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‘Chromosome painting’ by fluorescent *in situ* hybridization (FISH) in hybrids and introgressions of *Lolium* and *Festuca* species

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Fluorescent *in situ* hybridization (FISH) was used to characterize genomic constitution and chromosome rearrangements in amphiploid ×*Festulolium braunii* cultivars ‘Punia’ and ‘Rakopan’ (winter-persistent and random plants), and *Lolium perenne* × [*Festuca pratensis*] introgression lines $BC_{2\text{-}2}$ (freezing-tolerant and non-tolerant) together with triploid parental plants and the previous generations BC_1 and BC_2. The presence of *F. pratensis, L. perenne* and ribosomal DNA (rDNA) has been evaluated. Hybridization patterns in ‘Punia’ and ‘Rakopan’ revealed more *L. perenne* chromosomes in the winter-hardy than in random plants. In intogression lines, frost-resistant plants had a higher number of introgressed *F. pratensis* DNA fragments and more rDNA sites than non-resistant ones. In $BC_{2\text{-}2}$ plants, *F. pratensis* introgressions were very small and in most of the cases fragments were found next to rDNA sites.

Key words: FISH, GISH, *Festuca*, *Lolium, Festulolium,* species introgression, genome recombination, rDNA

INTRODUCTION

Lolium grasses are considered ideal grass species for livestock forage. However, their main disadvantage is that they lack winter persistency and in Northern countries are sensitive to hard abiotic stress, such as drought and freezing winter temperatures. Their close relatives from the genus *Festuca* have a lower nutritious value and digestibility, but they are much better adapted and tolerant to abiotic stress [1]. To combine the positive qualities together, hybrids of these two genera species have been created. *Lolium* and *Festuca* species hybridize easily and their chromosomes pair and recombine readily in the hybrids. One way to put together the traits of *Lolium* and *Festuca* is amphiploidy when whole genomes are combined. Another way is introgression breeding between species, when genes are being transferred by repeated backcrossing [2]. Introgression breeding is the most convenient way to transfer the *Festuca* stress-tolerance genes to the genus *Lolium*.

In *Festulolium* hybrids, chromosomes of *Lolium* and *Festuca* genomes can be easily distinguished using GISH (genomic *in situ* hybridization) by fluorochrome-labeled genomic DNA of one of the parents [3, 4]. Such genome differentiation by ‘chromosome painting’ is possible because of divergent dispersed repetitive sequences in these grass species.

The term “chromosome painting” defines the *in situ* labeling of chromosomes or chromosome regions by a fluorescent DNA probe (chromosome DNA hybridization). Analysis *in situ* differs from the analysis of nucleic acids by Southern or Northern hybridization in that the hybridization signal is localized *in situ* (directly on the chromosomes) and not on a solid support such as a nylon membrane [5]. Individual chromosomes can be painted using chromosome-specific sequences generated by PCR from microdissected or flow-sorted chromosomes or chromosome-specific dispersed repeats. Chromosome-specific contigs of large insert clones can also be used [6].

In situ hybridization enables: 1) hybrid genome constitution analysis; 2) construction of physical maps of chromosomes; 3) analysis of chromosome structure and aberrations; 4) investigation of chromosome structure, the function and evolution of chromosomes and genomes; 5) determination of spatial and temporal expression of genes; 6) identification and characterization of viruses, viral sequences and bacteria in tissues [5].

In this study, we report the patterns of ‘chromosome painting’ in the amphiploid ×*Festulolium braunii* cultivars ‘Punia’ (Lithuania) and ‘Rakopan’ (Poland), and introgression lines *L. perenne* × [*F. pratensis*] developed in the Norwegian Institute for Agricultural and Environmental Research.

MATERIALS AND METHODS

Plant material

1) Two ×*Festulolium braunii* cultivars were involved in this study: the Lithuanian cultivar ‘Punia’ (*F. pratensis* ×

L. multiflorum, 2n=4x=28) and the Polish cultivar 'Rakopan' (*L. multiflorum* × *F. pratensis,* 2n=4x=28). For comparison, two genotype accessions were tested in each cultivar: (i) plants grown in the greenhouse randomly sampled from the cultivar seed lot (random sample); (ii) best survival plants after four winters in the field (field sample).

