Characterization of alien chromosomes in backcross derivatives of *Triticum aestivum* × *Elymus rectisetus* hybrids by using molecular markers and sequential multicolor FISH/GISH

Quan-Wen Dou, Yunting Lei, Xiaomei Li, Ivan W. Mott, and Richard R.-C. Wang

Abstract: Wild Triticeae grasses serve as important gene pools for forage and cereal crops. Based on DNA sequences of genome-specific RAPD markers, sequence-tagged site (STS) markers specific for **W** and **Y** genomes have been obtained. Coupling with the use of genomic in situ hybridization, these STS markers enabled the identification of the **W**- and **Y**-genome chromosomes in backcross derivatives from hybrids of bread wheat *Triticum aestivum* L. ($2n = 42$; **AABBDD**) and *Elymus rectisetus* (Nees in Lehm.) Á. Löve & Connor ($2n = 42$; **StStWWYY**). The detection of six different alien chromosomes in five of these derivatives was ascertained by quantitative PCR of STS markers, simple sequence repeat markers, rDNA genes, and (or) multicolor florescence in situ hybridization. Disomic addition line 4687 ($2n = 44$) has the full complement of 42 wheat chromosomes and a pair of 1**Y** chromosomes that carry genes for resistance to tan spot (caused by *Pyrenophora tritici-repentis* (Died.) Drechs.) and *Stagonospora nodorum* blotch (caused by *Stagonospora nodorum* (Berk.) Castellani and Germano). The disomic addition line 4162 has a pair of 1**St** chromosomes and 21 pairs of wheat chromosomes. Lines 4319 and 5899 are two triple substitution lines ($2n = 42$) having the same chromosome composition, with 2**A**, 4**B,** and 6**D** of wheat substituted by one pair of **W**- and two pairs of **St**-genome chromosomes. Line 4434 is a substitution–addition line ($2n = 44$) that has the same **W**- and **St**-genome chromosomes substituting 2**A**, 4**B,** and 6**D** of wheat as in lines 4319 and 5899 but differs by having an additional pair of **Y**-genome chromosome, which is not the 1**Y** as in line 4687. The production and identification of these alien cytogenetic stocks may help locate and isolate genes for useful agronomic traits.

Key words: addition line, substitution line, genome, apomixis, disease resistance.

Résumé : Les graminées sauvages constituent un important réservoir de gènes pour les cultures fourragères et céréalières. En partant de marqueurs RAPD spécifiques d'un génome, des marqueurs STS (« sequence-tagged site ») spécifiques des génomes **W** et **Y** ont été obtenus. En utilisant ceux-ci conjointement avec de l'hybridation génomique in situ, ces marqueurs STS ont permis d'identifier les chromosomes provenant des génomes **W** et **Y** au sein des descendances de rétrocroisements réalisés entre le blé tendre *Triticum aestivum* L. ($2n = 42$; **AABBDD**) et l'*Elymus rectisetus* (Nees in Lehm.) Á. Löve & Conner ($2n = 42$; **StStWWYY**). La présence de six chromosomes étrangers au sein de cinq de ces descendants a été vérifiée par qPCR des marqueurs STS, de marqueurs microsatellites et de gènes d'ADNr ou par hybridation in situ en fluorescence multicolore. La lignée d'addition disomique 4687 ($2n = 44$) possède le complément chromosomique complet de 42 chromosomes du blé ainsi qu'une paire de chromosomes 1**Y**, lequel porte des gènes conférant une résistance à la tache bronzée [causée par le *Pyrenophora tritici-repens* (Died.) Drechs.] et à la tache des glumes [causée par le *Stagonospora nodorum* (Berk.) Castellani et Germano]. La lignée d'addition disomique 4162 porte une paire de chromosomes 1**St** ainsi que les 21 paires de chromosomes du blé. Les lignes 4319 et 5899 sont deux lignées de substitution triple ($2n = 42$) avec la même composition chromosomique, où les chromosomes 2**A**, 4**B** et 6**D** du blé ont été remplacés par une paire de chromosomes **W** et deux paires de chromosomes **St**. La lignée 4434 est une lignée d'addition–substitution ($2n = 44$) chez laquelle les mêmes chromosomes des génomes **St** et **W** ont remplacé 2**A**, 4**B** et 6**D** du blé, comme chez les lignées 4319 et 5899, mais au sein de laquelle se trouve une paire additionnelle de chromosomes du génome **Y**, laquelle n'est cependant pas le 1**Y** comme chez la lignée 4687. La production et l'identification de ces ressources cytogénétiques pourraient faciliter la localisation et le clonage des gènes qui codent pour des caractères d'intérêt agronomique.

Mots-clés : lignée d'addition, lignée de substitution, génome, apomixie, résistance aux maladies.

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Introduction

Perennial Triticeae grasses serve as important gene pools for forage and cereal crops (Dewey 1984). Knowledge of their genome compositions is pivotal for efficient utilization of this vast gene pool in germplasm enhancement programs. Despite the large amount of genome research on Triticeae species, many of the approximate 350 species have not had their genome compositions confirmed (http://herbarium.usu.edu/Triticeae/genomes.htm).

A number of genome-specific random amplified polymorphic DNA (RAPD) markers were identified and sequenced in perennial Triticeae species (Wei and Wang 1995; Zhang et al. 1998). Many species- or genome-specific repetitive sequences have also been reported (Rayburn and Gill 1986; Zhang and Dvorak 1990; Tsujimoto and Gill 1991; Anamthawat-Jónsson and Heslop-Harrison 1993; Li et al. 1995). Genome-specific molecular markers are useful to identify the genome constitution of the species in question (Svitashev et al. 1998).

Sequence-tagged site (STS) markers (Tragoonrung et al. 1992) are PCR-based markers generated by a pair of primers (each ~20 bases long) that are designed according to known DNA sequences. Ideally, only one DNA fragment of a specific length (STS marker) will be amplified from the template DNA containing the target sequence. These STS markers are more reproducible and specific than the original RAPD markers. The restriction fragment length polymorphism (RFLP) technique requires more genomic DNA and more time to run an assay compared with the RAPD or STS techniques. Therefore, PCR-based markers such as STS and cleaved-amplified polymorphic sequence (CAPS; Konieczny and Ausubel 1993) markers are preferred by most researchers. STS and (or) CAPS markers have been developed for the identification of species (Li et al. 2002), chromosomes (Talbert et al. 1994; Blake et al. 1996; Erpelding et al. 1996), and genomes (Li et al. 2007; Okito et al. 2009). Simple sequence repeats (SSR), especially those derived from expressed sequence tags (EST), are also useful molecular markers for identifying and mapping chromosomes (Somers et al. 2004; Bushman et al. 2008; Sim et al. 2009; Wu et al. 2009).

Genomic in situ hybridization (GISH) and florescence in situ hybridization (FISH) techniques have been used in genome and chromosome analyses (Anamthawat-Jónsson et al. 1990; Anamthawat-Jónsson 2001; Marín et al. 2008; Dou et al. 2009; Wang et al. 2010). When used properly, these methods can identify the genome constitution of an allopolyploid species, the introgressed alien chromosome(s) in interspecific hybrids and alien addition or substitution lines, or the structural organization of introgressed chromosome(s) in a translocation line.

