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ORIGINAL ARTICLE

Chromosome-specific painting in *Thinopyrum* species using bulked oligonucleotides

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Abstract

Key message **Chromosome-specific painting probes were developed to identify the individual chromosomes from 1 to 7E in *Thinopyrum* species and detect alien genetic material of the E genome in a wheat background.**

Abstract The E genome of *Thinopyrum* is closely related to the ABD genome of wheat (*Triticum aestivum* L.) and harbors genes conferring beneficial traits to wheat, including high yield, disease resistance, and unique end-use quality. Species of *Thinopyrum* vary from diploid ($2n=2x=14$) to decaploid ($2n=10x=70$), and chromosome structural variation and differentiation have arisen during polyploidization. To investigate the variation and evolution of the E genome, we developed a complete set of E genome-specific painting probes for identification of the individual chromosomes 1E to 7E based on the genome sequences of *Th. elongatum* (Host) D. R. Dewey and wheat. By using these new probes in oligonucleotide-based chromosome painting, we showed that *Th. bessarabicum* (PI 531711, E^bE^b) has a close genetic relationship with diploid *Th. elongatum* (E^eE^e), with five chromosomes (1E, 2E, 3E, 6E, and 7E) maintaining complete synteny in the two species except for a reciprocal translocation between 4 and $5E^b$. All 14 pairs of chromosomes of tetraploid *Th. elongatum* have maintained complete synteny with those of diploid *Th. elongatum* (Thy14), but the two sets of E genomes have diverged. This study also demonstrated that the E genome-specific painting probes are useful for rapid and effective detection of the alien genetic material of E genome in wheat–*Thinopyrum* derived lines.

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Introduction

Wild relatives of cultivated wheat in the tribe Triticeae are an economically important gene pool for genetic improvement of cereal and forage crops (Dewey 1984). *Thinopyrum* species are known to possess genes conferring resistance to various diseases, such as leaf rust, stem rust, and *Fusarium* head blight (Fedak and Han 2005; Li and Wang 2009). Five species of the genus, comprising the diploid *Th. bessarabicum* (Săvul. & Rayss) Á. Löve (syn. *Agropyron bessarabicum* or *Lophopyrum bessarabicum*, $2n=2x=14$, E^b genome), the diploid *Th. elongatum* (Host) D. R. Dewey (syn. *A. elongatum* or *L. elongatum*, $2n=2x=14$, E^e genome), the tetraploid *Th. elongatum* ($2n=4x=28$, E_1E_2 or E? genome), the hexaploid *Th. intermedium* (Host) Barkworth and D. R. Dewey (syn. *A. intermedium* or *Elytrigia intermedia*, $2n=6x=42$, StE^bE^e, StE^bJ^{vs} or StJ^rJ^{vs} genome), and the decaploid *Th. ponticum* (Podp.) Barkworth and D. R. Dewey (syn. *L. ponticum* or *E. pontica*, $2n=10x=70$, $StStE^eE^bE^x$ or $J^sJ^sE^bE^bE^b$ genome), have superior traits such as abiotic stress resistance and disease resistance. Thus, these species have been

widely used as alien genetic resources for wheat improvement. (Danilova et al. 2017; Li et al. 2018; Mago et al. 2019; Wang et al. 2020).

Rapid and accurate identification of alien genetic material in wheat backgrounds is an important objective of researchers. Morphological markers commonly used are plant height, grain color, thousand-kernel weights, and disease resistance. However, the morphological and physiological characteristics of organisms are influenced by the interaction of genes and the environment. Therefore, alien genetic material cannot be accurately identified only from phenotypic characteristics. Molecular markers used to detect the E genome in wheat backgrounds include expressed sequence tag–simple sequence repeat, single-nucleotide polymorphism (SNP), and resistance gene analog polymorphism markers (Cesh et al. 2019). However, few molecular markers are specific to the E genome, and the screening is time-consuming and laborious, making it impossible to effectively detect alien genetic material. In situ hybridization is commonly used to detect alien E chromosomes in wheat backgrounds. For example, Wang (2016) identified three wheat–*Th. intermedium* addition lines carrying novel protein subunits and highly resistant to stripe rust and powdery mildew by genomic in situ hybridization (GISH). Xi et al. (2019) designed two oligonucleotide (oligo) probes based on the published specific repeat sequences of *Th. ponticum*, which can be used to identify *Th. ponticum* chromosomes in a wheat background. Although the current in situ hybridization technology can intuitively and accurately visualize alien chromosomes, it is difficult to detect chromosomal structural variations, such as small-segment translocations, owing to the low detection resolution, and trace the source of specific chromosomal segments.

Understanding the structure and evolution of the E genome in the *Thinopyrum* genus will greatly facilitate the use of the member species to transfer agronomically useful genes to wheat. Unfortunately, chromosome identification is a challenge in the genus *Thinopyrum*. Fluorescence in situ hybridization (FISH) is a powerful tool for chromosome identification in plants (Jiang 2019). Linc et al. (2012) established a FISH karyotype of the diploid *Th. elongatum* E genome using pSc119.2, pTa71, and Afa-family probes, and revealed that the diploid *Th. elongatum* from different geographical provenances harbored significant intraspecific chromosome polymorphism. A FISH pattern was established for tetraploid *Th. elongatum* using the probes pTa535, pSc119.2, pTa713, and pTa71, allowing precise FISH characterization of the E genome chromosomes in wheat–tetraploid *Th. elongatum* progeny (Li et al. 2018). Although karyotypes have been developed for certain *Thinopyrum* species, individual chromosomes have not been reliably identified. Therefore, these reported karyotypes are not useful for comparative studies.

The genome composition of the tetraploid *Th. elongatum* is controversial. From the degree and pattern of chromosome pairing in hybrid progeny between diploid and tetraploid *Th. elongatum*, it was concluded that the tetraploid is an autotetraploid, with the two genomes having undergone a degree of differentiation (Dvořák 1981; Charpentier et al. 1986). This finding was further supported by the FISH karyotype using probes developed from single-chromosome microdissection and the pattern of chromosome pairing in F_2 hybrids between two wheat–tetraploid *Th. elongatum* amphiploids (Shi et al. 2023). However, phylogenetic analysis based on *PepC* and *Pgk1* sequences, and non-denaturing FISH (ND-FISH) analysis using repetitive sequence probes suggested that the tetraploid *Th. elongatum* might be an allotetraploid (Dai et al. 2021). Thus, the debate on the genomic composition and origin of tetraploid *Th. elongatum* is ongoing.

The relationship between the E^e and E^b genomes has long been controversial. Cytological studies have shown that the E ($E=E^e$) and J ($J=E^b$) genomes are closely related, and the differences suggest they are subtypes of the same genome (Dvořák 1981; Wang 1985; Wang and Hsiao 1989; Liu and Wang 1993; Xu and Conner 1994). However, karyotype analysis has shown that the J and E genomes are homoeologous (Jauhar 1990). In addition, phylogenetic analysis of *DMC1* sequences (Petersen and Seberg 2002), *GBSSI* sequences (Mason-Gamer 2004), and *β-amylase* sequences (Mason-Gamer 2005) suggest that the E^e and E^b genomes are not closely related. However, phylogenetic analysis of *Pgk1* and *PepC* sequence data indicates that the E^e and E^b genomes are closely related (Dai et al. 2021). At present, the relationship between the E^e and E^b genomes remains controversial.