2) Initial triploid parental hybrids (LpLpFp, 2n=3x=21) from the cross *L. perenne* (2n=4x=28) × *F. pratensis* (2n=2x=14), and backcross lines BC_1, $BC_{2\text{-}1}$ and $BC_{2\text{-}2}$ developed on the basis of diploid *L. perenne* (2n=2x=14). $BC_{2\text{-}2}$ plants were selected after freezing tests, the most and the least tolerant were taken for investigations. These plants were provided by the Norwegian Institute for Agricultural and Environmental Research.

Chromosome preparation

Mitotic chromosomes from root-tips were prepared on objective slides after pretreatment in ice-cold water for 24 h, followed by fixation in 1:3 acetic acid – ethanol. The roots were softened in a mixture of 0.1% pectolyase Y-23 and 0.1% cellulase R-10, and squashed in 45% acetic acid.

Probes

L. perenne, L. multiflorum and *F. pratensis* genomic DNA was sonicated for 5 min in an ELMA Transsonic T 460/H ultrasonic bath and labeled with rhodamine-11-dUTP (Roche) and fluorescein-12-dUTP (Fermentas). The pTA71 plasmid containing wheat 18S-5.8S-26S ribosomal DNA repeats [7] was cleaved with the restriction enzyme EcoRI to release the ribosomal DNA sequence and labeled with fluorescein-12-dUTP.

***In situ* hybridization**

Slides were soaked in 45% acetic acid for 5 min at RT and for 3 min at 48–50 ˚C. Denaturation of nuclear DNA was performed at 70 ˚C in 70% deionized formamide for 2 min, followed by dehydration with cold ethanol series (70%, 90% and 100%) 2 min each, and air-drying. Slides were incubated at 37 ˚C with 25 µl of denatured (10 min at 70 ˚C) hybridization mix (2 µg DNA probe, 60% formamide, 25% dextran sulphate, 10% 20 × SSC, 5% 5%SDS solution) for 16 h in a moist chamber. After hybridization, slides were washed in 20% formamide in 0.1 × SSC twice for 5 min at 42 ˚C, and 3 times for 3 min in 2 × SSC at 42 ˚C. Slides were mounted with Vectashield antifade and DAPI (4,6-diamidino-2-phenylindole) for the counterstaining of DNA.

Analysis of hybridization signal

Hybridization signals were analysed with a filter setoff (Nikon Eclipse E800 fluorescence microscope). Three filter sets were used for detection as follows: 1) DAPI (excitation 330–380 nm, beam 400 nm, barrier 420 nm); 2) rhodamine (excitation 510–560 nm, beam 575 nm, barrier 590 nm); 3) fluorescein (excitation 450–490 nm, beam 505 nm; barrier 520 nm). Photographs were taken with a Pixera Penguin digital 600CL camera. For processing the colour pictures, Image Pro-Discovery 4.5 and Adobe Photoshop Elements were used.

RESULTS AND DISCUSSION

Amphiploids

In our study, we analyzed genome composition in plants of ×*Festulolium braunii* cultivars produced from crosses between *L. multiflorum* and *F. partensis*. In total, the chromosome number was 27 or 28 in the cultivar 'Punia' and 28 or 29 in the cultivar 'Rakopan'. The number of pure *F. pratensis* chromosomes in these plants ranged from 0 to 6. There were more *Lolium* than *Festuca* chromosomes in most of the plants (Fig. 1). Only two hybrids (one of 'Punia' and one of 'Rakopan') had more *F. pratensis* than *L. multiflorum* chromosomes. A high number of recombinant chromosomes (>10) was found in all plants. In most cases, there was a majority of such chromosomes (Table 1).