Elymus rectisetus (Nees in Lehm.) Á. Löve & Connor is an allohexaploid species having **St**, **W**, and **Y** genomes (Torabinejad and Mueller 1993). Because this species is apomictic, it was crossed with bread wheat *Triticum aestivum* L. in an attempt to transfer apomixis into wheat (Wang et al. 1993; Liu et al. 1994). However, apomixis was not expressed in the 'Fukuho-komugi' wheat × *E. rectisetus* hybrid and backcross progenies (Peel et al. 1997). Nevertheless, some derivatives had been characterized using molecular and cytogenetic methods (Xue and Wang 1999). Some of these derivatives had been found to be resistant to wheat diseases, such as *Fusarium* head blight (FHB, or scab), tan spot, and *Stagonospora nodorum* blotch (SNB) (McArthur et al. 2005; Oliver et al. 2008). RFLP analysis revealed that lines A1026, A1057, and A1048 had alien chromosomes belonging to homoeologous group 1 (McArthur et al. 2005). Group-1 chromosomes of the **St** and **Y** genomes in Triticeae have the genes encoding 5S rRNA (Scoles et al. 1988). Mukai et al. (1990, 1991) labeled five chromosome pairs with 45S rDNA sites (1AS, 1BS, 6BS, 5DS, and 7DL) and six pairs with 5S rDNA sites (1AS, 1BS, 1DS, 5AS, 5BS, and 5DS) in common wheat chromosomes.

In this study, one RAPD marker each specific to the **St** and **W** genomes was converted into STS markers. These STS markers and the one for the **Y** genome (Okito et al. 2009) were used to detect alien chromosomes in derivatives of 'Fukuho-komugi' wheat × *E. rectisetus* hybrids. The added alien chromosomes and (or) substituted wheat chromosomes were further characterized by SSR markers and sequential multicolor FISH/GISH. Five different types of wheat–wheatgrass cytogenetic stocks involving six different alien chromosomes are reported here.

Materials and methods

Plant materials (Table 1) were raised from seeds and grown in a greenhouse at the United States Department of Agriculture, Agricultural Research Service, Forage and Range Research Laboratory (FRRL), Logan, Utah. All diploid and several polyploid species having known genomes were used in developing and screening converted STS markers. Backcross derivatives of *T. aestivum* × *E. rectisetus* hybrids were developed at FRRL.

RAPD marker sequences published in Wei and Wang (1995), Li et al. (1995), Zhang et al. (1998), and others cloned in R.R.-C. Wang's laboratory (unpublished) were used for the development of STS markers. Based on known sequences of RAPD markers specific to **St**, **W**, and **Y** genomes, three primer pairs were designed using the program Primer3 (Rozen and Skaletsky 1997) and tested (Table 2). The selected new primer sites may or may not partially overlap with the original RAPD primer sites. Procedures for DNA extraction, PCR amplification, and visualization of amplification products follow those described by Li et al. (2002) with some modifications. The amount of template DNA in the 25 μL PCR reaction mix was 40 ng for diploid, 80 ng for tetraploid, and 120 ng for hexaploid. PCR conditions, mainly the annealing temperature and number of amplification cycles, were tested and optimized for each assay.

Quantitative PCR (qPCR) assays followed the procedure previously described (Okito et al. 2009), except that the **W**-genome specific STS marker, $OPB03_{306}$, was also quantified.

Sequential multicolor FISH and GISH techniques described in Dou et al. (2009) were followed, with minor adjustments. Sequential FISH/GISH was conducted first by detection of pAs1 (labeled with tetramethy1-rhodamine-5-dUTP for red color) and $(AAG)_{10}$ microsatellite sequence (labeled with fluorescein-12-dUTP for green color); second using probes of **W**-genomic DNA (green) and **St**-genomic DNA (red); and last using probe of **StWY**-genomic DNA (green). No blocking DNAs were used in all GISH probing steps.

Table 1. Plant materials used in this study.

Genome symbol[a]	Species	ID No.	Source	Notes
E^b = J	*Thinopyrum bessarabicum* (Savul. & Rayss) Á. Löve	PI 531710	FRRL	
E^e = E	*Thinopyrum elongatum* (Host) D. Dewey	PI 531718	FRRL	
St^{sp}	*Pseudoroegneria spicata* (Pursh) Á. Löve	PI 236668	FRRL	
St^l	*Pseudoroegneria libanotica* (Hackel) Á. Löve	PI 338391	FRRL	
R	*Secale montanum* Guss	PI 531829	FRRL	
		PI 531835	FRRL	
H	*Hordeum bogdanii* Wilensky	PI 499501	FRRL	Perennial
I	*Hordeum vulgare* L. 'Walker'	PI 557000	USU	Annual
P^c	*Agropyron cristatum* (L.) J. Gaertner	PJ-3817	FRRL	
Ns^j	*Psathyrostachys juncea* (Fisch.) Nevski	PI 314521	FRRL	
Ns^h	*Psathyrostachys huashanica* Keng	PI 531823	FRRL	
Ns^f	*Psathyrostachys fragilis* (Boiss.) Nevski	PI 343190	FRRL	
W	*Australopyrum pectinatum* subsp. *retrofractum* (J.W. Vickery) Á. Löve	PI 531553	FRRL	
V	*Dasypyrum villosum* (L.) Candargy	D-2990	FRRL	Annual
StY	*Elymus longearistatus* (Boiss.) Tzvelev	PI 401282	FRRL	
StWY	*Elymus rectisetus* (Nees in Lehm.) Á. Löve & Connor	PI 533028	FRRL	
ABD	*Triticum aestivum* L. 'Chinese Spring'	CItr 14108	Missouri	Annual
	Triticum aestivum L. 'Fukuho-komugi'	6207	FRRL	Annual
	Triticum aestivum lines with *Elymus rectisetus* chromosomes	0290, 0293, 0294, 0295, 4162, 4431, 4434, 4687	FRRL	$2n = 44$
	Triticum aestivum lines with *Elymus rectisetus* chromosomes	0291, 0297, 4319, 4348, 5899	FRRL	$2n = 42$

Note: FRRL, Forage and Range Laboratory, Logan, Utah; USU, Utah State University, Logan, Utah.
[a]The genome symbols are those designated by Wang et al. (1994).

Table 2. Primers, GenBank identification numbers of target RAPD sequences, target genomes, expected sequence-tagged sites (STS) marker length in base pairs (bp), polymerase chain reaction (PCR) conditions, and results of STS assays with genomes in Triticeae.

Primer name	5′–3′	GenBank ID	Genome	Expected length	*T*	Cycles	Results
D15F	GTGCTGGTGCGGTCATAGA	BV679217	**St**	498	60	30	**St, StY, StWY**
D15R	ATCCGTGCTTAGAAAGGTAGCA						
B03F2	CCCCTGCCCGATAGATTTTA	BV679211	**W**	306	60	30	**W, StWY**
B03R2	CATCCCCCTGGATAAATAAGTG						
B14F1	TCCGCTCTGGGATGTGAC	BV679236	**Y**	269	55	30	**StY, StWY**
B14R1	TCCTGAAGGTAAAACTTTCTGTTTTT						

For confirmation of the presence of homoeologous group-1 alien chromosomes in lines 4162, 4687, and 4434 (derivatives of lines A1026, A1057, and A1048, respectively), mitotic chromosomes were analyzed by sequential FISH/GISH —first probed with 5S rDNA (PCR production followed Fukui et al. 1994; labeled in red) and 45S rDNA (pTa71, Gerlach and Bedbrook 1979; in green); then followed by probing with genomic DNA of *E. rectisetus* (in green). For sequential FISH/GISH analyses, *E. rectisetus* was probed by 5S rDNA and 45S rDNA, followed by pAs1 and $(AAG)_{10}$, and then lastly by genomic DNA of *E. rectisetus*.

