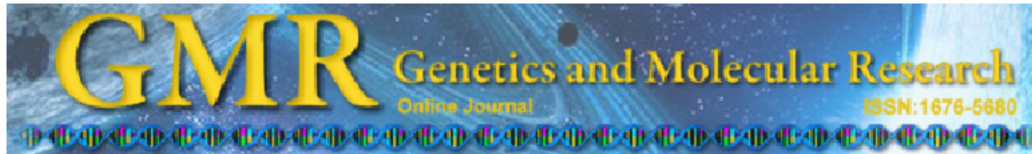

A novel SCAR marker for detecting *Psathyrostachys huashanica* Keng chromatin introduced in wheat

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ABSTRACT. In this study, we cloned and sequenced a 938-base pair polymorphic band, pHs27, in the tightly linked random amplified polymorphic DNA marker OPU10 and converted it into a sequence-characterized amplified region (SCAR) marker referred to as RHS141, which was specific for the Ns genome of *Psathyrostachys huashanica*. A GenBank basic local alignment search tool search showed that the sequence of pHs27 had no primary sequence homology with known sequences, and Southern blotting confirmed this result. This SCAR marker was used to detect Ns genome chromatin in wheat, and it was successfully amplified in *P. huashanica* itself, a complete set of wheat-*P. huashanica* disomic addition lines (1Ns-7Ns), and undetermined homoeologous group addition lines. This SCAR marker will be a powerful tool for the marker-assisted selection of *P. huashanica* chromosome(s) in a wheat background, and it should also allow wheat breeders to screen for the excellent traits found in *P. huashanica* chromatin.

Key words: Marker-assisted selection; *Psathyrostachys huashanica*; RAPD; Repetitive sequence; SCAR; Wheat

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INTRODUCTION

The production and utilization of polymerase chain reaction (PCR)-based markers is effective for tracing specific alien chromatin or genes during the breeding process (Landjeva et al., 2007). Amplified fragment length polymorphism (AFLP) markers and random amplified polymorphic DNA (RAPD) markers have been used successfully in recent years to develop reliable sequence-tagged, sequence-characterized amplified region (SCAR) markers in crops. However, the development of sequence-specific primers for SCAR markers based on AFLP markers is not an efficient process in wheat (Shan et al., 1999). In contrast, RAPD has the advantages of being rapid, cost effective, and supportive of high levels of polymorphism, and it has been used for the identification of crops (Scheef et al., 2003).

Psathyrostachys huashanica Keng ($2n = 2x = 14$, NsNs) is a perennial cross-pollinating plant that is found only on Huashan Mountain in the Shaanxi Province of China (Kuo, 1987) and is a potentially useful germplasm source in China. It is characterized by high resistance to cold, salinity, drought, and barren conditions, and it also exhibits a dwarfed stature, early maturity, and resistance to scab, stripe rust, take-all, and powdery mildew (Zhao et al., 2010; Wang et al., 2011).

In order to transfer desirable traits from *P. huashanica* into wheat, our research team successfully developed a complete set of wheat-*P. huashanica* disomic addition lines (1Ns-7Ns) and undetermined homoeologous group addition lines, which were supported by cytogenetic, genomic *in situ* hybridization (GISH), and molecular marker expressed sequence tag-simple sequence repeat (EST-SSR) and EST-sequence tagged site (STS) analysis (Du et al., 2013a,b,c,d; 1Ns, 2Ns, and 3Ns unpublished). However, no specific molecular markers have been reported to identify the Ns genome from *P. huashanica*. Therefore, new and specific molecular markers are urgently needed to rapidly and accurately detect *P. huashanica* chromosome(s). In this study, we isolated a novel repetitive DNA fragment from the Ns genome of *P. huashanica* using RAPD analysis, which we converted into a fast and reliable SCAR marker specifically for the Ns genome of *P. huashanica*. The main objective of this study was to develop and characterize a SCAR marker that could be used to distinguish wheat lines carrying chromosome(s) derived from *P. huashanica*.

