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Distribution of highly repeated DNA sequences in *Haynaldia villosa* and its application in the identification of alien chromatin

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Haynaldia villosa (L.) is a wild relative species of common wheat that possesses many beneficial genes that can be used for wheat improvement. The accurate detection of *H. villosa* chromosomes in the genetic background of wheat is critical for transferring its beneficial genes to common wheat by chromosome engineering. The aim of the present study was to investigate the distribution patterns of two repeated DNA sequences, pSc119.2 and pAs1, as well as two rDNA multigene family sequences, 45S rDNA and 5S rDNA, in the individual chromosomes of *H. villosa* for the future precise identification of alien chromatin in germplasm development and breeding programs. A set of common wheat-*H. villosa* disomic addition 1V–7V lines was used to determine these specific signals on individual chromosomes of *H. villosa*. The results showed that two rDNA probes, pTa71 (45S rDNA) and pTa794 (5S rDNA), were located on 1VS and 5VS, respectively, and the signal could be discriminated exclusively in the common wheat background as effective markers of 1VS and 5VS. Furthermore, all seven chromosomes of *H. villosa* could be distinguished clearly by fluorescence *in situ* hybridization using pSc119.2 and pAs1 as probes in combination. The utilization of these cytogenetic markers of repetitive sequences, combined with other molecular markers sometimes, will make it possible for a precise identification of alien chromosomes with high efficiency.

fluorescence *in situ* hybridization, *Haynaldia villosa*, repeated DNA sequence, wheat

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Wild relatives of common wheat (*Triticum aestivum* L., 2*n*=6*x*=42, AABBDD) are important sources of disease and pest resistance genes that can be employed in wheat improvement. *Haynaldia villosa* (L.) Schur (syn. *Dasypyrum villosum* (L.) Candargy) is an allogamous annual diploid relative (2*n*=2*x*=14, VV) that is native to the northeastern part of the Mediterranean region. *H. villosa* possesses many important agronomical traits, such as disease resistance to powdery mildew, leaf and stem rusts, take-all, eyespot and wheat streak mosaic virus (WSMV) [1–5], as well as some elite genes that can increase the amount of seed storage protein [6], lysine content and gluten strength [7]. Therefore, *H. villosa* is an important resource for increasing genetic diversity in wheat improvement programs.

The transfer of beneficial genes from *H. villosa* into common wheat was initiated in the 1970s at the Cytogenetics Institute, Nanjing Agricultural University, China. Since then, wheat-*H. villosa* hybrids, amphiploid, disomic addition lines (1V to 7V), substitution and translocation lines have been developed [2,3,8–10]. To better utilize the elite genes of *H. villosa* for wheat improvement through chromosome engineering, the development of approaches for the rapid and precise identification of *H. villosa* chromosomes or chromosome segments in wheat genetic background is particularly important in germplasm development and breeding processes.

Giemsa C- and N-banding have usually been used to detect alien chromosomes or chromosome segments in the wheat genetic background. Some researchers have distinguished individual *H. villosa* chromosomes by Giemsa C-

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and N-banding [11–13]. However, the distributed bands were not of a sufficient resolution for analysis and several chromosomes of *H. villosa* could not be distinguished accurately. The fluorescence *in situ* hybridization (FISH) technique, especially using highly repeated DNA sequences as probes, is an effective method for chromosome identification. With increasing numbers of commercially available fluorochromes, multi-color FISH offers the possibility of combining two or more DNA probes labeled with different fluorochromes in simultaneous and/or successive treatments on the same cells. The use of this technique has dramatically increased the resolution for the identification of all chromosomes within a species. In recent years, diverse repetitive sequences have been released with the development of genomics, and versatile probe combinations make it possible to establish a high resolution and unique karyotype of a species. Therefore, FISH-based chromosome identification methods are more powerful than the traditional chromosome banding techniques. The repetitive sequence pSc119.2 isolated from *Secale cereale* [14], the pAs1 isolated from *Triticum tauschii* [15], the ribosomal gene DNA sequence pTa71 (45S) and pTa794 (5S) [16] isolated from *T. aestivum* have been used to construct the chromosomal karyotype in common wheat and its relative species, while there has been a lack of investigation of their patterns in *H. villosa* chromosomes.

