RESEARCH NOTE

# Characterization of a genome-specific Gypsy-like retrotransposon sequence and development of a molecular marker specific for *Dasypyrum villosum* (L.)

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## Introduction

As an important gene resource for wheat improvement, genetic materials of *Dasypyrum villosum* (L.) ($2n = 14$, VV) have been introduced into wheat genome. Isolation of *D. villosum* specific DNA sequences, especially the genomewide interspersed repetitive sequences, is an effective way to trace *D. villosum* chromatin in wheat genetic background during breeding programmes. In this study, a Gypsy-like retrotransposon sequence was isolated by random amplified polymorphic DNA (RAPD), designated as C1-10. Fluorescence *in situ* hybridization (FISH) revealed that C1-10 could be specifically hybridized with all *D. villosum* chromosome arms of *Triticum durum – D. villosum* amphiploids, as well as segments of *D. villosum* chromosomes in wheat – *D. villosum* translocation lines, suggesting that C1-10 might be a *D. villosum* specific repetitive sequence, which could be used to distinguish V genome from A, B and D genomes of wheat. Based on sequence of C1-10, one pair of PCR primers Dv1 (Dv1-R and Dv1-F) was designed and could efficiently and specifically amplify the target from five accessions of *D. villosum*, five accessions of *T. durum – D. villosum* amphiploids, seven accessions of wheat – *D. villosum* additional lines, and three wheat lines containing *D. villosum* chromatins. Therefore, it could be used as a sequence-characterized amplified region (SCAR) marker to effectively trace the introgression of *D. villosum* chromatin in the wheat genome, and consequently will facilitate the utilization of useful genes of *D. villosum* in wheat improvement.

*D. villosum*, as a valuable gene resource for wheat improvement, shows resistance to biotic causal agents of several wheat diseases such as powdery mildew (Chen *et al.* 1995; Qi *et al.* 1995), rust (Yildirim *et al.* 2000), eyespot (Yildirim *et al.* 2000; Uslu *et al.* 1998), as well as resistance to abiotic stress (Scarascia Mugnozza *et al.* 1982). Thus, many wheat – *D. villosum* addition lines (Sears 1953; Blanco *et al.* 1987; Liu *et al.* 1988), substitution lines (Liu *et al.* 1988), translocation lines (Chen *et al.* 1995; Qi *et al.* 1996; Li *et al.* 2005; Bie *et al.* 2007) and amphiploids (De Pace *et al.* 1988; Liu *et al.* 1988; Minelli *et al.* 2005) have been developed for transferring useful genes from *D. villosum* to wheat.

With the introduction of alien chromatin into wheat, many useful markers such as morphological markers (Zhong and Qualset 1990), biochemical markers (Li *et al.* 2009), and cytological markers (Zhong *et al.* 1996) have been developed to monitor the behaviour of the alien chromosomes. However, the major limitation of these techniques is that they are highly technical and time-consuming. The species-specific PCR-based markers are useful and convenient tools for detecting alien chromosome segments incorporated into wheat genomes (Fu *et al.* 2010).

In the present study, to develop a reliable molecular marker for providing an access to molecular-assistant breeding in wheat breeding programmes, a novel repeated sequence, C1-10, of *D. villosum* were characterized and converted to a reliable SCAR marker specific to *D. villosum*. Further, the chromosomal distribution of this sequence was characterized using FISH image.

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**Keywords.** FISH; molecular marker; wheat; *Triticum aestivum* L.; chromatin mapping; *Dasypyrum villosum*.

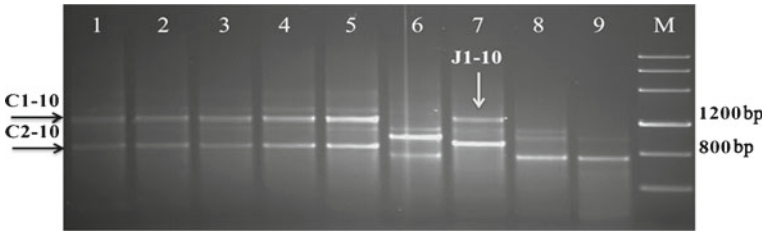


**Figure 1.** RAPD pattern amplified using primer OP-A5. Lane 1 to 9 indicated W67266, H.V078, W67290, H.V077, H.V076, CS, JZHM, *Ae. ventricosa* and *Ae. uniaristata*, respectively. Black arrow indicates C1-10 and C2-10, and white arrow indicates J1-10, respectively. M, DNA marker.

