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GENOMIC COMPOSITION OF AMPHIPLOID × *FESTULOLIUM BRAUNII* CULTIVARS 'PUNIA' AND 'RAKOPAN'

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Abstract

The aim of this study was to compare genomic composition of winter-persistent (sample of 4 years' winter survivors in the field) and random plants (random sample) in two ×*Festulolium braunii* tetraploid cultivars 'Punia' and 'Rakopan' (2n = 4x = 28). For this purpose, fluorescent in situ hybridization (FISH) was carried out using labeled probes of genomic DNAs of *Lolium multiflorum* and *Festuca pratensis,* and ribosomal DNA (rDNA) sites have been evaluated applying pTA71 fluorescent probe. In the plants' genome of both cultivars, there were more of pure *L. multiflorum* chromosomes present than of *F. pratensis* ones, on average about 6–7 and 2–3, respectively. The majority of chromosomes (between 18 and 20) in all plants were recombinant. The study also revealed significantly higher amount of *F. pratensis* chromatin in winter-persistent plants than in random ones, although *L. multiflorum* remains as a major part of genome. In addition, 'Punia' had shown more terminal *Festuca* fragments in the field plants. The rDNA site number ranged from 5–14 with a mean about 9 sites per chromosome set. None of the plants had 16 expected sites; that demonstrated tendency of rDNA reduction in ×*Festulolium braunii* tetraploids compared to parental *L. multiflorum* and *F. pratensis* species.

Key words: *Festulolium, Lolium, Festuca,* FISH, GISH, 'Punia', 'Rakopan', genome recombination, rDNA.

Introduction

Lolium grasses are considered ideal grass species for livestock forage. However, they lack resistance during harsh winters, especially in Northern countries. Their close relatives from *Festuca* genus have lower nutritious value and digestibility, but they are much better adapted and tolerant to abiotic stress /Humphreys, 2004/. To compose best qualities together hybrids of these two genera species have been created. *Lolium* and *Festuca* species hybridize easily and their chromosomes pair and recombine undergoing meiosis in the hybrids. One of the ways to put together the traits of *Lolium* and *Festuca* is amphiploidy when whole genomes are combined together /Humphreys et al., 2003/. In these plants, chromosomes of *Festuca* and *Lolium* can be easily distinguished by GISH (genomic *in situ* hybridization), using labelled *Lolium* or *Festuca* DNA as a probe

/Thomas et al., 1994; Pašakinskienė, Jones, 2005/. Such genome differentiation is possible because of divergent dispersed repetitive sequences in these grass species.

Since intergeneric hybrids between diploid *L. multiflorum*, *L. perenne* and *F. pratensis* are completely male sterile, it is more appropriate to use tetraploid forms for production of *Festulolium* hybrids. Many cultivars of allotetraploid hybrids *Lolium multiflorum* (4x) × *Festuca pratensis* (4x) and *F. pratensis* × *L. perenne* (4x) have been created. The first cultivars developed in the United Kingdom were 'Elmet' (*L. multiflorum* × *F. pratensis*) and 'Prior' (*L. perenne* × *F. pratensis*). Greater success in amphiploid breeding from reciprocal *L. multiflorum* × *F. pratensis* and *F. pratensis* × *L. multiflorum* hybrids has been achieved and several cultivars have been developed: 'Paulita' in Germany, 'Perun', 'Achilles', 'Perseus' and 'HŽ14DK' in Czech Republic /Kopecky et al., 2006/, 'Punia' and 'Pūga' in Lithuania /Nekrošas et al., 1995; 1997; 2007/, 'Felopa', 'Sulino', 'Rakopan' and 'Agula' in Poland /Zwierzykowski, 2004/.