A new type of chromosome-specific painting probe based on oligos has rapidly become the "new generation" of FISH probes (Jiang 2019). Using any plant species with a reference genome, oligos specific for a portion of a chromosome, or multiple regions of a chromosome or different chromosomes can be identified, synthesized, and labeled as FISH probes (Han et al. 2015). Chromosomal research based on pooled oligo probes has been reported for several plant species, such as potato, rice and maize (Han et al. 2015; Qu et al. 2017; Braz et al. 2018; Xin et al. 2018; Albert et al. 2019; Martins et al. 2019; Šimoníková et al. 2019; Bi et al. 2020; Liu et al. 2020; He et al. 2020; Yu et al. 2022). Recently, chromosomal evolution and syntenic relationships in Triticeae species have been investigated by cross-species chromosome painting using pooled oligo probes (Li et al. 2021; Zhao et al. 2022; Shi et al. 2023). However, the large genome size, high heterozygosity, frequency of repetitive sequences, and insufficient sequence information for wild Triticeae species (Rabanus-Wallace et al. 2019; Wang et al. 2020; Parisod and Badaeva 2020) currently hinder the

development of painting probes for chromosomal research on Triticeae species.

The objectives of this study were to develop a set of E genome-specific painting probes that can be used for identification of the individual chromosomes 1E to 7E and to use these probes to visually examine non-homologous chromosomal rearrangements and homoeologous chromosome relationships in *Thinopyrum* species.

Materials and methods

Plant materials

Materials employed in the current study are listed in Table 1. The diploid *Th. elongatum* (Thy14, E^eE^e) was kindly supplied by Prof. Hongwei Wang (Shandong Agricultural University, Shandong, China). The hexaploid *Trititrigia* 8801 ($2n=6x=42$, AABBEE) was kindly provided by Dr. George Fedak (Eastern Cereal and Oilseed Research Center, Ottawa, Canada). The seed materials with PI numbers were kindly provided by the U. S. National Plant Germplasm System (Pullman, WA, USA).

The *Trititrigia* 8801 ($2n=6x=42$), a genetically stable partial amphidiploid, was produced by crossing of *Triticum durum* ($2n=4x=28$, AABB) with tetraploid *Th. elongatum* (Guo et al. 2015). The wheat–*Th. elongatum* 1E (1D) disomic substitution line K17-841-1, which is highly resistant to stripe rust, was isolated from the F_5 progeny derived from crossing 8801 with the wheat cultivars 'SM482' and 'SM921' (Li et al. 2019). The wheat–*Th. elongatum* 4E (4D) chromosomal substitution line, K16-730-3, was identified from the F_5 progeny raised from the cross between 8801 and the wheat cultivar 'SM482' (Gong et al. 2019).

Bioinformatic pipeline for oligo selection

The Chorus2 software (Zhang et al. 2021) was used to design specific single-copy oligos for chromosomes 1E–7E based on the genomes of *Th. elongatum* (Wang et al. 2020) and wheat (IWGSC 2018). Initially, the two genomes were merged to form a single entity and repetitive sequences were removed. The remaining sequences were divided into oligos of 45 nucleotides, with an overlap of 20 nucleotides between adjacent oligos. Each oligo was then aligned to the genome to filter out duplicates that had more than 80% similarity. To further refine the oligo selection, we selected 17-nucleotide subsequences of each oligo, mapped them to the combined genome, and calculated the hit count. The hit count served as the *k*-mer score for each oligo, which represents its specificity. Each oligo was then ranked by its *k*-mer score, and the oligos with the lowest scores were eliminated as they may have originated from sequencing errors. Similarly, the oligos with the highest scores were also removed as they had a higher probability of appearing multiple times in the genome.

The same *k*-mer score method was used to filter *Th. elongatum* genomic shotgun sequences using ChorusNGSfilter and ChorusNGSselect, which are included in the Chorus2 software. Oligos with dTm < 10 °C and more than seven repeats of the same base, such as 'AAAAAAA' or 'TTT TTTT', were eliminated. Finally, on average, 12.1 oligos per megabase (Mb) were selected for each chromosome.

Probe preparation

Oligos specific for different chromosomes were flanked with specific primers. The bulked oligo libraries were synthesized by GenScript Biotech Corp (Nanjing, China). Primers with a tag (6-carboxyfluorescein, FAM; 6-carboxytetramethylrhodamine, TAMRA) at the 5′ end were synthesized by Sangon Biotech (Shanghai, China). The bulk oligos for whole were amplified as described by Han et al. (2015) and Bi et al. (2020). The PCR products were purified using the GeneJET PCR Purification Kit (Kit Code, K0701, Thermo Scientific) in accordance with the manufacturer's protocol and eluted with 50 µl solution buffer to obtain the labeled oligo probes. The tandem repeat-based oligo probes, pSc119.2 and pTa535, were synthesized by Sangon Biotech (Shanghai, China). The synthetic oligos were labeled with either FAM or TAMRA.

Table 1 Plant materials used in this study

Species	Accessions/lines	Chromosome number	Ploidy
Thinopyrum elongatum	Thy 14	14	2*x*
	PI 531718	14	2*x*
	PI 531750	28	4*x*
Th. bessarabicum	PI 531711	14	2*x*
Triticum durum—Tetraploid Th. elongatum amphidiploid	8801	42	6*x*
Wheat—*Tetraploid Th. elongatum* substitution line	K17-841-1	42	6*x*
Wheat—*Tetraploid Th. elongatum* substitution line	K16-730-3	42	6*x*

Fluorescence in situ hybridization

To prepare mitotic metaphase chromosomes, root tips from germinating seeds were harvested and treated with nitrous oxide for 2.5 h and 90% acetic acid for 10 min and then digested with pectinase and cellulase (Komuro et al. 2013). Sequential bulk painting with oligos was conducted according to the method of Li et al. (2021). The hybridization mixture contained 10 µl of 100% formamide, 2 µl of 20 × SSC, 4 µl of 50% dextran sulfate sodium salt in water, and 3 µl of oligo probes (200–300 ng). Non-denaturing FISH using the synthesized probes was performed in accordance with a published protocol (Fu et al. 2015). Chromosome images were captured using an Olympus BX-63 microscope equipped with a DP-70 CCD camera and processed using Adobe Photoshop CS software.

Results

Development of chromosome-specific painting probes in *Th. elongatum*

Based on the genome sequences of *Th. elongatum* (Wang et al. 2020) and wheat (IWGSC 2018), we developed a set of E genome-specific painting probes for the individual chromosomes 1E to 7E by using the Chorus2 software (Zhang et al. 2021). First, we retained the E genome-specific oligos based on the *Th. elongatum* and wheat genomes through *k*-mer scoring. The lengths of the seven *Th. elongatum* pseudomolecules ranged from 553 megabases (Mb) of chromosome 1 to 744 Mb of chromosome 7. Finally, we selected 6691, 8955, 8187, 7413, 7564, 7065, and 9000 oligos (each 45nt in length) from among the seven pseudomolecules (Table S1). A density of 0.012 oligos per kilobase (kb) was adopted for each chromosome in the *Th. elongatum* genome. A lack of oligos was observed in the putative centromeric region for most chromosomes (Fig. 1).

Chromosome painting in *Th. elongatum*

The seven painting probes (Chr1 to Chr7) were used in FISH for validation of the signal specificity and distribution for the *Th. elongatum* (Thy14) chromosomes (Fig. 2). For example, painting probes for chromosomes 1 and 2 were hybridized to a metaphase cell (Fig. 2A). The slides were washed and reprobed with the painting probes for chromosome 5 (Fig. 2B). All seven chromosomes were individually identified after two rounds of sequential FISH in two cells (Fig. 2A, B, D, E). Each of the seven painting probes produced distinct hybridization signals covering the entire length of the chromosome arms, suggesting that the painting probes were chromosome-specific. In addition, no or very weak signals were observed in the centromeric region of most Thy14 chromosomes (Fig. 2).