## Materials and methods

### *Plant materials*

In the present investigation, seven accessions of *T. aestivum*, five accessions of *D. villosum*, three accessions of *T. durum*, two accessions of *Secale cereale*, three accessions of *Aegilops*, five accessions of *T. durum – D. villosum* amphiploid and seven *T. aestivum* (Chinese Spring) – *D. villosum* additional lines were used and are listed in table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/. The *T. aestivum* accessions Mianyang 26 and Neimai 9 were obtained from Academy of Agricultural Science of Mianyang and Agricultural Science of Neijiang, respectively; wheat lines Xiaoyan 54 and Gaoyou 503 were obtained from Institute of Genetics and Developmental Biology, Chinese Academy of Sciences; wheat – *D. villosum* translocation lines 9R178 and 9R137 were supplied by Nanjing Agricultural University; *D. villosum* accessions W67266 and W67290, and *Aegilops ventricosa* accession As106 were obtained from Triticeae Research Institute, Sichuan Agricultural University; wheat – *D. villosum* additional lines Add 1V - 7V were introduced from School of Life Science and Technology, University of Electronic Science and Technology of China. The *T. durum – D. villosum* amphiploid accessions (Th1w, Th2w, Th3w, Th1, Th3) and *T. durum* accessions D311, Mo75 and 81086A were obtained from Prof. Xiao Chen, Chinese Academy of Agricultural Sciences. Other accessions were conserved by our laboratory.

### *RAPD analysis*

Total genomic DNA was extracted from young leaves using the CTAB method (Murray and Thompson 1980). DNA concentration was determined by using Multimode Reader (Varioskan Flash, Waltham, USA). A total of 100 RAPD primers were used and PCR reactions were conducted in a volume of 25 $\mu$L containing 2.5 $\mu$L of 10× buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, pH 8.3), 200 $\mu$M of each deoxynucleotide, 10 pM of each primer, 50 to 100 ng genomic DNA, and 1.0 U *Taq* DNA polymerase (TianGen, Beijing, China). The amplification reactions were carried at 94°C for 5 min, followed by 42 cycles at 94°C for 1 min, 36°C (primer set dependent) for 40 s, 72°C for 40 s, and a final extension at 72°C for 10 min. PCR products were separated on 2% agarose gels and visualized by EtBr staining.

### *Cloning and sequencing of the RAPD product*

RAPD products were excised from 2.0% agarose gels and purified by using Gel Extraction kit (TianGen). The purified products were ligated to the pMD19-T vector (TaKaRa,

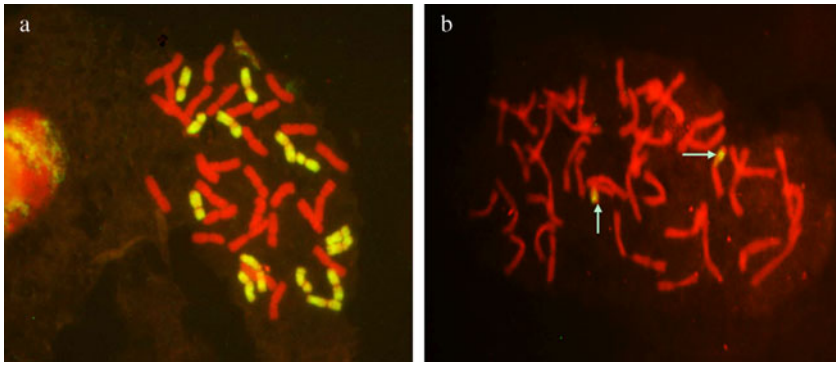


**Figure 2.** Part of nucleotide sequence alignment of the C1-10 to FN564432.1. The CAAAA (invert) motif is boxed, and the 12-mer direct nucleotide repeats are underlined. The arrows indicated the directions and positions of the designed primers DV1-F and DV1-R.

