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Research Article

Physical mapping of repetitive sequences and genome analysis in six *Elymus* species by *in situ* hybridization

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Abstract Genome constitution and genetic relationships between six *Elymus* species were assessed by physical mapping of different repetitive sequences using a technique of sequential fluorescence *in situ* hybridization and genomic *in situ* hybridization. The six *Elymus* species are all naturally growing species in northwest China, namely, *E. sibiricus*, *E. nutans*, *E. barystachyus*, *E. xiningensis*, *E. excelsus*, and *E. dahuricus*. An StStHH genome constitution was revealed for *E. sibiricus* and StStHHYY for the remainder species. Each chromosome could be clearly characterized by physical mapping with 18S-26S rDNA, 5S rDNA, Afa-family, and AAG repeats, and be allocated to a certain genome by genomic *in situ* hybridization. Two 5S rDNA sites, each in the H and St genomes, and three 18S-26S rDNA sites, two in the St genome and one in the Y genome, were uncovered in most of the species. The strong Afa-family hybridization signals discriminated the H genome from the St and Y genomes. The H and Y genome carried more AAG repeats than St. A common non-Robertsonian reciprocal translocation between the H and Y genomes was revealed in *E. barystachyus*, *E. xiningensis*, *E. excelsus* and *E. dahuricus*. Comparison of molecular karyotypes strongly suggests that they can be classified into three groups, namely, *E. sibiricus*, *E. nutans*, and others.
Key words *Elymus*, fluorescence *in situ* hybridization, repetitive sequences.

Elymus L. is the largest and most widely distributed genus within the grass tribe Triticeae, containing approximately 150 species (Dewey, 1984; Löve, 1984). The genus is entirely composed of polyploidy species containing different combinations of five basic genomes, namely, St, H, Y, P, and W. The St, H, P, and W genomes are derived from *Pseudoroegneria* (Neveski) Löve, *Hordeum* L., *Agropyron* Gaertn., and *Australopyrum* (Neveski) Löve, respectively, although the origin of the Y genome is still unknown (Jensen, 1990; Torabinejad & Mueller, 1993; Wang et al., 1995). *Elymus* species, *E. sibiricus* L., *E. nutans* Griseb., *E. excelsus* Turcz. ex Griseb., and *E. dahuricus* Turcz. ex Griseb. are widely distributed in Asia, whereas *E. barystachyus* L. B. Cai and *E. xiningensis* L. B. Cai are endemic to China, occurring in western mountainous regions (Cai, 1993). The genome constitution of *E. sibiricus* was reported as StStHH ($2n = 28$) (Dewey, 1984), and those of *E. nutans* and *E. dahuricus* as StStHHYY ($2n = 42$) (Lu, 1993; Dewey, 1984). The genome constitutions of the remaining species, however, remain unknown. Furthermore, even in species in which the genome is known, information on chromosome identification and chromosome allocation to the specific genome is rather limited.

Fluorescence *in situ* hybridization (FISH) has been an important tool in the physical mapping of chromosomes in cereals. FISH-based karyotypes can be generated using different repetitive sequences. Comparisons of FISH karyotypes of closely related species have provided chromosome evidence of their evolutionary relationship (Schwarzacher, 2003; Jiang & Gill, 2006). Both 18S-26S rDNA and 5S rDNA, encoding ribosomal RNA, have been widely applied as FISH probes in plants allowing identification of chromosomes, karyotyping, and supporting the revolutionary relationships among different closely related species (Jiang & Gill, 1994; Murata et al., 1997). The Afa-family repetitive sequence reportedly exists in a number of genomes of the tribe Triticeae (Nagaki et al., 1995, 1998). Afa-family sequences are usually formed by numerous blocks in several subtelomeric and interstitial chromosome regions, and therefore, have been extensively used as chromosome markers (Tsujimoto et al., 1997). The distribution of microsatellite AAG on the chromosomes of wheat, barley, and related species in Triticeae are well reported, and the distribution patterns corresponding

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to the N-band make them useful cytological markers for chromosome identification (Pedersen et al., 1996; Cuadrado & Schwarzacher, 1998).

Genomic *in situ* hybridization (GISH), which uses the total genomic DNA of an analyzer (a genomically known species) as a probe to detect the chromosome homology of the analyzer in the genome of a species with an unknown genome constitution (Ørgaard & Heslop-Harrison, 1994), is a powerful method for analyzing the origins, genomic composition, and intergenomic rearrangements of polyploid species.