Characteristic patterns of pAs1 and $(AAG)_{10}$ microsatellite sites on wheat chromosomes suggested the missing chromosomes being 2**A**, 4**B**, and 6**D** in 4319, 5899, and 4434. Wheat SSR markers for these three wheat chromosomes were tested to confirm the identities of substituted chromosomes. For Xcwem and Xbarc SSR markers, PCR products were visualized by polyacrylamide gel electrophoresis (PAGE) with separate lanes of size markers. To test the WMC47 SSR marker for 4**B** chromosome, PCR products were separated by capillary electrophoresis using GS500 LIZ internal size standard and ABI 3730 genetic analyzer (PE Applied Biosystems Inc., Foster City, Calif., USA). The PCR amplicons were compared using Genographer version 1.6.0 (Benham et al. 1999).

Results

One STS marker was successfully developed for each of the **St**, **W**, and **Y** genomes of *E. rectisetus* (Table 2). The **W**-genome specific STS marker B03-$\mathbf{W}_{306}$ (Fig. 1, top) was detected only in *Australopyrum retrofractum* (**W** genome) and *E. rectisetus* (**StWY**), while **Y**-specific B14-$\mathbf{Y}_{269}$ was present in *Elymus longearistatus* (**StY**) and *E. rectisetus* (Table 2) as reported by Okito et al. (2009). D15-$\mathbf{St}_{498}$ could be detected in *Pseudoroegneria* species (**St**), *E. longearistatus* (**StY**), and *E. rectisetus* (**StWY**).

Fig. 1. Top: Sequence-tagged site (STS) marker B03-$\mathbf{W}_{306}$ for the **W** genome in the tribe Triticeae, amplified at 60 °C for 30 cycles with primers 5′-CCCCTGCCCGATAGATTTTA-3′ and 5′-CATCCCCCTGGATAAATAAGTG-3′. Bottom: Sequence-tagged site (STS) markers B03-$\mathbf{W}_{306}$ of the **W** genome (A) and B14-$\mathbf{Y}_{269}$ of the **Y** genome (B) were used to detect the presence of alien-genome chromosomes in the backcross derivatives of *Triticum aestivum* × *Elymus rectisetus* hybrids: lines 4687 ($2n$ = 44; 4 plants), 0295 ($2n$ = 44; 5 plants), 0290 ($2n$ = 44; 5 plants), and 0291 ($2n$ = 42; 4 plants). Lines 0290 and 0291 probably had different **W**-genome chromosomes, whereas 4687 had **Y**-genome chromosomes. Bands in lane M are size markers (top to bottom) 1500, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200, and 100 bp.

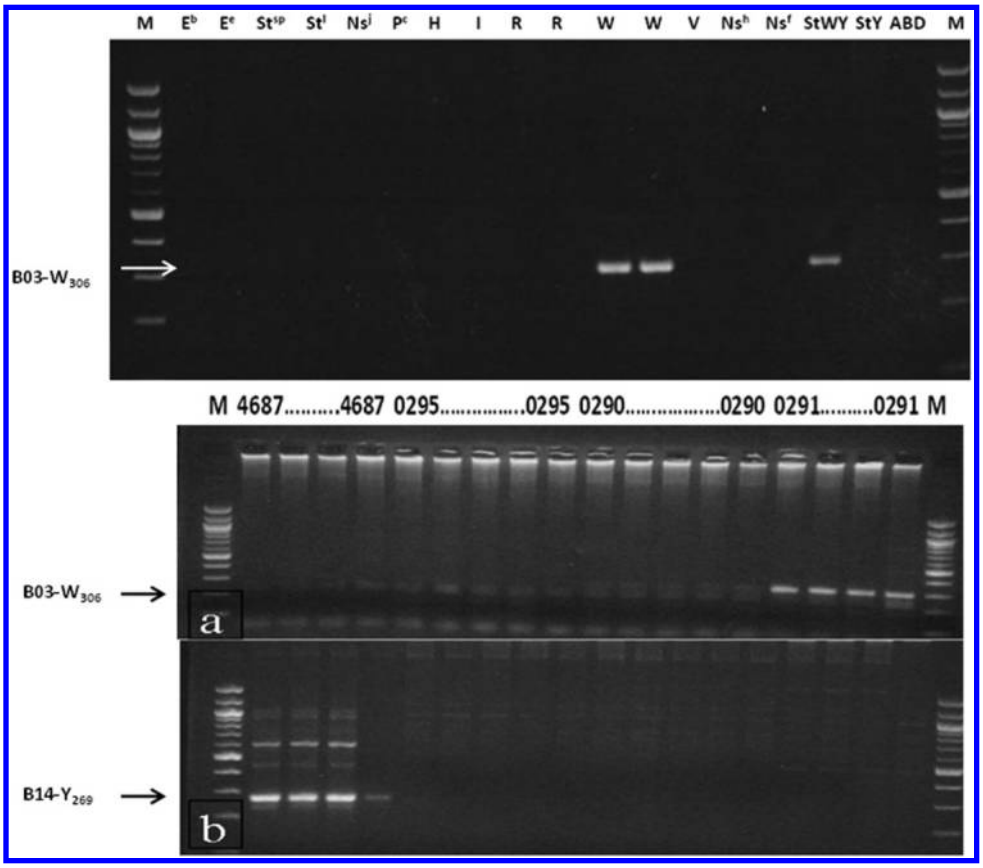


Table 3. Results of sequence-tagged site assays on *Triticum aestivum* × *Elymus rectisetus* backcross derivatives to detect **St**-, **W**-, or **Y**-genome chromosomes.

ID No.[a]	Origin	$2n$	B03-$\mathbf{W}_{306}$	B14-$\mathbf{Y}_{269}$	D15-$\mathbf{St}_{498}$
0290	A1040-1-3-3	44	+	–	–
0291	A1024-4-1-1	42	++	–	–
0293	A1057-1-2-1	44	–	+	–
0294	A1057-3-3-2	44	–	+	–
0295	A1026-1-4-1	44	–	–	–
0297	A1036-2-2-1	42	++	–	–
4162	A1026-1-5	44	–	–	–
4319	A1048-1-3	42	++	–	–
4348	A1048-4-6	42	++	–	–
4431	A1048-19	44	++	+	–
4687	A1057-7-4	44	–	+	–

[a]ID numbers in bold have been characterized by STS–qPCR, SSR–PCR, or sequential multicolor FISH/GISH.

Triticum aestivum × *E. rectisetus* backcross derivatives were tested for the presence of **St**-, **W**-, or **Y**-genome chromosomes using the STS markers. The **St**-genome marker D15-$\mathbf{St}_{498}$ was not detected in any of the hybrid derivatives tested (Table 3), even though some lines were shown to possess **St**-genome chromosomes by GISH (see later figures). Lines 0291, 0297, 4319, 4348, and 4431 tested positive for the **W**-genome marker B03-$\mathbf{W}_{306}$. Line 0290 possessed the B03-$\mathbf{W}_{306}$ marker in a much lower intensity than that in 0291 (Fig. 1, bottom). The presence of the **Y**-genome marker B14-$\mathbf{Y}_{269}$ was detected in lines 0293, 0294, 4431, and 4687.

