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GENETIC DIFFERENCES BETWEEN ANNUAL AND PERENNIAL RYEGRASS REVEALED BY ISSR MARKERS AND THEIR SEQUENCE CHARACTERISTICS

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Abstract

Lolium multiflorum varieties 'Adin' and 'Zenith' and *Lolium perenne* varieties 'Veja' and 'Riikka' were assessed for inter simple-sequence repeat (ISSR) diversity. Six dinucleotide repeat sequences, 5'-$(TC)_8$A, 5'-$(AC)_8$G, 5'-$(CA)_8$RC, 5'-$(TC)_8$RA, 5'-$(TG)_8$RT, 5'-$(CA)_7$GA and two tetranucleotide repeat sequences 5'-$(GAGA)_4$GA, 5'-$(GACA)_4$TC, were used in PCR reactions as primers to develop ISSR fingerprints. In total, 57 DNA fragments were amplified within the size interval of 250-2000 bp, and the number of fragments produced in the variety's DNA profile varied from 1 to 11. Thirty seven bands were differential between *L. multiflorum* and *L. perenne* species, 11 between *L. multiflorum* varieties 'Adin' and 'Zenith' and 11 between *L. perenne* varieties 'Veja' and 'Riikka'. Only in few cases DNA profiles were identical between *L. perenne* varieties when amplified by primers $(TG)_8$RT, $(CA)_7$GA and $(GAGA)_4$GA.

One hundred and two PCR fragments ranging in size between 400–2000 bp were selected and sequenced from their clones in the pJET1.2 vector. These ISSR fragment sequences were tested for sequence homology using MEGABLAST application at the National Centre for Biotechnology Information (NCBI) data base. Twenty two sequences were found to be highly similar to genomic sequences coming from other Poaceae plants, mostly from *Oryza sativa, Avena strigosa* and *Triticum turgidum*. The most significant homology was detected to beta-amyrin synthetase, cytochrome P450 CYP51H10 (Sad2) and HMW glutenin genes.

Key words: ISSR fingerprinting, *L. multiflorum*, *L. perenne*, varieties, sequence homology search.

Introduction

Perennial ryegrass and Italian ryegrass (annual), *L. perenne* and *L. multiflorum*, are the most important forage grass species in Europe. These two species cover 23 % of the grassland area (52 million ha) in Europe with the perennial *L. perenne* being the most prevalent grass species /Lübbersted et al., 2003/. Breeding of ryegrass varieties is aimed at high yielding capacity, forage quality, resistance to adverse climatic conditions and disease resistance /Nekrošas, 2003/. Knowledge of genes controlling morphological differences between annual and perennial types helps in understanding relationships among species, assists breeders in grass improvement, and permits more accurate seed purity testing /Brown et al., 2006/.

ISSR are markers suitable for plant genome analysis due to their widespread distribution and high levels of polymorphism. This method has been successfully applied in many crop species /Reddy et al., 2002/. Pašakinskienė et al. in 1999 reported the usefulness of anchored SSRs, tetra- and dinucleotide repeats for generating species-specific and variety-specific DNA markers of the most important cereal crops: wheat, barley, rye and oat. Inter-SSR fingerprinting was also identified as a valuable new marker system for genetic studies of the *Lolium/Festuca* complex /Pašakinskienė et al., 2000/. The ISSR data have provided evidence of a genetic diversity among the tested perennial ryegrass accessions /Reddy et al., 2002; Posselt at al., 2006/. A large number of polymorphic ISSR markers were generated using appropriate primers in Tunisian perennial ryegrass /Ghariani et al., 2003/.

Molecular homologies can reflect the phylogeny of either species or genes, and are a valuable tool for systematic studies, providing information that morphology alone might not be able to provide. Molecular data reveal complexity of underlying genome pattern providing potential advantages over traditional morphological data /Howis, 2006/. For example, Larsen /2003/ cloned and analysed *ENOD40* genes (coding short peptides with a signalling function) from perennial ryegrass (*Lolium perenne*) and barley (*Hordeum vulgare*). Alignment of the *ENOD40* cDNA sequences from ryegrass and barley revealed high (77 %) nucleotide homology. Jones et al. /2001/ detected cross-species amplification of SSRs in a number of related pasture and turfgrass species, with high levels of transfer to other *Lolium* species and members of the related genus *Festuca*.

