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MOLECULAR CHARACTERIZATION OF INTERSPECIFIC CLOVER HYBRIDS USING ISSR MARKERS

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Abstract

The aim of the present study was to employ ISSR (Inter-Simple Sequence Repeats) markers for the identification of hybrids in the crosses between the clover species *T. pratense* x *T. diffusum* and *Trifolium ambiguum* x *T. hybridum.* We evaluated ISSR profiles of the F_1 interspecific hybrids *T. pratense* x *T. diffusum, T. ambiguum* x *T. hybridum* and compared them with their parental species.

Thirteen primers out of 17 tested generated characteristic ISSR profiles which differentially marked the two clover species, *T. pratense* and *T. diffusum*. Four types of ISSR profiles were found in the interspecific hybrids of *T. pratense* x *T. diffusum*: 1 – representation of both *T. pratense* and *T. diffusum* fragments; 2 – representation of *T. pratense* and *T. diffusum* and 'novel' DNA fragments which are not present in the parental species; 3 – identical to *T. pratense*; 4 – identical to *T. diffusum*.

Primers used for analysis of *T. ambiguum* and *T. hybridum* generated species-specific ISSR fragments, but the DNA profile of the hybrids was identical, in most of the cases (82.5 %), to these of *T. ambiguum*. The trinucleotide primer $(TCC)_5GT$ produced discriminative specific fragments for the parents, and these specific fragments were retained and followed in most of the hybrids (97.5 %). Similar data was obtained when the dinucleotide primers $(GA)_8CT$ and $(TC)_8G$ were used. The $(TCC)_5GT$ and $(GA)_8CT$, and $(TC)_8G$ primers were found to be the most suitable for the identification of interspecific hybrids of *T. ambiguum* x *T. hybridum*.

Key words: *T. pratense* x *T. diffusum, T. ambiguum* x *T. hybridum,* ISSR fingerprinting, primers.

Introduction

Wide hybridization plays an important role in the development of novel forage grass varieties. It is a way of combining useful traits from both parents, and both intergeneric and interspecific hybrids have been successfully applied in forage grass breeding /Nekrošas, Sliesaravičius, 2004/. Clover breeders are now adopting a similar strategy to improve the cultivated species *T. pratense* L., *T. repens* L. and *T. hybridum* L. by introgressing into their genomes valuable traits from their wild relatives. Wild *T. ambiguum* Bieb. has useful agronomic traits of persistency, the capacity to form rhizomes, as well as drought and disease resistance; while *T. nigrescen*s Viv. could contribute high seed yield and resistance to nematodes /Meredith et al., 1995; Abberton et al., 2003/. Data on interspecific hybridization of clovers has been available for some

time, but there are still no varieties produced by such means. B_1 and B_2 populiations of *T. repens* x *T. ambiguum* are undergoing test evaluations for yield, persistency, forage quality in the mixtures with other /Marshall et al., 2002; 2004/.

Characterization and identification of true hybrids at an early stage is mandatory in studies involving interspecific/ intergeneric hybridization. Biochemical and morphological markers are used to ascertain hybridity in many crop species /Abberton et al., 1998; Malaviya, 2004/. While the sensitivity of these markers to environmental and developmental variations limits their applicability, molecular markers can be profitably utilized to screen and identify true hybrids at an early stage. In recent years, ISSRs (Inter Simple Sequence Repeats) have become an efficient tool to study genetic diversity, somaclonal variation and phylogenetic relationships in many crop species /Zehdi et al., 2004; Pharmawati et al., 2005/. They are highly polymorphic, reproducible and cost effective, requiring no prior information of the sequence /Bornet et al., 2002/. Genetic diversity of clover species has been estimated using various DNA fingerprints methods /Bennett, Mathews, 2003; Ulloa et al., 2003/. Recently, the attempts to employ DNA markers for marking of white clover morphological characters determining productivity have been reported /Abberton et al., 2003; Marschall et al., 2003/.