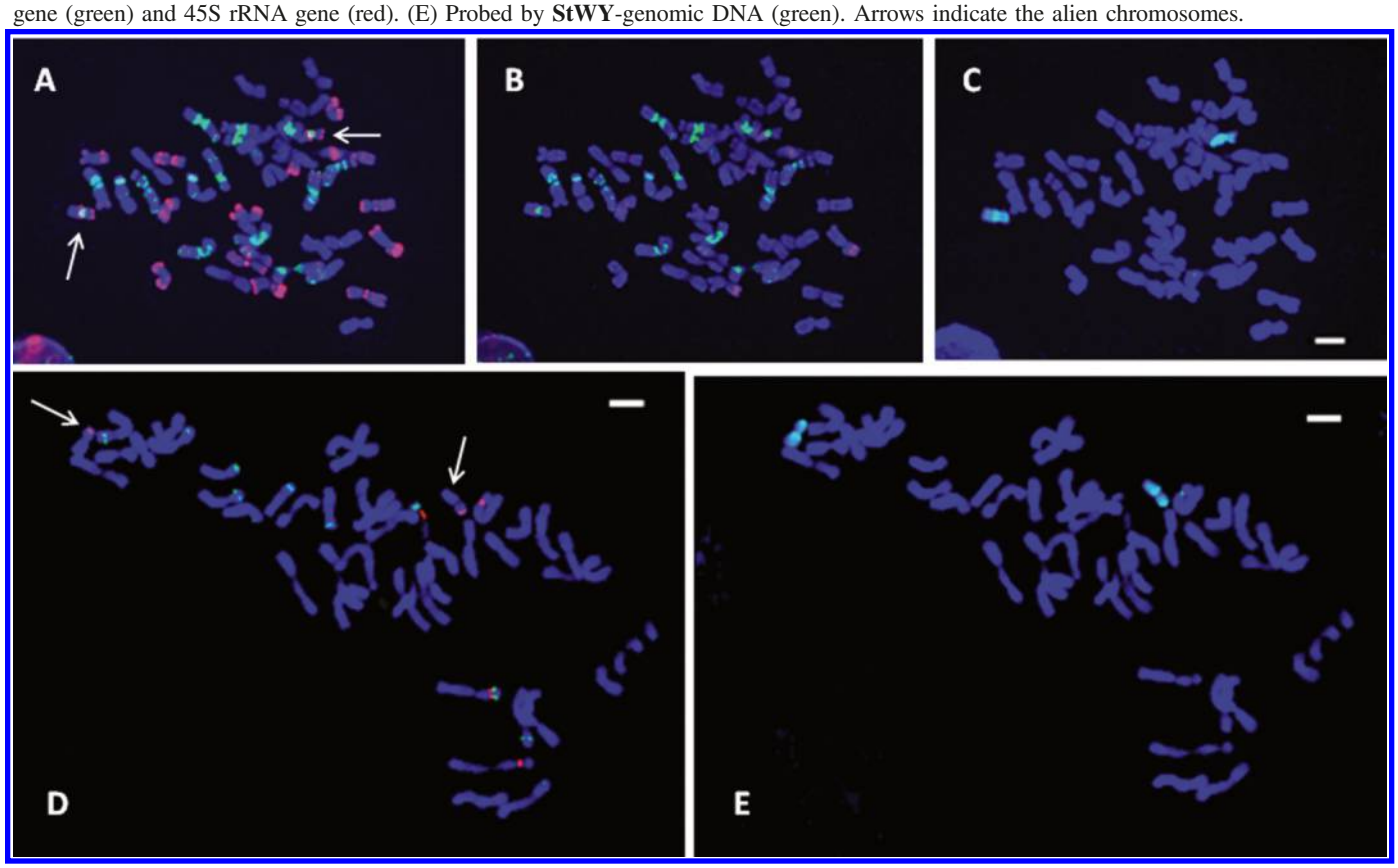
The presence of **W**- and (or) **Y**-genome chromosomes in

Table 4. Quantitative PCR for **W**- and **Y**-genome STS markers, B03-$\mathbf{W}_{306}$ and B14-$\mathbf{Y}_{269}$, from genomic DNA of wheat, *Elymus rectisetus*, *Elymus longearistatus*, *Australopyrum retrofractum*, and wheat × *E. rectisetus* derivatives.

					Relative copy number	
ID No.	Derived from	Species	2*n*	Genome and chromosome	B03-$\mathbf{W}_{306}$	B14-$\mathbf{Y}_{269}$
PI 531553		*Australopyrum retrofractum*	14	7″ **W**	6.70	0
PI 401282		*Elymus longearistatus*	28	14″ **StY**	0.00	58.23
PI 533028		*Elymus rectisetus*	42	21″ **StWY**	1.00	16.77
6207		Wheat 'Fukuho-komugi'	42	21″ **ABD**	0.01	0
4162	A1026-1-5	Wheat × *Elymus rectisetus*	44	21″ **ABD** + 1″ 1**St**	0.01	0
4319	A1048-1-3	Wheat × *Elymus rectisetus*	42	18″ **ABD** + 1″ **W** + 2″ **St**	1.89	0
4434	A1048-19	Wheat × *Elymus rectisetus*	44	18″ **ABD** + 1″ **W** + 2″ **St** + 1″ **Y**	8.95	1.51
4687	A1057-7-4	Wheat × *Elymus rectisetus*	44	21″ **ABD** + 1″ 1**Y**	0.01	1
5899	A1036-2	Wheat × *Elymus rectisetus*	42	18″ **ABD** + 1″ **W** + 2″ **St**	3.28	0

Note: The five wheat × *Elymus rectisetus* derivatives have been also characterized by sequential multicolor FISH/GISH to identify the number and origin of alien chromosomes.

Fig. 2. Mitotic chromosomes in line 4687 analyzed by sequential FISH and GISH techniques. (A) Chromosomes probed by pAs1 (labeled with tetramethy1-rhodamine-5-dUTP for red color) and $(AAG)_{10}$ microsatellite sequence (labeled with fluorescein-12-dUTP for green color). (B) Probed by **W**-genomic DNA (green) and **St**-genomic DNA (red). (C) Probed by **StWY**-genomic DNA (green). (D) Probed by 5S rRNA gene (green) and 45S rRNA gene (red). (E) Probed by **StWY**-genomic DNA (green). Arrows indicate the alien chromosomes.



substitution and (or) addition lines of wheat × *E. rectisetus* derivatives was further confirmed by qPCR assay (Table 4). The relative copy number varied widely for the **W**-genome specific sequence among lines containing chromosomes of this genome. The relative copy number for **Y**-genome specific sequence in the tetraploid **StStYY** species was more than three-fold of that in the hexaploid **StStWWYY** species.

Sequential FISH/GISH analyses revealed that lines 4687 and 4162 are disomic addition lines (2*n* = 44) with the full complement of wheat chromosomes, but the former has a pair of **Y**-genome chromosomes and the latter has a pair of **St**-genome chromosomes (Figs. 2 and 3). The alien chromosomes in line 4162 carried 5S rDNA, whereas those in line 4687 had 45S rDNA (Figs. 2 and 3). Alien chromosomes in line 4434 did not reveal either 5S or 45S rDNA signals (data not shown).

