance significantly increased	Guillén and Rejón (1984)
Brachycome lineariloba (2n = 2x = 2 + 0–3Bs)	Carter and Smith-White (1972)
Secale cereale experimental population (2n = 2x = 14 + 0–4Bs) but increase in between cell variance. Effects are chromosome specific	Jones and Rees (1967); White and Rees (1985)

Animals	
Chiasma frequency increased	
Dichroplus pratensis (2n = 2x = 19♂/20♀ + 0–4) Between cell variance also increased	Bidau (1987)
Euthystira brachyptera (2n = 2x = 17♂ + 0–1B) and between cell variance also increased	Fletcher and Hewitt (1980)
Myrmeleotettix maculatus (2n = 2x = 17♂ + 0–3Bs)	John and Hewitt (1965)
Rattus fuscipes (2n = 2x = 38 + 0–3Bs)	Thomson, Westerman, and Murray (1984)
Chiasma frequency reduced	
Trimerotropis pallidipennis (2n = 2x = 23♂ + 0–1)	Confalonieri (1992)
No effect	
Dichroplus elongatus (2n = 2x = 23♂ 24♀ + 0–6Bs) but change in pattern of chiasmata distribution within bivalents	Remis and Vilardi (1986)
Heteracris littoralis (2n = 2x = 23♂/24♀ + 0–3Bs)	Cano and Santos (1988)
Locusta migratoria (2n = 2x = 23♂ + 0–5Bs) and no effect on between cell variance either	Viseras, Salcedo, and Camacho (1988); Cabrero, Viseras, and Camacho (1984)

homologous chromosomes. B chromosomes from *L. rigidum* behave in the same way in hybrids with *L. temulentum* (Evans & Taylor, 1976), but this does not happen in all hybrids. Another potentially useful effect of Bs on chromosome pairing in hybrids was discovered when

X-irradiated inflorescences of *L. perenne* produced a centric B^A fragment that was found to have lost the capacity to undergo nondisjunction at pollen grain mitosis, and was transmitted in the normal mendelian manner (Evans & Macefield, 1977). In diploid hybrids of
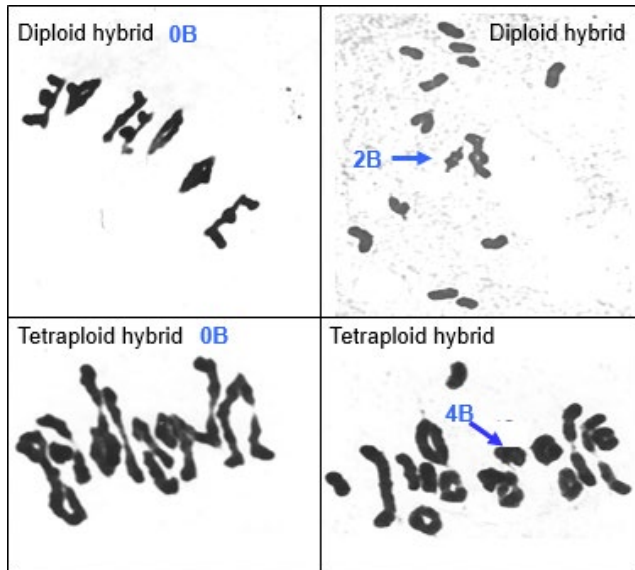
FIGURE 4 Chromosome pairing at metaphase I of meiosis in diploid and tetraploid hybrids of *Lolium temulentum* x *Lolium perenne*

L. temulentum × *L. perenne* it was found that this fragment could, to some extent, partially suppress homoeologous pairing at metaphase I, although this promising line of work has not been taken any further. It is exceptional to create B[A] fragments that are regular in their inheritance, since standard B chromosomes cannot normally be made to function in this way, which opens up many practical opportunities.

6 | B CHROMOSOMES CAN BE USED FOR UNDERSTANDING NONDISJUNCTION

Nondisjunction is a phenomenon of failed chromosome segregation either in mitosis or meiosis. In humans mitotic nondisjunction is often connected to tumorigenesis, while the inability of homologs to separate in meiosis leads to the formation of unbalanced gametes. Further combination of normal and unbalanced gametes results in aneuploid zygote formation. These zygotes are either non-viable or give rise to embryos with severe developmental abnormalities such as trisomy (Day & Taylor, 1998).

While nondisjunction of A chromosomes has harmful effects, B chromosomes use it for their own benefit. In many plants, as well as animal species, Bs possess a specific accumulation mechanism, also called B chromosome drive (Jones, 1991). The drive is essential for the successful maintenance of Bs in populations, and for the balancing of their loss due mitotic and/or meiotic elimination. The timing, and the way that the drive works, is different for Bs of different species, but in many cases it plays a key role in both premeiotic and post meiotic drive, as reviewed by Houben (2017).

Despite the fact that chromosome drive involving nondisjunction is widespread among B-carrying species, the understanding of its control is still limited. The parts of Bs responsible for nondisjunction are known only for rye and maize. In rye, a trans-acting control region is located at the end of the long arm and includes

two B-specific repeat families, E3900 and D1100, which are shown to be transcriptionally active in anthers where the nondisjunction process occurs (Banaei-Moghaddam et al., 2012; Carchilan et al., 2007). In addition, cis-acting pericentromeric sticking sites are required to ensure B-nondisjunction (Endo et al., 2008). The absence of one of these elements makes Bs behave in the same way as As and segregate regularly. The maize Bs are characterized by the presence of two factors on the long arm which act in trans, and which are responsible for the nondisjunction process (Birchler & Han, 2013). The centric heterochromatin of maize Bs was also shown to be involved into nondisjunction control (Carlson, 2006). For both rye and maize it seems that nondisjunction does not depend on centromere function, but rather on sequences located in centromeric and pericentromeric region (Banaei-Moghaddam et al., 2012; Han, Lamb, Yu, Gao, & Birchler, 2007). Now new technologies have opened up new possibilities for the detailed analysis of B-derived transcripts and for deciphering the exact way of nondisjunction control. Assuming that basic aspects of chromosome biology may be conserved among all eukaryots, the data obtained from Bs may be useful in understanding mechanisms of chromosomal nondisjunction in human cells.

7 | B CHROMOSOMES AS MINI-CHROMOSOMES

Artificial or engineered chromosomes (mini-chromosomes) do not require many components to function properly; essential components are centromere, telomeres, origin of replication and genes of interest. Engineered chromosomes are used as nonintegrating vectors. Since they were first successfully constructed in yeast in 1983 (Murray & Szostak, 1983) and in bacteria in 1989 (O'Connor, Peifer, & Bender, 1989), engineered chromosomes have proven to be useful in several situations. Bacterial and yeast artificial chromosomes have been used to clone large DNA inserts, which are still in high demand for sequencing and mapping. Currently, the use of mini-chromosomes in higher organisms appears to be most attractive to those studying in plants. It offers the next generation of crop genetic engineering, with the possibility to introduce multiple genes, or even whole metabolic pathways, in a single step without interfering in the functions of existing genes in the normal genome (Yu, Yau, & Birchler, 2016).