The probes pSc119.2 and pTa535 were used for FISH with the Thy14 chromosomes. The chromosomes displayed distinct pSc119.2 hybridization signals that were concentrated in the terminal regions, and strong hybridization signals for pTa535 were observed in the terminal

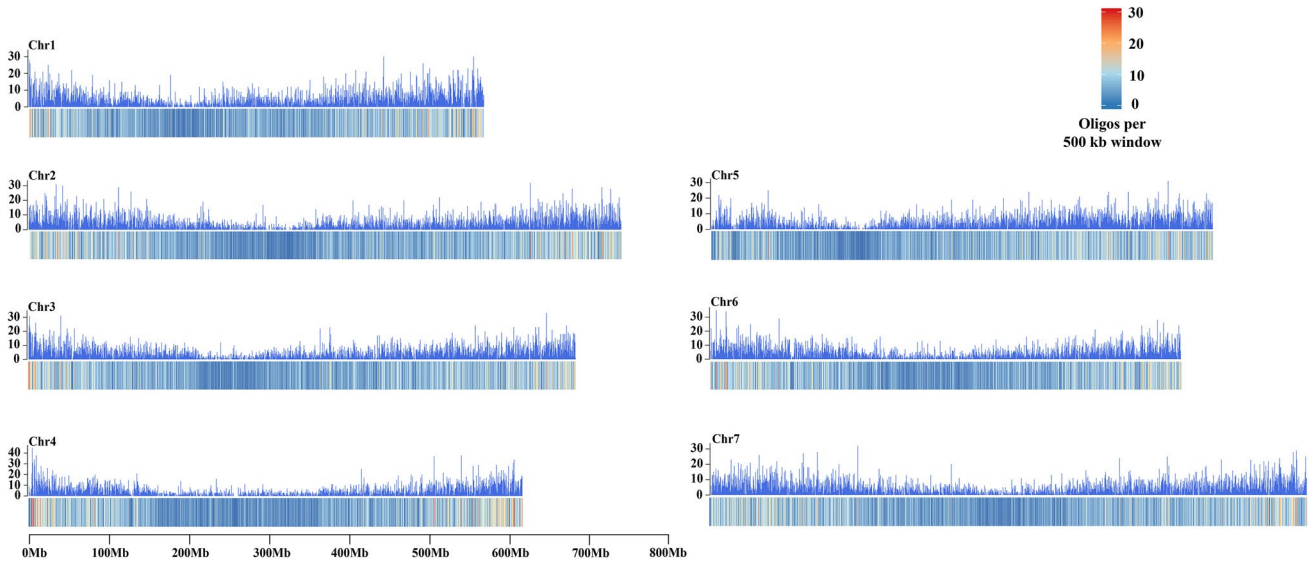

Fig. 1 Development of chromosome-specific painting probes based on the *Th. elongatum* and wheat genomes. The seven pseudomolecules of *Th. elongatum* were divided into 500 kb windows, and the number of oligos was calculated for each window. The distribution of the number of oligos is shown in the line plot and heat map. The *x*-axis is the position of the chromosome. The *y*-axis is the number of oligos in each 500 kb window

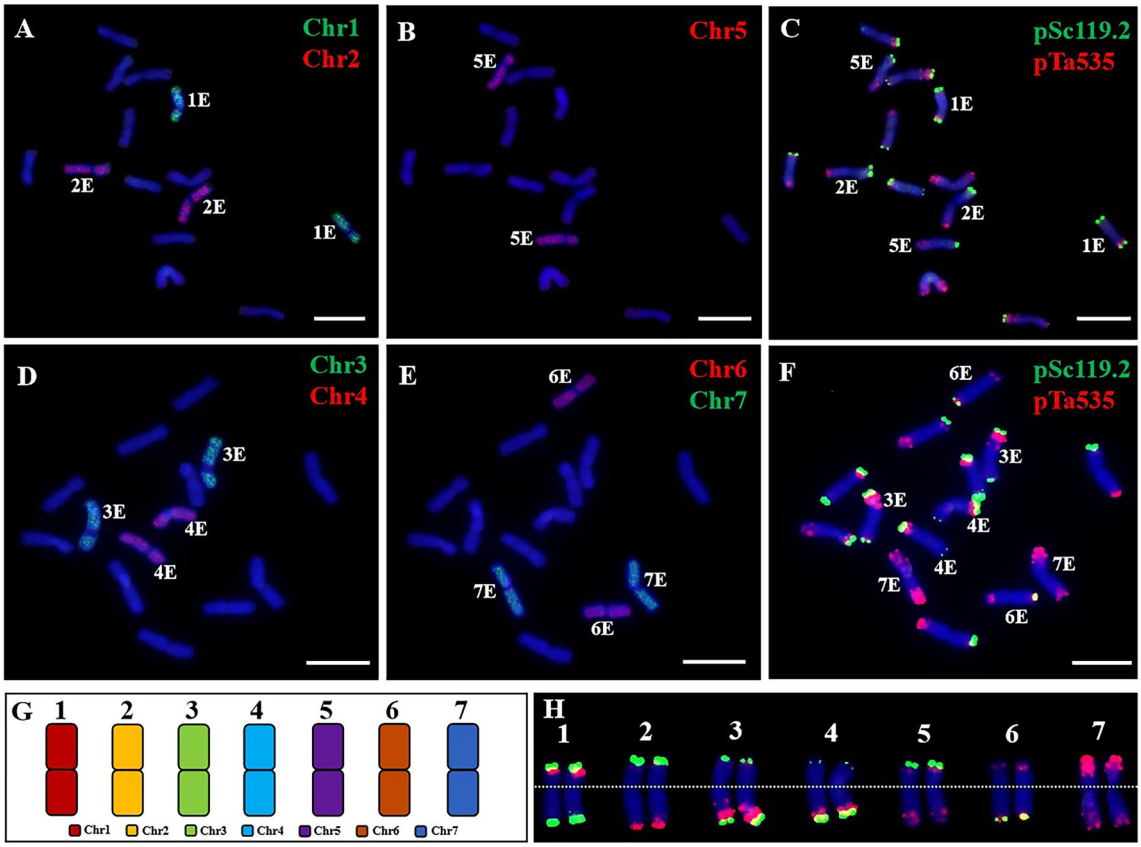

Fig. 2 FISH painting using bulked oligo probes on chromosomes of *Th. elongatum* (Thy 14). **A**–**C** FISH using probes Chr1 and Chr2 (**A**), Chr5 (**B**), and pSc119.2 + pTa535 (**C**) of Thy 14. **D**–**F** FISH using probes Chr3 + Chr4 (**D**), Chr6 + Chr7 (**E**), and pSc119.2 + pTa535 (**F**) of Thy 14. **G** An ideogram illustrating the chromosomes 1–7. **H** FISH patterns of probes pSc119.2 and pTa535. Bars = 10 µm

and sub-terminal regions (Fig. 2C, F). Although abundant hybridization signals with the probes pSc119.2 and pTa535 were detected, individual chromosome pairs could not be identified. Thus, a high-resolution FISH karyotype for diploid *Th. elongatum* was developed by applying oligo-based chromosome painting and tandem-repeat oligo probes for ND-FISH, which have previously proved useful for comparative karyotype studies.