In this paper, we first characterize the individual chromosomes of *E. sibiricus*, *E. nutans*, *E. barystachyus*, *E. xiningensis*, *E. excelsus*, and *E. dahuricus* using different repetitive sequences as probes for FISH, and second, allocate these chromosomes to the different genomes using GISH onto the same cell. This sequential FISH/GISH method allowed us to present detailed molecular karyotypes for these six *Elymus* species. The results could be very helpful in elucidating the origin and evolution of the St, H, and Y genomes. Furthermore, *E.sibiricus* and *E. nutans* are two important perennial forage crops in the Qinghai–Tibet plateau. The knowledge of genome constitutions could greatly contribute to the germplasm enhancement of the forage crops through interspecific hybridization, and through genetic manipulation.

1 Material and methods

1.1 Plant materials

Elymus sibiricus L., *E. nutans* Griseb., *E. barystachyus* L. B. Cai, *E. xiningensis* L. B. Cai, *E. excelsus* Turcz. ex Griseb., and *E. dahuricus* Turcz. ex Griseb. were collected in Xining, Qinghai, China. The local altitude is approximately 2300 m.

1.2 Probe DNA preparation and sequential *in situ* hybridization

The 5S rDNA and Afa-family repetitive sequences were amplified by polymerase chain reaction using genomic DNA of *E. sibiricus* as a template, as described by Fukui et al. (1994) and Nagaki et al. (1995), respectively. pTa71 including the 18S-26S rDNA of wheat (Gerlach & Bedbrook, 1979) and genomic DNAs of *Hordeum chilense* Roem. and Schult ($2n = 14$, HH genome) and *Pseudoroegneria stipifolia* (Czern. ex Nevski) A. Love ($2n = 14$, StSt genome) were fragmentized by autoclaving at 120 °C for 2 min before labeling. A 30-base length $(AAG)_{10}$ repetitive oligomer was synthesized by Fasmac (Kanagawa, Japan). Probe labeling and procedures of sequential FISH and GISH were followed according to Dou et al. (2009).

2 Results

Molecular karyotyping of the six *Elymus* species was carried out using sequential FISH and GISH. First, the 18S-26S rDNA and 5S rDNA hybridization sites were physically mapped (Fig. 1: A1–F1) and the signals stripped out, then FISH was carried out using AAG and Afa-family sequence probes to the same cell (Fig. 1: A2–F2). These signals were again stripped out and finally GISH carried out using genomic DNA probes of *H. chilense* (H genome) and *P. stipifolia* (St genome) (Fig. 1: A3–F3). All chromosomes were clearly classified into the genome St, H, or Y, and homologous chromosomes were clearly recognized by their FISH patterns. As a result, detailed molecular karyotypes of these six species were well described (Fig. 2).

The results showed that *E. sibiricus* is a tetraploid species ($2n = 28$) with the genome constitution StStHH, and the remaining five are hexaploid ($2n = 42$) having the common genome StStHHYY. The karyotype formulas of *E. sibiricus* were revealed as 5″m+2″sm in the H and 4″m+3″sm in the St genome, and those of *E. nutans* were 5″m+2″sm in the H, 5″m+2″sm in the St and 5″m+2″sm in the Y genome (where m represents metacentric, sm represents submetacentric, and ” represents a pair of chromosomes). *Elymus xiningensis*, *E. dahuricus*, *E. barystachyus*, and *E. excelsus* shared the common karyotype formulas of 5″m+2″sm in the H, 5″m+2″sm in th St, and 7″m in the Y genome (Fig. 2, Table 1).

Multiple sites of 18S-26S rDNA and 5S rDNA were revealed in the six species examined. Two 5S rDNA sites, each in the H and St genome, and three 18S-26S sites, two in the St genome and one in the Y genome, were uncovered with variations. One 5S rDNA site was uncovered in *E. sibiricus, E. nutans*, *E. barystachyus*, and *E. xiningensis* in the H genome, and moreover, the chromosomes carrying this site on the subtelomeric region of the short arm showed the biggest arm ratio and shared similar FISH patterns among the four species (Fig. 2). Another 5S rDNA hybridization site was positioned with the 18S-26S rDNA site of the metacentric chromosomes in the St genome. All six species except *E. barystachyus* were shown to carry two 18S-26S rDNA sites in the St genome. One site was located on the subtelomeric region of the metacentric chromosomes and shared a similar arm ratio and FISH patterns in all species except *E. sibiricus*, whereas the other was located on the telomeric region of the submetacentric chromosomes and presented the biggest arm ratio in the St genome in the same five species. This site was not detected in *E. barystachyus*. An extra 18S-26S rDNA site was detected in the Y genome in each hexaploid

