High frequency of karyotype variation revealed by sequential FISH and GISH in plateau perennial grass forage *Elymus nutans*

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Elymus nutans L. (StHY, $2n=6x=42$) is extensively selected from the natural population and domesticated as perennial grass forage in the Qinghai-Tibet plateau in China due to its high tolerance to environmental stresses, such as cold and drought. Karyotyping was conducted in 12 randomly selected plants by sequential fluorescence *in situ* hybridization (FISH) and genomic *in situ* hybridization (GISH). GISH discriminated St, H and Y genomes, indicating that *E. nutans* has retained its ancestral genome, and large chromosomal rearrangements have not occurred. However, FISH using an AGG satellite and *Afa*-family repetitive sequences, revealed marked variation in the signalling patterns of most of the chromosomes. Many of the plants carried chromosomes with a specific pattern in the homozygous state, indicating that self-pollination or sib-crossing occurs in small populations during selection for domestication. In addition, several intergenomic translocations appeared, possibly caused by homoeologous chromosome recombination. The nature of the polymorphisms seen in the chromosomes of the domesticated population of *E. nutans* is discussed.

Key Words: perennial grass forage, *Elymus nutans*, karyotype variation, FISH, GISH.

Introduction

The genus *Elymus* L., which includes as many as 150 species, is the largest and most widely distributed genus in the Triticeae tribe of family Poaceae (Löve 1984). *Elymus nutans* L. is a commonly distributed *Elymus* species in China and grows on grassland, bush land, river banks, mountain slopes, and swales, at altitudes from 1000 m up to 5000 m (Lu 1993) and is tolerant to various environmental stresses associated with high altitude, such as cold and drought. In the Qinghai-Tibet plateau, vigorous plants are selected from the natural population and used as perennial forage crops. A cytological study has suggested that *E. nutans* is hexaploid ($2n=6x=42$), and has three genomes: St, Y and H (Lu 1993). The St genome originates from the genus *Pseudoroegneria* and the H genome from *Hordeum*, but the origin of the Y genome is unknown (Wang *et al.* 1995). Recent molecular phylogenetic studies have suggested that the St and Y genomes may have originated from the same ancestors (Liu *et al.* 2006). In this species, chromosomal rearrangements and karyotype variations are shown by assessing intraspecific hybrids and mitotic chromosomes (Lu 1993, Zhang *et al.* 2008). Knowledge of karyotype variation is helpful in selecting stable plants for breeding forage crops. However, the cytological analysis of this species is very limited.

Molecular cytogenetic analysis using genomic *in situ* hybridization (GISH) and fluorescence *in situ* hybridization (FISH) has provided powerful methods to detect karyotype variation. GISH using the total genomic DNA of an analyzer (a species with a known genome) as a probe revealed intergenomic translocations, as well as the presence, or absence, of the analyzer genome in polyploid species (Ørgaard and Heslop-Harrison 1994). FISH with chromosome-specific repetitive sequences or microsatellites as AAG identifies each chromosome in the genomes (Tsujimoto *et al.* 1997, Cuadrado and Schwarzbacher 1998, Pedersen *et al.* 1996). We here adopted FISH/GISH analyses to the chromosomes of *E. nutans* to reveal intraspecific chromosomal variation.

Materials and Methods

Plant materials

Seeds of domesticated *E. nutans* were kindly donated by the Qinghai Grassland Management Station, China. The original materials were collected from the natural populations in regions around Qinghai Lake (altitude 3000–3500 m) in Qinghai about 10 years ago by the breeders. They were then selected and domesticated as forage crops.

Seeds of *Elymus sibiricus* L. (StStHH) were collected in Xining, Qinghai. *Hordeum chilense* (HH) and *Pseudoroegneria stipifolia* (StSt) are maintained in the Laboratory of Plant Genetics and Breeding Science, Tottori University, Japan.