Comparative chromosome painting in diploid and tetraploid *Th. elongatum*

To examine chromosome evolution in different populations and ploidies of *Th. elongatum*, we selected two additional accessions of *Th. elongatum* for FISH analyses, comprising a diploid (PI 531718) and a tetraploid (PI 531750). Mitotic metaphase spreads of PI 531718 and PI 531750 were subjected to FISH with the chromosome painting probes and sequential ND-FISH using the probes pSc119.2 and pTa535 (Figs. 3, 4). The painting probes Chr1, Chr2, Chr3, Chr4, and Chr6 painted the complete 1E, 2E, 3E, 4E, and 6E chromosomes in diploid *Th. elongatum* (PI 531718), indicating that the chromosomes had maintained their synteny with the corresponding Thy14 chromosomes (Fig. 3). In contrast, a reciprocal translocation involving chromosomes 5E and 7E was detected by the complex hybridization patterns with the Chr5 and Chr7 probes (Fig. 3E). The breakpoint on the centromere of 5E was situated close to the strong pTa535 signals, whereas the breakpoint in the telomeric section of the short arm of 7E was located near strong pSc119.2 signals.

The seven painting probes completely painted all 14 pairs of chromosomes in tetraploid *Th. elongatum* (PI 531750), seven of which displayed brighter signals while the other seven pairs of chromosomes showed weaker FISH signals (Fig. 4). This result suggested that all 14 pairs of chromosomes of tetraploid *Th. elongatum* have maintained complete synteny with those of diploid *Th. elongatum* (Thy14), but the two sets of the E genome had diverged. In addition, the hybridization patterns of the probes pSc119.2 and pTa535 with the two sets of genomes of PI 531750 were distinct, which indicated that the two sets of genomes of PI 531750 were differentiated.

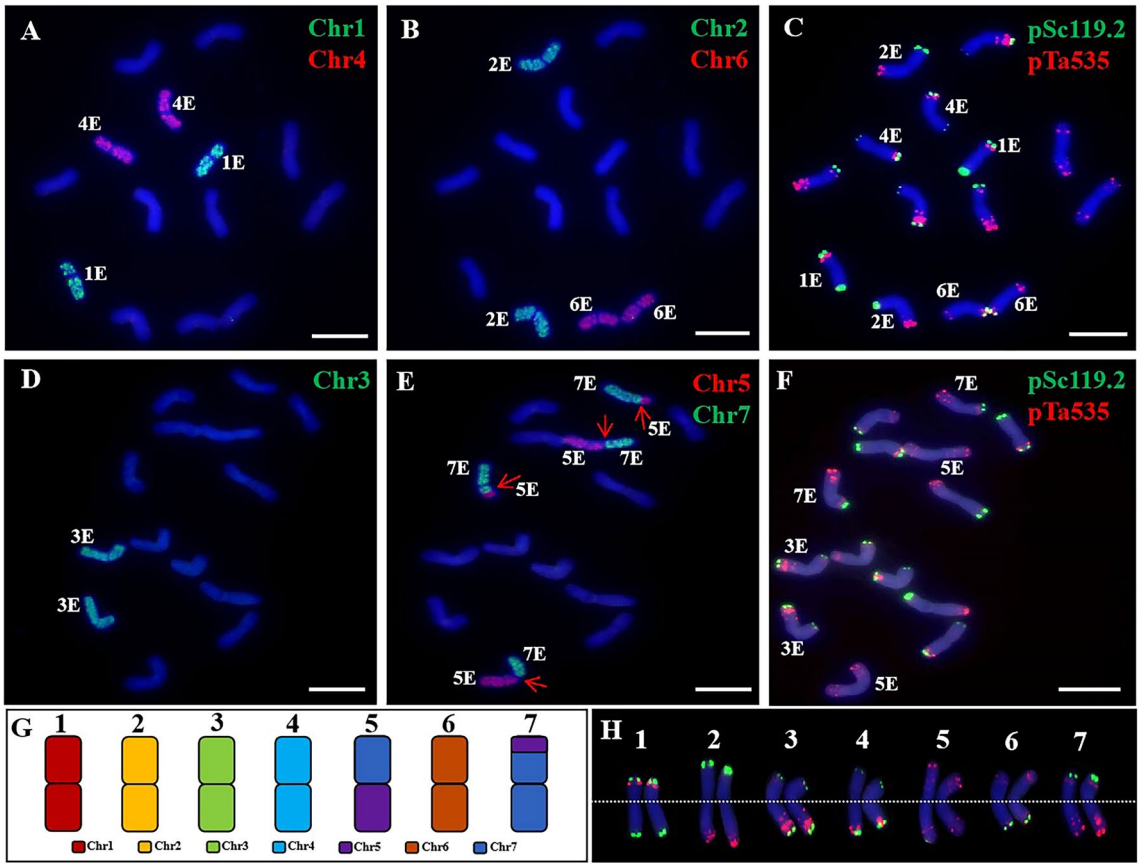

Fig. 3 FISH painting using bulked oligo probes on chromosomes of diploid *Th. elongatum* (PI 531718). **A**–**C** FISH using probes Chr1 + Chr4 (**A**), Chr2 + Chr6 (**B**), and pSc119.2 + pTa535 (**C**) of PI 531718. **D**–**F** FISH using probes Chr3 (**D**), Chr5 + Chr7 (**E**), and pSc119.2 + pTa535 (**F**) of PI 531718. Arrows indicate the translocation of 5E segments to 7ES and 7E segments to 5ES. **G** An ideogram illustrating the chromosomes 1–7. **H** FISH patterns of probes pSc119.2 and pTa535. Bars = 10 μm

Painting analysis of *Th. bessarabicum* chromosomes

We next evaluated the quality of the painting probes in hybridization to chromosomes from *Th. bessarabicum* (PI 531711, E^bE^b), which has a close genetic relationship with diploid *Th. elongatum* (E^eE^e). Painting with the probes Chr1, Chr2, Chr3, Chr6 and Chr7 resulted in distinct hybridization signals covering the entire lengths of the *Th. bessarabicum* chromosome arms, indicating that chromosomes $1E^b$, $2E^b$, $3E^b$, $6E^b$, and $7E^b$ had maintained complete synteny with the corresponding Thy14 chromosomes (Fig. 5). FISH using the probes Chr4 and Chr5 revealed the presence of a reciprocal translocation between the terminal region of $4E^bL$ and $5E^bL$ (Fig. 5A, D). The chromosomes of *Th. bessarabicum* exhibited strong pSc119.2 signals in the terminal regions, and the distinct signals for pTa535 were observed in the terminal and subterminal regions. Thus, each individual chromosome pair was distinguished by ND-FISH using the probes pSc119.2 and pTa535, and the chromosome-specific probes (Fig. 5C, F).

Identification of the E genome in the wheat background

To accurately identify alien genetic material in the wheat background, we used chromosome-specific painting probes for the E genome to precisely detect individual chromosomes of *Th. elongatum* in wheat–alien species derivatives.

Trititrigia 8801 ($2n = 6x = 42$, AABBEE), a genetically stable partial amphidiploid line produced by crossing of *T. durum* ($2n = 4x = 28$, AABB) with tetraploid *Th. elongatum*, is commonly used as a bridging parent for wheat improvement (Guo et al. 2015; Dai et al. 2017). Mitotic metaphase chromosomes of *Trititrigia* 8801 were sequentially probed with the painting probes pSc119.2 and pTa535 (Fig. 6). In *Trititrigia* 8801, the seven pairs of E genome chromosomes of tetraploid *Th. elongatum* were precisely identified using the painting probes and repeated oligo probes (Fig. 6).