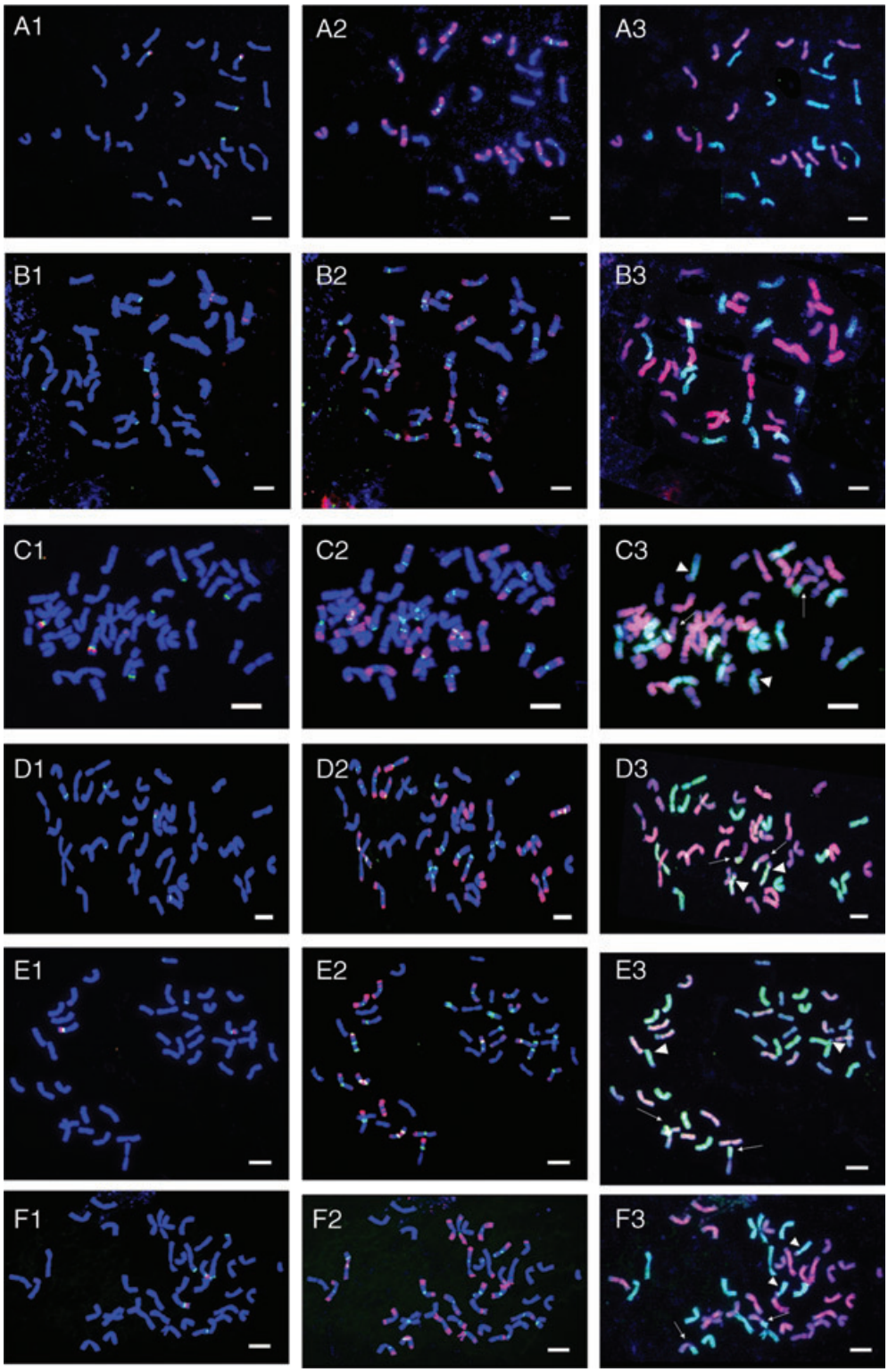

Fig. 1. Sequential fluorescence *in situ* hybridization (FISH) and genomic *in situ* hybridization (GISH) photographs of mitotic metaphase in (**A**) *Elymus sibiricus*, (**B**) *E. nutans*, (**C**) *E. xiningensis*, (**D**) *E. dahuricus,* (**E**) *E. barystachyus,* and (**F**) *E. excelsus*. **A1, B1, C1, D1, E1, F1:** First FISH probed with 18S-26S DNA (green) and 5S rDNA (red). **A2, B2, C2, D2, E2, F2:** Sequential FISH probed with Afa-family sequences (red) and $(AAG)_{10}$ (green). **A3, B3, C3, D3, E3, F3:** Sequential GISH probed with *Hordeum chilense* (green) and *Pseudoroegneria stipifolia* (red). Chromosomes of H, St, and Y genome were stained with green, red, and pink, respectively. Arrows and arrowheads indicate translocated chromosomes. (Bar = 10 μm).