The construction of mini chromosomes may be based on "top-down" or "bottom-up" approaches. The first involves endogenous chromosomes as basis for further modifications, including the truncation of end parts and the insertion of sequences of interest. The second approach involves the construction of entire artificial chromosome by assembling all the necessary parts of it *de novo*. The only success of the "bottom-up" approach in plants was the generation of a maize mini-chromosome, which was shown to be stable and to segregate in meiosis with a ratio close to Mendelian inheritance (Carlson et al., 2007). However, certain aspects of this work were subject to criticism (Houben, Dawe, Jiang, & Schubert, 2008) and require further experimental evidence. At the present time the most successful

methodology was developed for mini-chromosome construction in maize (Yu, Han, Gao, Vega, & Birchler, 2007). Both, maize A- and B chromosomes were used as a basis for the "top-down" generation of engineered chromosomes. To reduce the size and complexity of maize Bs during mini-chromosome construction, telomere mediated chromosomal truncation was adopted (Yu, Lamb, Han, & Birchler, 2006). However, the recovery of truncated chromosomes derived from A chromosomes is not possible in diploid backgrounds due to the lack of compensation for the loss of key genes, meaning that tetraploids are required at the primary stage of mini-chromosome generation (Gaeta, Masonbrink, Krishnaswamy, Zhao, & Birchler, 2012). Bs can tolerate truncation due to their dispensable nature, and can be recovered with higher frequency (Yu et al., 2007).

The main properties required for functional mini-chromosomes were defined in Yu et al. (2016) as follows: synthetic chromosomes should have minimal interference with host growth and development and possess no genes of their own; they should be stable in mitosis and meiosis and transmit from cell to cell and generation to generation; they should allow modification, addition, and deletion of genes on them. By definition, Bs do not interfere with the host phenotype if present in low numbers, and until recently Bs were supposed to have no genes of their own. Even so, the latest findings demonstrate that some Bs may not only possess gene sequences, but those sequences may even be functional (Akbari, Antoshechkin, Hay, & Ferree, 2013; Banaei-Moghaddam, Meier, Karimi-Ashtiyani, & Houben, 2013; Ma et al., 2016), nonetheless Bs may be the perfect system for integrating and expressing genes of interest without significant disturbance to the core genome. It was shown using a GUS gene expression cassette integrated on B- or mini B chromosomes of maize that Bs can accept and express foreign genes (Yu et al., 2007), but the most challenging part is the transmission and stability of the Bs. The transmission success of mini Bs can vary according to host genotype, and an understanding of B transmission control is thus important for the successful use of B-based mini-chromosomes (Houben & Schubert, 2007).

Once constructed for one species, mini-chromosomes will not necessarily be functional in other species, especially due to centromere identity. As discussed in Jin et al. (2004), centromeres of one parental genome in wide crosses are often weaker, causing chromosome elimination. Most likely, *de novo* generation of artificial chromosomes will be required for almost every plant species with the exception of closely related taxa.

8 | CONCLUDING REMARKS

B chromosomes are known in well over a thousand species (Jones, 2017), and are especially common in grasses and cereals. It comes as no surprise therefore that many researchers have looked for ways to find their usefulness, and practical applications, particularly in crop plants, and also to justify their widespread occurrence in nature. Thus far the story is not one of much success, and what has shown most promise is mainly confined to maize. The applications of maize Bs for

chromosome mapping are well established, and their potential as mini chromosomes also holds promise. The maize Bs are dispensable, and can be present in low numbers without any noticeable effects on the host phenotype. They are ideal candidates for plant artificial chromosomes, and can carry enough genes to code for a whole biochemical pathway without any conflict with the host genome. B chromosomes are useful, albeit to a limited extent and manly for one species, yet they continue to engage researchers and we look forward to further developments in the field.

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3.4. B Chromosomes of *Aegilops speltoides* are enriched in organelle genome-derived sequences

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PLOS ONE

B Chromosomes of *Aegilops speltoides* Are Enriched in Organelle Genome-Derived Sequences

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Abstract

B chromosomes (Bs) are dispensable components of the genome exhibiting non-Mendelian inheritance. Chromosome counts and flow cytometric analysis of the grass species *Aegilops speltoides* revealed a tissue-type specific distribution of the roughly 570 Mbp large B chromosomes. To address the question whether organelle-to-nucleus DNA transfer is a mechanism that drives the evolution of Bs, *in situ* hybridization was performed with labelled organellar DNA. The observed B-specific accumulation of chloroplast- and mitochondria-derived sequences suggests a reduced selection against the insertion of organellar DNA in supernumerary chromosomes. The distribution of B-localised organellar-derived sequences and other sequences differs between genotypes of different geographical origins.

Introduction

B chromosomes (Bs) are optional additions to the basic set of standard A chromosomes (As), and occur in all eukaryotic groups. They differ from the As in inheritance, and Bs are not required for normal growth and development of the host organism. Due to the dispensable nature of Bs, they can be present or absent among individuals of the same population in a species. It is widely accepted that B chromosomes derived from the A chromosomes and/or from sex chromosomes. However, there is also evidence suggesting that Bs can be spontaneously generated in response to the new genome conditions following interspecific hybridisation (for reviews, see [1–3]).

Sequence characterisation of the B chromosome of rye provided a unique opportunity for the analysis of the origin and evolution of this enigmatic genome component [4,5]. In contrast to the prevalent view that Bs do not harbor genic sequences, analyses showed that rye Bs are rich in partly transcriptionally active gene-derived sequences [6]. This allowed us to trace their origin from duplicated fragments of multiple A chromosomes [4]. Beside the amplification of B-located satellite repeats [5], this selfish chromosome has accumulated significantly greater amounts of chloroplast- and of mitochondrion-derived sequences than the A chromosomes [4]. Almost all parts of the chloroplast and mitochondrial genomes are found on the Bs, indicating that all sequences are transferable. The higher amount of organelle-derived DNA inserts in B than in A chromosomes and an increased mutation frequency of B-located organellar DNA

suggests a reduced selection against the insertion of organellar DNA in supernumerary chromosomes. To study whether the in rye observed B-specific accumulation of organelle-derived DNA can also be found in other species we analysed the B chromosomes of the grass *Aegilops speltoides*.

Aegilops speltoides Tausch (syn. *Triticum speltoides* (Tausch) Gren.) is an annual diploid species (2n = 2x = 14, genome type: S) which belongs to section *Sitopsis* (Triticeae, Poaceae). This species is mainly distributed in the Fertile Crescent and also occurs in south-eastern part of the Balkan peninsula [7]. The submeta-centric Bs of *Ae. speltoides* are about 2/3 of the average length of the A chromosomes [8]. The Bs of this species are absent in the roots but stably present in the aerial tissue of the same individual [9], and a maximum number of eight Bs per cell was reported [10]. The maintenance of Bs in natural populations is possible by their transmission at higher than Mendelian frequencies. Accumulation of Bs occurs due to the non-disjunction of B chromatids and the preferential transport of both B chromatids into the generative nucleus during the first mitosis in the male gametophyte [9]. *Aegilops* Bs probably originated from the standard set of As as a consequence of interspecific hybridisation and/or chromosomal rearrangement events. Proposed potential donors of the Bs are the A chromosomes 1, 4 and 5 of the *Ae. speltoides* genome [11,12]. Consequently, the Bs are also characterized by a number of A chromosome-localised repeats like *Spelt1*, pSc119.2 tandem repeats, 5S rDNA and *Ty3-gypsy* retroelements [11–14].
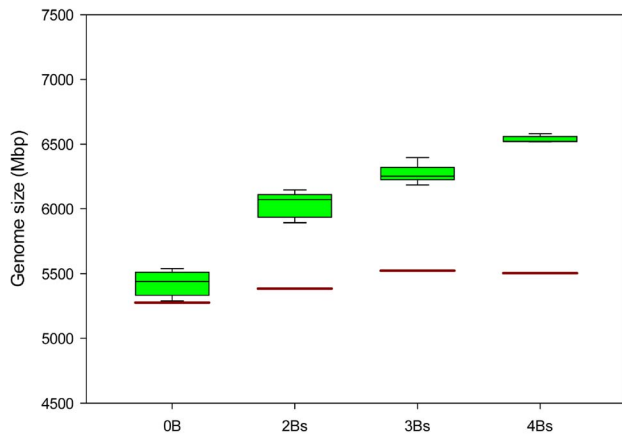
Figure 1. Size determination of the B chromosome. Boxplot representing the genome size distribution in plants without and with B chromosomes of *Ae. speltoides* from Katzir (Israel) measured by flow cytometry. In plants without Bs no difference was found between nuclei isolated from leaf (green boxes) and root (brown line) tissues, while it differed remarkably in plants with Bs. The box boundaries indicate the 75th and 25th percentiles and the error bars the 90th and 10th percentiles of the five replicates.
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Materials and Methods