The two disomic substitution lines, K17-841-1 ($2n = 42$, 1E/1D) and K16-730-3 ($2n = 42$, 4E/4D), were used in the FISH analysis. The line K17-841-1 contained the 1E

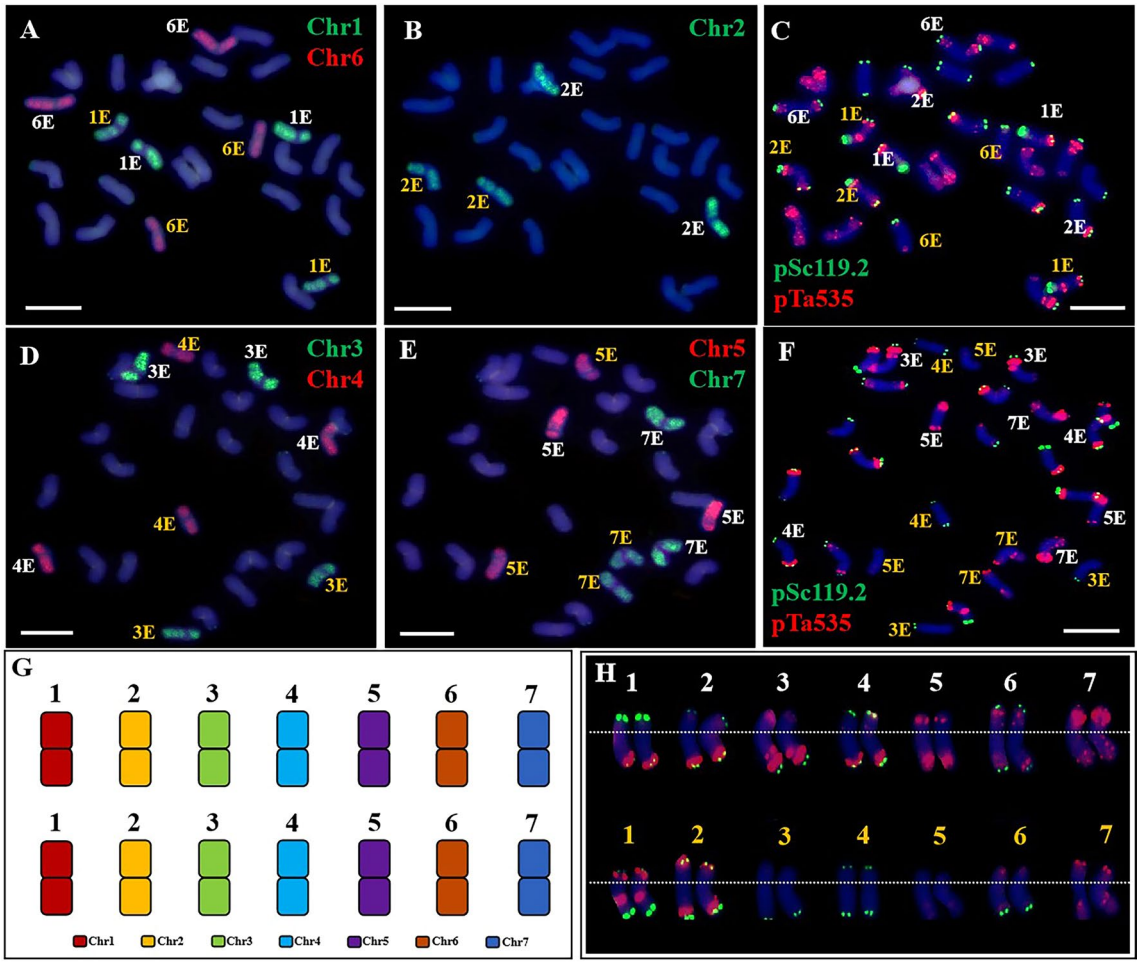

Fig. 4 FISH painting using bulked oligo probes on chromosomes of tetraploid *Th. elongatum* (PI 531750). **A**–**C** FISH using probes Chr1 + Chr6 (**A**), Chr2 (**B**), and pSc119.2 + pTa535 (**C**) of PI 531750. **D**–**F** FISH using probes Chr3 + Chr4 (**D**), Chr5 + Chr7 (**E**), and pSc119.2 + pTa535 (**F**) of PI 531750. **G** An ideogram illustrating the chromosomes 1–7. **H** FISH patterns of probes pSc119.2 and pTa535. Bars = 10 μm

chromosome for which hybridization signals of the painting probe Chr1 was present along the entire chromosome arms (Fig. 7). The remaining 40 wheat chromosomes identified by ND-FISH with pSc119.2 and pTa535 consisted of 14 A-chromosomes, 14 B-genome chromosomes, and 12 D-genome chromosomes. Based on the ND-FISH and chromosome painting results, we could accurately identify the 1E chromosome in K17-841-1 and trace its origin. The line K17-841-1 derived its 1E chromosome from *Trititrigia* 8801, which, in turn, acquired its 1E chromosome from the E genome with weaker painting signals of tetraploid *Th. elongatum* (Fig. 7C). This observation was consistent with the cytological analysis conducted by Li et al. (2019).

The Chr4 painting probe hybridized to the 4E chromosome in the K16-730-3 line (Fig. 8). The remaining 40 wheat chromosomes were identified by ND-FISH using pSc119.2 and pTa535. Combining the ND-FISH and chromosome painting, we could accurately identify the 4E chromosome in K16-730-3 and trace the origin of the alien 4E chromosome. The line K16-730-3 derived its 4E chromosome from *Trititrigia* 8801, which in turn derived its 4E chromosome from the E genome with brighter painting signals of tetraploid *Th. elongatum* (Fig. 8C). These findings were consistent with the results of the cytological and molecular marker analyses of Gong et al. (2019).

Discussion

Identification of individual chromosomes in plants such as *Thinopyrum* species has long been a difficult task. Traditional cytogenetic markers, such as the microsatellites $(CTT)_n$, and $(ACT)_n$, failed to show any signals on the chromosomes of the E genome (Sepsi et al. 2008; Linc et al. 2012; Li et al. 2018). The most common FISH probes used for E genome identification are repetitive

