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GENETIC DIVERSITY ASSESSMENT IN PERENNIAL RYEGRASS AND *FESTULOLIUM* BY ISSR FINGERPRINTING

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Abstract

An essential prerequisite to cultivar identification is to determine whether cultivars are differentiated genetically. We investigated genetic diversity among sixteen perennial ryegrass (*Lolium perenne* L.) cultivars and one breeding line (Lithuanian ʻVejaʼ, ʻAlduvaʼ, ʻElenaʼ, ʻRaminta', ʻSodrėʼ, ʻVerseka', ʻŽvilgėʼ, 3703 and foreign: ʻGladioʼ, ʻLimesʼ, ʻPimpernelʼ, ʻRastroʼ, ʻVincentʼ, ʻAcentoʼ, ʻAlligatorʼ, ʻCastelʼ, ʻToveʼ) and two breeding lines of *Festulolium* L. (3223, 3227) using inter simple sequence repeat (ISSR) markers, with the goal of determining whether cultivars could be differentiated on the basis of genetic data. Genetic similarity in the computed matrix (Nei, Li, 1979) between *L. perenne, Festulolium* L. cultivars and breeding lines ranged from 0.80–0.93. Cluster analysis (UPGMA) revealed two main groups of *L. perenne* and separated a group of *Festulolium.*

Our results indicated significant level of genetic diversity between cultivars and breeding lines assessed by ISSR polymorphism from varieties' bulked DNA samples. The use of the thirty three primers yielded 220 reproducible bands and 159 (71 %) of them were polymorphic. The study demonstrated that ISSR fingerprinting is an efficient approach for genotyping perennial ryegrass cultivars. These results provide important baseline for future improvement of grass breeding programs.

Key words: ISSR markers, *Lolium perenne*, *Festulolium* L., cultivar, genetic similarity, polymorphism.

Introduction

L. perenne is a diploid obligate outbreeder that maintains a high degree of genetic diversity in natural and agricultural populations /Gill et al., 2006/. Close relationships and considerable genome homology are found between the ryegrasses and fescues, and this has allowed breeders to make hybrids and to develop new types of forage grasses called *Festuloliums* /Nekrošas, Kemešytė, 2007/. Since the advent of turf-type cultivars in the 1960s, the development and release of perennial ryegrass cultivars has increased steadily, making cultivar identification and property right protection difficult. Firstly, cultivar improvement has been based on limited germplasm, and thus many cultivars are both phenotypically and genetically similar. Secondly, perennial ryegrass is a diploid (2n = 2x = 14), self-incompatible species, and therefore each cultivar is a heterogeneous scope of many genotypes /Kubik et al., 2001/. Many recent cultivars of *L. perenne* are tetraploids (2n = 4x = 28) produced by colchicine treatment

for chromosome doubling /Nekrošas, Kemešytė, 2007/. So far, description and classification of perennial ryegrass cultivars and ecotypes has been mainly based on morphological traits or isozymes, but it can be difficult and time consuming to distinguish perennial ryegrass cultivars by morphological characteristics alone, and for this reason it is important to develop molecular markers to aid cultivar identification /Bolaric et al., 2005/.

Nowadays, many molecular techniques are being used to distinguish cultivars in crop plants. Various DNA-based marker systems have been applied to several plant groups to assess their level of relatedness and for development of marker-assisted selection, and cultivar discrimination /Pašakinskienė et al., 2000; Ghariani et al., 2003; Paplauskienė et al., 2007; Xiong et al., 2007/. Over the recent years, DNA fingerprinting has significantly improved for the application in cross-pollinating crops. For example, RAPD markers have been successfully employed for the establishment of genetic diversity and phylogenetic relationships between 42 accessions of spring and winter rye from fourteen different countries /Ma et al., 2004/.

One of the most efficient molecular marker methods in terms of ability to produce polymorphic markers within a comparatively short time and with a limited budget is ISSR (Inter Simple Sequence Repeat) profiling for total genomic DNA. ISSRs have become widely used in many plant species and have a potential to contribute in breeding, genetics and systematics /Cekic et al., 2001; Reddy et al., 2002; Posselt et al., 2006/. ISSRs were the choice among the many classes of available molecular markers because they do not require preliminary sequence information, they are less prone to laboratory conditions than RAPDs, they have been successfully used in similar studies in plant species, and are amenable to be upgraded to co-dominant markers in the form of microsatellites /Meloni et al., 2006/. Blair et al., 1999/ proved that a higher percentage of polymorphic bands was produced with the ISSR technique than the AFLP method, based on a similar PCR reaction among rice cultivars.