Dalian, China) and the modified vector was used to transform competent cells of *Escherichia coli* strain JM109. Positive clones were screened by PCR amplification using M13 universal primers, and three positive clones were randomly chosen for DNA sequencing at Genome Research Institute (BGI), Shenzhen Wah, China. Sequence similarity analysis was performed using BLASTn program at http//www.ncbi.nlm.nih.gov.

### *Sequence analysis, primer design and PCR validation*

Based on the sequences alignment of RAPD products, PCR primers were designed using Primer Premier 5.0 (PREMIER Biosoft, Palo Alto, USA), and were synthesized by BGI. The PCR reaction, with 25 $\mu$L volume containing 40–100 ng genomic DNA, 0.2 $\mu$M of each primer, 200 $\mu$M $MgCl_2$ and 1 U *Taq* DNA polymerase, was performed at 5 min at 94°C, 35 cycles of 1 min at 94°C, 0.5 min at 57°C, 0.5 min at 72°C and a final extension at 72°C for 10 min. The PCR products were separated on 1% agarose gel.

### *Fluorescence in situ hybridization (FISH)*

For chromosome preparation, the root tip meristems of germinating seeds were cut and placed in saturated α-bromonaphthalene solution at 4°C for 14–18 h. The root

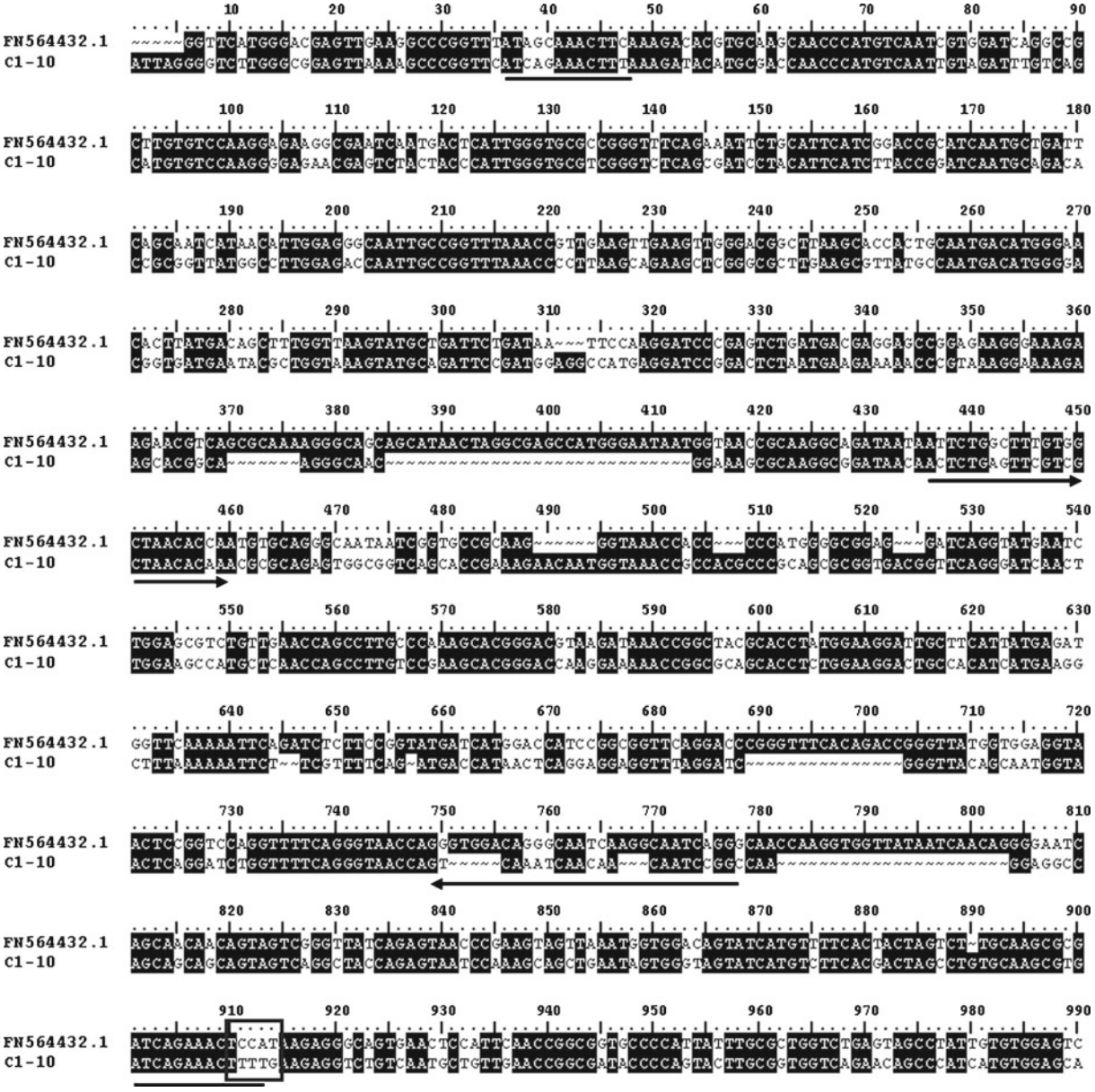


**Figure 3.** Fluorescence *in situ* hybridization (FISH) analysis using C1-10 as probe on mitotic metaphase chromosomes of *T. durum – D. villosum* amphipoild Th2w ($2n = 42$) (a) and wheat- *D. villosum* translocation line 9R178 ($2n = 42$). Fourteen *D. villosum* chromosomes of Th2w showed strong signals. Arrows shows *D. villosum* chromatin.

tips were subsequently fixed in ethanol : glacial acetic acid (3 : 1, v/v). The fixed root tips were squashed on microscope slides in 45% acetic acid. The coverslips were removed after the slides were frozen in liquid nitrogen and the slides were air-dried before hybridization.