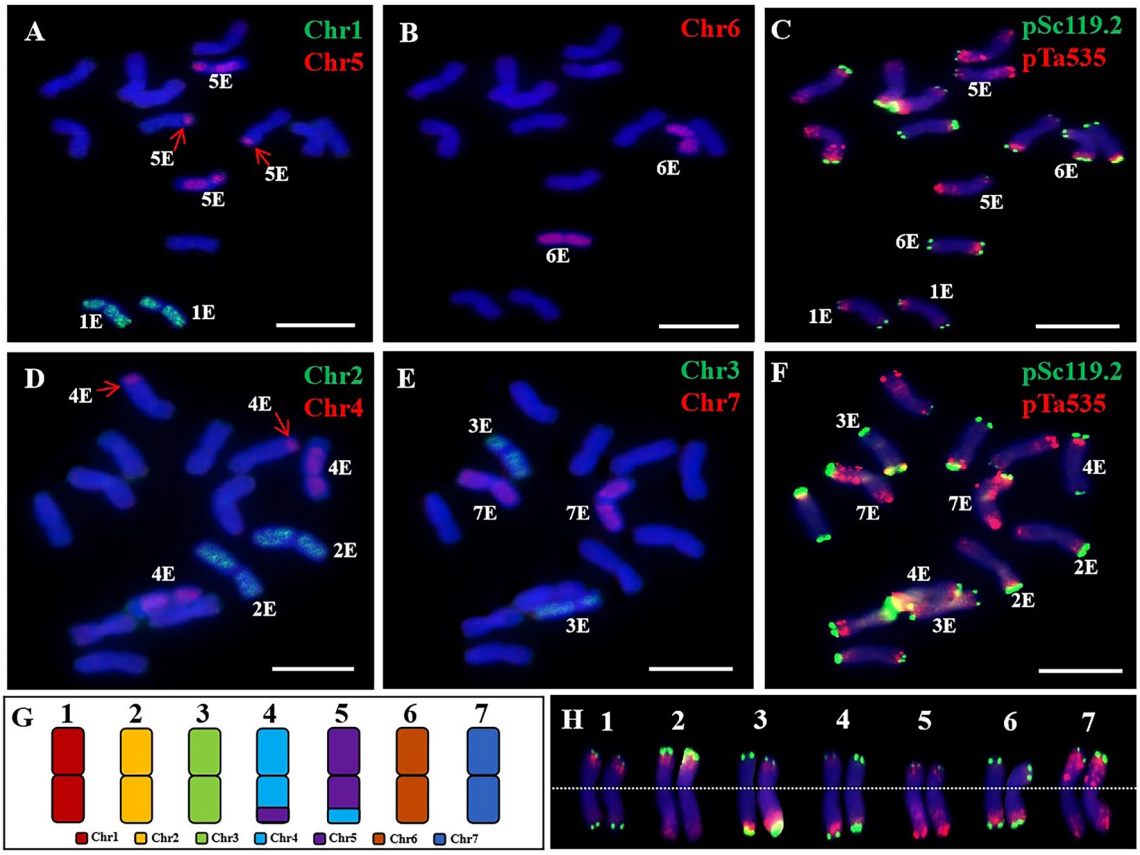

Fig. 5 FISH painting using bulked oligo probes on chromosomes of *Th. bessarabicum* (PI 531711). **A**–**C** FISH using probes Chr1 + Chr5 (**A**), Chr6 (**B**), and pSc119.2 + pTa535 (**C**) of PI 531711. Arrows indicate the position of translocation breakpoints. **D**–**F** FISH using probes Chr2 + Chr4 (**D**), Chr3 + Chr7 (**E**), and pSc119.2 + pTa535 (**F**) of PI 531711. **G** An ideogram illustrating chromosomes 1–7. **H** FISH patterns of probes pSc119.2 and pTa535. Bars = 10 µm

DNA elements (Lapitan et al. 1987; Charette and Andre 2003; Georgieva et al. 2011; Linc et al. 2012; Du et al. 2017; Li et al. 2018). For example, the combination of the repeated probes pSc119.2, pTa71, and Afa family enables the discrimination of E genome chromosomes in diploid *Th. elongatum* (Linc et al. 2012). One set of the E genome in the tetraploid *Th. elongatum* can be identified by the combination of the probes pSc119.2, pTa535, pTa713, and pTa71, but the second set of the E genome is not identified with these probes (Li et al. 2018). Developing such probes to identify all chromosomes in each species often requires considerable effort. In addition, repeat-based probes only label the specific regions of chromosomes and may not be suitable for identification of the same chromosome in different genotypes or homoeologous chromosomes from different species. Bulked oligo probes are superior in resolution and versatility to repetitive sequence- or microsatellite-based probes in many respects. A bulked oligo probe, specific for single-copy sequences, can be designed to target a chromosomal region or an entire chromosome in a variety of ways. Thus, probes can be specifically designed according to the objectives of the research project.

In the present study, seven E genomes-specific bulked oligos based on the *Th. elongatum* and wheat genomes were developed (with a mean density of 0.012 oligos per kb) and were applied to identify the individual chromosomes 1E to 7E. The oligo density of 0.012 oligos per kb in the present study was lower than the 0.052 oligos per kb in wheat (Song et al. 2020) and 0.014 oligos per kb in wheat (Li et al. 2021) in previous studies. A density of 0.012 oligos per kb is sufficient to achieve a balance between cost-effectiveness and a reasonable resolution level. The synthesized library and PCR products can also be used as templates for secondary amplification (Bi et al. 2020). Thus, the bulked oligos probes Chr1 to Chr7 can be used as a permanent resource to identify the E genome chromosomes in *Thinopyrum* species and wheat–alien species derivatives with high efficiency and low cost.

The karyotype can be used to understand phylogenetic relationships among genetically related plant species.

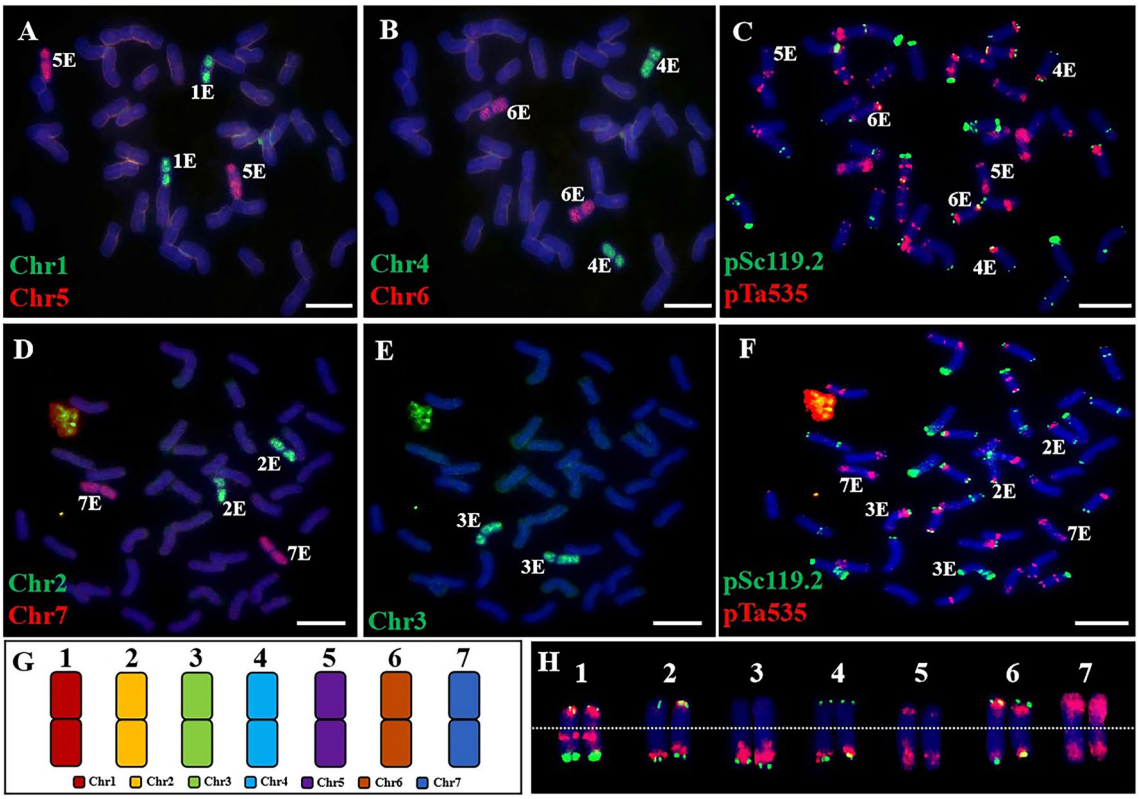

Fig. 6 FISH painting using bulked oligo probes on chromosomes of *Trititrigia* 8801. **A**–**C** FISH using probes Chr1 + Chr5 (**A**), Chr4 + Chr6 (**B**), and pSc119.2 + pTa535 (**C**) of *Trititrigia* 8801. **D**–**F** FISH using probes Chr2 + Chr7 (**D**), Chr3 (**E**), and pSc119.2 + pTa535 (**F**) of *Trititrigia* 8801. **G** An ideogram illustrating the chromosomes 1–7. **H** FISH patterns of probes pSc119.2 and pTa535. Bars = 10 μm

Construction of a karyotype in plants depends on the accurate identification of individual chromosomes. The E genome of *Thinopyrum* species exhibits chromosome structural variation and differentiation (Chen et al. 1998; Linc et al. 2012; Dai et al. 2021), which makes it difficult to identify individual chromosomes in the E genome. Thus, we developed E genome-specific painting probes for identification of the individual chromosomes 1E to 7E. The results of specific painting probes revealed the structural variation and differentiation of E genome chromosomes in *Thinopyrum* species among different populations and ploidies, as well as the E^e and E^b genomes.