The objective of this study was to carry out a search for ISSR markers suitable for the discrimination between the clover species *T. pratense* L., *T. diffusum* Ehrh., *T. hybridum* L. and *T. ambiguum*, and for the identification of interspecific hybrids *T. pratense* x *T. diffusum* and *T. ambiguum* x *T. hybridum*.

Materials and methods

The following clover species and hybrids were studied: *T. pratense* L. (2n = 14, cross-pollinator), variety 'Liepsna' – high forage yield, medium seed setting, non-resistant to diseases; *T. diffusum* Ehrh., (2n = 16, self-pollinator), wild accession – low forage yield, high seed setting, disease resistant; *T. ambiguum* Bieb., (2n = 16, 32, 48), which is rhizomatous, persistent and disease resistant; and *T. hybridum* L., 'Daubiai' (2n = 16), which is high-yielding, susceptible to diseases, and tolerant of acid soils; F_1 interspecific hybrids *T. pratense* L. x *T. diffusum* Ehrh. (2n = 15) and *T. ambiguum* Bieb. x *T. hybridum* L. (2n = 16, 24, 32).

Interspecific hybrids were developed by pollinating the emasculated *T. pratense* and *T. ambiguum* flowers with *T. diffusum* and *T. hybridum* pollen and using embryo-culture for embryo rescue /Dabkevičienė, 2000/.

DNA samples of *T. pratense*, *T. diffusum*, *T. ambiguum* and *T. hybridum* were isolated from the mixture of 30 plants. Twenty plants of interspecific hybrids were assayed with each primer. DNA was extracted from young leaves by a micro-method following the DNA extraction protocol of Doyle and Doyle /Doyle, Doyle, 1990/. Polymerase chain reactions (PCR) were carried out in 25 µl volume in an Eppendorf Master Cycler Gradient thermocycler. Amplification products were analysed in 1.5 % agarose gel, and electrophoresis was carried out in 1xTAE buffer. GeneRuler™ DNA Ladder Mix (Fermentas) was used as the DNA fragment size marker.

Results and discussion

DNA profiles of T. pratense, T. diffusum and their hybrids. For DNA fingerprinting analysis of *T. pratense, T. diffusum* and their hybrids we used 17 microsatellite tetra-, tri- and dinucleotide repeats as primers. Using tetranucleotide motif primers in the two parent species 1–6 fragments were obtained (Table 1). Species-specific fragments were obtained that allowed disrcrimination between the species, although some fragments were common to both species. However, low number of fragments were amplified by the primer $(GACA)_4GT$: one for *T. pratense* and two for *T. diffusum*. It was found that the profiles of hybrids obtained by using tetranucleotide repeats not only provide fragments specific to parental forms, but were also supplemented by 'novel' fragments which were not present in the DNA profile of parental clover species. Initially, tetranucleotide repeat primers were successfully adapted for DNA profiling and discrimination of closely related *Lolium* and *Festuca* species /Pašakinskienė et al., 2000/.

Table 1. Number and the range of sizes of ISSR fragments amplified in DNA profiles of *T. pratense, T. diffusum* and their hybrids
1 lentelė. *T. pratense, T. diffusum ir jų tarprūšinių F_1 hibridų DNR fragmentų skaičius ir dydžiai*