For FISH analysis, the whole plasmid with insert was labelled with digoxigenin-11-dUTP (Roche Diagnostics, Basel, Switzerland) by nick translation. The hybridization procedure was similar to that of Islam-Faridi and Mujeeb-Kazi (1995) with slight modification, before hybridization, preparations were treated with 0.05 M HCl for 2 min and incubated with pepsin (5 $\mu$g/mL) for 15 min at 37°C, and then the preparations were washed with water and 2× SSC for 2 min and 10 min, respectively. The digoxigenin labelled repeated DNA signal was detected with anti-digxigenin-rhodamine fab-fragment (Roche Diagnostics). Slide was finally counterstained with propidum iodide (0.25 $\mu$g mL$^{-1}$) and mounted on Vectashield Antifade solution (Vector Laboratories, Burlingame, USA). Microphotographs of FISH chromosomes were taken using Zeiss Axioplan2 microscope (Zeiss, Oberkochen, Germany).

## Results

### *Identification of retrotransposon sequences from D. villosum*

A total of 100 arbitrary 10-mer RAPD primers were screened, in which OP-A5 (5′-AGGGGTCTTG-3′) could specifically amplify a 1200-bp fragment in five accessions of *D. villosum* and JZHM, designated as C1-10 and J1-10, respectively. OP-A5 could also amplify an 850-bp product in all accessions of *D. villosum* specifically, designated as C2-10 (figure 1). All three bands were cloned and sequenced.

The C1-10 was 1224-bp long with AT content of 49.67%. One motif CAAAA (invert) and direct repeats were present in this sequence. BLAST analysis showed that C1-10 has 74% sequence identity to a long terminal repeat (LTR) Gypsy retrotransposon which was part of chromosome 3B-specific BAC library sequence of *T. aestivum* (FN 564432.1, Choulet *et al.* 2010). A low-homology region (positions 400 to 800) consisting of nucleotide variations and deletions was present in C1-10 (figure 2).