In a previous study, Linc et al. (2012) characterized the diploid *Th. elongatum* karyotype by applying highly repetitive DNA sequences as probes and uncovered extensive variation in the probe hybridization patterns in four accessions of diploid *Th. elongatum* of different geographical provenances. However, the extensive variation in the FISH patterns of diploid *Th. elongatum* severely hinders the accurate identification of E genome chromosomes. For example, Linc et al. (2012) observed that the pSc119.2 telomeric sites were distributed on all chromosomes in *Th. elongatum* (PI 531718), with the exception of chromosome 2E. Compared with the results of Linc et al. (2012), the current study revealed that the telomeric sites of pSc119.2 were distributed on all chromosomes except for chromosome 5E in *Th. elongatum* (PI 531718). We speculated that the similarity in the length and arm ratio of chromosomes 2E and 5E may have led to previous confusion regarding their identification. In addition, chromosome painting analysis revealed a reciprocal translocation involving chromosomes 5E and 7E in PI 531718.

Although FISH pattern polymorphisms in diploid species have been reported by several previous studies, it is difficult to use FISH to detect chromosomal rearrangements. We demonstrated that a complete set of E genome-specific painting probes can be used to visualize non-homologous chromosomal rearrangements in *Th. elongatum*. In the present study, a high-resolution FISH karyotype of diploid *Th. elongatum* was developed, which will be useful for comparative karyotype studies. The newly developed chromosome painting techniques provide a powerful tool for the identification of chromosomal rearrangements. Accurate identification of the origin of chromosomal translocations is beneficial for evaluation and utilization of the genetic diversity of *Th. elongatum* and will enhance its utility in wheat breeding.

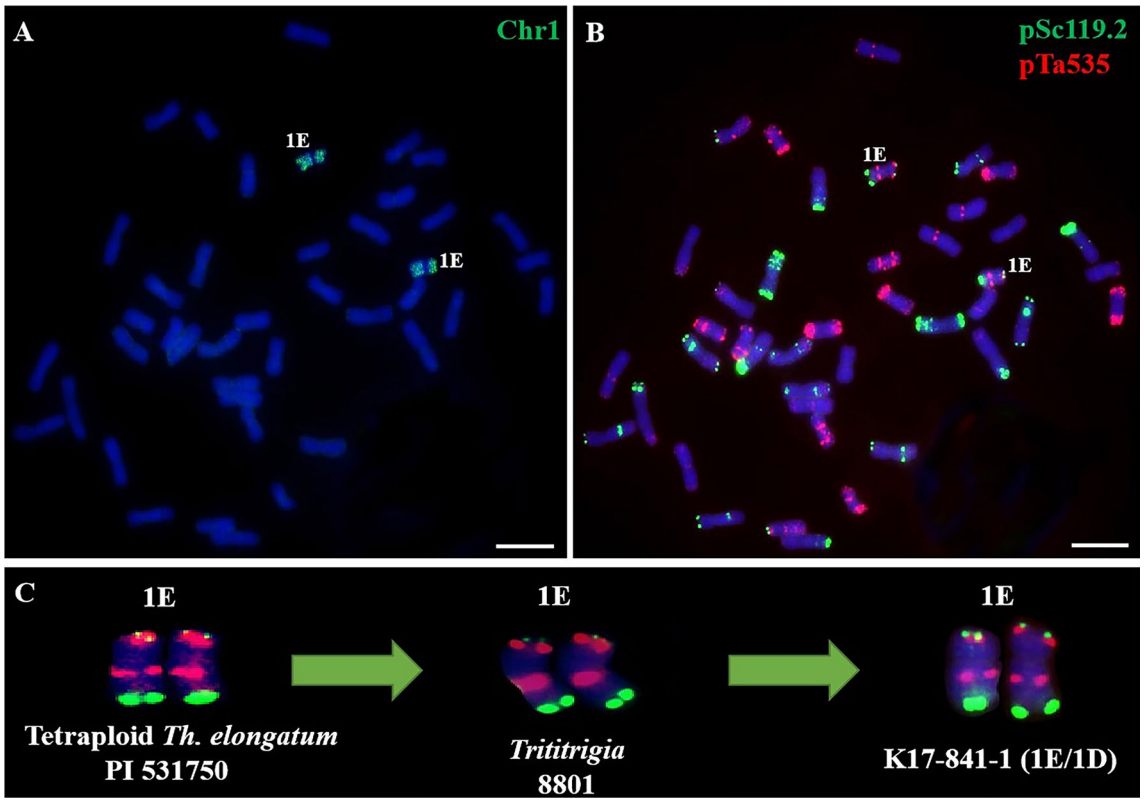

Fig. 7 FISH painting using bulked oligo probes on chromosomes of K17-841-1. **A** FISH using probes Chr1 of K17-841-1. **B** FISH using probes pSc119.2 + pTa535 of K17-841-1. **C** FISH patterns of probes pSc119.2 and pTa535 in K17-841-1, *Tritritrigia* 8801, and tetraploid *Th. elongatum* (PI 531750)

From a breeding standpoint, the present study also provides a useful strategy for obtaining good germplasm with high levels of genetic diversity and fitness through artificial hybridization.

Polyploidization, a crucial factor in species diversification, stimulates the occurrence of large-scale chromosomal rearrangement (Dubcovsky and Dvorak 2007; Otto 2007; Doyle et al. 2008; Hegarty and Hiscock 2008), which can be visualized by FISH (Badaeva et al. 2011; Dong et al. 2017; Winterfeld et al. 2018). For example, the distribution of pSc119.2 signals on the chromosomes of diploid and polyploid species of *Aegilops* differed among the accessions (Badaeva et al. 2011; Dong et al. 2017). In the present study, hybridization patterns with the probes pSc119.2 and pTa535 differed between diploid and tetraploid *Th. elongatum*, suggesting that the E genome had diverged after polyploidization. In general, ND-FISH polymorphisms in different accessions are a result of DNA sequence deletions, duplications, and base mutations (Jackson and Chen 2010; Salmon and Ainouche 2010). This may be one reason why we did not detect the same ND-FISH pattern in diploid and polyploid *Th. elongatum*. In addition, the chromosome painting results indicated that the two sets of genomes of tetraploid *Th. elongatum* had diverged (Fig. 4).

Given the high affinity between the E^e and E^b genomes, distinguishing chromosomes from the two genomes is extremely difficult by traditional chromosome banding, GISH, and FISH (Dai et al. 2021). In the present study, a high-efficacy FISH for *Th. elongatum* (Thy14, E^eE^e) and *Th. bessarabicum* (PI 531711, E^bE^b) was achieved by combining ND-FISH and chromosome painting (Figs. 2, 5). All seven chromosome pairs of Thy14 and PI 531711 were identified by chromosome painting, and a reciprocal translocation between the terminal region of $4E^bL$ and $5E^bL$ was detected in PI 531711. The hybridization patterns of the repeat probes were similar between the E^e and E^b genomes, particularly in chromosomes 1E, 3E, and 4E, where they were almost identical, indicating that the two genomes were closely related. Thus, FISH using painting probes and repeat oligo probes has provided new insights for understanding the relationship between the E^e and E^b genomes.