MATERIAL AND METHODS

Plant materials

The genomic materials used in this study are shown in Table 1, which also includes the controls used in the RAPD analysis. The wheat cultivar ‘7182’ and *P. huashanica* were used as parental materials for intergeneric hybridization, and 22 addition line plants were used to validate the SCAR marker RHS141, as shown in Table 2. These specimens were deposited at the Key Laboratory of Genetic Engineering for Plant Breeding of Shaanxi Province, College of Agronomy, Northwest A&F University, Shaanxi, China.

DNA extraction and RAPD assay

The total genomic DNA was extracted from fresh leaves using the modified cetyl

trimethyl ammonium bromide (CTAB) method (Cota-Sánchez et al., 2006). A RAPD assay was used to screen 200 random decamer primers. DNA amplification was performed following the procedure described by (Wu et al., 2010). The amplified products were fractionated on a 1.0% agarose gel using 1X Tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer. The gels were stained with ethidium bromide (EB) and visualized using an automatic gel imaging analysis system.

Table 1. Study species, ploidy level, genomic constitution, and origin.

	Species	Ploidy	Genome	Origin
Common wheat cultivars	7182 (*Triticum aestivum* L.)	6x	AABBDD	Our research group
Rare species	*Psathyrostachys huashanica* Keng	2x	NsNs	
	Triticum amyleum L.	2x	AA	
	Triticum dicoccoides	4x	AABB	
	Triticum araraticum Jakubz.	4x	AAGG	
	Triticum zhukovskyi Men. et Er.	6x	AAAAGG	
Wild relative species	*Aegilops markgrafii* (Greuter) Hammer	2x	CC	Chinese Academy of Agricultural Sciences
	Aegilops caudata L.	2x	CC	
	Aegilops tauschii (Coss.) Schmal.	2x	DD	
	Thinopyrum elongatum	2x	EE	
	Hordeum violaceum	2x	HH	
	Hordeum vulgare L.	2x	II	
	Crithopsis delileana (Schult) Roshev	2x	KK	
	Aegilops comosa Sm. in Sibth. & Sm.	2x	MM	
	Agropyron cristatum Gaertn.	6x	PPPPPP	
	Eremopyrum orientale	4x	B′B′C′C′	
	Triticum timopheevii Zhuk.	4x	AtAtGG	
	Secale cereale L.	2x	RR	
	Aegilops speltoides Tausch	2x	SS	
	Roegneria ciliaris (trin) Nevski	4x	SSYY	
	Elymus rectisetus	6x	SSYYWW	
	Pseudoroegneria strigosa A. Love	2x	StSt	
	Roegneria grandiglumis Keng	6x	StStPPYY	
	Aegilops umbellulata Zhuk.	2x	UU	
	Haynaldia villosa (L.) Schur.	2x	VV	
	Australopyrum coinitum retrofractum	2x	WW	
	Roegneria kamoji ohwi	2x	YY	

Table 2. Genetic constitution of the study species.

Plant code	2n	Chromosome composition	Homoeologous group	Plant code	2n	Chromosome composition	Homoeologous group
P. huashanica	14	14 Ns		18-1-1	44	42 W + 2 Ns	-
7182	42	42 W		21-4	43	42 W + 1 Ns	-
12-3	44	42 W + 2 Ns	1	23-2-1	44	42 W + 2 Ns	-
3-6-4-1	44	42 W + 2 Ns	2	24-5	44	42 W + 2 Ns	-
22-2	44	42 W + 2 Ns	3	25-4-4	44	42 W + 2 Ns	-
24-6-3	44	42 W + 2 Ns	4	27-4-4	43	42 W + 1 Ns	-
3-8-10-2	44	42 W + 2 Ns	5	28-1-2	43	42 W + 1 Ns	-
59-11	44	42 W + 2 Ns	6	30-2	44	42 W + 2 Ns	-
2-1-6-3	44	42 W + 2 Ns	7	31-6	44	42 W + 2 Ns	-
14-7	44	42 W + 2 Ns	-	33-1-10	44	42 W + 2 Ns	-
15-9	44	42 W + 2 Ns	-	34-8-6	43	42 W + 1 Ns	-
17-7	43	42 W + 1 Ns	-	38-2-4	43	42 W + 1 Ns	-

(-) = undetermined homoeologous groups. Ns and W were representives of *Psathyrostachys huashanica* and wheat chromosomes, respectively.