The recombinant chromosomes had one or more breakage points. Both in 'Punia' and 'Rakopan' cultivars, considerably more of *L. multiflorum* chromosomes were found in plants that survived four winters in comparison with those from a random sample (10.25 and 6.13, and 10.67 and 7.71, respectively). On the one hand, this provides an explanation for the mechanism of the *Lolium* dominant behaviour in these amphiploids. Previously, this tendency was recorded in F_8 generation of the amphiploid

Table 1. **GISH analysis in ×*Festulolium braunii* cultivars 'Punia' and 'Rakopan'**

Cultivar	Sample	No. of plants	No. of chromosomes	No. of Fp chromosomes		No. of Lm chromosomes		No. of recombinant chromosomes		No. of rDNA sites		
				Range	Mean	Range	Mean	Range	Mean	No. of plants	Range	Mean
Punia	field	4	27–28	1–4	3.00	8–13	10.25	11–16	14.50	5	6–10	8.40
	random	8	27–28	0–4	2.50	2–11	6.13	16–22	19.13			
Rakopan	field	3	28–29	0–4	1.67	10–12	10.67	14–19	16.00	5	6–11	8.60
	random	7	28–29	0–6	2.71	4–14	7.71	12–20	17.71			

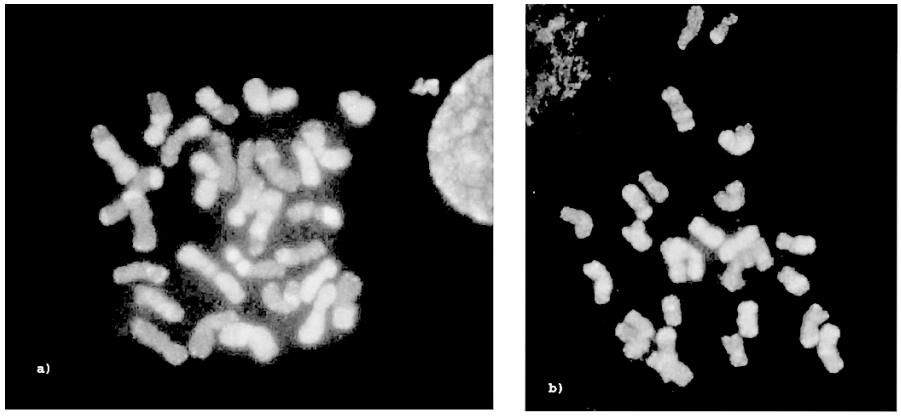

Fig. 1. Mitotic metaphase plates of x*Festulolium braunii* cultivars 'painted' by GISH: a) 'Rakopan' (2n=4x+1=29). Total genomic DNA of *Festuca pratensis* labeled with fluorescein-12-dUTP (bright grey) and genomic DNA of *Lolium multiflorum* labeled with rhodamine-11-dUTP (dark grey) were used as probes. The genome consists of 8 *F. pratensis*, 6 *L. multiflorum* and 15 recombinant chromosomes with different size and number of recombinant fragments. b) 'Punia' (2n=4x–1=27). Genomic DNA of *F. pratensis* was labeled with rhodamine-11-dUTP (bright grey) and all chromosomes were counterstained with DAPI (dark grey). The plant has 4 *F. pratensis*, 5 *L. multiflorum* and 18 recombinant chromosomes.