Plant material and plant cultivation

Plants from natural populations of *Aegilops speltoides* ssp. *aucheri* (Boiss.) Chennav. from Katzir, Israel (TS 89 Mediterranean, 233–250 m, 32829′N, 35805′E), Technion, Haifa, Israel (2.36 Mediterranean, 224 m, 32846′N, 35800′E), Ramat Hanadiv, Israel, (2.46 Mediterranean, 100–125 m, 32833′N, 34856′E) and Tartus, Syria (PI 487238 Mediterranean, 600 m, 35807′N, 36807′E) were cultivated first under greenhouse conditions (16 h light, 22°C day/16°C night) and were further cultivated in a garden (Gatersleben, Germany) under natural condition. The plant material was provided by USDA, Aegean Agricultural Research Institute (Turkey) and the Institute of Evolution, Haifa (Israel).

Flow cytometric genome size determination

The genome size was determined using a CyFlow Space (Partec) or a FACSAria Flow Cytometer (BD Biosciences) following the protocol of Dolezel et al. [15]. The analysis is based on the mean of five independent measurements of individuals with known number of Bs. For all measurements, *Secale cereale* L. (Genebank Gatersleben, accession number R 737) with an estimated genome size of 16.19 pg/2C was taken as internal reference standard.

Chromosome preparations derived from shoot meristems

Young secondary shoots before stem elongation were used for chromosome preparation. After an ice-water treatment of 24 hours the material was fixed in 3:1 (ethanol:acetic acid) and stored at 4°C until use from 4 days to several weeks. The fixative was changed after first hour of fixation and then each few weeks during storage. The basal parts of shoots were excised and washed first in ice-cold water and next twice in 1× citric buffer. Meristematic tissue was treated with an enzyme mixture (0.7% cellulase R10, 0.7% cellulase, 1.0% pectolyase, and 1.0% cytohelicase in 1× citric buffer) for 50 min at 37°C. Afterwards material was washed in 1× citric buffer and twice in ice-cold

water. The shoot meristem was fragmented in 7 µl of 60% freshly prepared acetic acid into smaller pieces with the help of a needle on a slide. After, another 7 µl of 60% acetic acid was added the specimen was kept for 2 min at room temperature. Next, a homogenization step was performed with an additional 7 µl 60% acetic acid and the slide was placed on a 50°C hot plate for 2 min. The material was spread by hovering a needle over the drop without touching the hot slide. After spreading of cells, the drop was surrounded by 200 µl of ice-cold, freshly prepared 3:1 fixative. More fixative was added and the slide was shortly washed in fixative, then dipped in 60% acetic acid for 10 min and rinsed 5 times in 96% ethanol. A quality check of the air dried slides was performed by phase contrast microscopy. The slides were stored until use in 96% ethanol at 4°C.

Fluorescence *in situ* hybridisation (FISH) and microscopy

The following probes were used: barley BACs encoding chloroplast DNA (BAC clone ChHB 100G01) and mitochondrial DNA (BAC clone MnHB 0205G01) [4,5] and the *Arabidopsis*-type telomere repeat [16]. Amplicons for the 5S ribosomal DNA, which include the coding as well as the flanking spacer region, were generated by PCR as described by Fukui et al. [17]. The *Spelt-1* tandem repeat probe was generated by PCR according to Salina et al. [18]. FISH probes were labelled with ChromaTide Texas Red-12-dUTP or Alexa Fluor 488-5-dUTP (http://www.invitrogen.com) by nick translation. FISH was performed as described by Ma et al. [16]. Fluorescence signals were observed by standard epifluorescence microscopy or to achieve a resolution of ~100 nm by structure illumination microscopy (SIM) using an Elyra super resolution microscope (Zeiss). All images were collected in grey scale and pseudocoloured.

Results and Discussion

Tissue type-specific distribution of B chromosomes

Since B chromosomes of *Ae. speltoides* are known to possess 5S rDNA sites, plants carrying B chromosomes were screened based on additional hybridisation signals after FISH on interphase nuclei of leaf tissue using a corresponding hybridisation probe. This allowed an unambiguous identification of B-positive plants at an early stage of development as the Bs of this species are absent in roots (Figure S1). The tissue-type specific distribution pattern of Bs was confirmed by flow cytometric analysis of 0B, 2B, 3B and 4B plants from the Katzir (Israel) population (Table S1). The average size of a single unreplicated B chromosome was estimated to be ~570 Mbp, ranging from 550 to 600 Mbp, for (1C). Hence, one B equals ~10% of the genome size of a 0B plants (5,400 Mbp). The genome size of nuclei isolated from roots was similar in all analysed plants, while it increased in leaf nuclei depending on the number of Bs (Figure 1). An analysis of Bs in differentiated tissues has not been reported before. A comparable situation of tissue-type specific B chromosome distribution is also known for species as, *Agropyron cristatum* [19], *Poa alpina* [20], or *Aegilops mutica* [21]. The absence of Bs in some organs or tissues could be caused by a specific elimination process of Bs during an early stage of embryo differentiation.