From this points of view, we studied and elucidated the relationships among the Lithuanian cultivars and one breeding line of *L. perenne* and the cultivars of foreign origin, and two breeding lines of *Festulolium* L. using ISSR genotyping.

Materials and Methods

Plant material and DNA extraction

A total of 17 perennial ryegrass accessions (*L. perenne*), six Lithuanian cultivars, one breeding line (3703), and eight cultivars of foreign origin were analysed in this study (Table 1). For comparison, two *Festulolium* L. breeding lines (*L. multiflorum* x *F. pratensis*) were included in this experiment. The bulk sample of leaf tissue of about 3 g was taken from 25–30 plants of each cultivar or breeding line. Three replicates of bulked total genomic DNA for each cultivar was isolated from 0.7 g of leaf tissue. DNA samples were extracted following the DNA extraction protocol of Doyle and Doyle (1990).

Table 1. Description of perennial ryegrass and *Festulolium* cultivars and breeding lines
1 lentelė. *Daugiamečių svidrių ir eraičinsvidrių veislių ir selekcinių linijų charakteristika*

No. *Nr.*	Cultivar / breeding line *Veislė / selekcinė linija*	Chromosome number *Chromosomų skaičius*	Country of origin *Kilmės šalis*
		Perennial ryegrass / *Daugiametės svidrės*	
		Lithuanian / *Lietuviškos*	
1.	‘Veja’	2n = 2x = 14	Lithuania
2.	‘Alduva’	2n = 4x = 28	Lithuania
3.	‘Elena’	2n = 4x = 28	Lithuania
4.	‘Raminta’	2n = 4x = 28	Lithuania
5.	‘Sodrė’	2n = 4x = 28	Lithuania
6.	‘Verseka’	2n = 4x = 28	Lithuania
7.	‘Žvilgė’	2n = 4x = 28	Lithuania
8.	3703	2n = 4x = 28	Lithuania
		Foreign / *Užsienio šalių*	
9.	‘Gladio’	2n = 2x = 14	Germany
10.	‘Limes’	2n = 2x = 14	Germany
11.	‘Pimpernel’	2n = 2x = 14	Estonia
12.	‘Rastro’	2n = 2x = 14	Germany
13.	‘Vincent’	2n = 2x = 14	United Kingdom
14.	‘Acento’	2n = 4x = 28	Germany
15.	‘Alligator’	2n = 4x = 28	Germany
16.	‘Castel’	2n = 4x = 28	Germany
17.	‘Tove’	2n = 4x = 28	Italy
		Festulolium / *Eraičinsvidrės*	
1.	3223	2n = 4x = 28	Lithuania
2.	3227	2n = 4x = 28	Lithuania

DNA amplification and electrophoresis

PCR amplifications were performed in 20 µl reaction contained 1 µl (50 ng) of genomic DNA, 2 µl (10xPCR) reaction buffer, 0.8 µl (50 mM) $MgCl_2$, 0.4 µl (10 mM) dNTP, 2 µl (2.5 µM) of each primer and 1 µl (2 units) of DyNAzyme™ II DNA polymerase (Finnzyme, Finland). PCR was performed in a Thermocycler (Applied Biosystems, USA) using one of the following profile, 95 °C initial denaturation for 2 min., then 40 cycles of 95 °C for 30 s, a 1 minute annealing step at either 50, 52, 54 °C, depending on the Tm value of the primer pair, and 72 °C for 1 min. (Table 2). The PCR was finished with a 6 min. elongation step at 72 °C (Table 2).

ISSR fragments were separated by gel electrophoresis in 1.5 % agarose gel and stained with ethidium bromide. Samples were run for 2 h at 4V/cm in TAE buffer. Each prime profile was estimated and scored from 3 or more PCR replications. Fragment sizes were estimated relative to GeneRuler™ DNA Ladder Mix (Fermentas, Vilnius).

Thirty three anchored primers were used to generate ISSRs: 21 primers were composed of di-nucleotide repeats, 6 of tri-nucleotide repeats and 6 of tetra-nucleotide repeats listed in Table 2.

Statistical analysis

The gel images were scored in a dominant manner recording the presence or absence as 1 and 0, respectively. The data were exported into a spreadsheet and was calculated based on the genetic similarity matrix /Nei, Li, 1979/. A dendrogram for 16 cultivars and one breeding line of *L. perenne* and two breeding lines of *Festulolium* L. was constructed from the similarity matrix using Unweighted Pair Group of Arithmetic Mean (UPGMA) procedure of the SAHN clustering method /Sneath, Sokal, 1973/. All computations were performed with the Numerical Taxonomy and the Multivariate Analysis System, NTSYSpc v. 2.2 cluster analysis software /Rohlf, 2005/.