In this study we report the patterns of 'chromosome painting' and chromosome recombination between *L. multiflorum* and *F. pratensis* in two × *Festulolium braunii* cultivars 'Punia' (Lithuania) and 'Rakopan' (Poland). We used GISH to determine the numbers of parental and recombinant chromosomes in individual plants and counted the chromosome breakage points per genome. We measured relative length of *L. multiflorum* and *F. pratensis* chromatin in genomes of these plants which is a new approach compared to our previous study /Lideikytė et al., 2006/. FISH using ribosomal DNA-specific probe pTA71 was used to determine the numbers of rDNA sites.

Materials and Methods

Plant material. Two ×*Festulolium braunii* cultivars were involved in this study: Lithuanian cultivar 'Punia' (*F. pratensis* × *L. multiflorum*, 2n = 4x = 28) and Polish cultivar 'Rakopan' (*L. multiflorum* × *F. pratensis,* 2n = 4x = 28). Two accessions of these cultivars 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 4 winters in the field (field sample).

Chromosome preparation. Mitotic chromosomes from root-tips were prepared on objective slides after pre-treatment 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. multiflorum and *F. pratensis* genomic DNA was sonicated for 5 min in ELMA Transsonic T 460/H ultrasonic bath and labelled 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 restriction enzyme EcoRI to release the ribosomal DNA sequence and labelled 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, 0.05 % 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 counterstaining of DNA.

Analysis of hybridization signal. Hybridization signals were analysed by filters' set under the 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 by Pixera Penguin digital 600CL camera. For processing colour pictures, Image Pro-Discovery 4.5 and Adobe Photoshop Elements were used.

Chromosome measuring and fragment counting. Relative *F. pratensis* and *L. multiflorum* chromatin measuring was performed manually on the photographs of labelled metaphase plates using Image-Pro Discovery 4.5 program (Media Cybernetics). The lengths of *F. pratensis* and *L. multiflorum* chromatins were measured separately (in pixels) and the percentage of their chromatins in each metaphase plate was calculated. In recombinant chromosomes, numbers of breakpoints and translocated terminal and interstitial fragments were counted.

Statistics. Microsoft Excel 2003 was used for statistical processing of the data. P-value was calculated for chromosome numbers, *L. multiflorum* and *F. pratensis* chromatin length and numbers of translocated fragments.

Results and Discussion

'Punia' and 'Rakopan' are tetraploids (2n = 4x = 28) with a high frequency of aneuploids, from 21.74 to 29.17 % (Table 1). In total, chromosome numbers ranged from 25 to 29 in 'Punia' plants and from 21 to 29 in 'Rakopan' plants. There was a majority of recombinant chromosomes (having one or more breakpoints) in all plants. Mean number of recombinant chromosomes was from 18 to 20 per plant which exceeded total sum of pure *Lolium* or *Festuca* chromosomes. In all plants, there were more pure chromosomes of *L. multiflorum* than the ones of *F. pratensis*, about 6-7 and 2-3 on average, respectively (Table 2). Previously, the same tendencies in *Festulolium* hybrids were recorded by Kopecky et al. (2006). Their research showed similar numbers of pure and recombinant chromosomes in these plants.

Table 1. Chromosome number in the plants of ×*Festulolium braunii* cultivars 'Punia' and 'Rakopan'

1 lentelė. *Chromosomų skaičius* × *Festulolium braunii 'Punia' ir 'Rakopan' veislių augaluose*

Cultivar *Veislė*	Accession *Ėminys*	No. of plants *Augalų sk.*	No. of chromosomes *Chromosomų sk.*						Aneuploids % *Aneuploidai %*
			21	25	26	27	28	29	
'Punia'	Field / *Lauko*	24			1	5	17	1	29.17
	Random / *Atsitiktinis*	25		1		3	20	1	24.00
'Rakopan'	Field / *Lauko*	23	1		1	3	19		21.74
	Random / *Atsitiktinis*	35			2	5	26	2	25.71