The objective of this study was to construct a molecular karyotype of *H. villosa* chromosomes using pSc119.2, pAs1, 45S rDNA and 5S rDNA as FISH probes for the identification of individual *H. villosa* chromosomes or chromosome segments in a wheat genetic background, which would be helpful for the utilization of beneficial genes of *H. villosa* in future wheat improvement programs.

1 Materials and methods

1.1 Plant materials

The *H. villosa* parental line (91C43), originally introduced from the Cambridge Botanical Gardens, UK, was used as the donor parent in the production of wheat-*H. villosa* alien chromosome addition lines. The *T. aestivum* cultivar, Chinese Spring (CS), *Triticum durum*, *T. durum-H. villosa* amphiploid (2*n*=42, AABBVV) and a set of *T. aestivum-H. villosa* disomic addition lines (AD 1V–7V) were all maintained at the Cytogenetics Institute, Nanjing Agricultural University (CINAU), China.

1.2 DNA probes and labeling

The repeated DNA sequences, pSc119.2, pAs1, pTa71 (45S rDNA) and pTa794 (5S rDNA) were used for the FISH analyses. The probes were labeled with digoxigenin-11-dUTP (Roche Diagnostics GmbH, Germany) or biotin-16-dUTP (Roche Diagnostics GmbH, Germany) by the nick-translation according to the manufacturer's protocols.

1.3 Cytogenetic analyses

The chromosome preparations of root-tip cells (RTCs) were made according to the method described by Gill et al. [17]. Genomic *in situ* hybridization (GISH) and fluorescence *in situ* hybridization (FISH) were performed as described by Zhang et al. [18] with minor modifications. The biotinylated probe was detected with FITC-conjugated avidin (Roche Diagnostics GmbH) and digoxigenin-dUTP was detected with anti-digoxigenin rhodamine conjugate (Roche Diagnostics GmbH). The slides were counterstained with 0.6 ng/μL of propidium idodide (PI) for GISH and 2.5 ng/μL of 4′,6-diamidino-2-phenylindole (DAPI) for FISH, and were mounted with Vectashield (Vector Laboratories, USA). Signals were then visualized using an Olympus BX60 fluorescence microscope (Olympus Corporation, Japan) with appropriate filters.

1.4 Molecular markers analysis

Two 3V-specific molecular markers and two 7V-specific molecular markers were used to identify the alien chromatin. Primer information is shown in Table 1. Genomic DNA was extracted using the SDS method described by Sharp et al. [19]. PCR amplifications were conducted in a 10 μL reaction mixture containing 1×*Taq* DNA polymerase buffer, 0.8 mmol/L $MgCl_2$, 0.8 mmol/L dNTPs, 200 μmol/L primers, 2 U DNA polymerase and 50 ng genomic DNA as the template. The samples were denaturated at 94°C for 3 min and subjected to 34 cycles of 30 s of denaturation at 94°C, 50 s annealing at T_m and 1.2 min elongation at 72°C. A final

Table 1 Specific molecular markers used for the identification of chromosomes 3V and 7V of *H. villosa*

Marker	Type	EST ID	Chromosome arm	Primer sequence (5′→3′)
$X_{CINAU38\text{-}250}$	EST-STS	BJ216564	3VS	Forward: CGACGACGTAGATCCAGATG Reverse: TGCTCATGATCGTCATCTCC
$X_{HVM64\text{-}200}$	Barely-SSR		3VL	Forward: GATGTGAAGGCTGCCCTG Reverse: ACACGCCCTATTACCCAGTG
$X_{CINAU256\text{-}800}$	EST-STS	BE446380	7VS	Forward: TCACCTCCCTACCTGTCA Reverse: ACGAAATGCTTGGTCCTT
$X_{CINAU261\text{-}1300}$	EST-STS		7VL	Forward: GGTTTGGACTGAATGGTGCT Reverse: TGCATTTGCCTTGCTGATAG

cycle with an extension of 10 min at 72°C was applied to complete the reactions. The PCR products were analyzed on 8% polyacrylamide gels in 1× TBE buffer.