Cloning and sequence analysis

P. huashanica-diagnostic RAPD bands were excised from 1% agarose gels using a gel extraction kit. The purified products were cloned into the pMD19-T vector and transformed into *Escherichia coli* DH5a competent cells by heat shock transformation. Plasmids from randomly selected white colonies were extracted using a plasmid kit. DNA sequencing was performed at Sangon Biotech (Shanghai, China). The plasmid construction was confirmed by enzyme digestion and DNA sequencing. The sequences were submitted to the National Center for Biotechnology Information (NCBI) network service to screen for the nonexistence of similar sequences or low similarity sequences based on comparisons. The nucleotide sequences that were unique to the Ns genome were submitted to GenBank and registered under the accession number HR614226.

Southern blot hybridization

The probe was labeled using a digoxigenin (DIG) High Prime DNA Labeling & Detection Starter Kit (Roche, company, Germany) and hybridized overnight at 37°C. The membrane transfer and washing procedures have been described previously (Wu et al., 2010). Images were acquired using an automatic gel imaging analysis system.

SCAR primer design and specific amplification in various genomes

Based on the nucleotide sequences of the RAPD products, a pair of SCAR marker primers was designed and synthesized for the specific amplification of loci identified by previous RAPD markers in various genomes (Table 3). The primers were used to amplify the specific marker under the following conditions: 2 μL 10X polymerase chain reaction (PCR) buffer, 2 μL primer (2.5 mM), 2 μL DNA template (50-100 ng/μL), 1.6 μL dNTPs (2.5 mM), 1.6 μL $MgCl_2$ (2.5 M), 0.2 μL, *Taq* polymerase (5 U/μL), and 10.6 μL ddH_2O. The amplification procedure comprised an initial denaturation at 94°C for 4 min; 40 cycles at 94°C for 50 s, 60°C for 50 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. The amplified products were resolved by electrophoresis using 1% agarose gels, as described earlier.

Table 3. Characteristics of the SCAR marker developed for the Ns genome of *Psathyrostachys huashanica*.

Marker	Type	Accession No.	Primer sequence (5'-3')	Size (bp)	Annealing temperature (°C)
OPU10	RAPD		ACCTCGGCAC	938	34
RHS141	SCAR	HR614226	F: CTCGGCACCATAAACTAT R: CTCGGCACTAGAGGAAAC	938	60

Verifying the SCAR marker in wheat-*P. huashanica* addition lines

The SCAR marker RHS141 was used to test the validity of the molecular marker in the wheat cultivar ‘7182’, *P. huashanica*, a complete set of wheat-*P. huashanica* disomic addition lines (1Ns-7Ns), and undetermined homoeologous group addition lines. The conditions for the SCAR reactions were the same as those used for the specific amplifications in various genomes.

RESULTS

Species diagnosis and RAPD markers

After screening 200 RAPD primers, we selected 32 primers that produced distinct *P. huashanica* genome-specific bands. The RAPD primer OPU10 diagnostic band was clearly observed in *P. huashanica*, and it was missing from the genomes of other species (Figure 1), which confirmed that primer OPU10 was a good candidate for the specific detection of the *P. huashanica* genome. The other RAPD primer diagnostic bands are not shown.