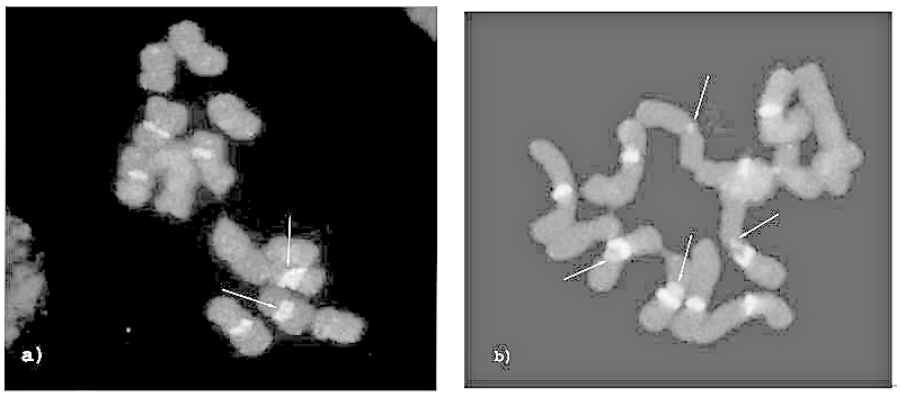

Fig. 2. Metaphase plates of two plants from the diploid introgression lines of *L. perenne* x (*L. perenne* × *F. pratensis*) (2n=2x=14) visualized by GISH. Total genomic DNA of *F. pratensis* labeled with rhodamine-11-dUTP was used as a probe to highlight *Festuca* DNA and pTA71 plasmid labeled with fluorescein-12-dUTP was used to visualize rDNA sites: a) $BC_{2\text{-}2}$ 9 (non-tolerant) – 2 bands of *Festuca* DNA visible (arrowed) located close to pTA71 bands (bright grey) and 6 rDNA sites detected; b) BC2-2 19 (freezing-tolerant) – 4 fragments of *Festuca* DNA (arrowed) and 3 of them located near rDNA sites (arrowed).

cultivar 'Prior' from the cross of *L. perenne* × *F. pratensis*, where numerous of *F. pratensis* chromosomes were replaced by *L. perenne* ones [9]. On the other hand, it is surprising that winter survivals of ×*Festulolium braunii* carry more chromosomes of *L. multiflorum* than of *F. pratensis*. No significant difference was found between the amounts of *F. pratensis* and recombinant chromosomes in random samples and field samples. However, the number of recombinant chromosomes was slightly higher in random samples. Five plants in each cultivar were studied for rDNA sites. The number of rDNA sites ranged from 6 to 11 in the 'Rakopan' plants (mean, 8.6) and from 6 to 10 in 'Punia' plants studied (mean, 8.4).

Previously, we have tested whether genomic *Lolium* DNA hybridizes equally to *Lolium* chromosomes. For this test, *L. perenne* F6-13 line and *L. perenne* genomic DNA were used. The hybridization was bright and equal at all chromosomes, showing that *L. perenne* and *L. multiflorum* DNA hybridization is reliable (results not shown).

The genomic *in situ* hybridization (GISH) method is used to verify the allopolyploid origin of some species, also to elucidate chromosome rearrangements between the parental genomes [8]. Even a small introgression of alien chromatin can be identified, except in cases when parental DNA sequences are too similar or extensive homogenization between the parental genomes has occurred within the hybrids [6].

Introgression lines

Using *L. perenne* or *F. pratensis* genomic DNA labeled probes genomic composition of 7 Lp×Fp triploids was revealed. These triploids were used as initial plants in the backcrosses with diploid *L. perenne* for production of introgression lines. As expected, in

Table 2. **GISH analysis of introgression lines**

Backcross line	No. of plants	No. of translocated chromosomes		No. of small segments		No. of larger segments		rDNA sites	
		Range	Mean	Range	Mean	Range	Mean	Range	Mean
BC_1	6	2–6	3.33	2–6	3.33	0	0.00	7–8	7.60
$BC_{2\text{-}1}$	5	2–4	3.60	2	2.00	0–2	1.60	6–8	6.40
$BC_{2\text{-}2}$ -9	15	0–2	1.87	0–2	1.87	0	0.00	6–8	6.80
$BC_{2\text{-}2}$-19	9	1–4	2.11	1–4	2.11	0	0.00	7–9	7.71

most of the cases 7 non-recombinant chromosomes of fescue were found and 14 chromosomes of ryegrass. Five triploids did not have recombinant chromosomes at all, but 2 plants had a single *F. pratensis* type recombinant chromosome with small terminal fragment of *L. perenne*. All plants had 9 rDNA sites.