2 Results

2.1 Distribution of highly repeated DNA sequences in *H. villosa* chromosomes

The repeated DNA sequences were used as FISH probes to identify individual *H. villosa* chromosomes. Figure 1(a), (c) and (d) shows the somatic metaphase chromosomes of *H. villosa* after FISH with pSc119.2, 45S rDNA and 5S rDNA as probes, respectively, and after counterstaining with 4′, 6-diamidino-2-phenylindole (DAPI). The pSc119.2 as a probe for FISH has a relatively simple distribution among each pair of chromosomes, its signals are mainly located on the terminal or sub-terminal sites of both arms in five pairs of chromosomes, but are located on the short arms in the other two pairs of chromosomes (Figure 1(a)). The signals generated by the pSc119.2 probe differed in position and their signal intensity could be identified uniquely. The hybridization sites of the 45S rDNA are located on the short arm of chromosome 1V, which is a satellite (SAT) chromosome (Figure 1(b) and (c)), while the hybridization sites of 5S rDNA are located on the short arm of chromosome 5V, whose arm ratio is the biggest in all seven chromosomes of *H. villosa* (Figure 1(d)). These results were in accordance with the findings of Yuan et al. [20] in that only chromosome 1VS and 5VS could be identified using the 45S rDNA probe and 5S rDNA probe, respectively. It was found that pAs1 hybridization signals were distributed on all the chromosome arms of *H. villosa* (Figure 1(e)), including signals at the terminal, sub-terminal or interstitial sites and, occasionally, at centromeric positions. Furthermore, when pSc119.2 and pAs1 were used as probes to hybridize with the *H. villosa* chromosomes in dual-color FISH, the pAs1 signals were more widely dispersed than those of pSc119.2 (Figure 1(f)).

2.2 Assignment of the FISH signals to specific chromosomes using a set of wheat-*H. villosa* disomic addition lines

A set of *T. aestivum-H. villosa* disomic addition lines, which were confirmed by molecular marker analysis (Table S1 and Figure S1), were used to assign the FISH signals distributed on individual chromosomes of *H. villosa* through sequential GISH (Figure 2(a)–(g), left) and dual-color FISH using pSc119.2 (green) and 45S rDNA (red) as probes (Figure 2(a)–(g), right). A pair of *H. villosa* chromosomes in each disomic addition lines appeared as a unique FISH pattern, and therefore chromosomes 1V–7V could be distinguished. An idiogram of *H. villosa* chromosomes was made as shown in Figure 3. Using pSc119.2 and pAs1 as probes in combination, all of the chromosome arms of *H. villosa* could be distinguished clearly. For example,