Lines 4319 and 5899 are disomic triple substitution lines (2*n* = 42) having one pair of **W**-genome chromosomes and two pairs of **St**-genome chromosomes (Fig. 4). Although derived from the same lineage, line 4434 (2*n* = 44) differed

Fig. 3. Mitotic chromosomes in line 4162 analyzed by sequential FISH and GISH techniques. (A) Chromosomes probed by pAs1 (labeled with tetramethy1-rhodamine-5-dUTP for red color) and $(AAG)_{10}$ microsatellite sequence (labeled with fluorescein-12-dUTP for green color). (B) Probed by **W**-genomic DNA (green) and **St**-genomic DNA (red). (C) Probed by **StWY**-genomic DNA (green). (D) Probed by 5S rRNA gene (green) and 45S rRNA gene (red). (E) Probed by **StWY**-genomic DNA (green). Arrows indicate the alien chromosomes.

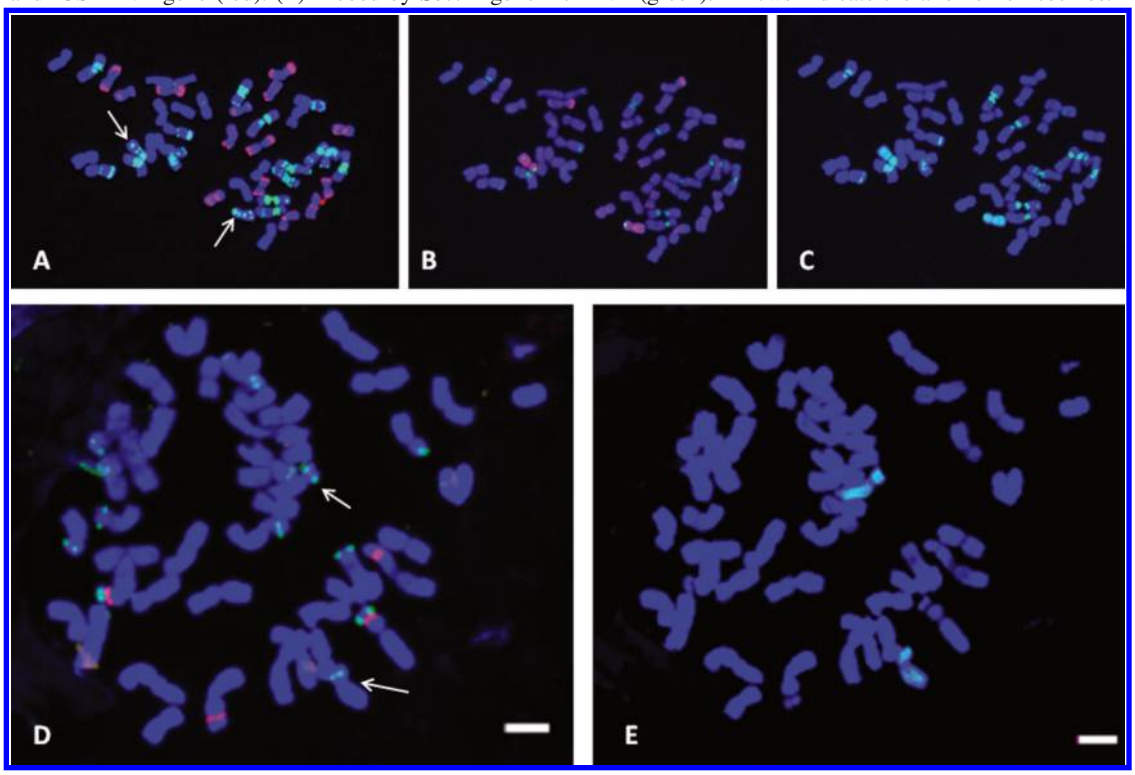


Fig. 4. Mitotic chromosomes in lines 4319 (top), 5899 (middle), and 4434 (bottom) following sequential FISH/GISH analyses. Left to right: chromosomes probed by pAs1 (labeled with tetramethy1-rhodamine-5-dUTP for red color) and $(AAG)_{10}$ microsatellite sequence (labeled with fluorescein-12-dUTP for green color); probed by **W**-genomic DNA (green) and **St**-genomic DNA (red); and probed by **StWY**-genomic DNA (green).

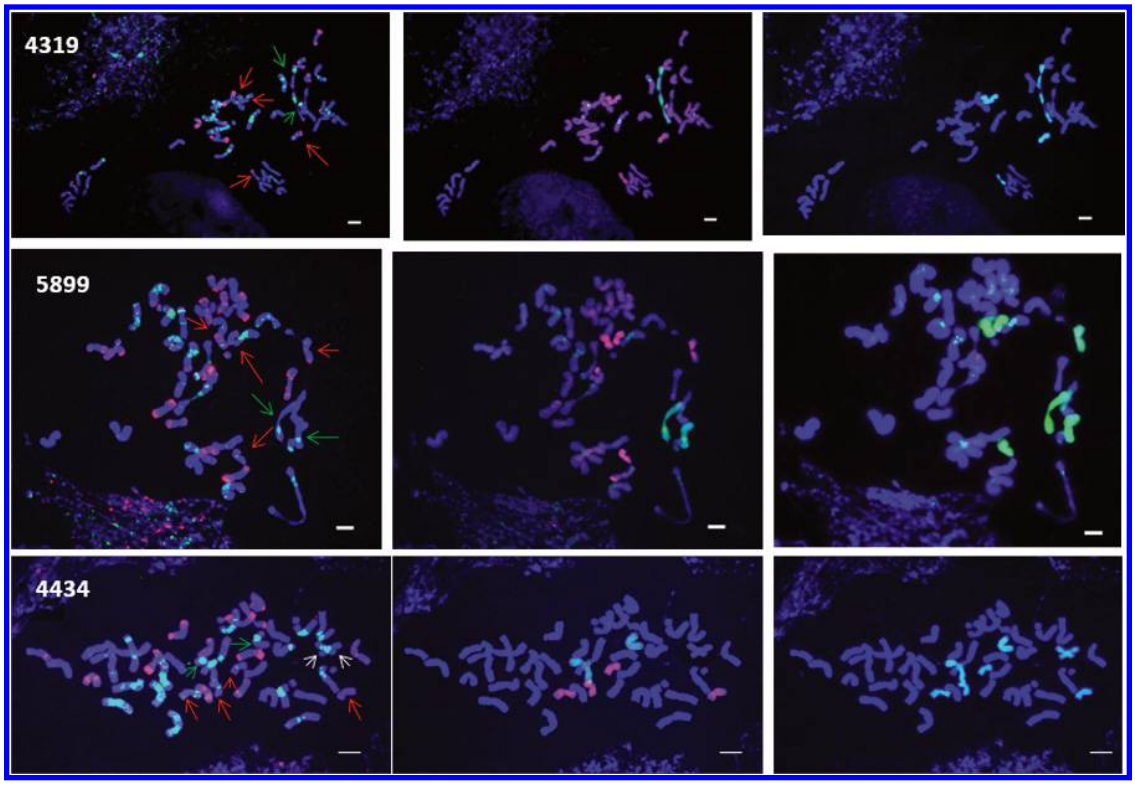


Fig. 5. Mitotic chromosomes in *Elymus rectisetus* (PI 533028) analyzed by sequential FISH and GISH techniques. (A) Chromosomes probed by 5S rDNA (labeled with fluorescein-12-dUTP for green color) and 45S rDNA (labeled with tetramethy1-rhodamine-5-dUTP for red color). (B) Probed by pAs1 (red) and $(AAG)_{10}$ microsatellite sequence (green). (C) Probed by **W**-genomic DNA (green) and **St**-genomic DNA (red). (D) FISH karyotype of **W**-, **St**-, and **Y**-genome chromosomes arranged according to length and arm ratios. Chromosomes having rRNA genes or intergenomic translocations are underlined. Note that the 14 chromosomes of each genome could not be paired into seven homologous groups.