Results and Discussion

Genetic variability

The information obtained by the analysis of the banding pattern is summarized in Table 2. Thirty three ISSR primers used in this study yielded 220 reproducible amplification products in the range 290–2000 bp after amplification of total genomic DNA of seventeen accessions of *L. perenne* L. Six primers, namely 155H, UBC 824, UBC 826, UBC 856, G03, 104H generated the highest ISSR banding pattern polymorphism (100 %) among the perennial ryegrass cultivars. UBC 811 and UBC 851 gave the lowest polymorphism (14–20 %). Primers 155H, UBC 810, UBC 824, UBC 822, UBC 827, UBC 847, UBC 856, G03, G11, G02, G04 and105H revealed from 9 to 6 polymorphic bands.

A. P. Davierwala et al. (2000) reported that ISSR primers such as UBC 807, 808, 809, 810, 811, 812, 814, 834, 835, 836, 847 and 850 were useful in rice studies and produced good amplification and polymorphic patterns. The primers UBC 807 and UBC 808 produced nine bands each, all of which were polymorphic. The primer UBC 811 resulted in amplification of seven bands, all of which were polymorphic in their study. In our study, the primer UBC 807 resulted seven bands in total and only three of them were polymorphic among *L. perenne* cultivars, and primer UBC 811 gave seven bands in total and only one of them was polymorphic.

The degree of polymorphism detected was high, showing that the ISSR markers were efficient in detecting genetic variability between the cultivars studied. I. Paša-kinskienė et al. (2000), S. Ghariani et al., (2003) and U. K. Posselt et al. (2006) have already reported on the efficiency of ISSR markers in identifying cultivars and genotypes of perennial ryegrass. In comparison, P. M. Sweeney and T. K. Danneberger (2000) indicated that useful segregating markers were difficult to find using Restriction Amplification Fragment Length Polymorphism (RFLP) in perennial ryegrass.

Table 2. ISSR products generated by 33 primers in ryegrass *L. perenne*
2 lentelė. *Svidrių L. perenne rezultatai, gauti naudojant 33 ISSR pradmenis*

ISSR primer / code *ISSR pradmuo / kodas*	Anneal. temp. °C *Kait. temp. °C*	Size range of DNA fragments bp *DNR fragmento ribos bp*	No of total fragments *Bendras fragmentų skaičius*	No of polymorphic fragments *Polimorfiškų fragmentų skaičius*	Poly-morphism % *Polimorfizmas %*
Di-nucleotide repeats / *dinukleotidiniai pasikartojimai*					
G10 $(AG)_{12}$	54	500–1031	4	2	50
155 H $(CA)_7GA$	50	500–1200	8	8	100
UBC 807 $(AG)_8T$	50	400–1031	7	3	43
UBC 810 $(GA)_8T$	50	490–1300	8	7	86
UBC 811 $(GA)_8C$	50	290–1100	7	1	14
UBC 814 $(CT)_8A$	50	710–1400	5	4	80
UBC 822 $(TC)_8A$	50	500–1200	9	8	89
UBC 823 $(TC)_8C$	50	500–1200	6	4	67
UBC 824 $(TC)_8G$	50	550–1200	7	7	100
UBC 825 $(AC)_8T$	50	700–1200	6	3	50
UBC 827 $(AC)_8G$	50	390–1350	8	6	75
UBC 826 $(AC)_8C$	50	350–700	5	5	100
UBC 834 $(AG)_8YT$	50	300–800	6	3	50
UBC 847 $(CA)_8RC$	50	390–1500	7	6	87
UBC 848 $(CA)_8RG$	50	300–1200	7	5	71
UBC 851 $(GT)_8YG$	50	900–1850	5	1	20
UBC 852 $(TC)_8RA$	50	400–950	5	4	80
UBC 856 $(AC)_8YA$	52	400–1300	6	6	100
UBC 857 $(AC)_8YG$	50	510–1500	5	2	40
UBC 860 $(TG)_8RA$	50	400–1300	7	3	43
UBC 858 $(TG)_8RT$	50	700–1031	5	2	40
Tri-nucleotide repeats / *trinukleotidiniai pasikartojimai*					
G03 $(TCC)_5GT$	52	460–2000	8	8	100
G07 $(GAA)_5CG$	50	590–1500	7	3	43
G08 $(ATG)_5GA$	52	490–900	5	4	80
G11 $(CAA)_5GC$	50	500–1250	8	7	86
UBC 864 $(ATG)_6$	52	490–1031	3	2	67
UBC 866 $(CTC)_6$	50	400–1200	6	4	67
Tetra-nucleotide repeats / *tetranukleotidiniai pasikartojimai*					
77 H $(AGAC)_4GC$	50	680–2000	6	5	83
78 H $(GACA)_4AC$	50	550–1500	8	6	75
G02 $(ACTG)_4GA$	50	550–1800	10	7	70
G04 $(GACA)_4TC$	50	600–1300	10	9	90
104 H $(GACA)_4GT$	50	700–600	6	6	100
105 H $(GAGA)_4GA$	50	600–1300	10	8	80
In total / *Iš viso*		290–2000	220	159	
Mean / *Vidurkis*			6.7	5	71