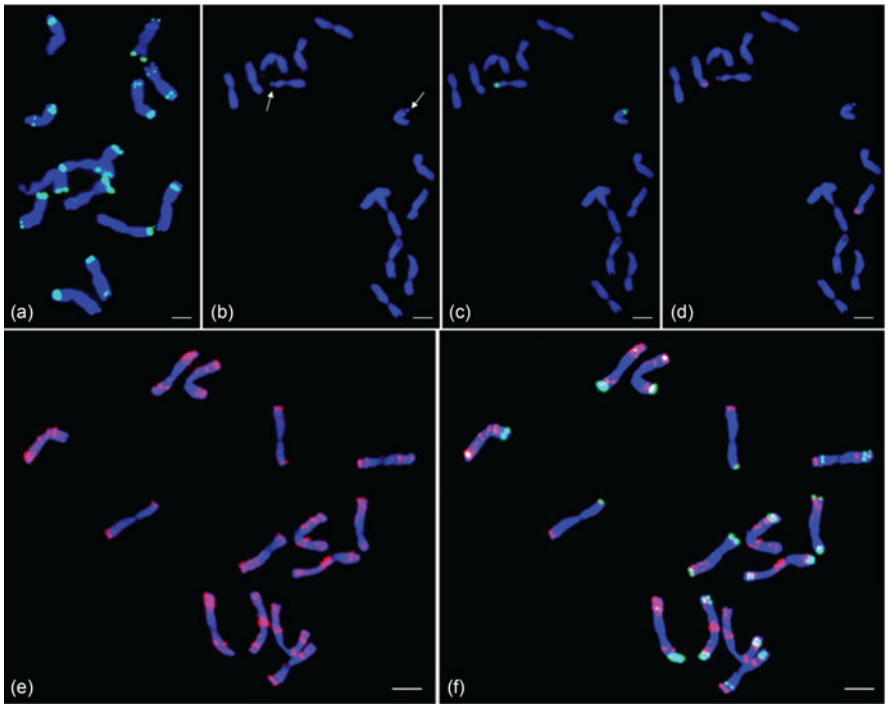

Figure 1 Fluorescence *in situ* hybridization analysis of chromosome in *H. villosa*. (a) Biotin-labeled pSc119.2 (green), (b) counterstained with DAPI (the SAT chromosomes are arrowed), (c) biotin-labeled 45S rDNA (green), (d) digoxigenin-labeled 5S rDNA (red), (e) digoxigenin-labeled pAs1 (red), (f) dual-color FISH with pSc119.2 (green) and pAs1 (red). Scale bars, 10 μm.

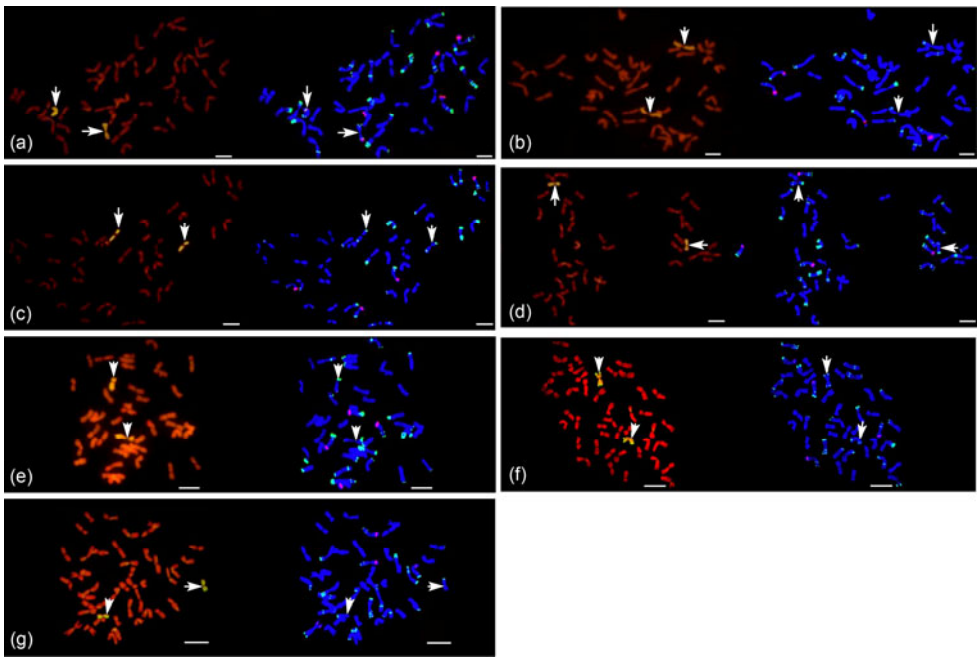

Figure 2 Chromosome sequential genomic *in situ* hybridization (left) and fluorescence *in situ* hybridization (right) on root tip cells of a set of wheat-*H. villosa* disomic addition lines, arrows show one pair of *H. villosa* chromosomes in each figure, simultaneous visualization of hybridization sites to pSc119.2 (green) and 45S rDNA (red) in FISH, signals of 45S rDNA on wheat chromosomes are 1B and 6B in each figure. (a)–(g) shows 1V, 2V, 3V, 4V, 5V, 6V and 7V addition lines, respectively. Scale bars, 10 μm.

the signals of pSc119.2 only appeared on the short arm of 2V and 7V, but the signals of pAs1 on 2V were more widely dispersed than those of pAs1 on 7V. Consequently, both the chromosome arms of 2V and 7V could be distinguished by utilizing pSc119.2 and pAs1 as probes in combination (Figure 3).

2.3 The application of FISH landmarks in the identification of alien chromatin

The pollen of *T. durum-H. villosa* amphiploid was irradiated with ^{60}Co γ-rays [21,22] in order to develop more wheat-*H. villosa* translocations that are involved in different chromosomes and chromosome segments of *H. villosa*. We screened numerous *H. villosa* chromosome structural changes in the offspring that included whole-arm translocations, small alien segment translocations, large alien segment translocations and the terminal deletion of *H. villosa* chromosomes by GISH. However, some wheat-*H. villosa* translocations, especially small alien segment translocations, could not be identified accurately with the C-banding method. Therefore, some chromosome structural changes were used to test the usefulness of these FISH landmarks for the identification of chromosome segments of *H. villosa*.