In this study, we examined the genetic ISSR polymorphism between *L. perenne* and *L. multiflorum* and provide a new data on relationship of these genomes, and demonstrate homologies of these ISSRs within the sequence data of other organisms'. The knowledge of the genetic structure of annual and perennial ryegrasses would be useful for development of new molecular tools and for improvement of existing screening methods for ryegrass breeding and testing.

Materials and methods

Plant material and DNA extraction

Two varieties of *Lolium perenne* L. and two varieties of *Lolium multiflorum* L. were obtained from the Genetic Resources collections at the Lithuanian Institute of Agriculture (Table 1). DNA samples were extracted from 2-week-old leaves following the DNA extraction protocol of Doyle and Doyle (1990). Pooled DNA samples taken from each variety comprised 15 genotypes.

PCR amplification and electrophoresis

A set of 8 oligonucleotides comprised of simple sequence repeats were tested for amplification of ISSRs. The primers' set consisted of 2 tetra-nucleotide repeats and 6 di-nucleotide repeats. Table 2 summarizes the primer sequences.

PCR amplification was performed in Thermocycler (Applied Biosystems). Each 20 μl reaction contained 50 ng of genomic DNA, 10xPCR reaction buffer, 50 mM $MgCl_2$, 10 mM dNTP, 2,5 μM of each primer and 2 unit of DyNAzyme^{TM} II DNA Polymerase (FINNZYMES). The thermal profile for ISSR amplification by PCR was: 95 ^{o}C initial denaturation for 2 min, then 40 cycles of 95 ^{o}C for 30 s, 50 ^{o}C for 1 min,

and 72 ^{o}C for 1 min. A final extension step of 6 min at 72 ^{o}C was followed by hold at 10 C. ISSR amplification products were separated by gel electrophoresis in 1.5 % agarose gels and stained with ethidium bromide. Each prime profile was estimated and scored from 3 or more of PCR replications.

Table 1. List of plant material
1 lentelė. *Augalų sąrašas*

No *Nr.*	Species *Rūšis*	Variety *Veislė*	Chromosome number *Chromosomų skaičius*	Country of origin *Kilmės šalis*
1	*L. multiflorum*	Adin	2n = 2x = 14	Belgium *Belgija*
2		Zenith	2n = 2x = 14	The Netherlands *Nyderlandai*
3	*L. perenne*	Veja	2n = 2x = 14	Lithuania *Lietuva*
4		Riikka	2n = 2x = 14	Norway *Norvegija*

Cloning and sequencing

Amplified inter-SSR fragments were excised and purified using the GenElute™ Gel DNA Extraction Kit from Sigma. Fragments were cloned using pJET1.2 cloning vector and the CloneJET™ PCR Cloning Kit (Fermentas) and transformed into *E. coli* DH5ά ultracompetent cells with the ligation mix and selected for ampicillin resistance. Plasmid DNA was extracted from overnight cultures using the QIAprep Spin Miniprep kit (QIAGEN). A total of 102 colonies representing different primer combinations were arbitrarily selected and sequenced using the ABI PRISM 377 (Applied Biosystems) at the Sequencing Centre (Institute of Biotechnology, Vilnius).

Computer analysis of sequences

DNA sequence alignment was performed using MEGABLAST program application at the website of National Centre for Biotechnology Information (NCBI) (http:\\www.ncbi.nlm.nih.gov\).

Results and discussions

Species and variety differentiation by ISSR fingerprints

Among the 8 primers tested for their ability to detect genetic polymorphism in *L. perenne* and *L. multiflorum*, all of them have generated polymorphic ISSR fragments. A total of 57 amplified bands were scored at a size range between 250–2000 bp. Out of 57 fragments 37 bands were found to be differential between *L. multiflorum* and *L. perenne* species, 11 between *L. multiflorum* varieties ‘Adin' and ‘Zenith' and 11 between *L. perenne* varieties ‘Veja’ and ‘Riikka’ (Table 2). Earlier 136 polymorphic bands were detected out of 141 amplified and considered as ISSRs markers suitable for assessing the genetic diversity in Tunisian ryegrass ecotypes /Ghariani et al., 2003/.