The Bs are polymorphic and enriched in organellar - derived DNA sequences

To determine whether the Bs of *Ae. speltoides* from Katzir accumulated mitochondria- and plastid-derived sequences as demonstrated for the Bs of rye [4], we hybridised labelled BACs encoding barley plastid and mitochondrial DNA with *Ae. speltoides*
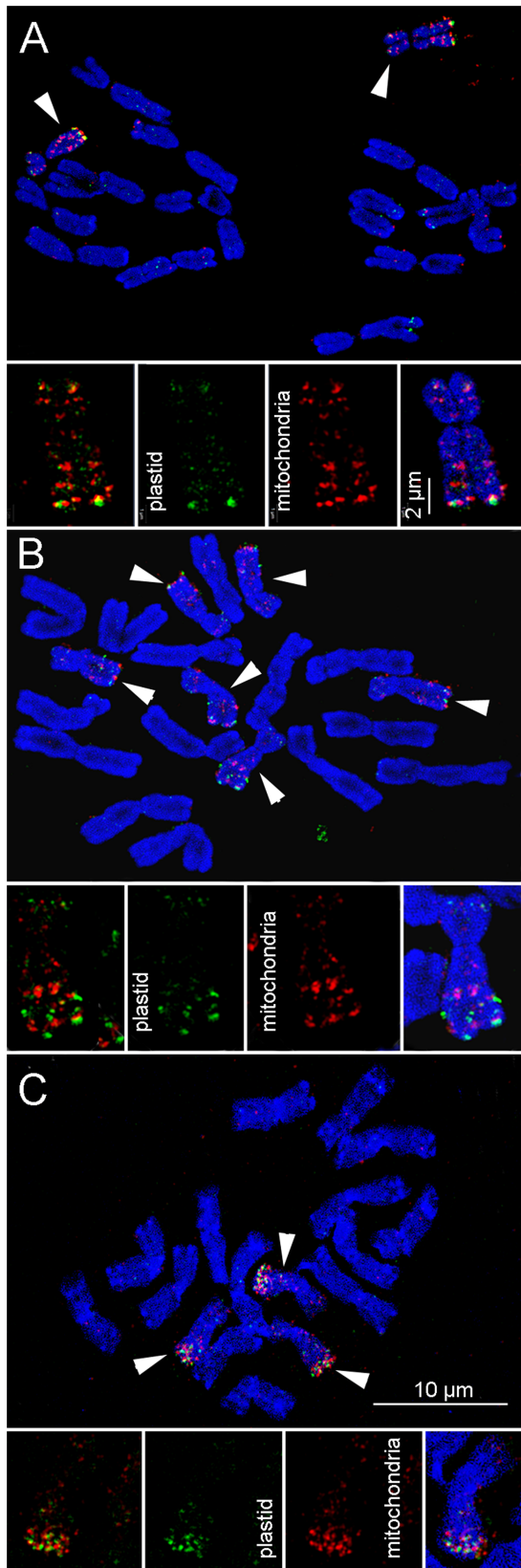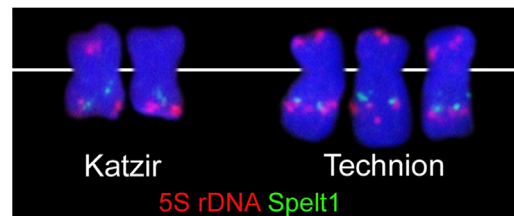
Somatic metaphase cells with 2 Bs from Katzir (Israel), (B) with 6 Bs from Tartus (Syria), and (C) with 3 Bs from the Technion (Israel) population. Note the geographical origin-dependent hybridisation patters along the Bs (arrowed).
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mitotic chromosomes. Both types of probes revealed B-enriched hybridisation signals (Figure 2A). Multiple mitochondria-derived DNA insertions were found along both arms of the B chromosomes except at the pericentromere. The global distribution of plastid DNA-specific signals was similar but of less amount, likely due to a lower abundance. The A chromosomes revealed only minor insertions of mitochondrial origin mainly (Figure S2).

To address whether Bs present in geographically distinct population of *Ae. speltodies* are polymorphic we analysed the chromosomal distribution patterns of organellar DNA in Bs derived from different accessions. As B chromosomes are dispensable, it is expected to observe polymorphisms among populations. The distribution of B-localised organellar-derived signals differed between the tested genotypes. Bs from Tartus showed less organellar-derived signals, although exhibiting a similar distribution as for the Bs from Katzir (Figure 2B). In contrast, Bs from the Technion population displayed a strong accumulation in the subtelomeric region of the long arm for both types of probes (Figure 2C). A partial colocalisation of both mitochondria and plastid DNA-specific signals was found in all Bs independently of their geographical origin. In contrast, a comparative analysis of rye Bs coming from geographically distinct populations did not identify polymorphisms regarding the distribution of accumulated organellar DNA and different B-specific repeats [22]. We conclude that the Bs of *Ae. speltoides* accumulated a significant amount of organellar DNA and that differences of the molecular composition exist between the Bs of different geographical origin. These differences between populations are also reflected by the distribution of FISH signals in interphase nuclei (Figure S3).

The observed polymorphism was further tested by applying the *Triticeae*-specific tandem repeat *Spelt-1*, which is a dynamic component of the *Ae. speltoides* genome [11,18], as well as of the 5S rDNA. Bs of the Technion and Katzir population revealed 5S rDNA signals localised in the terminal part of short arm and in the long arm near the *Spelt-1* cluster. In some plants of the Katzir population the 5S rDNA locus in the short arm is absent (Figure 3). *Ae. speltoides* Bs are submetacentric, but in one plant from the Katzir population we found a metacentric type of Bs. Likely due to terminal deletion in the long arm, former interstitial 5S rDNA and *Spelt-1* clusters became terminal (Figure 3). Apparently, the repeat clusters of the B are hot spots of chromosomal rearrangements. Comparable intraspecific differences in the patterns of repeat clusters were reported previously for the A chromosomes of *Ae.*

Figure 2. B chromosomes of *Aegilops speltoides* are enriched in organelle genome-derived sequences. Distinct chromosomal localisation of plastid (in green) and mitochondria (in red) derived sequences in three *Ae. speltoides* populations after applying SIM. (A)

Figure 3. Localisation of 5S rDNA and *Spelt-1* tandem repeat along selected Bs. Deficient-B type from the Katzir population and standard Bs from the Technion population.
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speltoides [13]. The localisation of *Arabidopsis*-type telomeric repeats is identical on the A- and B-chromosomes of all populations analysed (Figure S4).

What mechanism could account for the accumulation of organellar DNA on the Bs of *Ae. speltoides*? The first possibility is that insertion into B chromosome DNA has fewer deleterious genetic consequences than their counterparts on As. Insertions into A chromosomes may disrupt gene function with lethal consequences. In contrast, Bs which are not required for growth and development can tolerate more mutations. The second possibility is that the nuclear integration of organellar sequences may be dependent on the formation of double strand breaks and if Bs are particularly prone to double strand breaks, this could facilitate the preferred integration of promiscuous DNA in Bs.

Plastid DNA fragments are very numerous in some tissues such as the developing pollen gametophyte [23] or after stress [24]. Hence, uncontrolled insertion would be expected to result in the accumulation of plastid- and, by analogy, mitochondria-derived sequences. Alternatively, the mechanisms that prevent nuclear genome expansion may be impaired on the Bs. Transfer of organellar DNA to the nucleus is very frequent [25], but much of the promiscuous DNA also is rapidly lost again within one generation [26]. If this elimination mechanism (e.g. illegitimate recombination) is impaired in Bs, the high turnover rates that prevent such sequences from accumulating on the A chromosomes would be absent. Thus, the dynamic equilibrium between frequent integration and rapid elimination of organellar DNA could be impaired for B chromosomes. Alternatively, a B-specific amplification process increased the copy number of integrated organellar DNA.

The observed partial overlapping of plastid and mitochondria DNA-specific hybridisation patterns suggests that both types of promiscuous DNA are chromosomally inserted under similar constrains in *Ae. speltoides* Bs. Future analyses of other B-bearing species such as maize will be needed to address the question whether organelle-to-nucleus DNA transfer is an important mechanism that drives the evolution of B chromosomes [27].