Primer code *Pradmens kodas*	Oligonucleotide sequence *Oligonukleotidų seka*	*T. pratense*		*T. diffusum*		F_1 hybrids / *F_1 hibridai*	
		Number of bands *Fragmentų skaičius*	Band size range, bp *Ribos, bp*	Number of bands *Fragmentų skaičius*	Band size range, bp *Ribos, bp*	Number of bands *Fragmentų skaičius*	Band size range, bp *Ribos, bp*
Tetra-repeat primers / *Tetranukleotidinių motyvų pradmenys*							
77H	$(AGAC)_4GC$	6	700–3000	6	800–3000	4–11	600–3000
78H	$AC(GACA)_4$	4	700–2500	5	700–2500	3–7	700–2500
104H	$(GACA)_4GT$	1	1050	2	900–950	2–5	550–1050
Total / *Iš viso*		11	700–3000	13	700–3000	9–23	550–3000
Tri-repeat primers / *Trinukleotidinių motyvų pradmenys*							
GO8	$(ATG)_5GA$	2	450–950	1	450	2–4	450–800
UBC864	$(ATG)_5$	3	400–750	3	300–700	3	400–750
UBC807	$(GAA)_5CG$	3	500–750	2	580–850	2	450–550
UBC866	$(CTC)_5$	4	400–1250	3	1000–1350	6	400–1350
Total / *Iš viso*		12	400–1250	9	300–1350	13–15	400–1350
Di-repeat primers / *Dinukleotidinių motyvų pradmenys*							
UBC845	$(AC)_8G$	7	750–1500	5	650–1500	5–7	750–1500
UBC825	$(AC)_8T$	2	600–800	2	600–800	2	600-800
UBC857	$(AC)_8CG$	5	550–2000	8	300–1500	3–8	300–2000
UBC856	$(AC)_8YA$	6	500–1600	8	500–1600	7–10	500–1600
GO7	$(AG)_8T$	3	600–1200	2	650–950	2–4	600–1200
155H	$(CA)_7GA$	3	850–1100	2	1000–1300	2	1000–1300
UBC847	$(CT)_8RG$	2	550–600	4	400–1900	3	400–600
UBC822	$(TC)_8A$	3	400–700	7	400–900	4–5	400–900
UBC823	$(TC)_8C$	4	500–900	4	500–900	4	500–900
UBC824	$(TC)_8G$	1	400	1	850	1	850
Total / *Iš viso*		36	400–2000	43	300–1900	33–46	300–2000

Using trinucleotide repeat primers 1–4 fragments were obtained in the fingerprints of *T. pratense* and *T. diffusum*. These primers also generated species-specific fragments. The profiles of the hybrids amplified by the primers $(ATG)_5GA$ and $(CTC)_5$ had species-specific and 'novel' DNA fragments. Profiles of hybrids amplified by $(ATG)_5$ primer were identical to the maternal parent *T. pratense*, consequently this primer was considered as unsuitable for the identifcation of hybridity. Profiles of species and hybrids obtained by the primers $(GAA)_5CG$ had species-specific fragments that were weakly expressed, which made assessment of hybridity difficult.

Twelve primers out of 17 di-, tri- and tetranucleotide repeats tested by PCR provided us with a useful ISSR fingerprints for hybridity confirmation of *T. pratense* and *T. diffusum* hybrids. Our results suggest that ISSR profiles of interspecific hybrids of *T. pratense* x *T. diffusum* can be of 4 types: 1 – representation of both *T. pratense* and *T. diffusum* fragments; 2 – representation of *T. pratense* and *T. diffusum* and 'novel' DNA fragments which are not present in the parental species; 3 – identical to *T. pratense*; 4 – identical to *T. diffusum* (Table 2). One third of trinucleotide motif primers generated DNA profiles of *T. pratense* x *T. diffusum* hybrids identical to the maternal *T. pratense* species, the rest of the primers generated profiles composed of fragments specific to parental forms as well as new type fragments. When using dinucleotide repeats as primers, hybridity was confirmed by the DNA profiles of three types: identical to *T. diffusum*; representing *T. pratense* and *T. diffusum* – specific DNA fragments, and showing *T. pratense* and *T. diffusum* – specific and 'novel' fragments.