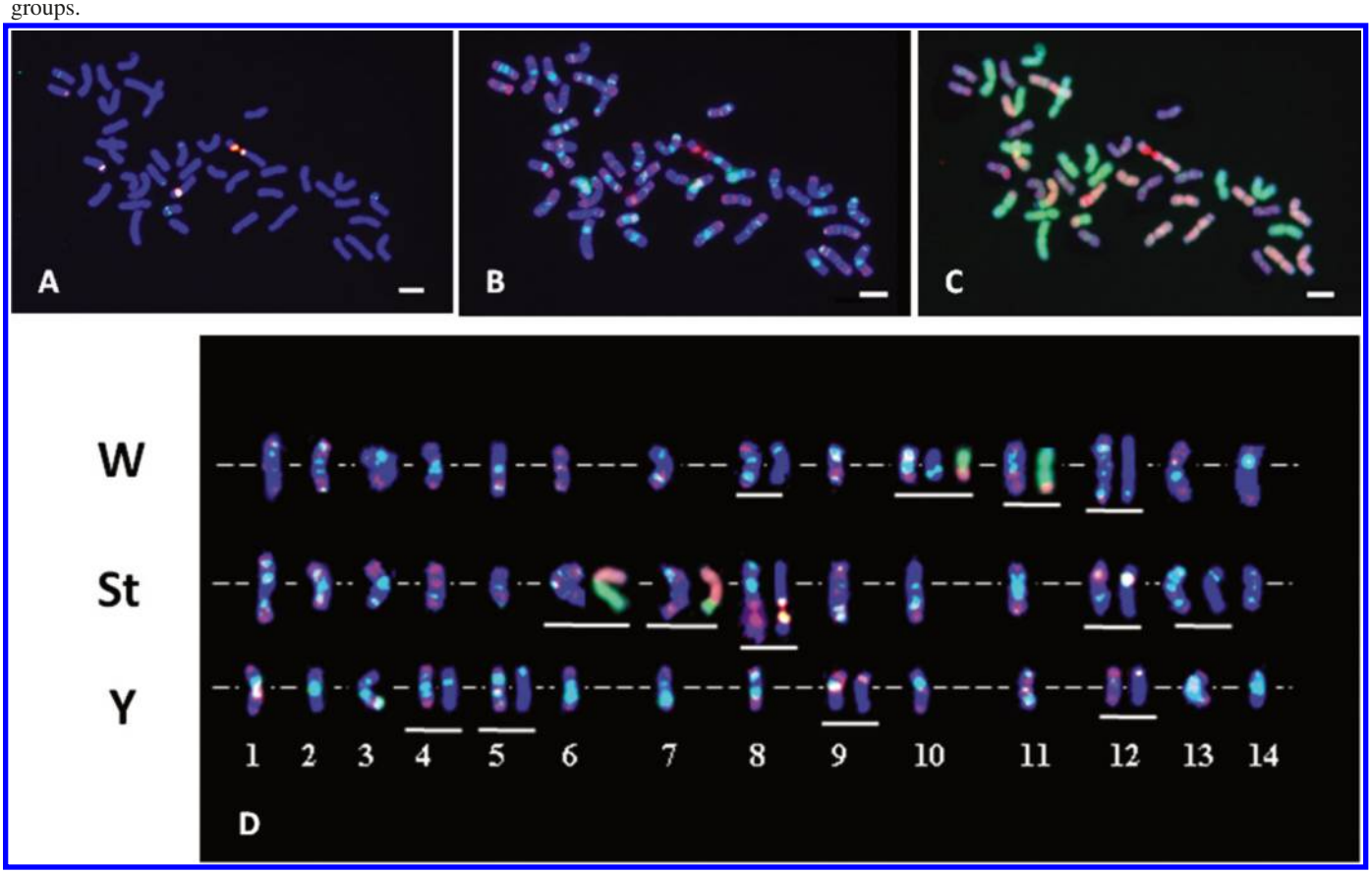


Fig. 6. Alien chromosomes, originated from the **W**, **St**, or **Y** genome of *Elymus rectisetus*, in the backcross derivatives of *Triticum aestivum* × *E. rectisetus* hybrids. Lines 4687 and 4162 possess 1**Y** and 1**St** that has the 45S and 5S rDNA, respectively. Lines 4319, 5899, and 4434 might share the same one pair **W**- and two pairs of **St**-genome chromosomes. m**W** is **W**-14, n**St** is **St**-4, p**St** is **St**-3, and q**Y** is **Y**-2 in Fig. 5D. 1**Y** and 1**St** are corresponding to **W**-9 and **St**-13 in Fig. 5D, respectively.