Supporting Information

Figure S1   FISH of isolated *Ae. speltoides* nuclei labelled with 5S rDNA. (A) Nucleus of a plant without Bs and (B) with Bs (arrowed). (B) The large 5S rDNA signals are of A chromosome origin, while the arrowed minor signals are B chromosome derived. Scale bar equals 10 μm.
(TIF)

Figure S2   Localisation of mitochondrial- and plastid-derived sequences on *Ae. speltoides* metaphase chromosomes of a plant without Bs from the Ramat Hanadiv population. Scale bar equals 10 μm.
(TIF)

Figure S3   Localisation of mitochondria (in red)- and plastid (in green) derived sequences on *Ae. speltoides* interphase nuclei. (A) Nucleus of a plant with 2Bs from Katzir. (B) Nucleus of a plant with 3Bs from the Technion population. Scale bar equals 10 μm.
(TIF)

Figure S4   Localisation of *Spelt-1* tandem repeat (in green) and *Arabidopsis*-type telomeric repeat (in red) sequences on *Ae. speltoides* metaphase chromosomes. The Bs are marked with arrows. Scale bar equals 10 μm.
(TIF)

Table S1   Genome size determination of *Ae. speltoides* plants with and without Bs. Flow cytometry was used to determine the genome size of nuclei isolated from leaf tissue.
(DOCX)

Acknowledgments

Author Contributions

Conceived and designed the experiments: EB AH. Performed the experiments: AR JF AM VS. Analyzed the data: AR JF AH. Contributed reagents/materials/analysis tools: AS OR. Wrote the paper: AR EB AH.

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3.5. Nondisjunction and unequal spindle organization accompany the drive of Aegilops speltoides B chromosomes

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3.6. The supernumerary B chromosomes of *Aegilops speltoides* undergo precise elimination in roots early in embryo development

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4. Discussion

4.1. B chromosomes of three Triticeae species share a common repeat and accumulate a high amount of organellar DNA

During the last years, significant progress was made in the investigation of the B chromosome origin in different species. With cost efficient sequencing methods and advanced bioinformatics tools, it became possible to look deeper into the organization and composition of B-located sequences. There are different strategies to access and to analyze the DNA of B chromosomes (Ruban et al., 2017). As B chromosomes are often smaller than the chromosomes of the basic karyotype (Jones and Rees, 1982), a flow cytometric approach seems to be a convenient way to isolate Bs. However, the main requirement for successful flow sorting of chromosomes is a high mitotic index. Increasing metaphase synchrony up to the sufficient level is not easy in plants in general and by now the most suitable for it are the root meristems of young seedlings (Doležel et al., 2012). In the case of *Ae. speltoides*, B chromosomes are already absent in primary roots (Mendelson and Zohary, 1972), which makes it impossible to perform their isolation by flow sorting. For this reason, an alternative approach was chosen for the identification of B-located high copy sequences. Application of *in silico* graph-based clustering of sequence reads allows the comparative analysis of the repetitive fraction of genomes (Novák et al., 2010). This approach has been used to identify B chromosome-specific repeats of rye and *Plantago lagopus* (Martis et al., 2012; Kumke et al., 2016). Using the tool RepeatExplorer for the *in silico* characterization of repetitive elements (Novák et al., 2013), we identified in *Ae. speltoides* the B-specific repeats AesTR-183 and AesTR-205 as well as the B-enriched repeats AesTr126 and AesTR-148 (Wu et al., 2019).

Surprisingly, one of these repeats, AesTR-183, was found to be B-specific in two other species, *Ae. mutica* and rye, too. In all three species, this repeat locates in the distal part of the B long arm, and in rye in the nondisjunction control region (Wu et al., 2019). AesTR-183 shows high similarity to the previously described rye B-specific repeat Sc26c38 (Klemme et al., 2013). Its location in the nondisjunction control region raises the question about the possible contribution of this repeat in the functioning of the nondisjunction mechanism. However, there were no corresponding

transcripts identified neither in rye anthers, where nondisjunction of Bs is occurring nor in roots and leaves (Klemme et al., 2013). Another B-specific repeat AesTR-205 of *Ae. speltoides* shows no similarity to any known sequence.

Among the two B-enriched repeats, only AesTR-126 (previously named Aesp_SAT86 (Badaeva et al., 2015)) shows similarity to the repetitive sequence pTa-713 of hexaploid wheat (Komuro et al., 2013). This repeat is also present in other *Aegilops* species (Badaeva et al., 2017; Ruban and Badaeva, 2018). Repeat AesTR-126 localizes in the pericentromeric region of the *Ae. speltoides* B and forms interstitial clusters on the short and long arms. Its localization is highly polymorphic on *Ae. speltoides* A chromosomes. Only two loci were observed in all analyzed accessions, while other loci showed variation between and within different genotypes (Ruban and Badaeva, 2018). Opposite to *Ae. speltoides*, all other S-genome bearing species have species- and chromosome-specific localization of AesTR-126 repeat clusters.

Altogether, comparison of our comprehensive chromosome analysis results of all species from *Aegilops* section Sitopsis with previously available molecular data indicates a distinct position of *Ae. speltoides* not only within the section but in the genus itself (Ruban and Badaeva, 2018). Being probably one of the species which diverged earliest from a common ancestor before the split of *Aegilops* and *Triticum* (Feldman and Levy, 2015), *Ae. speltoides* remains a very dynamic species from the point of its DNA repeat composition (Pollak et al., 2018; Ruban and Badaeva, 2018). *Ae. mutica* together with *Ae. speltoides* are bearing a range of ancestral traits, including allogamy and B chromosomes, suggesting their basal position in the wheat group (Feldman and Levy, 2015).

The accumulation of organellar DNA insertions in the B chromosome was first discovered in rye (Martis et al., 2012). Similarly, *Ae. speltoides* Bs possess a high amount of plastid- and mitochondrial sequences. However, while in rye insertions of organellar DNA are clustered preferentially in the pericentromeric region (Martis et al., 2012), in *Ae. speltoides* they are either scattered all along the B chromosome or accumulate in the distal part of its long arm. The distribution pattern of organellar DNA insertions differs between geographically distinct populations (Ruban et al., 2014). The observed intraspecific differences in the chromosomal distribution of repeats and organellar DNA insertions suggests that the Bs of *Ae. speltoides* have a

dynamic structure (Ruban et al., 2014) in contrast to the rye Bs, which exhibit a conserved structure across geographically distinct populations and at the level of rye subspecies (Marques et al., 2013). *Ae. mutica* revealed a similar B-specific accumulation of organellar sequences. However, while mitochondrial DNA insertions were abundant and distributed along the B chromosome, chloroplast DNA insertions were hardly detectable (unpublished data).

It is commonly accepted and experimentally proven, that the transfer of organellar sequences into the nuclear genome was occurring in the past and that this process is continuing (Timmis et al., 2004). Many genes, transferred from plastids and mitochondria to the nucleus, are still controlling the biogenesis and function of organelles, however, some of them acquired new functions (Rousseau-Gueutin et al., 2012). Despite the ongoing organelle-to-nucleus gene transfer with the frequency close to the natural mutation rate, as it was demonstrated, e.g., in tobacco (Sheppard et al., 2008; Wang et al., 2012a), the overall abundance of organellar DNA insertions is rather low on A chromosomes. A counterbalancing mechanism of sequence elimination must exist in such case to regulate the number of insertions and to neutralize their negative effects when inserted into genic sequences (Timmis et al., 2004). The accumulation of high amount of organellar DNA insertions on rye B chromosomes in contrast to A chromosomes was assumed to happen due to fewer deleterious effects of such insertions when occurring on supernumerary chromosomes. In addition, the mechanism responsible for the expulsion of newly inserted DNA may be impaired on B chromosomes (Houben et al., 2013b). The observed accumulation of organellar DNA sequences on Bs of three Triticeae species may be an indication that this process is an important part of B chromosome evolution. The different distribution patterns of chloroplast- and mitochondrial DNA insertions along the Bs of rye, *Ae. speltoides* and *Ae. mutica* suggests that the accumulation of organellar DNA occurred during the independent evolution of Bs in these species. However, it does not exclude the possibility that the proto-B was already formed in the common ancestor and the sequences accumulation and reshuffling were continued after speciation.