Table 2. Hybridity confirmation in the interspecific *T. pratense* x *T. diffusum* hybrids by ISSR fingerprinting presented by characteristic profile frequency (%) for different primer motifs

2 lentelė. *Tarprūšinių T. pratense x T. diffusum F_1 hibridų DNR profiliai ir jų pasikartojimų dažnumas %*

Primer motif *Pradmens tipas*	Number of primers *Pradmenų skaičius*	DNA profiles, % / *DNR profiliai %*			
		T. pratense and *T. diffusum* fragments are present *T. pratense ir T. diffusum fragmentai*	*T. pratense* and *T. diffusum* and 'novel' fragments are present *T. pratense ir T. diffusum bei 'nauji' fragmentai*	Identical to *T. pratense Identiškas T. pratense*	Identical to *T. diffusum Identiškas T. diffusum*
Tetra-repeats *Tetranukleotidiniai pasikartojimai*	3	14.3	0	0	0
Tri-repeats *Trinukleotidiniai pasikartojimai*	4	33.3	0	33.3	0
Di-repeats *Dinukleotidiniai pasikartojimai*	10	53.1	29.5	0	29.5
Total / *Iš viso*	17	33.6	9.8	11.1	9.8

DNA profiles of T. ambiguum, T. hybridum and their hybrids. For DNA tests of *T. ambiquum* and *T. hybridum* by ISSR method 4 tetranucleotide motif primers were used. In total 17 fragments were produced in *T. ambiguum* and 22 – In *T. hybridum* DNA profiles (Table 3). Primers, $(AGAC)_4GC$, $AC(GACA)_4$, $(GACA)_4GT$ and $(ACTG)_4GA$, generated different fragments for *T. ambiguum* and *T. hybridum*, but in the hybrids only fragments of the maternal parent, *T. ambiguum* were displayed. Similar results were obtained in DNA profile of *Brassica* species F_1 hybrids *B. juncea x B. campestris* /Gupta et al., 2004/.

Fragments amplified by $(TCC)_5GT$ were in size 750–1400 bp. A specific fragment of 1100 bp was obtained for *T. hybridum* and one of 750 bp for *T. ambiguum*. These specific fragments were retained and followed in most of the hybrids, but some (2.5 %) individuals showed fingerprints identical to *T. ambiguum*.

Table 3. Number and the range of sizes of ISSR fragments amplified in DNA profiles of *T. ambiquum, T. hybridum* and their hybrids
3 lentelė. *T. ambibuum, T. hybridum ir jų tarprūšinių F_1 hibridų DNR fragmentų skaičius ir dydžiai*

Primer code *Pradmens kodas*	Oligonucleotide sequence *Oligonukleotidų seka*	*T. ambiquum*		*T. hybridum*		F_1 hybrids / *F_1 hibridai*	
		Number of bands *Fragmentų skaičius*	Band size range, bp *Ribos, bp*	Number of bands *Fragmentų skaičius*	Band size range, bp *Ribos, bp*	Number of bands *Fragmentų skaičius*	Band size range, bp *Ribos, bp*
Tetra-repeat primers / *Tetranukleotidinių motyvų pradmenys*							
77H	$(AGAC)_4GC$	4	400–2000	3	700–1900	4	400–2000
78H	$AC(GACA)_4$	3	750–1300	7	400–2500	3	750–1300
104H	$(GACA)_4GT$	5	600–1300	7	550–1900	5	600–1300
GO2	$(ACTG)_4GA$	5	550–1300	5	650–2400	5	550–1300
Total / *Iš viso*		17	400–2000	22	550–2500	17	400–2000
Tri-repeat primers / *Trinukleotidinių motyvų pradmenys*							
GO3	$(TCC)_5$ GT	3	750–1400	2	900–1100	3–4	750–1400
GO8	$(ATG)_5GA$	3	500–850	3	450–700	6	450–850
GO7	$(GAA)_5CG$	4	450–1100	1	950	4	450–1100
UBC866	$(CTC)_5$	2	600–850	2	600–950	2	600–850
Total / *Iš viso*		12	500–1400	8	450–1100	15–16	450–1400
Di-repeat primers / *Dinukleotidinių motyvų pradmenys*							
UBC825	$(AC)_8T$	5	400–1000	6	500–1200	5	400–1000
UBC827	$(AC)_8G$	6	600–1700	5	600–1000	6	600–1700
UBC857	$(AC)_8CG$	4	500–1200	6	450–1200	4	500–1200
UBC856	$(AC)_8YA$	5	450–1200	5	400–1200	5–7	450–1200
155H	$(CA)_7GA$	2	700–1000	5	500–1000	2	700–1000
105H	$(GA)_8CT$	5	650–1700	3	1000–1400	5–6	650–1400
UBC823	$(TC)_8C$	1	400	4	650–1400	4–6	400–1400
UBC824	$(TC)_8G$	2	650–850	1	380	2–3	380–850
UBC822	$(TC)_8A$	1	700	2	1000–1200	1	700
Total / *Iš viso*		31	400–1700	37	400–1400	34–40	380–1700