In our study the number of amplified products generated by a primer varied from 1 to 11, and most of the primers yielded more than 6 bands per primer. The primers $(TC)_8RA$, $(CA)_7GA$ and $(GAGA)_4GA$ generated 6 bands ranging between 400–2000 bp

in size. The highest number, 11 reproducible and polymorphic bands was produced by the primer $(AC)_8G$. In comparison, Pašakinskienė et al. /2000/ found $AC(GACA)_4$ primer of greatest potential for identifying genotypic variation based on differences in ISSR fragment length within *L. perenne* and *F. pratensis.*

Table 2. ISSR products generated by 8 primers in ryegrasses *L. perenne* and *L. multiflorum*
2 lentelė. *Svidrių L. perenne ir L. multiflorum ISSR rezultatai, gauti naudojant 8 pradmenis*

Primer sequence *Pradmens seka*	MW range bp *Fragmentų ilgis bp*	No of reprod. bands *Reprd. fragmentų sk.*	Differential bands between *L.multiflorum* and *L. perenne* *Skirtingų fragmentų sk. tarp L.multiflorum ir L. perenne*	Differential bands between varieties *Skirtingų fragmentų sk. tarp veislių*	
				Adin/Zenith	*Veja/Riika*
$(TC)_8A$	500–1300	5	5	2	3
$(AC)_8G$	350–1120	11	9	1	2
$(CA)_8RC$	250–900	8	4	3	1
$(TC)_8RA$	400–1400	6	3	1	3
$(TG)_8RT$	490–1080	8	2	1	0
$(CA)_7GA$	600–1200	6	5	1	0
$(GAGA)_4GA$	500–2000	6	5	1	0
$(GACA)_4TC$	700–1500	7	2	1	2
Total / *Iš viso*	350–1500	57	37	11	11

DNA profiles of ryegrasses produced by primer $(AC)_8G$ consisted of 11 fragments ranging in size between 350–1120 bp. Comparing *L. multiflorum* and *L. perenne* profiles 9 polymorphic differential bands were found, which is the highest number of differential bands between *L. multiflorum* and *L. perenne* determined in this study. *L. multiflorum* varieties' profiles were almost identical, there was only one distinct band uniqe to 'Adin'. In *L. perenne* varieties, 2 distinct differential fragments (400 bp and 1120 bp) were present in 'Riikka', but not in 'Veja'.

Using the tetranucleotide primer $(GAGA)_4GA$, 6 reproducible bands were obtained in DNA profiles. We found 5 polymorphic fragments between *L. multiflorum* and *L. perenne*. *L. multiflorum* had 500 bp, 590 bp, 900 bp, 1650 bp and 2000 bp bands which made distinguishable it from *L. perenne.* No difference was found between varieties 'Adin' and 'Zenith', but *L. perenne* varieties had one differential fragment 1031 bp present in 'Veja'.

Another tetranucleotide primer $(GACA)_4TC$ generated 7 reproducible bands between 700–1500 bp in size. Two differential bands were obtained in the profiles of *L. multiflorum* and *L. perenne.* Two differential bands were distinguished between 'Veja' and 'Riikka' profiles (1200 bp and 1500 bp), and one between 'Adin' and 'Zenith' (1031 bp).

While using $(TC)_8A$ primer in PCR, 5 reproducible bands were found in DNA profiles of ryegrasses, their size ranging from 500 to 1300 bp. The DNA profiles were clearly differential between species – 5 distinct fragments. Two distinct fragments were found between 'Adin' and 'Zenith' (1200 bp and 1300 bp) and 3 distinct fragments between 'Veja' and 'Riikka' (700 bp, 1031 bp and 1200 bp).

$(CA)_8RC$ primer generated fragments within a narrow range, between 250–900 bp. Four differential bands were determined between species. Differences were found between ISSR profile of varieties: 'Veja' had a distinct 600 bp fragment comparing to 'Riikka', and 'Zenith' had distinct fragments of 700 bp and 800 bp comparing to 'Adin', whereas 'Adin' had 750 bp fragment which was not present in 'Zenith'. $(TC)_8RA$ primer generated 6 reproducible bands, three differential fragments (800 bp, 1031 bp and 1200 bp) were found between species and one between 'Adin' and 'Zenith' – 600 bp. Three polymorphic fragments were found between 'Veja' and 'Riikka'(500 bp, 600 bp and 800 bp).

$(TG)_8RT$ primer produced eight reproducible bands. Only two distinct fragments were found between species and one distinct fragment was between 'Adin' and 'Zenith' (1080 bp), but no discriminating fragments were found between *L. perenne* varieties.

$(CA)_7GA$ generated 6 reproducible bands, ranging from 600 bp to 1200 bp. Five differential bands were found between species and one between varieties 'Adin' and 'Zenith' – 750 bp. The DNA profiles of *L. perenne* varieties were identical.