The B-located organellar DNA insertions most likely represent genomic ballast. However, due to reduced selection pressure and higher mutation rate of B-located sequences, B chromosomes constitute a favourable source for the further

formation of functional sequences. These sequences theoretically may be transferred to the A chromosomes and became fully functional genes (Houben et al., 2013b) or they may contribute into B-located gene content.

4.2. Why is the B-specific repeat AesTR-183 conserved?

Accumulation of B chromosomes by means of nondisjunction during either first or second pollen mitosis was described to occur in many species (Jones and Rees, 1982). However, the factors involved in the control of nondisjunction are not known. Among plant species, the most comprehensive studies on this subject were performed in rye and maize resulting in the identification of chromosome regions responsible for the control of nondisjunction. These regions are (peri-)centromere and the distal part of the long B arm in both species (Endo et al., 2008; Lima-de-Faria, 1962; Lin, 1978). In rye, the nondisjunction control region at the end of the long arm is composed of the tandem repeats D1100, E3900 (Blunden et al., 1993; Sandery et al., 1990) and eight others, which were identified later (Klemme et al., 2013). Whether these sequences are involved in the regulation of nondisjunction is not known. Although transcription analysis revealed that D1100 and E3900 repeats are transcribed and forming long-noncoding RNA, there is no direct evidence that these transcripts control the nondisjunction process except the fact that the transcription level is higher in anthers, where nondisjunction occurs, than in leaves and roots (Carchilan et al., 2007).

The postmeiotic drive during pollen development occurs in a similar way in rye, *Ae. speltoides* and *Ae. mutica* (Banaei-Moghaddam et al., 2012; Ohta, 1995b; Wu et al., 2019). The presence of the highly conserved repeat AesTR-183 in the same B chromosome region of the three related species is intriguing. If we assume that the mechanism of nondisjunction is conserved, we could assume some similarity in the DNA composition of the nondisjunction controlling region in different species, too. However, to answer whether AesTR-183 is functionally involved in the control of nondisjunction, additional experiments are required. On the other hand, the conserved presence of the AesTR-183 repeat in the Bs of rye, *Ae. speltoides* and *Ae. mutica* points to the existence of a proto-B chromosome in an ancestral species. This, in turn, does not exclude a function for AesTR-183.

4.3. B chromosome elimination in *Ae. speltoides* is a highly specific and strictly controlled process

The absence of B chromosomes in the root tissue of B chromosome carrying *Ae. speltoides* plants is known for a long time (Mendelson and Zohary, 1972), but the process of their elimination was never studied. However, it was assumed that a preferential distribution of Bs to the plant apex occurs during the first zygotic division (Vardi and Dover, 1972). Our results demonstrate that the elimination starts with the beginning of embryo differentiation and that the Bs are present in the basal and apical cells after the first zygotic divisions. Seventeen DAP old embryonic root cells are completely free of B chromosomes. Bs are also eliminated in the cells of the root cap, which are essential for the root meristem establishment and maintenance (Jiang and Feldman, 2005).

Taking into account, that elimination of Bs occurs not only in the embryonic but also in adventive roots and in the root cells during callus differentiation, we can conclude that the B chromosome elimination is a strictly organ-specific process. Obviously, elimination of Bs starts when the cell fate is defined. The observation of a different B chromosomes behaviour in common background of F1 hybrids between hexaploid wheat with additional rye Bs and *Ae. speltoides* with Bs showed, that the elimination of *Ae. speltoides* Bs does not affect the Bs of rye. It indicates the presence of a specific factor involved in the elimination mechanism of *Ae. speltoides* Bs, but not of rye Bs. Theoretically, the control of B-elimination may work similarly to the control of non-disjunction via trans-acting elements and receptor sites (Endo et al., 2008).

Unfortunately, there is still not enough data to determine what exactly triggers the elimination of Bs from roots. It could be due to the B themselves, due to the interaction between the B chromosome and the host genome, or it could have a physiological reason. For instance, it was shown that rye plants with Bs have a longer cell cycle than plants without Bs (Ayonoadu and Rees, 1968). Thus, the absence of Bs in roots may have an advantage regarding speed and efficiency of root growth, while the retention of Bs in the shoot apex is a prerequisite for their successful transmission into the progeny.

No matter what are the reasons for the elimination of B chromosomes from roots, the process itself shows that programmed chromosome elimination exists in plants too, although it affects only a dispensable part of the genome.

4.4. Are the programmed chromosome elimination in animals and the organ-specific elimination of B chromosomes in *Ae. speltoides* similar?

Programmed DNA elimination may be considered as a mechanism for irreversible silencing of genes, which expression is undesirable in certain tissues or organs, and/or may serve to reduce the costs of maintaining large amounts of extra DNA (Streit, 2012). It is usually subdivided into two categories: chromatin diminution and chromosome elimination (Wang and Davis, 2014). In the first case, it is associated mainly with germline-soma differentiation, for example, in the sea lamprey *Petromyzon marinus* L. (Smith et al., 2012). Beside repetitive sequences (Degtyarev et al., 2002; Smith et al., 2009), also a number of gene-coding sequences (Smith et al., 2012; Wang et al., 2012b) are subjected to elimination. Chromosome elimination is often involved in sex determination in insects (Sanchez, 2014). In the bird species, *Taeniopygia guttata* Vᴇɪʟʟᴏᴛ germline-restricted chromosomes are eliminated from soma of both sexes and during male meiosis (Pigozzi and Solari, 1998, 2005).

Most of the known cases of programmed chromosome elimination in animals affect chromosomes which are present in all individuals of a species, like sex chromosomes or germline-restricted chromosomes (Pigozzi and Solari, 2005; Singh and Belyakin, 2018). Whether programmed elimination of Bs exists in animals is not easy to define mainly due to the lack of information. For example, the B chromosomes of the planaria *Polycelis tenuis* Iᴊɪᴍᴀ. are eliminated from somatic cells, but accumulate during ovarial meiosis (Melander, 1950). In the marsupial *Echymipera kalabu* Fɪsᴄʜᴇʀ, B chromosomes are absent in certain somatic tissues, but present in the corneal epithelium and testis, similarly to the sex chromosomes (Hayman et al., 1969). However, there are no further studies performed to investigate the mechanism and frequency of the B elimination in this species. In two grasshopper species, *Eyprepocnemis plorans* Cʜᴀʀᴘᴇɴᴛɪᴇʀ with mitotically stable and *Eumigus monticola* Rᴀᴍʙᴜʀ with mitotically unstable B chromosomes, Bs are eliminate postmeiotically during spermiogenesis, but it happens sporadically and does not affect all spermatids (Cabrero et al., 2017). Additionally, there is also one

example among animals when paternally transmitted B chromosomes do not eliminate themselves, but trigger the elimination of paternal chromosomes, turning *Nasonia vitripennis* WALKER females into males (Akbari et al., 2013). This process results in a sex ratio shift in populations where Bs are present.