Apis mellifera caucasica race discriminating fragments were obtained by $(TCC)_5GT$ primer /Paplauskienė et al., 2006/. Using the other trinucleotide primers, species specific fragments were obtained in the DNA profiles of parental clover species, but DNA profiles of interspecific hybrids amplified with $(GAA)_5CG$ and $(CTC)_5$ primers were identical to *T. ambiguum*. When the $(ATG)_5GA$ primer was used, the fingerprints of the hybrids, in most cases – 94.7 %, were identical to that of *T. ambiguum*, 5.3 % to – *T. hybridum* (Table 4).

Most of the dinucleotide motif primers used were composed of AC repeat sequence with different anchors. These primers generated from 4 to 6 fragments in the DNA profiles of clover species. Most of DNA profiles of *T. ambiguum* x *T. hybridum* hybrids amplified with these primers were identical to *T. ambiguum,* and only by used $(AC)_8YA$ primer a small representation (5.3 %) of both parental species fragments was obtained. $(GA)_8CT$ amplified fragments within the range 650–1700 bp. *T. hybridum* had a strong specific band of 1000 bp and *T. ambiguum* was characterized by three faint specific fragments. The hybrids displayed specific fragments from both parents in most of the cases, but in some individuals 'novel' DNA fragments appeared which had not been present in the parental species. Substitution of an anchor for TC repeat had an effect on the number of fragments. Similar results were obtained when amplifying rice DNA fragments with the above-mentioned primer /Joshi et al., 2000/.

Table 4. Hybridity confirmation in the interspecific *T.ambiguum* x *T hybridum* hybrids by ISSR fingerprinting presented by characteristic profile frequency (%) for different primer motifs

4 lentelė. *Tarprūšinių T. ambiguum x T. hybridum F_1 hibridų DNR profiliai ir jų pasikartojimų dažnumas %*

Primer motif *Pradmens tipas*	Number of primers *Pradmenų skaičius*	DNA profiles, % / *DNR profiliai* %			
		T. ambiguum and *T. hybridum* fragments are present *T. ambiguum ir T. hybridum fragmentai*	*T. ambiguum* and *T. hybridum* and 'novel' fragments are present *T. ambiguum ir T. hybridum bei 'nauji' fragmentai*	Identical to *T. ambiguum* *Identiškas T. ambiguum*	Identical to *T. hybridum* *Identiškas T. hybridum*
Tetra-repeats *Tetranukleotidiniai pasikartojimai*	4	0	0	100	0
Tri-repeats *Trinukleotidiniai pasikartojimai*	4	24.3	0	70.4	5.3
Di-repeats *Dinukleotidiniai pasikartojimai*	9	20.4	0.3	79.3	0
Total / *Iš viso*	17	14.9	0.1	83.2	1.8

While amplifying DNA with these primers, *T. ambiguum* and *T. hybridum* were represented by species specific fragments. DNA profiles of hybrids amplified with $(TC)_8A$ and in most case with $(TC)_8C$ were identical to maternal clover species. $(TC)_8G$ primer generated DNA profiles specific to *T. ambiguum* and *T. hybridum* species. Part (15.8 %) *T. ambiguum* x *T. hybridum* hybrids had DNA profiles identical to *T. ambiguum,* but most (84.2 %) had representations of *T. ambiguum* and *T. hybridum* fragments (Table 4).