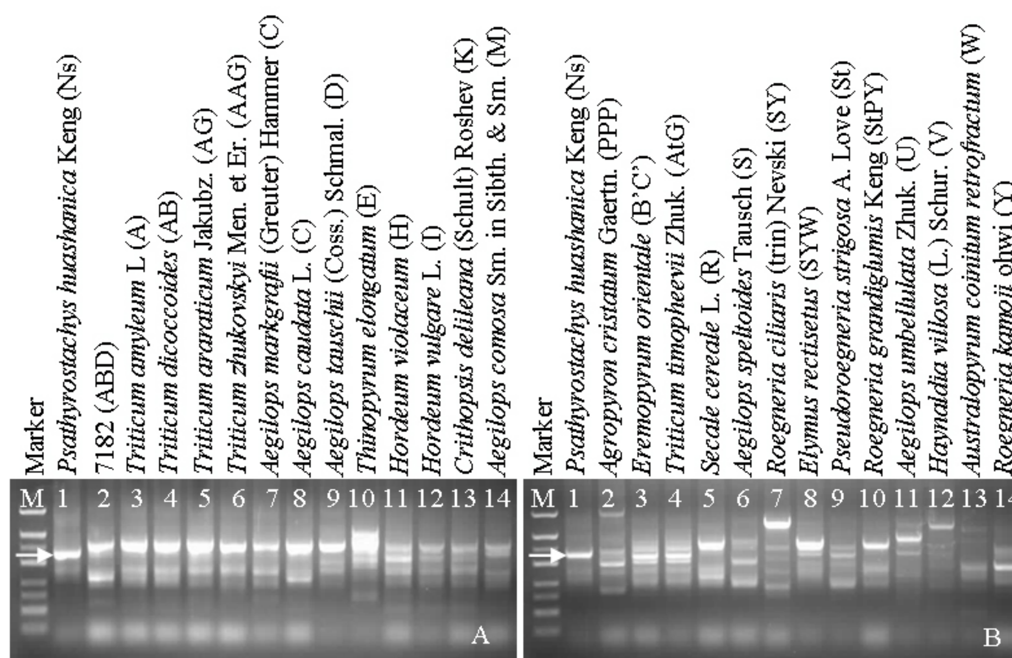

Figure 1. *Psathyrostachys huashanica* genome-specific RAPD fragments isolated by amplification using the OPU10 decamer as a primer in: **A.** *Lane M* = marker; *lane 1* = Ns; *lane 2* = ABD; *lane 3* = A; *lane 4* = AB; *lane 5* = AG; *lane 6* = AAG; *lane 7* = C; *lane 8* = C; *lane 9* = D; *lane 10* = E; *lane 11* = H; *lane 12* = I; *lane 13* = K; *lane 14* = M. **B.** *Lane M* = marker; *lane 1* = Ns; *lane 2* = PPP; *lane 3* = B'C'; *lane 4* = AtG; *lane 5* = R; *lane 6* = S; *lane 7* = SY; *lane 8* = SYW; *lane 9* = St; *lane 10* = StPY; *lane 11* = U; *lane 12* = V; *lane 13* = W, and *lane 14* = Y genomes. The arrows indicate the diagnostic amplification product of *P. huashanica*.

Sequence analysis

The *P. huashanica* genome-specific RAPD PCR product was cloned, sequenced, and designated pHs27, the full length of which was 938 bp (Figure 2). It had no special characteristics such as long terminal repeats, inverted repeats, or palindromes. Further sequence homology searches were conducted using the nucleotide basic local alignment search tool

(BLASTn) and translated BLAST (BLASTx) programs. The results showed that the sequence of pHs27 shared no similarity with sequences in the NCBI database. We hypothesized that pHs27 was a new *P. huashanica* repetitive DNA sequence.

```
1     ACCTCGGCAC  CATAAACTAT  GAAGAACGGT  GTATAGCCCG  TGGATCGGTT  AGGTGTGGTG
61    CGCAAGCCCC  ACAGCACGAA  GTCGAGCTCC  TCAACCCATT  TATTGTCAGC  CTCCTCGACC
121   CATTTATTGT  CAGCCTCCTC  TAAAGATCGA  ATCAGGCGAG  GTTTTATGCC  GCTTCAAATT
181   AACCCATTGG  CCCATTTAAC  CTGCCCGTTT  GTTTGCGGGT  GGTAGACAGA  GGCGTAGTCA
241   AGCTTAATGT  TTTGCTTGGC  GCACCACGTT  TTTACTTCCT  CGGTCATGAA  ATTGGATCCA
301   TTATCCATGA  TGATGCTGTG  GGGTGCCCCA  AAACGATGCA  CCACACCAGA  CATAAATTTG
361   ATCATTGGTT  TGGCTTTCGC  TGATGCCACA  GGTTTTGCCT  CTATCCATTT  TGTGAACTTG
421   TCGACCATGA  CCAGAAGATA  TTTCTTCTTG  CCCGTCCCTC  TACGGAGTGG  TCCGACCATG
481   TACAGCCCCC  AGACCGTGAA  CGACCAGGTT  ATTGGGATGG  TTTTCAGAGC  TGTGGCCGGC
541   ATGTTACTTT  GTCTGGCAAA  TAGCTGGCAA  CCCACACATG  CGTCCACCAA  GTTGCGTGCA
601   TCGGCTCGCG  CTGTAGGCCA  ATAAAACCCT  GTTCAGAACA  CTTTGCTTAC  CAAAGTCCGA
661   GGGGGTGCGT  GGTGCCCGCA  CATGCCGGAG  TGGATTTCTG  AGAGGAGTTC  TTTTCCTTCT
721   TCCTCGGAAA  TGCAGCGTTG  GAGCACACCT  GTAACACTAC  GCTTGTATAG  TTCCCCTTCA
781   TGGATTTTAT  ATGCTTTTGA  TCGTCGAACA  ATTCGCCGCG  CATCTGTCTG  GTCCTTGGGT
841   AATTCCTTTC  GTATTAAATA  TGCAAAGATT  GGCTCGGTCC  ATGGCGCGAT  TATTGCCATG
901   ATTTCATGGG  CCGAGGGGGT  TTCCTCTAGT  GCCGAGGT
```