In this study, we found that the plants that survived 4 winters in the fields had a tendency to have more pure *L. multiflorum* chromosomes and less recombinant chromosomes than random plants. The difference between *L. multiflorum* chromosome numbers in random and field samples is significant ($P \leq 0.05$): the mean number of *Lolium* chromosomes is 6.55 in the field samples and 5.65 in random samples, counting for both varieties together. Previously, the dominant behaviour of *Lolium* chromosomes was found in F_8 generation of *Festulolium* cultivar 'Prior' from the cross of *L. perenne* × *F. pratensis* /Canter et al., 1999/. So far, the reasons of *F. pratensis* chromatin substitution by *L. multiflorum* or *L. perenne* have not been elucidated. In 'Punia' plants, there is significantly lower number of translocated chromosomes in field than in random sample ($P \leq 0.05$), and there is the same tendency in 'Rakopan' plants.

Table 2. Chromosome analysis in the plants of ×*Festulolium braunii* cultivars 'Punia' and 'Rakopan'
2 lentelė. *Chromosomų tyrimai 'Punia' ir 'Rakopan' veislių augaluose*

Cultivar *Veislė*	Accession *Ėminys*	No. of plants *Augalų sk.*	Mean chromosome No. *Chromosomų sk. vidurkis*	No. of Fp chromosomes *Fp chromosomų sk.*		No. of Lm chromosomes *Lm chromosomų sk.*		No. of recombinant chromosomes *Rekombinantinių chrom. sk.*		No. of rDNA sites *rDNR saitų sk.*	
				Range *Ribos*	Mean *Vidurkis*	Range *Ribos*	Mean *Vidurkis*	Range *Ribos*	Mean *Vidurkis*	Range *Ribos*	Mean *Vidurkis*
'Punia'	Field *Lauko*	24	27.75	1-6	2.79	3–12	6.92	11–24	18.00	5–13	8.92
	Random *Atsitiktinis*	25	27.80	0-4	2.12	2–11	5.64	16–24	20.16	6–13	9.04
'Rakopan'	Field *Lauko*	23	27.48	0-5	2.39	3–11	6.17	13–24	18.83	7–11	8.76
	Random *Atsitiktinis*	35	27.80	0-5	2.20	2–11	5.66	16–23	19.94	6–14	9.34

Both cultivars had various numbers of rDNA sites, ranging from 5 to 13 in 'Punia' (mean 8.92 in field plants and 9.04 in random plants) and from 6 to 14 in 'Rakopan' (mean 8.76 in field plants and 9.34 in random plants) (table 2). Diploid *L. multiflorum* is known to have six 18S-5.8S-26S rDNA sites, and diploid *F. pratensis* – 2 of these sites /Thomas et al., 1996; 1997/, so the tetraploid *Festulolium* would be expected to have 16 rDNA sites. However, in our study all investigated plants had lower numbers of rDNA sites, and this result shows that in *Festulolium*, chromosomes tend to lose some of rDNA sites.

These findings are consistent with our previous study in Lideikytė et al. (2007), where we found the same tendency in chromosome numbers of 'Punia' and 'Rakopan' plants, as well as in rDNA site numbers. Since now we used more plants than in previous study, we found a higher variation in chromosome and rDNA site numbers; however, the tendencies remain the same.

For the first time we applied a new approach in *Festuliolium* study by measuring chromatin length in the GISH painted metaphase chromosome plates by using Image-Pro Discovery 4.5 program. We found that *L. multiflorum* chromatin exceeds the length of *F. pratensis* chromatin by 1.6–1.8 times on average in the plants of 'Punia' and 'Rakopan' cultivars (Table 3). Chromatin length variation was between 22–52 % for *Festuca* and 48–78 %. Only in several plants, *Festuca* chromatin length exceeded the one of *Lolium*. Previous genome recombination studies performed by Zwierzykowski et al. /2006/ showed a dominant *Lolium* DNA behaviour in the hybrids of *Lolium perenne* and *Festuca pratensis* – from F_2 to F_6 generations, the total length of *Lolium* chromatin increased and the one of *Festuca* chromatin decreased. These findings are consistent with our results. In addition, we found that there was significantly more *F. pratensis* and less *L. multiflorum* chromatin($P \leq 0.05$) in field plants that survived 4 winters than in random samples, counting for both cultivars together (Table 3). This may show that, nevertheless *Festuca* chromatin remains as minor part of hybrid plant genome the amount of *Festuca* genome tends to increase together with increased winter hardiness of ×*Festulolium braunii*.