In total, from the results with all simple repeat sequence primers most of the hybrids (83.2 %) had fragments of the female parent, *T. ambiguum*, part (14.9 %) – from both parental species. The $(TCC)_5GT$, $(GA)_8CT$ and $(TC)_8G$ primers were found to be the most suitable for the identification of interspecific hybrids of *T. ambiguum* x *T. hybridum*.

Conclusions

1. DNA profiles of interspecific hybrids *T. pratense* x *T. diffusum* and *T. ambiguum* x *T. hybridum* can be of 4 types: representation of both parental species fragments*;* representation of both parental species and 'novel' DNA fragments which are not present in the parental species; identical to maternal species; identical to paternal species.

2. Part of *T. pratense* x *T. diffusum* (11.1 %) and most of *T. ambiguum* x *T. hybridum* (83.2 %) hybrids had DNA profiles identical to maternal *T. pratense* and *T. ambiguum* species.

3. Twelve primers out of 17 di-, tri- and tetranucleotide repeats tested in PCR provided with a useful ISSR fingerprint system for hybridity confirmation of *T. pratense* and *T. diffusum* hybrids.

4. The $(TCC)_5GT$, $(GA)_8CT$ and $(TC)_8G$ primers were found to be the most suitable for the identification of interspecific hybrids of *T. ambiguum* x *T. hybridum*.

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TARPMIKROSATELITINIŲ (ISSR) ŽYMENŲ TAIKYMAS DOBILŲ TARPRŪŠINIAMS HIBRIDAMS CHARAKTERIZUOTI

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Santrauka

Įvertinta tarpmikrosatelitinių DNR sekų metodo (ISSR) panaudojimo galimybė dobilų tarprūšiniams hibridams *T. pratense* x *T. diffusum* ir *Trifolium ambiguum* x *T. hybridum* identifikuoti. Palyginti tarprūšinių hibridų ir jų tėvinių formų DNR profiliai.

Iš panaudotų 17 pradmenų 13 generavo fragmentus, leidžiančius atskirti *T. pratense* ir *T. diffusum* rūšis, 12 pradmenų tiko ir tarprūšiniams hibridams identifikuoti. *T. pratense* x *T. diffusum* hibridų DNR profiliai buvo keturių tipų: 1 – turintys abiejų tėvinių rūšių DNR fragmentus, 2 – abiejų tėvinių rūšių ir naujus DNR fragmentus, 3 – DNR profiliai tapatūs motininei *T. pratense* rūšiai, 4 – DNR profiliai tapatūs *T. diffusum* tėvinei rūšiai.

Pradmenys, naudoti hibridų tarp *T. ambiguum* ir *T. hybridum* DNR analizei, generavo dobilų rūšims specifinių fragmentų susidarymą, bet jų hibridų DNR profiliai daugumoje atvejų (82,5 %) tapatūs motininei *T. ambiguum* rūšiai. Trinukleotidinių motyvų $(TCC)_5GT$ pradmeniu gauti dobilų *T. ambiguum* ir *T. hybridum* rūšims specifiniai fragmentai, kurie buvo ir daugelio hibridų (97,5 %) DNR profiliuose. Panašūs rezultatai gauti naudojant $(GA)_8CT$ ir $(TC)_8G$ pradmenis. Hibridų tarp *T. ambiguum* ir *T. hybridum* hibridiškumui patvirtinti tiko $(TCC)_5GT$, $(GA)_8CT$ ir $(TC)_8G$ pradmenys.

Reikšminiai žodžiai: *T. pratense* x *T. diffusum, T. ambiguum* x *T. hybridum,* tarpmikrosatelitinai žymenys (ISSR), pradmenys.