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In situ hybridization

An *Afa*-family repetitive sequence was amplified by PCR, as described previously by Nagaki *et al.* (1995), with a template DNA of *E. sibiricus* genomic DNA. The sequence was separated using agarose-gel electrophoresis. Genomic DNAs of *H. chilense* and *P. stipifolia* for the probes of GISH were fragmented by autoclaving at 120°C for 2 min before labelling. DNA was labelled with fluorescein-12-dUTP or tetramethyl-rhodamine-5-dUTP using the random primer method (Gene Images™ Random Prime Labelling Module, Amersham Bioscience). The synthesized microsatellite sequence (AAG)₁₀ was labelled with tetramethyl-rhodamine-5-dUTP using the 3'-end method (Gene Images™ 3'-Oligolabelling Module, Amersham Bioscience).

The FISH and GISH procedures were followed as described previously by Kishii *et al.* (1999), with minor modifications. For the first hybridization, DNA denaturation was carried out in 0.2 M NaOH in 70% ethanol at room temperature for 10 min. The sequential hybridization procedure was as follows: after photography of the first probing, cover slips were removed and the slides were washed in 2×SSC for 20 min at room temperature and briefly dried; the mixture of the second hybridization was applied to the slide without denaturation. The following steps were the same as for the first hybridization. Several hybridizations can be conducted on the same chromosome preparation by repeating the steps above. GISH probing with labelled genomic DNAs of *H. chilense* (HH) and *P. stipifolia* (StSt) was carried out separately to avoid interference between the probes. Chromosome preparation was first detected by the *H. chilense* probe that was labelled with fluorescein-12-dUTP. After post-hybridization washing, the chromosome preparation was second detected by the *P. stipifolia* probe labelled with tetramethyl-rhodamine-5-dUTP. No blocking DNAs were added in either of the GISH probing steps. Images were acquired using a fluorescence microscope (Olympus BX61) with a CCD camera (Cool Snapper, Photometrics), and processed using Photoshop ver. 6.0 (Adobe).

Results

Sequential FISH and GISH analysis was conducted on 12 randomly selected samples (E1–E12) of the domesticated *E. nutans*. First, FISH was carried out using AAG and *Afa*-family probes (Fig. 1a–c, g–i, m). GISH was carried out on the same cells using genomic DNA probes of *H. chilense* and *P. stipifolia*, respectively (Fig. 1d–f, j–l, n, o).

All plants carried 42 chromosomes. Sequential GISH clearly classified all chromosomes into three genomes: H, St, or Y. As a result, we found that the H, St, and Y genomes include 3 (Nos. 1, 2, 3), 5 (Nos. 1, 2, 3, 4, 5) and 5 (Nos. 1, 2, 3, 4, 5) metacentric chromosomes, respectively (Fig. 2). Other chromosomes in these genomes were sub-metacentric.

Most of the chromosomes showed signals of AAG satellite and/or *Afa*-family sequences. Specific hybridization of these chromosome markers discriminated each chromo-

somes. Well marked chromosomes were arbitrarily designated as 1–7 by their arm ratio and the relative length (Fig. 2). Since some of the chromosomes were polymorphisms among different individuals, the polymorphisms showing the greatest similarity in the distribution of hybridization signal, arm ratio and length were allocated to the same number (Fig. 2). As the genome to which the chromosome belongs is known by GISH, the chromosomes can be designated as, for example, H1, H2, St2, and Y5. In this designation, the numerals do not correspond with those of the homoeologous groups as used in the nomenclature system of the chromosomes of wheat and barley.

The FISH/GISH analyses revealed large polymorphisms in the chromosome pattern (Fig. 2 and Table 1). We designated these polymorphisms as a, b, c, and etc (Table 1). The chromosomes shown with the same letter indicate identical patterns of FISH/GISH signals. As *E. nutans* has 42 chromosomes ($2n = 6x = 42$), we observed a total of 504 chromosomes for the 12 plants. We found at least two polymorphisms in all of the 21 chromosomes, except for in H1 and Y6 (Table 1). The H, St, and Y genomes demonstrated 36, 42, and 33 polymorphic patterns of chromosomes, respectively. Most of the plants carried the pattern in the homozygous state, although some were in the heterozygous state (5.1%; 13 in 252 chromosome pairs; Table 1).