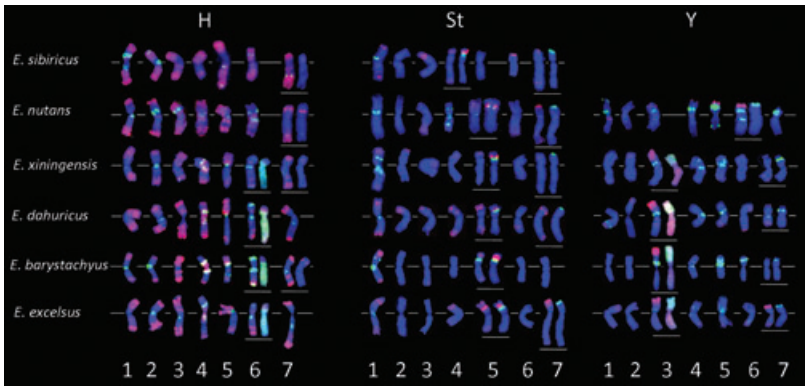

Fig. 2. Molecular karyotypes of *Elymus sibiricus*, *E. nutans*, *E. xiningensis*, *E. dahuricus*, *E. barystachyus*, and *E. excelsus*. Patterns of most chromosomes were characterized by Afa-family sequences (red) and $(AAG)_{10}$ (green). Underlined juxtaposed chromosomes indicate those involving 18S-26S (green) and (or) 5S rDNA (red) sites, and translocations.

Table 1 Karyotype features of six *Elymus* species

Genome	Species	Karyotype formula	No. of chromosomes with				
			Afa family	AAG	18S-28S rDNA	5S rDNA	Intergenomic translocation
H	*E. sibiricus*	5″m + 2″sm	7″	5″	—	1″sm(7)	—
	E. nutans	5″m + 2″sm	7″	6″	—	1″sm(7)	—
	E. xiningensis	5″m + 2″sm	7″	7″	—	1″sm(7)	1″sm(6)
	E. dahuricus	5″m + 2″sm	7″	5″	—	—	1″sm(6)
	E. barystachyus	5″m + 2″sm	7″	6″	—	1″sm(7)	1″sm(6)
	E. excelsus	5″m + 2″sm	7″	6″	—	—	1″sm(6)
St	*E. sibiricus*	4″m + 3″sm	5″	1″	1″m (4) + 1″sm (7)	1″m(4)	—
	E. nutans	5″m + 2″sm	4″	3″	1″m (5) + 1″sm (7)	1″m(5)	—
	E. xiningensis	5″m + 2″sm	7″	2″	1″m (5) + 1″sm (7)	1″m(5)	—
	E. dahuricus	5″m + 2″sm	7″	1″	1″m (5) + 1″sm (7)	1″m(5)	—
	E. barystachyus	5″m + 2″sm	3″	2″	1″m (5)	1″m(5)	—
	E. excelsus	5″m + 2″sm	5″	1″	1″m (5) + 1″sm (7)	1″m(5)	—
Y	*E. nutans*	5″m + 2″sm	5″	6″	1″sm (6)	—	—
	E. xiningensis	7″m	6″	5″	1″m (7)	—	1″m(3)
	E. dahuricus	7″m	6″	5″	1″m (7)	—	1″m(3)
	E. barystachyus	7″m	1″	4″	1″m (7)	—	1″m(3)
	E. excelsus	7″m	7″	4″	1″m (7)	—	1″m(3)

—, no chromosomes; ″, a pair of chromosomes; m, metacentric; sm, submetacentric. Chromosome numbers designated in Fig. 2 are described in parentheses.

species, positioned in a submetacentric chromosome in *E. nutans* and in a metacentric chromosome in the others (Fig. 2).

The Afa-family sequence was physically mapped in the H, St, and Y genomes. In particular, strong hybridization signals in subtelomeric, interstitial, or paracentric regions in all chromosomes of the H genome allowed clear discrimination between the chromosomes of the H genome and those of the St and Y genomes (Fig. 2, Table 1). Relatively faint Afa-family signals were detected in the subtelomeric regions in some of the St and Y genome chromosomes (Fig. 2, Table 1). Furthermore, similar distributions of the Afa-family in each chromosome shared the same number of the H genome in *E. xiningensis*, *E. dahuricus*, *E. barystachyus*, and *E. excelsus* were uncovered (Fig. 2).