The J1-10 from JZHM was 1228 bp with AT content of 60.26%. BLAST analysis showed that it has 93% sequence identity to a LTR Copia retrotransposon (CT009735.1) from *T. aestivum* (data not shown). Sequence alignment indicated that J1-10 shared only 33.44% homology with C1-10. C2-10 was highly similar (89%) with a Type2/Copia retrotransposon (FN564436) of *T. aestivum*, but showed low degree of homology with C1-10 (25.87%) and J1-10 (28.04%).

### *FISH analysis*

Metaphase chromosomes of *T. durum – D. villosum* amphiploid were hybridized with C1-10 which was labelled as probe. The hybridization appeared in all *D. villosum* chromosomes, while no signal was detected in *T. durum* chromosomes. Further, hybridization signals were strong throughout the chromosomal arms and weaker at the centromere region of *D. villosum* (figure 3).

### *Development and validation of V genome-specific SCAR marker*

One pair of PCR primers Dv1 (Dv1F: 5′-ACTCTGAGTTCGTCGCTAACACAA-3′; Dv1R: 5′-GCCGGATTGTTGTTGATTTGAC-3′) (figure 2) was designed from the low-homologous region of C1-10. It could successfully amplify a target band of 317 bp in five accessions of *D. villosum*, *T. durum – D. villosum* amphiploid, and CS-*D. villosum* additional lines. However, no band could be generated in CS, *T. durum*, *Ae. tauschii*, JZHM, and *Ae. ventricosa* (figure 4a). Due to the high homology (89%) to *T. aestivum* sequence, the C2-10 failed to be converted to SCAR marker for targeting *D. villosum* chromatin. To explore possible use of C1-10 in tagging the chromatin of *D. villosum* in wheat, two groups of wheat lines, containing *D. villosum* chromatins (9R178, 9R137, Neimai9) and without any genetic background of *D. villosum* (CS, Xiaoyan 54, Mianyang 26, Gaoyou 503), were assayed by the primer pair

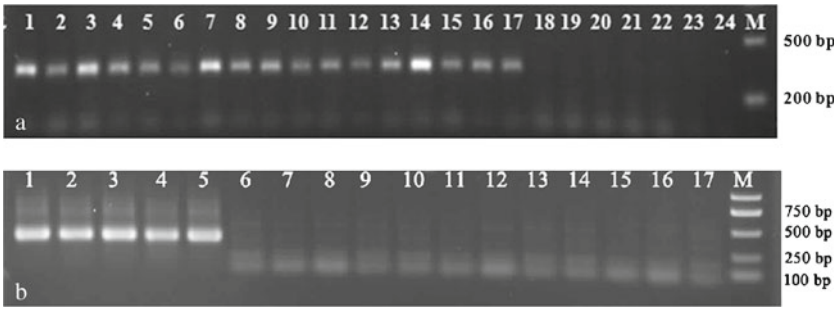


**Figure 4.** PCR bands amplified using the primer pair DV1-F and DV1-R. a) 1–5, *D. villosum*; 6–10, *T. durum – D. villosum* amphiploids; 11–17, CS-*D. villorum* additional lines; 18–20, *T. durum*; 21, *Ae. tauschii*; 22, JZHM; 23, *Ae. ventricosa*; 24, CS. b) 1, *D. villosum*; 2, *T. durum – D. villosum* amphiploid, 3–5, 9R178, Neimai 9, and 9R137 (common wheat varieties containing *D. villosum* chromatin); 6–8, *T. durum*; 9, *Ae. tauschii*, 10, 501; 11, JZHM; 12, *Ae. ventricosa*; 13, *Ae. uniaristata*; 14–17, CS, Xiaoyan 54, Mianyang 26, and Gaoyou 503 (the common wheat varieties without the genetic materials from *D. villosum*).

Dv1 (figure 4b). The result showed that it could amplify target band in all materials containing *D. villosum* chromatins but the materials without *D. villosum* chromatins.