In addition to the polymorphism of FISH patterns, translocation was observed in 7 of the 12 plants. These were non-Robertsonian translocations, and were possibly produced by homoeologous chromosome recombination. The translocations were grouped into four types. Type I involved two reciprocal translocations between chromosomes H6a and Y5a (Fig. 1g, j and Fig. 2). Plants E1, E6, and E7 included the pair of translocations in the homozygous state, whereas E12 carried it in the heterozygous state (Table 1 and Fig. 1m, n, o). Type II involved reciprocal translocations between the H and Y genome chromosomes. However, the FISH patterns of the AAG satellite and the *Afa* family, and also the translocation break-point by GISH, clearly demonstrated that the type II translocation was different from the type I translocation (Fig. 1b, e and Fig. 2 H5f, Y1c). Only one plant, E3, carried the type II translocation (Table 1). Type III was a translocation between the H and St genome chromosomes (Fig. 2 H4b). However, no other counterpart was observed in the plants. One plant, E4, contained only one such translocation (Fig. 1c, f). Similar to Type III, Type IV was a translocation between the H and St genome chromosomes; however, it is mostly the St genome chromosome with a small chromosome segment of an H genome chromosome (Fig. 2 St3g). Plant E11 included one chromosome of type IV translocation (Fig. 1i, l).

The karyotypes of the 12 plants were different from each other, and most chromosomes possessed polymorphisms at a high frequency. However, the heterozygosity was relatively low, suggesting that, in the domesticated strains of *E. nutans* used here, propagation was mainly by self-pollination or in a sib-crossing manner.

Fig. 1. Sequential FISH and GISH patterns of representatives of grass forage *E. nutans*. A, B, C, G, H, I, and M: FISH patterns of plants E2, E3, E4, E7, E8, E11, and E12 probed with AAG (red) and *Afa* family (green). D, E, F, J, K, and L: Sequential GISH patterns of E2, E3, E4, E7, E8, and E11 probed with genomic DNAs of *H. chilense* (H genome; green) and *P. stipifolia* (St genome; red). N, O: Sequential GISH patterns of E12 probed separately with *H. chilense* (green) and *P. stipifolia* (red). Arrows and arrowheads indicate translocation chromosomes. Scale bar=10 μ m.

Fig. 2. Karyotypes of the 12 *E. nutans* plants. Different variants are annotated by the different roman letters. Translocations are indicated by I–IV.

Discussion

In this study, genomes H, St, and Y of *E. nutans* were successfully analyzed and each chromosome was assessed by sequential FISH and GISH. The Y genome is a basic genome occurring in the genus *Elymus*, but its progenitor is still unknown. Based on the molecular phylogenetic data, Liu *et al.* (2006) suggested that the Y genome probably shares a common progenitor with the St genome. We showed that the Y genome could be well distinguished from the St genome in *E. nutans* by GISH. This suggests that, despite sharing the same progenitor, there is genetic divergence between the St and Y genomes.

Using FISH with microsatellite and repetitive sequences, the karyotypes were shown to be largely polymorphic in most chromosomes. In addition, many were present in a homozygous state. This indicates that the plants of the domesticated strains of *E. nutans* propagated in a self-pollinated or a sib-crossing manner. The high seed setting in isolated spikes of *E. nutans* indicates a self-pollination habit (inbreeder), but it is also common to find spontaneous hybrids between

E. nutans and other *Elymus* taxa in nature (Lu 1993). *E. nutans* is originally a possible out-crossing species in the natural population, and this can be confirmed by the rate of polymorphisms of the karyotype. During the domestication process, there was marked selection pressure in a small population. Exceptions are seen in chromosomes H1 and Y6, which are conservative and monomorphic. This may suggest the presence of key genes on these two chromosomes.