Pašakinskienė et al. /2000/ demonstrated that overall *L. multiflorum* and *L. perenne* had the most bands in common, but distinct *L. perenne* markers of 1.2 kb, amplified using $(GACA)_4GT$ and of 0.78 kb were amplified by $(AGAC)_4GC$; in addition it was shown that diploid varieties of *L. multiflorum* had apparently the same DNA profiles as tetraploid varieties.

Sequence homology assessment

We selected and cloned 102 ISSR fragments from *L. multiflorum* and *L. perenne*, and sequenced them at the Sequencing Centre (Institute of Biotechnology, Vilnius). Obtained sequences were examined for the sequence homology at the National Centre for Biotechnology Information (NCBI) data base using MEGABLAST search which is optimized for highly similar sequences. In total, sequence alignments had shown 134 high score matches to the known genomic sequences from plants, animals or microbes. Most important matching sequences which come from Poaceae plants are summarized in Table 3. Particularly high-scoring segment pairs were produced from plants such as *Avena strigosa, Hordeum vulgare, Triticum turgidum* and *Oryza sativa*. Armstead et al. /2004/ identified a region of genetic syntheny between rice and *L. perenne,* which contains the Hd3 heading-date in *L. perenne.* Recently, it was demonstrated that the crown rust resistance region (which had been introgressed from meadow fescue into the Italian ryegrass background) of *Lolium/Festuca* genome has a degree of conserved genetic syntheny either in chromosomes 11 and 12 of rice /Armstead et al., 2006/. Such identification of syntheny between rice and *L. perenne* demonstrates the direct applicability of the rice genome to the understanding of biological processes in other species. We found that 766 bp DNA fragment amplified from 'Veja' with primer $(AC)_8G$) was similar to genomic DNA *Oryza sativa*, chromosomes: 1, 2, 4, 7, 8 and 11. Such fragment which appears throughout many chromosomes could possibly derive from highly dispersed repetitive sequence.

Table 3. Summary of the search of ISSR fragment homologies in NCBI sequence data bases
3 lentelė. *Sekvenuotų ISSR fragmentų homologijos su žinomomis NCBI sekomis (paieškos rezultatų santrauka)*

Primer/ Variety *Pradmuo/ Veislė*	Length of PCR product (bp) *PGR fragm. ilgis (bp)*	Genebank accession number *Sekos registracijos Genų banke nr.*	Similarity to coding regions (MEGABLAST search) *Panašumas į koduojančius regionus (MEGABLAST paieška)*
$(TG)_8RT$ Veja	707	DQ680849.1	*Avena strigosa* beta-amyrin synthetase (Sad1) and cytochrome P450 CYP51H10 (Sad2) genes, complete cds, E=5e-98, I=355/426
		DQ249273.1	*Hordeum vulgare* subsp. vulgare cultivar Morex BAC 631P8, complete sequence, E=3e-56, I=328/421
Riikka	800	Q680949.1	*Avena strigosa* beta-amyrin synthase (Sad1) and cytochrome P450 CYP51H10 (Sad2) genes, complete cds, E=2e-92, I=234/256
		X64100.1	*S.cereale* DNA for dispersed repeat sequence (R173-1), E=3e-86, E=234/261
		AY146587.2	*Triticum turgidum* subsp. durum Pm3 locus, genomic sequence, E=1e-45, I=216/266
		AY94981.1	*Triticum turgidum* A genome HMW glutenin A gene locus, sequence, E=1e-44, I=210/258
$(AC)_8G$ Veja	766	AP008210.1	*Oryza sativa* (japonica cultivar-group) genomic DNA, chromosome 4, E=2e-33, I=114/128
		AL662960.3	*Oryza sativa* genomic DNA, chromosome 4, BAC clone: OSJNBa0021F22, complete sequence, E=2e-33, I=114/128
$(GACA)_4TC$ Veja	809	DQ680849.1	*Avena strigosa* beta-amyrin synthase (Sad1) and cytochrome P450 CYP51H10 (Sad2) genes, complete cds, I=620/733
		AY494981.1	*Triticum turgidum* A genome HMW glutenin A gene locus, sequence, E=7e-38, I=290/381
		AF459639.1	*Triticum monococcum* BAC clones 116F2 and 115G1 gene sequence, E=7e-38, I=300-396
		AY354123.1	*Aegilops tauschii* transposons Caspar, XJ, Angela, and XJ, complete sequence; complete cds; transposons Ophelia2, Angela3s, and XJ3, complete sequence; LRR protein WM1.3 (WM1.3) gene, complete cds; E=1e-34, I=288/381
		EF459639.1	*Triticum urartu* clone BAC 404H6 genomic sequen ce, I=1e-35
$(CA)_8RC$ Adin	575	AP008209.1	*Oryza sativa* (japonica cultivar-group) genomic DNA, chromosome 3, E=4e-14, I=105/131
		AP008209.1	*Oryza sativa* (japonica cultivar-group) genomic DNA, chromosome 3, E=4e-14, I=105/131
Zenith	606		
$(TC)_8A$ Veja	788	AP008210.1	*Oryza sativa* (japonica cultivar group) genomic DNA, chromosome 4, I=2e-33