Taking into account the dispensable nature of B chromosomes, it is hard to draw a connection between the processes of B elimination in *Ae. speltoides* and programmed chromosome elimination in animals. Nevertheless, there are certain similarities observed in how Bs in *Ae. speltoides* and supernumerary germline-restricted chromosomes of insects (Singh and Belyakin, 2018; Staiber, 2006) undergo elimination. In animals, it was proposed that elimination of chromosomes may serve to remove the genes which are necessary for proper germline development from somatic cells. Misexpression of these genes may potentially lead to cancer development (Smith, 2018). If we assume the presence of B-located active genes in *Ae. speltoides*, it also could be the case that some of them may be deleterious when expressed in roots.

It is difficult to answer the question of what exactly triggers the elimination process in *Ae. speltoides* and why does it occur in developing roots only. If the mechanism of DNA elimination is evolutionarily conserved among taxa, understanding of this process could help to unravel the universal mechanism by which undesirable DNA is eliminated from the host genome, as it was proposed by (Gernand et al., 2006).

4.5. Are there similar mechanisms responsible for the processes of B chromosome drive and elimination in *Ae. speltoides*?

In plants, chromosome elimination is mainly known to occur in interspecific hybrids where chromosomes of one parental species are eliminated during the development of hybrid embryos. For example, in unstable wheat x pearl millet (Gernand et al., 2005) and *Hordeum vulgare* L. x *Hordeum bulbosum* L. (Gernand et al., 2006) hybrids, elimination of the pollinator genome occurs. Such elimination process results in haploid plants instrumental for plant breeding (Davies, 1974; Dunwell, 2010). In the case of uniparental chromosome elimination, loss of centromere function, which was demonstrated for unstable *H. vulgare* x *H. bulbosum* hybrid embryos (Sanei et al. 2011) and for CENH3 mutants in *Arabidopsis*

thaliana (L.) HEYNH. (Ravi and Chan, 2010), is the driving force. Loss of CENH3 disturbs the interaction between kinetochores and tubulin fibres, which leads to the lagging of chromosomes with subsequent formation of micronuclei (Sanei et al., 2011). The crossing of *Arabidopsis* plants expressing different variants of CENH3 was also shown to induce chromosome elimination (Tan et al., 2015).

In the case of *Ae. speltoides* we did not observe the absence of CENH3 on lagging Bs or an abnormal interaction between tubulin fibres and the B-centromeres. Disturbance of other centromere components is also unlikely, because centromeres of B sister chromatids seem to function, meaning that they separate and move towards opposite poles under the tension of microtubules. The lagging of B chromosomes is caused rather by the inability of the chromatid arms to separate in time.

Strictly controlled nondisjunction of B sister chromatids also occurs during first pollen grain mitosis, when the B chromosome drive takes place. The asymmetry of microtubule spindle ensures the successful inclusion of nondisjoint B-chromatids into the generative nucleus (Banaei-Moghaddam et al., 2012; Wu et al., 2019). Interestingly, a similar situation was observed in the insect *Acricotopus lucidus* carrying germ line limited chromosomes (Staiber, 2012, 2013). Unequal segregation of different types of chromosomes appears in the last gonial mitosis prior meiosis. Germline limited chromosomes migrate as unseparated sister chromatids towards one pole while the other chromosomes remain at the metaphase plane and divide equally between two daughter cells only when all germ line limited chromosomes moved successfully to one pole. As a result, two cells with different chromosome constitution are formed during this process (Staiber, 2013). Post-transcriptional histone modifications were shown to be involved here (Staiber, 2012; Staiber, 2017).

Apparently, the nondisjunction mechanism is strictly controlled and specific for certain cell types, which however does not exclude the possibility, that it could be involved not only in the accumulation of Bs but also in their elimination. Potentially, if nondisjunction of centromere active chromosomes occurs in somatic cells, where the mitotic spindle is symmetrical, it may cause severe lagging of nondisjoint chromatids. In *Poa alpina*, telocentric Bs did not become eliminated from adventitious root cells, while standard Bs did (Müntzing and Nygren, 1955). It may resemble the cases when Bs do not undergo nondisjunction due to the lack of its control region, as

demonstrated in rye (Lima-de-Faria, 1962). Nondisjunction of Bs in somatic tissues was shown in maize plants having a high number of Bs. Whether this is related to the nondisjunction process in gametophytes in this species is an open question (Masonbrink and Birchler, 2010).

5. Conclusions and outlook

In the present study, *Ae. speltoides* B chromosome-specific and enriched tandem repeats were identified. One of these repeats, AesTR-183, has a B-specific localization not only in *Ae. speltoides*, but also in *Ae. mutica* and rye. The possible functional importance and involvement of this repeat in the nondisjunction control mechanism were discussed. However, additional experimental data are necessary to confirm or disprove this assumption. The B chromosomes of rye, *Ae. speltoides* and *Ae. mutica* are shown to accumulate a large number of organellar DNA insertions. The similar sequence composition of B chromosomes in these three closely related species is indicative of a common origin and/or common way of B chromosome evolution. An extended comparison of the Bs at the sequence level with a focus on genic sequences, including Bs of further related species, will help to understand how B chromosomes originated and evolved among Triticeae.

In this study, it was shown for the first time that programmed chromosome elimination occurs in plant species. The elimination of B chromosomes in *Ae. speltoides* is a strictly root-specific process, irrespectively of the root origin. Elimination of Bs begins at the onset of embryo differentiation and occurs via micronucleation. The mechanism responsible for the elimination of *Ae. speltoides* Bs works in a hybrid background as well, but does not affect the B chromosomes of rye. Hence *Ae. speltoides* Bs may possess a specific factor involved in the elimination process. What is the causal reason for the elimination of *Ae. speltoides* Bs and why it occurs exclusively in roots are questions to be addressed in future.

The lagging of B chromosomes at anaphase, probably due to an impaired release of cohesion, is the reason for micronuclei formation. Unexpectedly, Bs exhibit active centromeres and a normal spindle microtubules attachment prior elimination. Additional experiments are required to understand the factors involved in the mechanism of programmed B chromosome elimination and how the cohesion of B sister chromatids is controlled. Comparison of transcriptomes of embryos with and without B chromosomes at the same developmental stage could be a way to identify transcripts and pathways related to the process of chromosome elimination.

6. Summary

The basic chromosome set is essential for normal plant growth and development. The loss or gain of even one chromosome can cause changes of the normal developmental course. However, a class of extra chromosomes exists, which can occur as an optional addition to the standard chromosome set. These supernumerary B chromosomes (Bs) do not affect significantly the development and fitness of an organism, at least in low numbers, and are therefore generally considered as non-functional and without any essential genes. In most cases the number of Bs is stable within individual organisms, but numerical instability of Bs may exist in different plant and animal species. In B chromosome-carrying *Aegilops speltoides* plants, Bs are constant in the aerial organs and totally absent in primary and adventitious roots.