Table 3. *L. multiflorum* and *F. pratensis* chromatin relative length in the plants of × *Festulolium braunii* cultivars 'Punia' and 'Rakopan'
3 lentelė. *L. multiflorum ir F. pratensis chromatino santykinis ilgis veislių 'Punia' ir 'Rakopan' augaluose*

Sample *Ėminys*	Fp chromatin % *Fp chromatino %*		Lm chromatin % *Lm chromatino %*	
	Mean *Vidurkis*	Range *Ribos*	Mean *Vidurkis*	Range *Ribos*
Field ('Punia' and 'Rakopan') *Lauko ('Punia' ir 'Rakopan')*	38.7	21.7–51.8	61.3	48.2–78.3
Random ('Punia' and 'Rakopan') *Atsitiktinis ('Punia' ir 'Rakopan')*	35.4	22.1–51.4	64.6	48.6–77.9

The numbers of breakpoints in both cultivars ranged from 12 to 46 per genotype (23.38–25.96 on average). Both varieties were approximately equal in this aspect. However, in 'Punia' had shown more terminal *Festuca* fragments in field plants, and there were significantly less terminal *Lolium* fragments in field than in random plants ($P \leq 0.05$), There were also more interstitial translocated fragments in random plants (Table 4). The higher number of breakpoints in 'Punia' genotypes of random samples is not surprising, since it was shown above that random 'Punia' plants have more recombinant chromosomes than the ones taken from the field (Table 2), but it is not easy to interpret why they favour terminal *Lolium* translocations. In previous studies, it was shown that the lowest frequency of recombination in *Lolium* and *Festuca* chromosomes is located between the centromere and nucleolar organizer regions (NORs), and relatively high frequency of recombination was observed at the distal parts of chromosomes /King et al., 2002/. The reason why there are more of terminal segments of *Festuca* and less of terminal *Lolium* genome recombinations in field plants could be that some of the freezing-tolerance and winter-hardiness genes are located on the distal parts

of *Festuca* chromosomes. From the previous studies, it is known that some of such genes are located on the terminal region of *Festuca* chromosome 3 /Humphreys, 2004/.

Table 4. Characteristic features of recombinant chromosomes in the plants of × *Festulolium braunii* cultivars 'Punia' and 'Rakopan'
4 lentelė. *Rekombinantinių chromosomų ypatybės* × *Festulolium braunii veislių 'Punia' ir 'Rakopan'* augaluose

Cultivar *Veislė*	Chromosome breakpoints *Chromosomų trūkiai*		Terminal Fp fragments *Galiniai Fp fragmentai*		Interstitial Fp fragments *Vidiniai Fp fragmentai*		Terminal Lp fragments *Galiniai Lp fragmentai*		Interstitial Lp fragments *Vidiniai Lp fragmentai*	
	Range *Ribos*	Mean *Vidurkis*	Range *Ribos*	Mean *Vidurkis*	Range *Ribos*	Mean *Vidurkis*	Range *Ribos*	Mean *Vidurkis*	Range *Ribos*	Mean *Vidurkis*
'Punia' Field / *Lauko*	12-41	23.38	3–19	8.25	0–10	3.87	1–12	6.17	0–7	2.50
Random *Atsitiktinis*	12-46	25.96	4–15	7.81	1–13	5.15	1–19	8.81	0–9	3.30
'Rakopan' Field / *Lauko*	17-41	25.65	4–14	8.09	1–8	4.09	2–19	6.78	0–9	3.65
Random *Atsitiktinis*	15-40	25.36	3–17	8.36	0–11	4.09	3–12	6.85	0–8	3.18

Conclusions

1. In the plants of × *Festulolium braunii* cv. 'Punia' and 'Rakopan' in both field and random samples there are more recombinant than pure *Lolium* or *Festuca* chromosomes, i.e. about 18–20 recombinant chromosomes are present per chromosome set, and pure *L. perenne* chromosomes exceed *F. pratensis* ones in numbers with an average of 6–7 and 2–3, respectively.