Figure 2. Nucleotide sequence of pHs27, which has an accession No. of HR614226. The primer regions are marked while the underlined and boxed regions indicate the RAPD primer sequence and SCAR primer sequence, respectively.

Southern blot hybridization

Southern blot hybridization detected a strong, smeared signal of the probe pHs27 (Figure 3). A prominent band corresponded to the Ns genome of the *P. huashanica* species, which also suggested that the sequence was dispersed in the Ns genome of *P. huashanica*. However, the signal was not present in the other genomes, which was consistent with the PCR amplification results. These data confirmed that the pHs27 clone is a novel, Ns genome-specific DNA sequence in *P. huashanica*.

Verification of the SCAR marker RHS141 in various genomes

The polymorphic SCAR marker RHS141 was amplified in various genomes. However, it was only present in the Ns genome of *P. huashanica*, and it was absent from all of the other genomes (Figure 4). This specificity of the SCAR marker will provide valuable information for tracing *P. huashanica* chromatin in a wheat background.

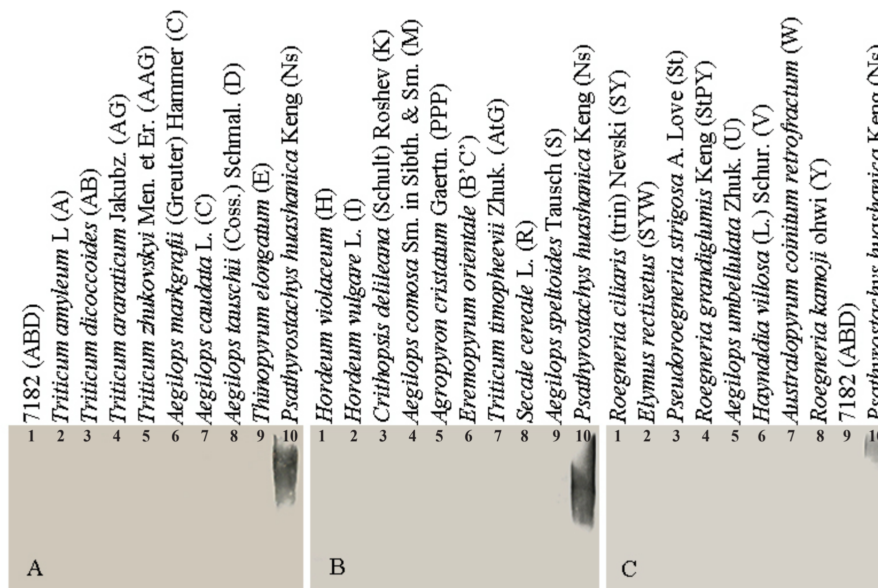

Figure 3. Southern hybridization using pHs27 as a probe with a range of total DNA samples. **A.** *Lane 1* = ABD; *lane 2* = A; *lane 3* = AB; *lane 4* = AG; *lane 5* = AAG; *lane 6* = C; *lane 7* = C; *lane 8* = D; *lane 9* = E; *lane 10* = Ns. **B.** *Lane 1* = H; *lane 2* = I; *lane 3* = K; *lane 4* = M; *lane 5* = PPP; *lane 6* = B'C'; *lane 7* = AtG; *lane 8* = R; *lane 9* = S; *lane 10* = Ns. **C.