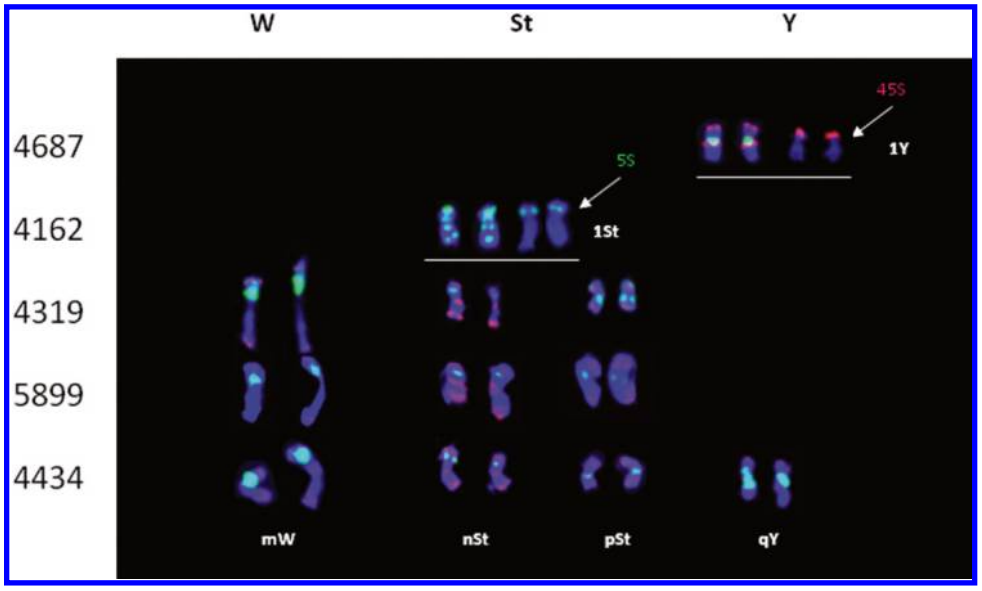
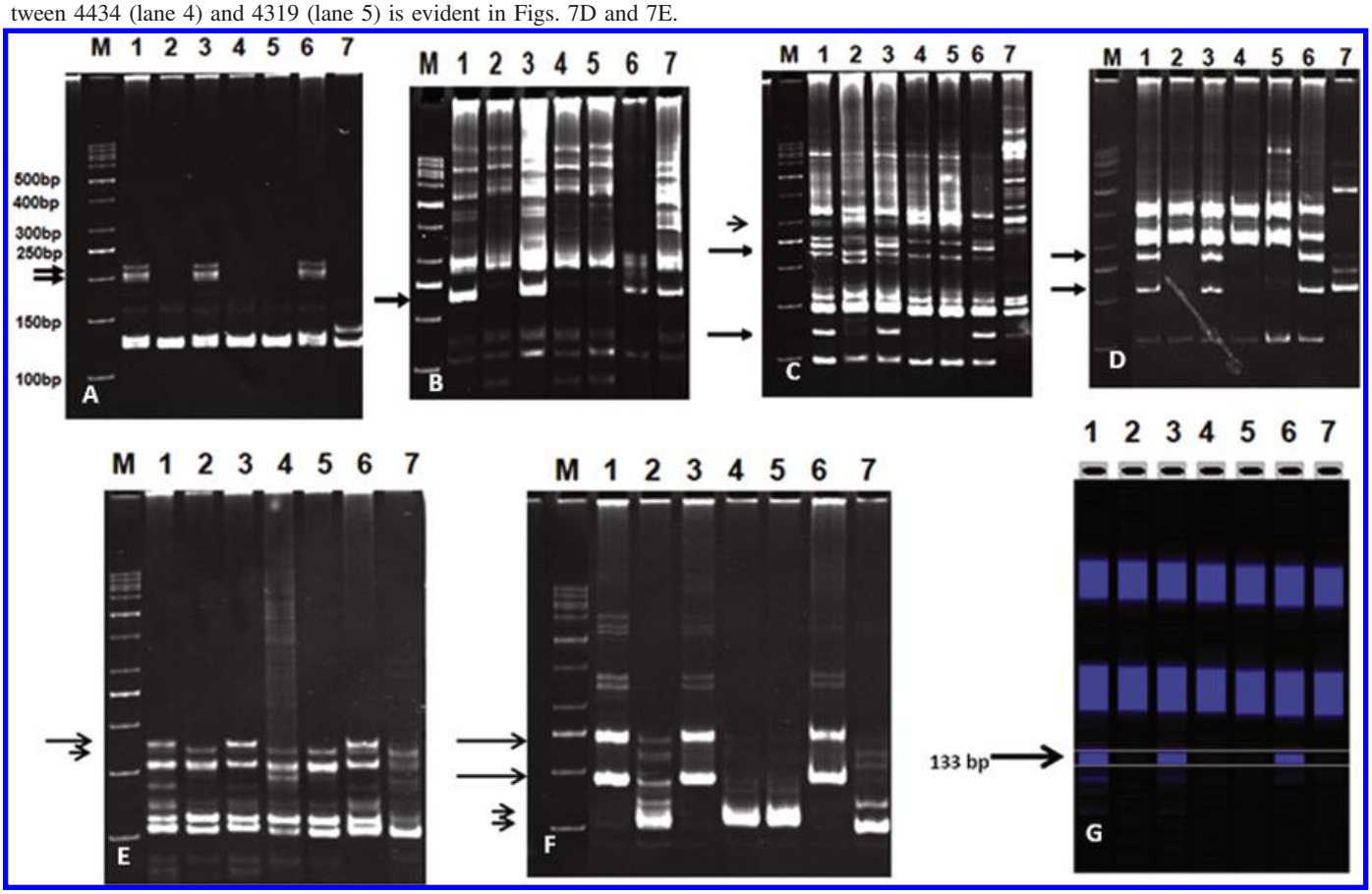


Fig. 7. Electrophoresis profiles of SSR markers amplified by primer pairs specific for wheat chromosomes 6**D** (A: Xcwem48 and B: Xbarc175), 2**A** (C: Xcwem39n and D: Xbarc5), and 4**B** (E: Xcwem38c, F: Xbarc20, and G: Wmc47) in lines 4687 (lane 1), 5899 (2), 4162 (3), 4434 (4), 4319 (5), wheat (6), and *Elymus rectisetus* (7). Figs. 7A–7F are images taken from polyacrylamide gels; Fig. 7G is an image generated by Genographer version 1.6.0 (Benham et al. 1999). Long arrows are pointing to bands that are present in wheat but absent in lines 5899, 4434, and 4319. Short arrows identify bands that were transferred from *E. rectisetus* into lines 5899, 4434, and 4319. Differences between 5899 (lane 2) and 4319 (lane 5) are observable in group-2 (Figs. 7C and 7D) and group-4 chromosomes (Fig. 7F). Difference between 4434 (lane 4) and 4319 (lane 5) is evident in Figs. 7D and 7E.



from 4319 by having one additional pair of **Y**-genome chromosomes (Fig. 4). The alien chromosomes in *E. rectisetus* were distinguished by sequential FISH/GISH and arranged into three genomic groups (Fig. 5). Based on the pattern and intensity of pAs1 (red color) and $(AAG)_{10}$ (green) hybridization sites, presence of 5S rDNA (green) or 45S rDNA (red), as well as the chromosome size and arm ratio, six different alien chromosomes were recognized (Fig. 6).

The missing wheat chromosomes in lines 5899, 4434, and 4319 were determined by SSR assays (Fig. 7), including two each for 2**A** (Xcwem39n and Xbarc5) and 6**D** (Xcwem48 and Xbarc175), and three for 4**B** (Xcwem38c, Xbarc20, and Wmc47). Some amplification products were present in wheat (lane 6) but absent in lines 5899, 4434, and 4319 (lanes 2, 4, and 5, respectively). Differences between 5899 (lane 2) and 4319 (lane 5) were noticeable for SSR amplicons of 2**A** (Figs. 7C and 7D) and 4**B** (Fig. 7F).

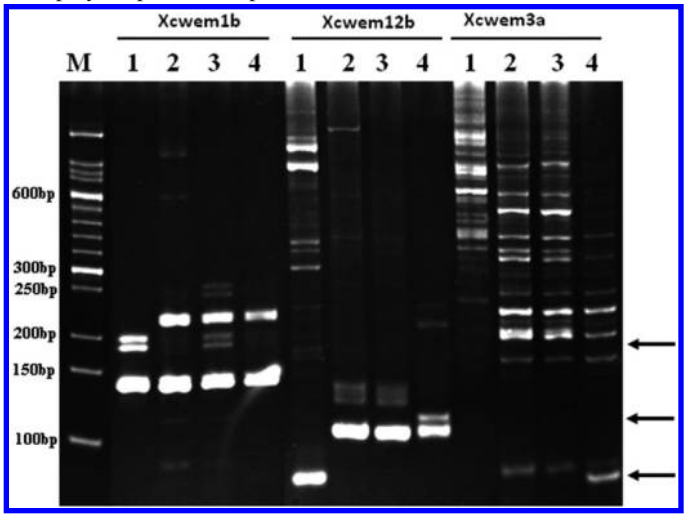
The group-1 alien chromosomes in 4687 and 4162 were also confirmed by three SSR loci (Fig. 8). Line 4687 had four polymorphic bands for Xcwem1b that were absent in 'Fukuho-komugi' wheat, two of which were present in *E. rectisetus* and two were de novo. Line 4162 had two bands from Xcwem12b and one band from Xcwem3a, all of which were not detected in the sample of *E. rectisetus*.