This study, firstly, addresses the molecular structure of the *Ae. speltoides* B chromosome with the focus on repetitive sequences. Two B-specific and two B-enriched repeats, as well as a high amount of mitochondrial and chloroplast DNA insertions were identified on *Ae. speltoides* Bs. One of these B-specific repeats, named AesTR-183, and insertions of organellar DNA are also present on the Bs of two other Triticeae species, *Ae. mutica* and rye. Similarities in the composition of B chromosomes of these species are discussed under the aspect of their evolutionary and functional significance. Localization of the AesTR-183 repeat in the nondisjunction control region of rye B chromosomes might indicate a possible functional involvement of this repeat in the nondisjunction process. The different distribution patterns of chloroplast and mitochondrial DNA insertions along the Bs of rye, *Ae. speltoides* and *Ae. mutica* suggest that the accumulation of organellar DNA occurred after their speciation. The second part of the study addresses how the root-specific elimination of B chromosomes occurs in *Ae. speltoides*. The elimination process is strictly controlled and genetically programmed. The present work is the first report about programmed chromosome elimination in a plant species. In contrast to plants, in animals programmed DNA/chromosome elimination is known since long and was described for broad range of taxa. The process of B chromosome elimination in *Ae. speltoides* is unique, as it does not involve the inactivation of centromeres, which often accompanies chromosome elimination. Here we show that

the process of elimination starts when the root cell fate is defined and occurs due to the lagging of Bs during anaphase. As the centromere of the B chromosome is functional, the most probable reason for B chromosome lagging is the extended or unresolved sister chromatid cohesion. At the end of the mitoses lagging Bs are micronucleated and further subjected to stepwise degradation. The presence of an *Ae. speltoides* B chromosome-specific factor involved in the elimination process was confirmed by comparison of the behavior of *Ae. speltoides* and rye Bs together in a hybrid background.

The possibility of using B chromosomes for crop improvement, their potential application as chromosome vectors and advanced methods suitable for the analysis of B chromosome sequence composition are discussed.

7. Zusammenfassung

Die Stabilität des Chromosomensatzes ist essentiell für normales Pflanzenwachstum und Entwicklung. Der Verlust eines einzigen Chromosoms kann enormen Einfluss auf die Entwicklung des Organismus haben. Nichtsdestotrotz existieren Chromosomen, welche zusätzlich zu dem normalen Chromosomenbestand optional vorkommen können. Diese sogenannten B-Chromosomen haben, zumindest so lange sie in niedriger Stückzahl vorkommen, keinen signifikanten Einfluss auf die Entwicklung und die Fitness des Trägers. Deshalb werden sie auch als nicht-funktional angesehen. In den meisten Fällen ist die Zahl der B-Chromosomen pro Organismus stabil. Andererseits gibt es sowohl einzelne Pflanzen- als auch Tierarten, die durch eine zahlenmäßige Instabilität gekennzeichnet sind. In *Aegilops speltoides* ist die Zahl der B-Chromosomen in allen überirdischen Organen konstant, während in den Wurzeln gar keine B-Chromosomen vorkommen.

Der erste Teil der vorliegenden Arbeit beschäftigt sich mit der Aufklärung der molekularen Struktur der B-Chromosomen von *Ae. speltoides* mit besonderem Fokus auf repetitive Sequenzen. Zwei B-spezifische und zwei auf den B-Chromosomen stark angereicherte Repeats konnten zusammen mit einem hohen Anteil an mitochondrialer und Chloroplasten-DNA in den B-Chromosomen von *Ae. speltoides* identifiziert werden. Eines der B-spezifischen Repeats, AesTR-183, und Insertionen von Organellen-DNA wurden auch auf B-Chromosomen von zwei weiteren Triticeae-Arten, *Ae. mutica* und Roggen, gefunden. Diese Ähnlichkeiten in der Zusammensetzung der B-Chromosomen der drei Arten werden hinsichtlich ihrer evolutionären und funktionalen Signifikanz diskutiert. Die Lokalisation von AesTR-183 in der Non-Disjunction-Kontrollregion der Roggen B-Chromosomen könnte auf eine mögliche funktionale Rolle dieses Repeats im Non-Disjunction-Prozess hindeuten. Die unterschiedlichen Verteilungsmuster der Insertionen von Chloroplasten- und mitochondrialer DNA entlang der B-Chromosomen von Roggen, *Ae. speltoides* und *Ae. mutica* weisen auf eine Akkumulation dieser Sequenzen nach der Aufspaltung dieser Arten hin.

Der zweite Teil der Arbeit beschäftigt sich mit der wurzelspezifischen Eliminierung der B-Chromosomen in *Ae. speltoides*. Der Eliminierungsprozess

unterliegt einer strengen Kontrolle und ist genetisch programmiert. Die vorliegende Arbeit ist der erste Beleg für eine programmierte Chromosomeneliminierung in Pflanzen. Anders als bei Pflanzen sind programmierte DNA/Chromosomen-Eliminierungen bei Tieren schon seit längerem bekannt und für eine Reihe von Arten beschrieben. Bisher beschriebene Chromosomenelimierungs-prozesse gehen häufig mit einer Reduktion bzw. einem Verlust der Zentromerfunktion einher. Der Prozess der B-Chromosmen-eliminierung in *Ae. speltoides* ist sofern einzigartig, da er unabhängig von einer Zentromerinaktivierung funktioniert. Mit der hier vorliegenden Arbeit konnte gezeigt werden, dass die Eliminierung mit der Definition des Zellschicksals als Wurzelzelle beginnt und mit zurückhängenden („lagging") Chromosomen in der Anaphase einhergeht. Da die Zentromere der B-Chromosomen funktionell aktiv sind, ist das Zurückhängen der Chromosomen sehr wahrscheinlich auf eine verstärkte oder nicht aufgelöste Schwesterchromatidenkohäsion zurückzuführen. Am Ende der mitotischen Teilung werden die zurückhängenden Chromosomen als Mikrokerne eliminiert und anschließend einem schrittweisen Abbau unterworfen. Durch den Vergleich des Verhaltens von B-Chromosomen aus *Ae, speltoides* und Roggen im Hybridhintergrund konnte die Präsenz eines *Ae. speltoides* B-Chromosomen-spezifischen Faktors bestätig werden.

Die Möglichkeiten des Einsatzes von B-Chromosomen zur Verbesserung von Kulturpflanzen, ihre potentielle Nutzung als Chromosomenvektoren und aktuelle Methoden zur Analyse der B-Chromosomen-Sequenzzusammensetzung werden diskutiert.

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9. Abbreviations

As – A chromosomes

Bs – B chromosomes

CENH3 – centromere-specific histone H3

DAP – days after pollination

DNA – deoxyribonucleic acid

FISH - fluorescent *in situ* hybridization

10. Acknowledgements

Eidesstattliche Erklärung / *Declaration under Oath*

Ich erkläre an Eides statt, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst, keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

I declare under penalty of perjury that this thesis is my own work entirely and has been written without any help from other people. I used only the sources mentioned and included all the citations correctly both in word or content.

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Datum / Date   Unterschrift des Antragstellers / *Signature of the applicant*

Erklärung über bestehende Vorstrafen und anhängige Ermittlungsverfahren /
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Hiermit erkläre ich, dass ich weder vorbestraft bin noch dass gegen mich Ermittlungsverfahren anhängig sind. / *I hereby declare that I have no criminal record and that no preliminary investigations are pending against me.*

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