The most important homologies were determined for *Avena strigosa* amyrin-synthetase (*Sad1*) and cytochrome P450 CYP51H10 (*Sad2*) genes, *Triticum turgidum* A genome HMW glutenin A gene locus, and *Aegilops tauschii* transposon sequences Caspar, XJ, Angela and others.

Conclusions

1. The genetic differences between two closely related *Lolium* species, *L. multiflorum* and *L. perenne*, were reveald by ISSR fragment length polymorphism using $(TC)_8A$, $(AC)_8G$, $(CA)_8RC$, $(TC)_8RA$, $(TG)_8RT$, $(CA)_7GA$, $(GAGA)_4GA$ and $(GACA)_4TC$ primers; primer $(AC)_8G$ generated the highest number of differential fragments, 9 out of 11in total.

2. ISSR profiles of *L. multiflorum* varieties 'Adin', and 'Zenith' were richer in fragments than the ones of *L. perenne* varieties 'Veja' and 'Riikka'. *L. perenne* varieties' DNA profiles were more identical than *L. multiflorum* varieties' DNA profiles.

3. As assessed by sequence alignment in NCBI data base, obtained ISSR fragments were found highly matching to the genomic sequences coming from other Poaceae plants, mostly from *Oryza sativa, Avena strigosa* and *Triticum turgidum*; significant homology was detected to beta-amyrin synthetase, cytochrome P450 CYP51H10 (*Sad2*) and HMW glutenin genes, and some transposon sequences.

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GAUSIAŽIEDĖS IR DAUGIAMETĖS SVIDRĖS GENETINIS SKIRTINGUMAS PAGAL ISSR ŽYMENIS IR JŲ SEKŲ ĮVERTINIMAS

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Santrauka

Dviejų svidrių rūšių *Lolium multiflorum* ir *Lolium perenne* veislių ‘Adin’ ir ‘Zenith’, ‘Veja’ ir ‘Riikka’ tyrimams buvo panaudoti aštuoni paprastų pasikartojančių sekų pradmenys. PGR metodu amplifikuoti 57 DNR fragmetai. Skirtingose svidrių veislių DNR profiliuose fragmentų skaičius kito nuo 1 iki 11, o jų dydžiai nuo 250 iki 2000 bp. *L. multiflorum* ir *L. perenne* rūšys skyrėsi 37 gautais DNR fragmentais, o *L. multiflorum* ir *L. perenne* veislėse buvo rasta po 11 skirtingų DNR fragmentų. Identiški DNR profiliai rasti tarp *L. perenne* amplifikacijai naudojant šiuos pradmenis: $(TG)_8RT$, $(CA)_7GA$ ir $(GAGA)_4GA$.

Svidrių DNR profiliuose buvo pasirinkti 102 ISSR fragmentai, kurie buvo klonuoti transformacijos būdu į plazmidę pJET1.2 ir sekvenuoti. MEGABLAST paieška DNR duomenų bazėse rado reikšmingų panašumų tarp gautų ISSR fragmentų ir *Oryza sativa, Avena strigosa, Triticum turgidum* žinomų sekų koduojančiose genomo srityse. Reikšmingiausios homologijos nustatytos su beta-amirino sintetazės, citochromo P450 CYP51H10 (Sad2) ir DMM gliuteninų genų sekomis, taip pat su kai kuriomis transpozoninėmis sekomis.

Reikšminiai žodžiai: ISSR DNR atspaudai, *Lolium multiflorum*, *Lolium perenne*, veislės, sekų holomologijos, MEGABLAST.