Findings on the chromosome composition of tetraploid *Th. elongatum* have differed in previous studies. Chromosome pairing analysis indicated that the tetraploid *Th. elongatum* is an autotetraploid (Dvořák 1981; Charpentier et al. 1986). This finding was further supported by a FISH karyotype and chromosome pairing analysis (Shi et al. 2023). However, analysis of isozyme and SSR

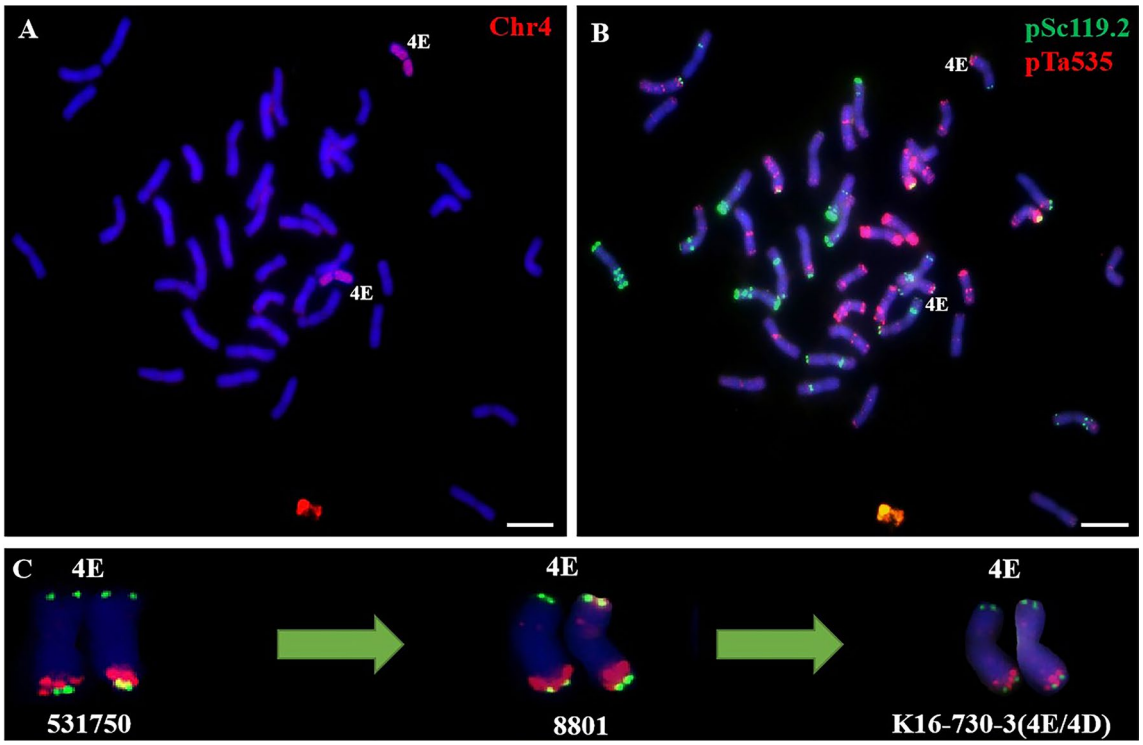

Fig. 8 FISH painting using bulked oligo probes on chromosomes of K16-730-3. **A** FISH using probes Chr4 of K16-730-3. **B** FISH using probes pSc119.2 + pTa535 of K16-730-3. **C** FISH patterns of probes pSc119.2 and pTa535 in K16-730-3, *Trititrigia* 8801, and tetraploid *Th. elongatum* (PI 531750)

markers showed that the tetraploid *Th. elongatum* was an allotetraploid (Li et al. 2005). Li et al. (2018) identified one set of the E genome of tetraploid *Th. elongatum* using the probes pSc119.2, pTa535, pTa713, and pTa71, but the other E genome could not be identified. In the present study, the hybridization patterns with the probes pSc119.2 and pTa535 were distinct between the two sets of E genomes of tetraploid *Th. elongatum*, indicating differentiation between the two sets of genomes. However, it was unclear whether the tetraploid *Th. elongatum* was an autotetraploid or allotetraploid. To further elucidate the genomic composition of tetraploid *Th. elongatum*, FISH analysis was performed using the developed E genome specific painting probes, which can only hybridize to the E chromosomes. The results showed that all 14 pairs of chromosomes of tetraploid *Th. elongatum* were painted by chromosome painting, seven of which had brighter signals and the other seven pairs of chromosomes had weaker signals (Fig. 4), suggesting that the two sets of genomes of tetraploid *Th. elongatum* were the E genome and were differentiated. Based on the ND-FISH and chromosome painting results, we developed a complete FISH karyotype for tetraploid *Th. elongatum* and speculated that tetraploid *Th. elongatum* was autotetraploid, but that the two genomes had differentiated. The FISH karyotype may help to understand the genomic composition of tetraploid *Th. elongatum*, which will facilitate its application in wheat breeding.

The efficient identification of alien genetic material directly affects wheat breeding. To improve the efficiency of identification, the selection of appropriate methods to shorten the breeding cycle is required. At present, the commonly used methods for identification of alien genetic material include molecular markers and in situ hybridization. For example, an SNP array was developed for *Th. intermedium* using *Th. intermedium* and 187 wheat–*Th. intermedium* introgression lines, and 634 SNP markers of *Th. intermedium* were developed for the identification of alien chromosomes (Cseh et al. 2019). However, few molecular markers are specific to the E genome, and the screening of markers is time-consuming and laborious, which is not conducive to the effective detection of alien genetic material. Wang (2016) used GISH to identify three wheat–*Th. intermedium* addition lines carrying genes encoding new protein subunits and conferring high resistance to stripe rust and powdery mildew in the hybrid progeny between common wheat and *Th. intermedium*. Although the traditional in situ hybridization technology is intuitive and accurate, its low detection resolution hinders the identification of small chromosomal rearrangements. Additionally, it does not allow for tracing

the origin of alien chromosomes. In the present study, we developed a set of E genome-specific painting probes suitable for identification of the *Th. elongatum* chromosomes in *Trititrigia* 8801, K17-841-1 (1E/1D), and K16-730-3 (4E/4D). Based on ND-FISH and chromosome painting results, *Th. elongatum* chromosomes in wheat–alien species derivatives could be accurately identified and traced. Chromosome painting can provide important technical support for the rapid and effective detection of alien genetic material in wheat-derived lines, which will facilitate the germplasm innovation and breeding of wheat.

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Author contribution statement HQZ and TZ designed the research. CC, HX, BCZ, and YSH performed the experiments. YSH and DDW designed the E genome oligo probes. LNS, CRY, SQL, and YRC analyzed the data. YW, HYK, XF, and YHZ provided intellectual input on image processing. CC, HX, BCZ, and DDW managed materials in the field. HQZ and CC wrote the manuscript. All authors read and approved the final manuscript.

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Data availability Data supporting the findings of this work are available within the paper and its Supporting Information files. All other data and probes generated and analyzed during the current study are available from the corresponding author upon request.

Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethical approval On behalf of all authors, the corresponding author states that the experiments comply with the current laws of the country in which they were performed.

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