Discussion

Triticum aestivum × *E. rectisetus* hybrids were synthesized and backcrossed to wheat (Liu et al. 1994). Some backcross derivatives have been characterized using RAPD and GISH (Xue and Wang 1999). Three types of addition lines were identified by RAPD markers: (1) 1048, (2) 1057, and (3) 1026 and 1034. Furthermore, lines 1036 and 1048 had been observed to possess four to six alien chromosomes in GISH studies (Xue and Wang 1999). In this study, we further verified that 1048 derivatives had **W**-genome chromosomes and 1057 derivatives had **Y**-genome chromosomes. Derivatives 4162, 4319, and 4431 that had three different **St**-genome chromosomes were negative for the D15-$\mathbf{St}_{498}$ assay. The fact that this **St**-genome STS marker worked with the whole **St** genome but not with individual **St** chromosomes suggests that it is a repeated sequence occurring in few of the seven chromosomes, limiting its usefulness.

The **W**- and **Y**-genome STS markers, B03-$\mathbf{W}_{306}$ and B14-$\mathbf{Y}_{269}$, were more effective for detecting the presence of alien chromosomes belonging to these two genomes. Using B14-$\mathbf{Y}_{269}$ as the probe, about 28 chromosomes of *E. rectisetus* had strong in situ hybridization signals (data not shown). Because this STS marker had only been detected in all species

Fig. 8. Polyacrylamide gel profiles of EST–SSR markers amplified by primer pairs specific for wheat homologous group-1 chromosomes, Xcwem1b, Xcwem12b, and Xcwem3a, in *Elymus rectisetus* (lane 1), 'Fukuho-komugi' (2), 4687 (3), and 4162 (4). Arrows indicate polymorphic bands present in 4687 or 4162.



containing **Y** genome and some **St**-genome species (Okito et al. 2009), the 28 chromosomes having hybridization signals were probably those of these two genomes and the remaining 14 chromosomes were those of **W** genome. Thus, STS assay for B14-$\mathbf{Y}_{269}$ sequence would be an efficient test to detect the presence of **Y**-genome chromosomes.

Although some plants analyzed in the qPCR (Table 4) and FISH/GISH (Fig. 6) had identification numbers differing from those used in STS assays (Table 3), they were siblings that could be considered as the same. Thus, line 4434 (Table 4) is the same as 4431 (Table 3) and 5899 (Table 4) is the same as 0297 (Table 3). Because the three different **St**-genome chromosomes in lines 4162, 4319, 4434, and 5899 (Fig. 6) could not be detected by the STS assay for D15-$\mathbf{St}_{498}$ (Table 3), this STS marker could only exist on four or less chromosomes of the **St** genome.

The results of qPCR for B14-$\mathbf{Y}_{269}$ with tetraploid *E. longearistatus* and hexaploid *E. rectisetus* (Table 4) are intriguing. The relative copy number for this sequence is much higher in the tetraploid than the hexaploid. Because no diploid **Y**-genome species had been found in Triticeae, Okito et al. (2009) speculated that the **Y** genome originated from a **St** genome that contains the B14-$\mathbf{Y}_{269}$ repetitive sequence. This repetitive sequence could have undergone rapid amplification and transposition, which typically occur following wide hybridization and polyploidy (Liu et al. 1998*a*, 1998*b*; Han et al. 2003), during the speciation of **StStYY** species. The lower copy number of this sequence in hexaploid **StStWWYY** species could be attributed to (*i*) *E. rectisetus* originated from the hybridization between a **W**-genome diploid and an **StStYY** species containing a lower copy number of the B14-$\mathbf{Y}_{269}$ sequence than *E. longearistatus* or (*ii*) diminution of this repetitive sequence occurred after the **StStYY** × **WW** hybridization event if all **StStYY** species had about the same copy number as in *E. longearistatus*.

In this study, pAs1 and $(AAG)_{10}$ were labeled in red and green, respectively; whereas, pAs1 and GAA repeats were labeled in green and red, respectively, in the study on *Elymus grandiglumis* (syn. *Kengyilia grandiglumis* $2n = 42$; **StStPPYY**) by Wang et al. (2010). Therefore, reversing the red and green colors would allow the comparisons of the FISH patterns for **St**- and **Y**-genome chromosomes in this study and those in Wang et al. (2010). The four **St**- and two **Y**-genome chromosomes in our *T. aestivum* × *E. rectisetus* derivatives could hardly be matched with those characterized by Wang et al. (2010). Therefore, the number and distribution of pAs1 and $(GAA)_n$ sites are too variable to identify chromosomes of the same basic genome in different species, or even in different accessions/ecotypes of the same species (Dou et al. 2009).

Another important finding in this study is that the 14 chromosomes in each of the three genomes of *E. rectisetus* PI 533028 could not be paired into seven homologous groups, unlike those in *E. grandiglumis* studied by Wang et al. (2010). This difference can be attributed to the fact that *E. rectisetus* PI 533028 is an apomict, thus highly heterozygous (Peel et al. 1997), while *E. grandiglumis* is a self-pollinating homozygous species.

Lines 4687 and 4434 having different **Y**-genome chromosomes (Fig. 6) had also been suggested by the observation of resistance to tan spot and SNB in 4687 (Oliver et al. 2008). RFLP analysis revealed that progenitor lines of 4162, 4687, and 4434 had alien chromosomes belonging to homoeologous group 1 (McArthur et al. 2005). The presence of homoeologous group-1 alien chromosomes in lines 4162 and 4687 was confirmed by the detection of 5S rDNA and 45S rDNA on the alien chromosomes, respectively (Fig. 6). However, we could not confirm the presence of group-1 alien chromosome in 4434 by the test of rDNA sites with sequential FISH/GISH.

The production and identification of these alien cytogenetic stocks may be useful in locating and isolating genes for desirable traits, such as perenniality and apomixis. A single chromosome 4**E** of *Thinopyrum elongatum* confers a polycarpic perennial habit to annual wheat (Lammer et al. 2004). If synteny holds true for this trait, the homoeologues of **St**, **W**, and **Y** genomes would also carry genes for this trait. There is a possibility that lines 4319, 4434, and 5899 carry the group-4 homoeologues of 4**E**, if these lines were compensating substitutions with 4**B** substituted by 4**W**, 4**St**, or 4**Y**. To determine if these lines are compensating substitutions, we will carry out further tests using PCR-based Landmark Unique Gene (PLUG) markers (Ishikawa et al. 2009).

In conclusion, we have identified (*i*) 4687 as a disomic wheat addition lines having a pair of 1**Y** chromosomes; (*ii*) 4162 as a disomic addition line with a pair of 1**St** chromosomes; (*iii*) 4319 and 5899 as wheat substitution lines having a pair of **W**-genome chromosomes and two pairs of **St** chromosomes that substituted wheat chromosomes 2**A**, 4**B**, and 6**D**; and (*iv*) 4431 or 4434 as a wheat substitution–addition line with the same **W**- and **St**-genome chromosomes as in 4319 but adding of a pair of **Y**-genome chromosomes. There are six different alien chromosomes in these five cytogenetic stocks, i.e., 1 **W**-, 3 **St**-, and 2 **Y**-genome chromosomes.

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