Lu (1993) conducted meiosis analysis of the single intraspecific hybrid and its parental accessions, which belonged to two different populations of *E. nutans*. The results showed that there was a low frequency of univalency and multivalency in parental accessions, and an increased frequency of multivalency at metaphase I. In addition, there were chromatid bridges and fragments at anaphase I in the intraspecific hybrid. This, it was suggested that certain chromosome rearrangements occurred in different populations of the species.

Four types of translocation were observed in the plants. These were probably produced by recombination between homoeologous chromosomes belonging to different genomes

Table 1. Chromosome variants included in karyotypes of 12 investigated samples (E1–E12)

		E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12	No. of variants	Total
H	1	a	a	a	a	a	a	a	a	a	a	a	a	1	36
	2	a	b	de	a	d	a	a	f	c	fg	b	f	7	
	3	a	b	e	a	d	a	a	d	c	d	c	d	5	
	4	a	c	d	ab ^T	e	a	a	e	cd	f	c	f	6	
	5	a	c	f ^T	b	e	a	a	e	d	e	d	e	6	
	6	a ^T	c	b	b	d	a ^T	a ^T	e	c	bd	c	a ^T b	5	
	7	a	a	c	a	c	a	a	d	b	c	b	ef	6	
St	1	a	c	b	b	b	b	b	b	c	b	b	b	3	42
	2	a	c	e	b	f	b	a	ef	d	fg	d	g	7	
	3	a	e	h	b	h	c	d	h	e	i	fg ^T	i	9	
	4	a	e	e	b	f	c	d	gh	e	e	e	g	8	
	5	a	c	e	a	f	a	b	e	c	e	d	e	6	
	6	a	b	b	a	b	b	a	a	b	b	a	b	2	
	7	a	c	d	b	e	b	a	fg	c	e	c	e	7	
Y	1	a	a	c ^T	a	a	a	a	a	a	a	a	b	3	33
	2	a	a	c	b	c	a	a	a	a	c	a	d	4	
	3	a	b	e	a	d	a	a	a	c	a	b	e	5	
	4	a	d	e	b	f	c	c	c	c	g	c	h	8	
	5	a ^T	c	f	b	g	a ^T	a ^T	h	d	b	e	ga ^T	8	
	6	a	a	a	a	a	a	a	a	a	a	a	a	1	
	7	a	c	d	b	b	b	b	b	b	b	b	b	4	

* letters with superscript as “T” indicate translocations; columns containing two letters indicate the heterozygote for the chromosomes.

because many plants carried the translocations in the homozygous state. If the translocation had occurred between non-homoeologous regions of certain chromosomes, pollen with non-compensating translocations that appeared after meiosis would have a lesser chance for fertilization during pollen competition and thus would not be homozygous. The translocations, possibly by homoeologous recombination, might be induced in the process of hybridization between different species, due to the misbalance in the pairing system of homoeologous chromosomes (*ph*) after hybridization. Although homoeologous translocations occurred at a high frequency via this process, only limited translocations that developed a new, balanced *ph* system and adapted well to the environment were selected by nature. It is confirmed that most plants retained their basic chromosome structure without translocations.

High genetic diversity is beneficial for *E. nutans* to deal with environmental hardness. However, karyotypes with homoeologous recombination cause aberration in meiosis and this affects seed setting. Seed products are a very important characteristic of forage production. Thus, the seed yield of the domesticated forage plant *E. nutans* can be improved by genetic homogeneity selection.

The plant materials of *E. nutans* used in this study were collected and domesticated from the natural population by breeders. Information relating to their original population is now lacking. Detailed studies of the genetic composition and variation in natural populations of this species may provide valuable information for the evolution, adaptation, and utilization of this species for a forage crop.

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