Genetic diversity

The clearest 220 amplified bands were treated as dominant genetic markers. For each sample ISSR bands were scored as 1 (present) or 0 (absent) and these binary data were used to assemble a rectangular matrix. The use of UPGMA algorithm permitted to cluster the data and to draw the relationships between the tested accessions.

The relationships among the *L. perenne* cultivars and breeding line, and *Festulolium L.* breeding lines are shown in the dendrogram (Figure). The computed genetic similarity between cultivars and breeding lines ranged from 0.80–0.93. The dendrogram reported in Figure 1 indicated the genetic divergence and supports the varietal clustering. The identified groups supported three main divergent clusters significantly. The first cluster consists of ʻPimpernelʼ and ʻVejaʼ. The second cluster is composed of three subclusters (A, B, C): the subcluster A consists of ʻAcentoʼ, ʻGladioʼ, ʻCastelʼ, ʻRaminta', and ʻLimesʼ; the subcluster B consists of ʻŽvilgėʼ, ʻAlduvaʼ and ʻToveʼ, and the subcluster C is composed of ʻSodrėʼ and 3703. The third cluster is composed of two breeding lines of *Festulolium*, and it is clearly separated from all accessions of *L. perenne*.

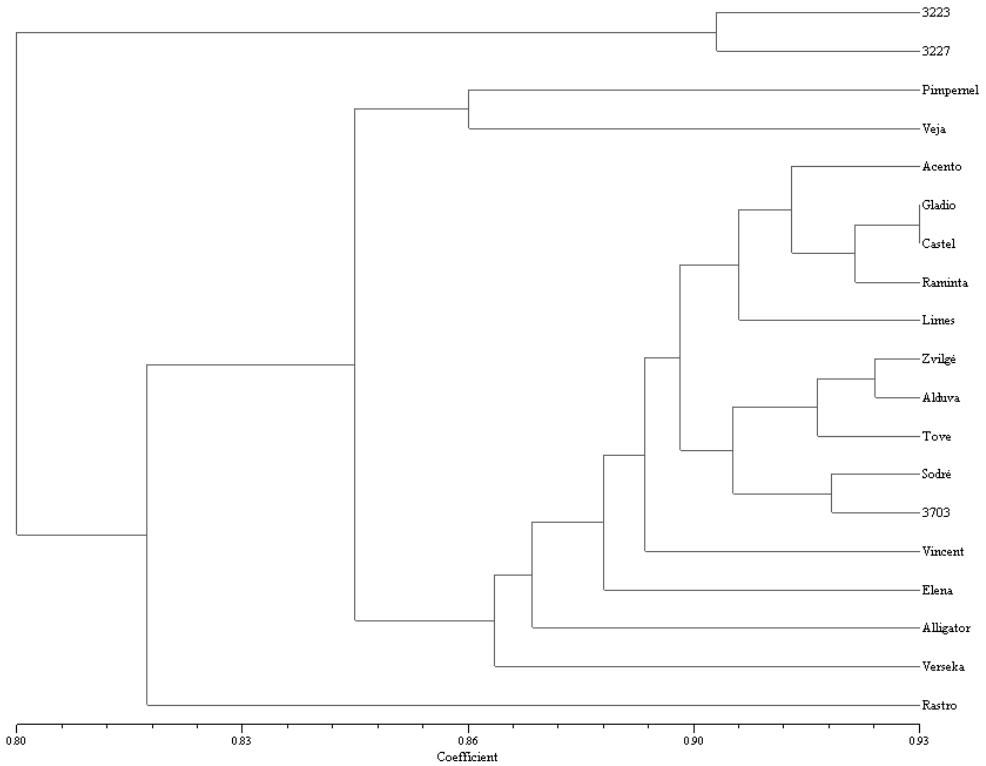

Figure. A dendrogram of the 16 ryegrass cultivars, one breeding line and two breeding lines of *Festulolium* L. generated from ISSR data and estimated according to the Nei & Li's formula (1979)