** *Lane 1* = SY; *lane 2* = SYW; *lane 3* = St; *lane 4* = StPY; *lane 5* = U; *lane 6* = V; *lane 7* = W; *lane 8* = Y; *lane 9* = ABD; *lane 10* = Ns. All DNAs were digested with *Hin*dIII.

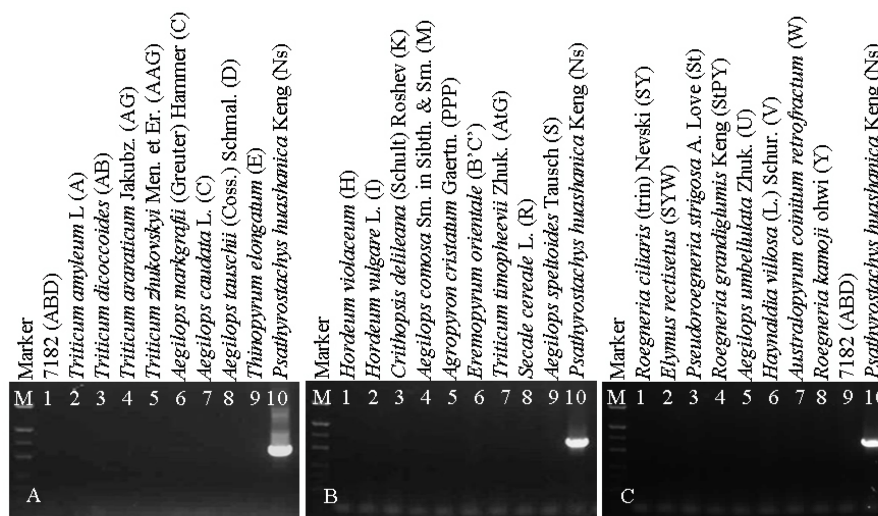

Figure 4. Validation of the SCAR marker RHS141 in the genomes of various species. **A.** *Lane M* = marker; *lane 1* = ABD; *lane 2* = A; *lane 3* = AB; *lane 4* = AG; *lane 5* = AAG; *lane 6* = C; *lane 7* = C; *lane 8* = D; *lane 9* = E; *lane 10* = Ns. **B.** *Lane M* = marker; *lane 1* = H; *lane 2* = I; *lane 3* = K; *lane 4* = M; *lane 5* = PPP; *lane 6* = B'C'; *lane 7* = AtG; *lane 8* = R; *lane 9* = S; *lane 10* = Ns. **C.** *Lane M* = marker; *Lane 1* = SY; *lane 2* = SYW; *lane 3* = St; *lane 4* = StPY; *lane 5* = U; *lane 6* = V; *lane 7* = W; *lane 8* = Y; *lane 9* = ABD; *lane 10* = Ns.

Validation of the SCAR marker RHS141 in wheat-*P. huashanica* addition lines

The specific Ns genome SCAR marker RHS141 produced a very intense band at 938 bp, which was absent in the female parent of 7182. Moreover, it was present in wheat-*P. huashanica* disomic addition lines carrying *P. huashanica* chromosomes 1Ns, 2Ns, 3Ns, 4Ns, 5Ns, 6Ns, and 7Ns, and it was also present in *P. huashanica* addition lines carrying undetermined *P. huashanica* chromosomes (Figure 5A and B). This was a fast and robust assay for tracking the presence of *P. huashanica* chromatin in wheat.