## Discussion

*D. villosum* is considered as a valuable gene resource of wheat improvement for its resistance to a range of wheat diseases. Thus, introgression of *D. villosum* chromatin into wheat genome has been widely utilized to introduce useful genes of *D. villosum* into hexaploid wheat (Minelli *et al.* 2005). To assay the alien chromatin in wheat genetic background, several strategies have been designed, among which PCR-based identification of the species-specific repeated sequences, without relying on the time-consuming procedures, such as Southern hybridization and *in situ* hybridization (Lapitan 1992; Katto *et al.* 2004), thought to be the most efficient way. De Pace *et al.* (1992) isolated a *D. villosum*-specific sequence with 380 bp in length; Li *et al.* (1995) obtained a species-specific sequence pHvNAU62 from *D. villosum*; Yuan and Tomita (2009) cloned a 350 family sequence pDvTU383 from *D. villosum*. However, the sequences mentioned above have not been transferred to PCR-based markers. Tang *et al.* (2007) isolated a 388 bp sequence pDv848/838 from *D. villosum* using ISSR primer UBC848. However, this marker can only trace the chromosome 5V. By RAPD analysis, Liu *et al.* (1999) found a DNA fragment of 1400 bp, $OPH17_{1400}$, which was linked to *Pm21*, and then developed a reliable SCAR marker. Liu *et al.* (2003) obtained a Gypsy-like sequence $OPF02_{757}$ from *D. villosum*. A SCAR marker based on this sequence can tag the *D. villosum* chromosomes in genetic materials of *T. aestivum* – *D. villosum* addition, *T. aestivum* – *D. villosum* substitution, *T. aestivum* – *D. villosum* amphidiploid, and *T. durum* – *D. villosum* amphidiploid. However, this marker has not been further evaluated, especially its sensitivity in assaying the introgression of *D. villosum* chromatin in wheat genetic background.

In the present study, we isolated a *D. villosum*-specific Gypsy-like retrotransposon sequence C1-10 by RAPD. It lacks homologies to $OPF02_{757}$ (Liu *et al.* 2003), indicating that it might be a novel Gypsy-like retrotransposon specific to *D. villosum*.

The C1-10 contains one copy of the motif CAAAA (invert) which was responsible for the breakage–reunion mechanism (Appels *et al.* 1986) and the basic characteristic of direct repeats, indicating the intra-element recombination contributing to the genome expansion of *D. villosum* (Yang *et al.* 2006) (figure 2). However because the whole sequence of C1-10 as retrotransposon is not known, we can not figure out which Ty3/Gypsy lineage it belongs to.

FISH study showed C1-10 was distributed throughout the whole chromosome arms of *D. villosum*, while no signal was detected in A, B and D genomes of wheat, indicating that the retrotransposon element occurs in all chromosomes and might be used to track the *D. villosum* chromatin efficiently no matter which chromosomal segment of *D. villosum* was introduced in wheat.

In addition, hybridization signals were strong throughout all the chromosomal arms and weaker at the region of centromere, which displayed different FISH pattern of Gypsy-like elements in genomes of sunflower species (Staton *et al.* 2009), rice, oats, maize, barley, wheat and rye (Jiang *et al.* 1996; Presting *et al.* 1998). This suggested that the species-specific expansion pattern of Gypsy-like retrotransposon is probably associated with the different lineages they belong to or the evolution of chromatin organization of their hosts. The size and intensity of the FISH signals were not uniform across chromosomes, suggesting that the obtained retrotransposon sequence differs in copy number in different chromosomes of *D. villosum*.

The primer pair based on C1-10 could reliably and easily monitor the presence of *D. villosum* chromatin in *T. Durum* – *D. villosum* amphidiploid, CS-*D. villosum* additional lines, as well as the wheat lines of 9R178, 9R137 and Neimai 9, containing *D. villosum* chromatin. Thus, it could be used as efficient molecular marker in wheat breeding programme targeting alien *D. villosum* chromatin containing useful genes for wheat improvement.

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