Paveikslas. *16 svidrių veislių, vienos selekcinės linijos ir dviejų eraičinsvidrių selekcinių linijų dendrogama ISSR duomenis įvertinus pagal M. Nei ir W. H. Li formulę (1979)*

The cultivar ʻŽvilgėʼ showed the highest genetic similarity to the cultivar ʻAlduvaʼ. According to the breeding development methods, the cultivar ʻŽvilgėʼ was developed by individual selection and hybridization method from the seed sample of Dutch origin in 1978. The cultivar ʻAlduvaʼ was developed by self-pollination method from Dutch varieties ʻEliteʼ 502ʼ and ʻBerlatanaʼ and Lithuanian breeding lines 869 and 870 in 1991 /Nekrošas, Kemešytė, 2007/. The cultivars ʻŽvilgėʼ and ʻAlduvaʼ sheared a set of 181 fragments in total and only 23 (13 %) were present or absent differently. Two German cultivars, ʻGladioʼ and ʻCastelʼ, were founded to be closely related as well, they sheared a set of 180 fragments in total and only 17 (9 %) were present or absent differently.

Recently, ISSR fragments were mapped to five out of seven linkage groups of *L. perenne* /Pivorienė et al., 2008/. In this study, we applied the ISSR profiling technology in order to enlarge the number of molecular markers that are suitable in the molecular characterization and examination of the genetic relationships between the cultivars of perennial ryegrass. The present work provides evidence that the ISSRs appear to be effective to explore the molecular polymorphism and to assess the genetic relationships in perennial ryegrass.

Using ISSR assessment, it was demonstrated that most of the cultivars can be easily distinguished. Moreover, some fragments were uniquely amplified or absent in some of the cultivars. These fragments are of great interest in optimal management and genetic identification of *L. perenne* accessions in the germplasm collection. Over all our data extends the knowledge of ISSR application as a molecular tool in perennial ryegrass as reported in previous studies /Ghariani et al., 2003; Posselt et al., 2006; Pivorienė, Pašakinskienė, 2007/.

Conclusions

1. The ISSR markers were polymorphic at the level of 71 %, and they are shown to be useful markers for the bulk total DNA profiling in assessment of genetic polymorphism of perennial ryegrass cultivars.

2. Six primers, namely 155H, UBC 824, UBC 826, UBC 856, G03 and 104H, generated the highest ISSR banding pattern polymorphism (100 %) among the perennial ryegrass cultivars.

3. Our data provide evidence of a genetic diversity between the tested *L. perenne* cultivars. Cluster analysis (UPGMA) revealed two main groups of *L. perenne* accessions and clearly distinguished them from *Festulolium* L.

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DAUGIAMEČIŲ SVIDRIŲ IR ERAIČINSVIDRIŲ GENETINĖS ĮVAIROVĖS ATSKLEIDIMAS NAUDOJANT ISSR ŽYMENIS

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Santrauka

Identifikuojant veisles labai svarbu nustatyti genetinius skirtumus. Naudojant įprastų pasikartojančių sekų intarpų (ISSR) žymenis buvo tirta šešiolikos daugiamečių svidrių veislių ir vienos selekcinės linijos (lietuviškų 'Veja', 'Alduva', 'Elena', 'Raminta', 'Sodrė', 'Verseka', 'Žvilgė', 3703 ir užsienietiškų 'Pimpernel', 'Rastro', 'Acento', 'Gladio', 'Castel', 'Tove', 'Vincent', 'Limes', 'Alligator') bei dviejų *Festulolium* L. selekcinių linijų (3223, 3227) genetinė įvairovė. Pagal M. Nei ir W. H. Li formulę (1979) apskaičiuotas genetinis panašumas tarp *L. perenne*, *Festulolium* L. veislių ir selekcinių linijų siekė nuo 0.80 iki 0.93. Gauti rezultatai (UPGMA) atskleidė dvi pagrindines *L. perenne* grupes ir atskyrė vieną atskirą *Festulolium* L. grupę.

Panaudojus trisdešimt tris pradmenis gauta 220 fragmentų, iš kurių 159 (71 %) buvo polimorfiški. Tyrimai atskleidė reikšmingą veislių ir selekcinių linijų genetinę įvairovę ir parodė, kad ISSR metodas yra efektyvus daugiamečių svidrių veislių DNR polimorfizmui nustatyti iš jungtinio veislės DNR mėginio. Ateityje gauti duomenys gali būti pritaikyti kuriant pašarinių žolių selekcines programas.

Reikšminiai žodžiai: ISSR žymenys, *Lolium perenne*, *Festulolium* L., veislė, genetinis panašumas, polimorfizmas.