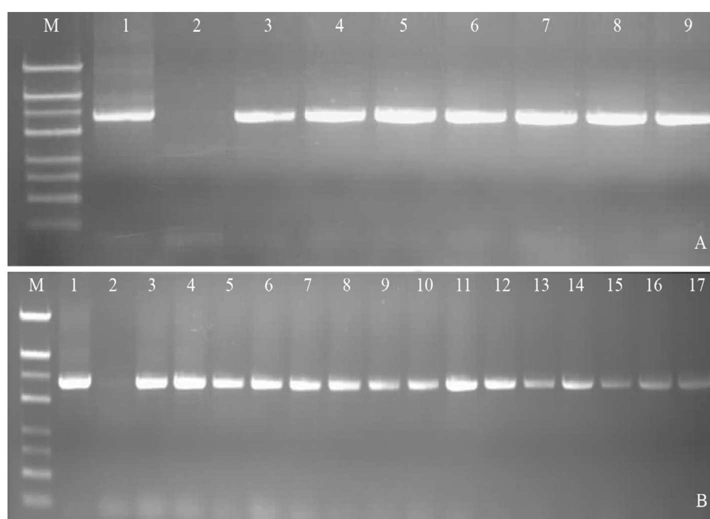

Figure 5. Identification of *Psathyrostachys huashanica* chromatin using the SCAR marker. RHS141 amplified specific bands in seven wheat-*P. huashanica* disomic addition lines (1Ns-7Ns) and undetermined homoeologous group addition lines. **A.** *Lane M* = marker; *lane 1* = *P. huashanica*; *lane 2* = 7182; *lanes 3-9* = seven wheat-*P. huashanica* disomic addition lines (1Ns-7Ns). **B.** *Lane M* = marker; *lane 1* = *P. huashanica*; *lane 2* = 7182; *lanes 3-17* = undetermined homoeologous group addition lines.

DISCUSSION

The identification of exogenous chromosome(s) or chromosome segments is very important after alien species are introduced successfully into wheat. The question of how accurately and effectively alien differentiate chromosomes and fragment is of great theoretical and practical significance because it could provide a means to transfer exotic genes into cultivated wheat, develop translocation lines, and function as a bridging material for breeding (Hernández et al., 1999). RAPD is a simple and inexpensive technique that may facilitate the analysis of a large proportion of the genome and the identification of bands suitable for conversion to SCAR, which makes it suitable for marker-assisted selection (Hernández et al., 2001).

P. huashanica has attracted considerable attention among wheat breeders because of its excellent agronomic traits. Thus, many researchers have successfully transferred useful traits from *P. huashanica* into hexaploid wheat via intergeneric hybridization (Chen et al., 1991; Kang et al., 2008). Therefore, the development of a fast and reliable technique for iden-

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tifying *P. huashanica* chromatin in a wheat background is very important. We used the specific SCAR marker RHS141 of *P. huashanica* as a practical tool for the detection of the alien chromatin of *P. huashanica* in a wheat background (Figure 5A and B). Our results suggest that it is possible to use the marker as a specific molecular marker for the chromosome(s) of *P. huashanica*. In addition, the unique Ns genome SCAR marker of *P. huashanica* could be used during marker-assisted selection to study genome evolution in wheat-*P. huashanica* offspring.

Identifying and cloning genome-specific DNA segments is valuable for determining the provenance, evolution, and source of alien hereditary substances during distant hybridization breeding (Wei and Wang, 1995; Zhang et al., 1998). RAPD markers usually display high polymorphism in alien species; therefore, they are useful tools for genetic diversity studies (Guadagnuolo et al., 2001). However, there have been no previous reports of a specific SCAR marker for the Ns genome based on a RAPD primer that could be used to detect the presence of *P. huashanica* chromatin in a common wheat background. In this study, we developed the *P. huashanica* genome-specific SCAR marker RHS141 and used it to screen test materials. We successfully identified those that contained *P. huashanica* chromatin. Our experimental data suggest that this pair of primers can be used to detect the presence of *P. huashanica* chromatin in a wheat background. In summary, the Ns genome-specific SCAR marker RHS141 will be particularly useful during the early stages of breeding when selecting hybrids between wheat and *P. huashanica*. This will provide an efficient alternative to laborious and time-consuming genetic testing, which are currently a prerequisite for breeding.

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