Using *F. pratensis* rhodamine-labeled DNA plants of BC_1, the $BC_{2\text{-}1}$ and $BC_{2\text{-}2}$ introgression lines that have a *L. perenne* genomic background were screened for the presence of *F. pratensis* chromosomal fragments. Plants from the final lines $BC_{2\text{-}2}$ were selected after a series of freezing tests. $BC_{2\text{-}2}$-19 represented the most stress-tolerant accession, and $BC_{2\text{-}2}$-9 had the lowest tolerance to freezing. *F. pratensis* chromosomal segments were found in 34 plants out of the 35 analyzed. No large *F. pratensis* fragments were detected. Most of the plants had two small interstitial chromosomal fragments of *F. pratensis* (Table 2). BC_1 and $BC_{2\text{-}1}$ plants had two to six *F. pratensis* fragments located on different chromosomes. Both the interstitial and terminal fragments were detected, and some of these introgressions were quite significant in size.

In total, the number of *F. pratensis* introgressions in $BC_{2\text{-}2}$ plants was lower and segments were smaller in comparison to the earlier backcross generations BC_1 and $BC_{2\text{-}1}$. The $BC_{2\text{-}2}$-19 line (freezing-tolerant) had a slightly larger number of *F. pratensis* genome introgressions in comparison to $BC_{2\text{-}2}$-9 (non-tolerant) – 2.11 and 1.87, respectively. In the $BC_{2\text{-}2}$-19 line, all plants had at least one *F. pratensis* introgression, and in some genotypes this number reached four. In $BC_{2\text{-}2}$-9, one plant occurred with no *F. pratensis* segments at all. Some of the introgressions could be *F. pratensis* chromosome segments that carry environmental stress-resistance genes.

Using fluorescein label, we applied FISH with a specific rDNA probe. It is known from the literature that *L. perenne* has seven sites of 18S-5.8S-26S rDNA [10], while *F. pratensis* has only two [11]. Despite having a genetic background of *L. perenne*, the number of rDNA sites varied within the backcross lines. It was found to be higher in the $BC_{2\text{-}2}$-19 line, in which most of the plants had 7–8 sites and one plant had nine. Therefore, the mean rDNA site number was found slightly lower in the $BC_{2\text{-}2}$-9 than in $BC_{2\text{-}2}$-19 line (6.80 and 7.71, respectively).

A new tendency was revealed regarding the location of *Festuca* fragments on *Lolium* chromosomes, namely most of *Festuca* introgressions appeared very close to rDNA sites (Fig. 2). This shows that recombination frequency increases nearer to rDNA sites. These parts of the chromosomes are very active and may induce higher recombination frequencies near rDNA sites. In *Lolium* × *Festuca* introgression lines, this could be one of the reasons for resistance to environmental stresses, since the high rate of rDNA might favour plant acclimation to hard climatic conditions.

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CHROMOSOMŲ DAŽYMAS FLUORESCENCINĖS *IN SITU* HIBRIDIZACIJOS (FISH) BŪDU *LOLIUM* IR *FESTUCA* RŪŠIŲ HIBRIDUOSE BEI INTROGRESINĖSE LINIJOSE

Santrauka

Fluorescencinė *in situ* hibridizacija (FISH) buvo naudojama tiriant amfiploidinių ×*Festulolium braunii* veislių 'Punia' ir 'Rakopan' (atsparių žiemai bei atsitiktinių augalų), *Lolium perenne* × [*Festuca pratensis*] introgresinių linijų $BC_{2\text{-}2}$ (atsparių ir neatsparių šalčiui), taip pat triploidinių tėvinių augalų bei ankstesnių kartų BC_1 ir BC_2 genomų sudėt[ir chromosomų persitvarkymą. Buvo vertinama bendroji *F. pratensis, L. perenne* ir ribosominės DNR (rDNR). Hibridizacijos pobūdis 'Punia' ir 'Rakopan' veislėse atskleidė, kad per žiemą išsilaikiusiuose augaluose išlieka daugiau *L. perenne* chromosomų negu atsitiktiniuose. Šalčiui atsparūs introgresinių linijų augalai turėjo daugiau *F. pratensis* DNR fragmentų ir rDNR saitų negu neatsparūs. $BC_{2\text{-}2}$ augaluose *F. pratensis* introgresijos buvo labai mažos ir daugeliu atvejų randamos šalia rDNR saitų.