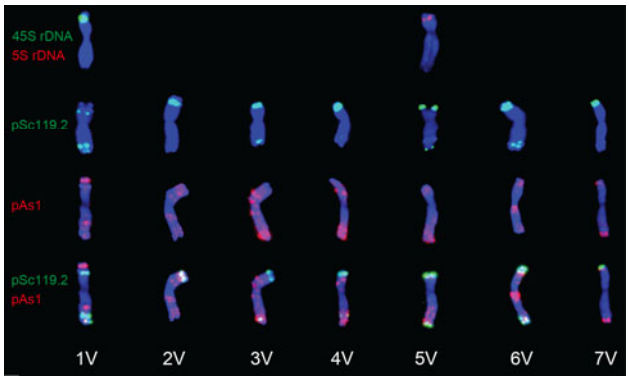

Figure 3 Idiogram of the chromosomes of *H. villosa* showing locations of 45S rDNA (green), 5S rDNA (red), pSc119.2 (green), pAs1 (red) and simultaneous visualization of pSc119.2 (green) and pAs1 (red). The chromosome nomenclature was based on their homology to wheat and *H. villosa* chromosomes.

As 45S rDNA and 5S rDNA were marked for 1VS and 5VS, respectively, the chromosome structural changes of *H. villosa* were initially analyzed using 45S rDNA and 5S rDNA as probes. These results showed that three chromosome structural changes (TV277-7, TV288-4 and TV54-4-1) that involved 1VS and four (TV49-1-6, TV08-3, TV179-3-3 and TV330-6) that involved 5VS (Figure 4) could be easily identified. Subsequently, the seven chromosome structural changes were confirmed to be chromosome specific molecular markers of 1V and 5V (Tables S2 and S3), and the results were consistent with those of sequential GISH/FISH assays.

The second example for the successful use of the established karyotype to determine the identity of chromosomes was to characterize two alien telocentric chromosomes in two plants, TV60-1-8-18 and TV60-1-8-27, identified in the offspring of irradiated *T. durum-H. villosa* amphiploid. To characterize the two telocentric chromosomes, the somatic

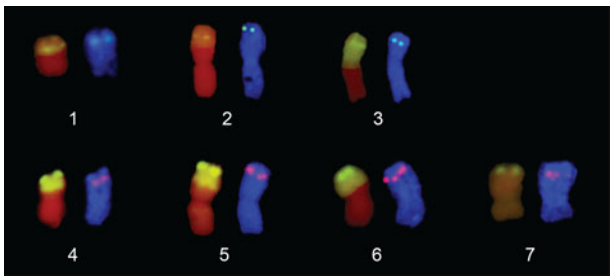

Figure 4 Chromosome sequential genomic *in situ* hybridization and fluorescence *in situ* hybridization of *H. villosa* chromosome structural changes involving 1VS (1–3) and 5VS (4–7). Total genomic DNA of *H. villosa* labeled with fluoresein-12-dUTP (green, GISH, left), 45S rNDA labeled with biotin-16-dUTP (green, FISH, right) and 5S rDNA labeled with digoxigenin-11-dUTP (red, FISH, right). 1, TV277-7; 2, TV288-4; 3, TV54-4-1; 4, TV49-1-6; 5, TV08-3; 6, TV179-3-3; 7, TV330-6.

chromosomes at metaphase were hybridized with biotin-labeled pSc119.2 and digoxigenin-labeled pAs1 (Figure 5). The result of the comparison of FISH-patterns showed that a telocentric chromosome in TV60-1-8-27 plants was a short arm of 3V (Figures 5(a) and 6) and a telocentric chromosome in TV60-1-8-18 plants was a long arm of 3V (Figures 5(b) and 6). Furthermore, the two telosomic plants were analyzed with the chromosome arm-specific molecular markers of 3VS and 3VL, $X_{CINAU38\text{-}250}$ and $X_{HVM64\text{-}200}$ (Figure 7), and the results were consistent with those of sequential GISH/FISH assays.