2. There are more *L. multiflorum* chromosomes in field samples of 'Punia' and 'Rakopan' plants than in random samples (6.55 and 5.65 on average, respectively, $P \leq 0.05$). In 'Punia', there are also significantly less recombinant chromosomes in the field samples (mean 18.00) than in the field ones (mean 20.16).

3. The amount of *L. multiflorum* chromatin measured by relative length in the genome of × *Festulolium braunii* plants is found to be higher from 1.6 to 1.8 times than the one *F. pratensis*, however, there are more of *F. pratensis* chromatin present in field than in random samples ($P \leq 0.05$); this finding is consistent with the better winter-resistance of *F. pratensis* than *L. multiflorum*.

4. In 'Punia' plants more terminal *Festuca* fragments are found in the field plants, and there are significantly less of *Lolium* terminal fragments in field than in random plants (6.17 and 8.81 on average, respectively).

5. In all plants, numbers of rDNA sites are lower than expected: from 5 to 14 (9 on average), instead of 16 expected coming form the parental *L. multiflorum* and *F. pratensis* species.

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BRAUNO ERAIČINSVIDRIŲ (×*FESTULOLIUM BRAUNII)* VEISLIŲ ‘PUNIA’ IR ‘RAKOPAN’ GENOMO STRUKTŪROS YPATUMAI

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Santrauka

Tyrimo tikslas buvo palyginti dviejų ×*Festulolium braunii* tetraploidinių veislių ‘Punia’ ir ‘Rakopan’ (2n = 4x = 28) augalų genominę sudėtį. Dalis šių veislių tyrimams paimtų augalų buvo atsparūs žiemojimui (4 metus lauke išgyvenę augalai) ir dalis – atsitiktinai paimti. Atliekant tyrimus buvo naudota fluorescencinė *in situ* hibridizacija (FISH), kaip zondus naudojant žymėtą *Lolium multiflorum* ir *Festuca pratensis* genominę DNR. Ribosominės DNR (rDNR) saitai buvo nustatomi fluorescencine pTA71 žyme. Abiejų veislių augalų genomuose rasta daugiau *L. multiflorum* negu *F. pratensis* chromosomų, kurių vidurkis buvo atitinkamai 6–7 ir 2–3. Visuose augaluose dauguma chromosomų nuo 18 iki 20 buvo rekombinantinės. Chromatino ilgio matavimai parodė, kad daugiau *F. pratensis* chromatino yra gerai žiemojančiuose (lauko ėminys) negu atsitiktiniuose augaluose ($P \leq 0.05$), nors visuose augaluose didesnę genomo dalį sudarė *L. multiflorum*. Taip pat veislės ‘Punia’ daugiau buvo terminalinių *F. pratensis* fragmentų lauke išgyvenusiuose augaluose. rDNR saitų buvo nuo 5 iki 14 (vidutiniškai 9), bet nė vienas augalas neturėjo 16, kiek buvo tikimasi. Tai rodo, kad ×*Festulolium braunii* augalų genomas turi polinkį prarasti rDNR saitus, vertinant pagal jų skaičių tėvinėse *L. multiflorum* ir *F. pratensis* rūšyse.

Reikšminiai žodžiai: *Festulolium, Lolium, Festuca,* FISH, GISH, ‘Punia’, ‘Rakopan’, genomų rekombinacija, rDNA.