AAG repeat hybridization sites were revealed in paracentric and interstitial regions in the H, St, and Y genomes. Five to seven chromosomes in the H genome and 4–6 chromosomes in the Y genome presented AAG sites in the tested species, but only 1–3 chromosomes did so in the St genome. The conserved distribution of AAG repeats in most H genome chromosomes and partial St and Y genome chromosomes was also revealed in *E. xiningensis*, *E. dahuricus*, *E. barystachyus*, and *E. excelsus* (Fig. 2, Table 1).

Special non-Robertsonian reciprocal translocation between the H and Y genome chromosomes was detected in *E. xiningensis*, *E. dahuricus*, *E. barystachyus*, and *E. excelsus.* The similar FISH and GISH patterns imply an identical origin for this translocation in these species.

3 Discussion

Chromosome allocation of the 18S-26S rDNA and 5S rDNA sites was relatively conserved with few variations in the six *Elymus* species. Chromosome No. 7 in the H genome showed the largest arm ratio and a similar distribution pattern of Afa-family sequences in the six species. Likewise, this was also the case for chromosome No. 7 in the St genome. This implies that these chromosomes are strongly conserved, with the lack of 5S rDNA sites in the No. 7 chromosomes of the H genome in *E. dahuricus* and *E. excelsus*, and 18S-26S rDNA in the No. 7 chromosome of the St genome of *E. barystachyus*, being the result of a copy number decrease during evolution. One chromosome pair in the St genome included both 5S rDNA and 18S-26S rDNA in all species. However, the arm ratio and FISH patterns suggest that the chromosomes (4St) in *E. sibiricus* differ from those (5St) in other species. This implies that chromosome rearrangement occurred in these chromosomes between *E. sibiricus* and the remaining five species during evolution. Similarly, chromosomes carrying an 18S-26S rDNA site in the Y genome represent variation between *E. nutans* and the others. In most cases, the 18S-26S and 5S rDNA in Triticeae are located in homoeologous 1 and 5 groups, whereas chromosomes of homoeologous group 5 always present the largest arm ratio in the genome (Mukai et al., 1990). In our study, the chromosomes showing the largest arm ratio in the H and St genomes carried 5S rDNA and 18S-26S rDNA, respectively. This suggests that the chromosomes of homoeologous group 5 in the H and St genomes were strongly conserved in these *Elymus* species during evolution.

The distribution of Afa-family sequences and AAG repeats in the St, H, and Y genomes revealed in our study suggests that they are good cytological markers for characterization of individual chromosomes in *Elymus*. The strong hybridization of Afa-family sequences makes it easy to discriminate H genome chromosomes from all others. Both the St and Y genomes had fewer and fainter Afa-family sequence signals. It has been reported that the St and Y genomes have a close relationship and may share a common progenitor (Liu et al., 2006). Here, it was revealed that the Y genome chromosomes carried more AAG sites than the St genome chromosomes. As the origin of the Y genome is still unknown, the distribution pattern of Afa-family sequences and AAG repeats of the Y genome unveiled in our study may be adopted as genomic characteristics of the Y genome in testing other polyploid *Elymus* species that possibly possess the Y genome.

Although polymorphism of the FISH signals of the repetitive sequence were observed in the H genomes of all six species, the chromosome shape and distribution of Afa-family sequences imply that the H genome in these species might come from the same ancestor. The St genome in *E. sibiricus* showing large genetic diversity compared to the others suggests a different origin. The difference between the Y genome of *E. nutans* and the other five species implies that they may have a different Y genome ancestor. *Elymus dahuricus*, *E. xiningens*is, *E. barystachyus*, and *E. excelsus* presented great similarity in their molecular karyotypes. In particular, all four species had the same reciprocal translocation chromosomes. This strongly implies that they share the same origin. Morphologically, *E. sibiricus* and *E. nutans* have nodded spikes making them different from the other species examined, which have erect spikes. To some extent, the molecular karyotyping data are therefore in agreement with the morphology. These results strongly suggest that these six species can be genetically grouped into three groups. The first includes *E. sibiricus*, the second *E. nutans*, and the third *E. dahuricus*, *E. xiningensis*, *E. barystachyus*, and *E. excelsus*.

Detailed analyses of meiotic behavior in interspecific hybrids have played an important role in our understanding of the genome constitutions of the genus *Elymus.* However, use of such analyses in the identification of chromosomes and in their allocation to a specific genome is rather limited. Sequential FISH and GISH not only identifies each chromosome using different chromosome markers, but also allocates the identified chromosome to a certain genome. In particular, the types of intergenomic translocation can be clearly determined. This method is therefore a powerful tool for analysis of genome constitution and phylogenetic affinities in the genus *Elymus.*

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