In addition, two whole-arm translocation plants were identified by *in situ* hybridization using biotin-labeled pSc119.2 and digoxigenin-labeled pAs1 as probes (Figure 8). The result of the comparison of FISH-patterns showed

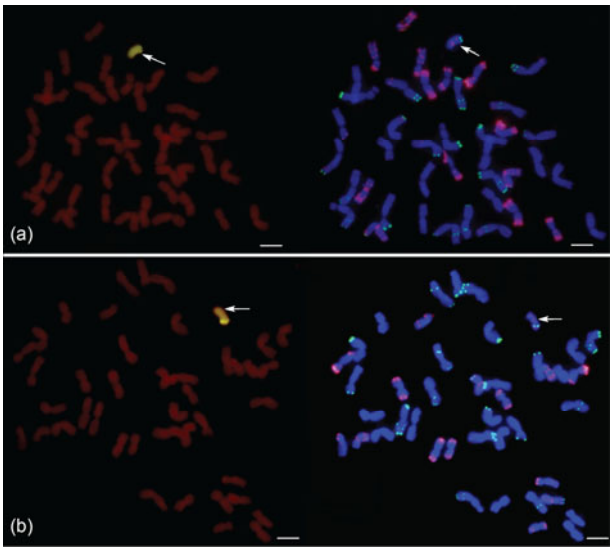

Figure 5 Chromosome sequential GISH (left) and FISH (right) on root tip cells of lines TV60-1-8-27 and TV60-1-8-18, where the pSc119.2 signal is green and pAs1 signal is red, and arrows show a telocentric chromosome. (a) The TV60-1-8-27 plant is a mono-telosomic addition line MAt.3VS, while (b) the TV60-1-8-18 plant is a mono-telosomic addition line MAt.3VL. Scale bars, 10 μm.

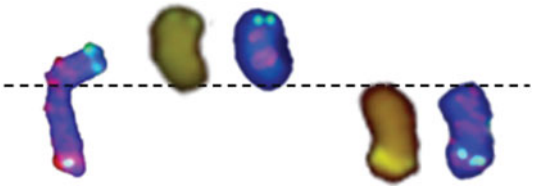

Figure 6 The pattern of dual-color FISH of 3V chromosome and two telocentric chromosomes, from left to right, chromosome 3V, short arm of 3V telocentric chromosome (in TV60-1-8-27) and long arm of 3V telocentric chromosome (in TV60-1-8-18), the pSc119.2 is green and pAs1 is red.

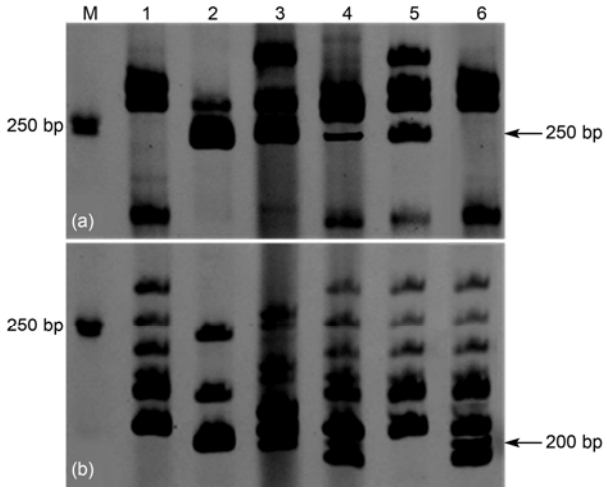

Figure 7 An example of PCR analysis of the two telocentric chromosomes using marker $X_{CINAU38\text{-}250}$ (3VS) and $X_{HVM64\text{-}200}$ (3VL). (a) Amplification of primer CINAU38, (b) amplification of primer HVM64, arrows show a specific band for chromosome 3V. M, DNA Marker DL2000; 1, Chinese Spring; 2, *H. Villosa*; 3, *T. durum-H. villosa* amphiploid; 4, DA3V; 5, TV60-1-8-27; 6, TV60-1-8-18.

that the translocation chromosome in the TV54-5-5-22 plant involved 7VS (Figures 8(a) and 9), while the translocation chromosome in TV54-5-5-21 plant involved 7VL (Figures 8(b) and 9). Furthermore, the two whole-arm translocations were analyzed with chromosome arm-specific molecular markers of 7V, $X_{CINAU256\text{-}800}$ (7VS) and $X_{CINAU261\text{-}1300}$ (7VL) (Figure 10), and the results were consistent with those of sequential GISH/FISH assays.

3 Discussion

Plant genomes usually consist of a high percentage of repetitive sequences, which are generally dispersed along the plant chromosomes [23]. However, some special repetitive families are only distributed in particular chromosome regions [24]. The distinct sequence composition and distribution of these repetitive families may be due to duplications, diversifications, homogenization and deletions. Therefore, the characterization of the repetitive families makes it possible to distinguish different genomes and chromosomes according to their unique distribution patterns or sequence poly

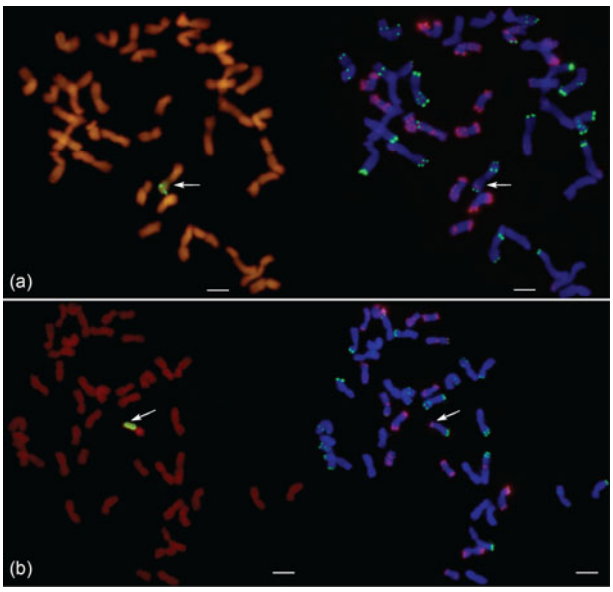

Figure 8 Chromosome sequential GISH (left) and FISH (right) on root tip cells of lines TV54-5-5-22 and TV54-5-5-21, the pSc119.2 signal is green and pAs1 signal is red, arrows show a translocation chromosome. (a) The TV54-5-5-22 plant with a translocation chromosome involved in 7VS, while (b) the TV54-5-5-21 plant with a translocation chromosome involved in 7VL. Scale bars, 10 μm.

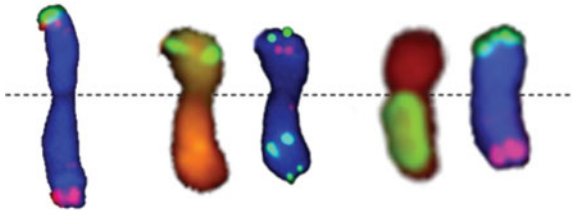

Figure 9 The pattern of dual-color FISH of 7V chromosome and two translocation chromosomes, from left to right, chromosome 7V, short arm translocation of chromosome 7V (in TV54-5-5-22) and long arm translocation of chromosome 7V (in TV54-5-5-21), the pSc119.2 is green and pAs1 is red.

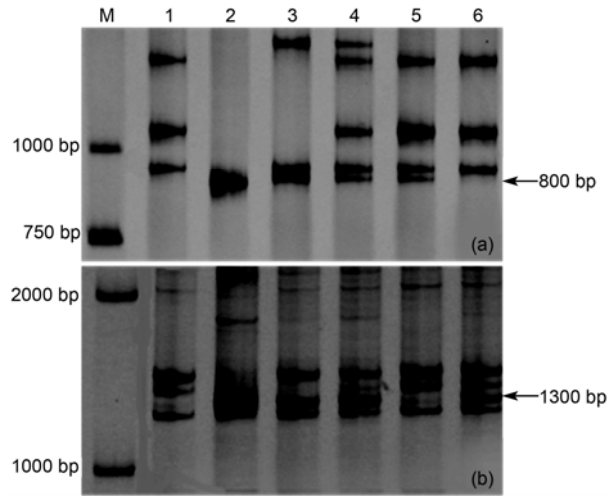

Figure 10 An example of PCR analysis of the two translocation chromosomes using markers $X_{CINAU256\text{-}800}$ (7VS) and $X_{CINAU261\text{-}1300}$ (7VL). (a) Amplification of primer CINAU256, (b) amplification of primer CINAU261, arrows show a specific band for chromosome 7V. M, DNA Marker DL2000; 1, Chinese Spring; 2, *H. villosa*; 3, *T. durum-H. villosa* amphiploid; 4, DA7V; 5, TV54-5-5-22; 6, TV54-5-5-21.

morphisms.

FISH has been developed for the physical mapping of repeated DNA sequences on chromosomes and genomes. In the tribe Triticeae, localized tandem repeated sequences are useful as cytogenetic markers for chromosome identification [25–29] and can be applied for the characterization of additions, substitutions and translocations [30–32].

The distributions of repeated sequences pSc119.2 and pAs1 in wheat and its related species have been described widely. In hexaploid wheat, the repeated sequence pSc119.2 is mainly located on the B genome, while pAs1 is mainly located on the D genome [33]. However, both of these repeated sequences are distributed on all the chromosomes (1R–7R) of *S. cereale* [34]. In our study, it was found that the repeated sequences, pSc119.2 and pAs1, were also distinctly distributed on all the individual chromosomes (1V–7V) of *H. villosa*. For example, pSc119.2 was mainly distributed at the terminal ends of all chromosomes from 1V to 7V, while pAs1 was located not only on the terminal regions, but also on the interstitial regions of all the *H. villosa* chromosomes. The similar distribution of both repetitive sequences in the V and R genomes implied that the relationship between the V and R genomes may be much closer than that between the V genome and A, B or D genomes.

Satellite chromosomes of alien species could be easily detected by carmine staining owing to the obvious cytogenetic marker, secondary constriction. However, when the satellite chromosomes of alien species, such as 1V of *H. villosa* and 1R of *S. cereale*, were transferred to the wheat background, the secondary constriction of the satellite chromosomes could not be expressed and detected by carmine staining. In this study, 45S rDNA not only appeared on the short arm of satellite chromosome 1V in the *H. villosa* background, but it also appeared in the 1VS whose secondary constriction was hidden in the wheat background (Figure 4). Therefore, for the identification of alien satellite chromosomes in the wheat background, the molecular-cytogenetic marker, 45S rDNA, is a good choice.

This study also used the repeated sequence, 5S rDNA. It was found that this signal could be observed only on chromosome 5VS in *H. villosa*, and the signal could be stably detected in the common wheat background (Figure 4). Elite genes that control the grain end-product quality, such as endosperm storage proteins and grain hardness, have been located on 1VS and 5VS [6,35], but these good genes have not been used in commercial wheat varieties. To transfer this quality related genes from *H. villosa* to common wheat, the development of chromosome translocations that involve 1V and 5V should be effective. In such chromosome engi-

neering processes, the precise identification of the alien chromosomes in the wheat background is a critical step, and the distributed pattern of 45S rDNA and 5S rDNA in the 1VS and 5VS, as revealed in this study, will be very helpful.

The combination of GISH and FISH assays using a set of wheat-*H. villosa* disomic addition lines could provide a unique strategy for assigning the FISH signals of repetitive sequences to individual chromosomes in order to yield a *H. villosa* molecular karyotype (Figure 3). As a demonstration of the utility of these FISH landmarks, two telocentric chromosomes and two whole-arm translocation chromosomes were identified with the use of pSc119.2 and pAs1 as probes. The result of this determination was confirmed with the simultaneous use of chromosome arm-specific molecular markers. Currently, FISH landmarks combined with molecular marker analysis strategies is being used to identify various translocation lines with different breakpoints that cover the entire genome of *H. villosa*. It appears that this is a highly efficient and high-throughput approach that should promote progress in alien chromosome manipulation, gene mapping and utilization.

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Supporting Information

Table S1 Chromosome arm-specific molecular markers for *Haynaldia villosa*
Figure S1 Amplification of specific primer on DNA of parents and wheat-*H. villosa* addition lines.
Table S2 Specific molecular markers used for the identification chromosome 1V and 5V of *H. villosa*
Table S3 The result of identification of chromosome specific molecular markers of 1V and 5V

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