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A study of genetic diversity in *Trifolium hybridum* varieties using morphological characters and ISSR markers

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Abstract

The nutritive value of alsike clover (*Trifolium hybridum* L.) is slightly lower than that of red and white clovers; however, it exhibits some advantages – persists longer in a sward, performs well on acid and wet soils, suits for forage and silage production, can be used as green manure, and is a melliferous plant. With declining liming volumes, the soils are rapidly acidifying, which may result in an increasing alsike clover production area.

To reveal plant diversity, the methods currently employed encompass not only research into agro-biological traits but also biochemical-molecular assays. The inter-simple sequence repeats (ISSR) technique is one of the DNA assay methods widely used in genetic diversity studies, genetic evolution and systematic specification, and gene mapping. The current study was aimed to assess individual plants of *T. hybridum* varieties ('Daubiai', 'Lomiaiai', 'Poliai', 'Skriveru') for morphological traits, quality parameters and genetic diversity and to establish the correlations between the ISSR markers and characteristics of agro-biological traits.

Morphological diversity was estimated according to 10 traits. The coefficient of variation varied within a wide range (CV 0.6–71.9%). The parameters of feeding value were found to be most stable (CV 0.6–16.6%), while seed yield was the most variable indicator (CV 38.7–71.9%). According to the values of agro-biological traits, the individual plants conditionally split into 4 sub-clusters, encompassing different varieties.

Five primers were used to reveal the DNA diversity of alsike clover. A total of 37 fragments were amplified, of which 62.2% were polymorphic. Genetically most distant were individuals of 'Lomiaiai' and 'Daubiai' cultivars. The dendrograms produced according to the values of agro-biological and genetic distances were not identical. The ISSR markers were found to correlate with the following alsike clover traits: seed yield, stem number and height, crude protein and water soluble carbohydrate contents.

Key words: *Trifolium hybridum* varieties, genetic diversity, ISSR markers, morphological characters.

Introduction

With the recent development of organic farming, increasing fertilizer and energy costs, long-term swards with a high ratio of legumes are becoming indispensable. One of the most valuable legumes are white clover (*Trifolium repens* L.), red clover (*Trifolium pratense* L.) and alsike clover (*Trifolium hybridum* L.). Although nutritive value of *T. hybridum* is slightly lower than that of red clover, it persists longer in swards and of all clover species performs best on acid, wet soils, exhibits a good over-winter survival and is tolerant of frosts (Daugėlienė, 2000). It suits for green forage and silage production. It can be ploughed-in as green manure. Alsike clover is commonly grown in mixtures with timothy, because of its lower competitive power than that of red clover. *T. hybridum* is distributed all over Lithuania and occurs in the communities of swampy meadows. It is a melliferous plant, which produces more nectar than red and white clovers and its nectar production is more stable.

With declining liming volumes, the soils are rapidly acidifying, which reduces the chances of acid-sensitive plant species to be grown and increases the likelihood that *T. hybridum* production will increase (Sprainaitis, 2005).

The methods currently used for plant diversity studies include not only analyses of agro-biological traits but also biochemical-molecular techniques. Special emphasis is attached to the research into nucleic acids, which are direct agents of genetic information transfer (Ghariani et al., 2003). Most of the DNA assay methods are based on polymerase chain reaction (PCR). This method has become common and its modifications (RFLP, RAPD, AFLP, ISSR and other marker systems) are being applied for the solution of various issues (Busti et al., 2004). The inter-simple sequence repeats (ISSR) technique is one of the DNA assay methods widely used in genetic diversity studies, genetic evolution and systematic specification, and gene mapping. Molecular marker analysis is being successfully applied in the studies of many important agricultural crops: wheat, barley, potatoes, lupine, oilseed rape, maize, grapes, ornamental plants, sugar beet, cotton, rice etc. (Suvendu et al., 2008). At Institute of Agriculture, Lithuanian Research Centre for Agriculture and Forestry the ISSR markers are used for the identification of polymorphism and interspecific and intergeneric hybrids as well as gene expression studies in cereals and perennial forage grasses (Brazauskas, Ruzgas, 2009;

Jonavičienė et al., 2009). The present study was designed to assess individual plants of alsike varieties for morphological traits, quality indicators and genetic diversity and to establish the correlations between ISSR markers and characters of agro-biological traits.

Material and methods

The experiment was carried out at the Institute of Agriculture, Lithuanian Research Centre for Agriculture and Forestry in 2008–2011.

Plant material. The study involved four alsike clover (*Trifolium hybridum* L.) varieties ‘Daubiai’, ‘Lomiaiai’, ‘Poliaiai’, ‘Skriveru’. The ‘Daubiai’ is diploid, cultivated in Lithuania since 1962, developed from the local commercial seed sample (or from local populations) by a mass selection method. The tetraploid variety ‘Skriveru’, developed in Latvia, has also been grown in Lithuania for many years. The new Lithuanian varieties – diploid ‘Lomiaiai’ and tetraploid ‘Poliaiai’ were released in 2001. Five genotypes of different geographical origin were used for their development (Sprainaitis, 2005). Plants were grown in a greenhouse and transferred into a climatic chamber prior to flowering (22–25°C, 13,000 lux, 16 h photoperiod).

ISSR analysis. DNA was extracted from 0.35–1.5 g young leaves following the CTAB-based extraction protocol (Doyle, Doyle, 1990). Polymerase chain reactions (PCRs) were carried out in 25 µl volume in an “Eppendorf Mastercycler Gradient” thermocycler (USA) using the following primers: (AC)₈T – UBC825, (AC)₈CG – UBC857, (AC)₈G – UBC827, (AGAC)₄GC – 77H, AC(GACA)₄ – 78H. Twenty five microlitre aliquots or PCR mixtures contained 10 × Mg²⁺-free buffer, 2.0 mM dNTP mix, 50 mM MgCl₂, 2.5 µM primer, 50 ng DNR, 2.0 U DyNAzyme II polymerase (“Finnzymes OY”, Finland). Reaction was repeated at least twice. Amplification products were analysed in 1.5% agarose gel, and electrophoresis was carried out in 1 × TAE buffer. “GeneRuler™

DNA Ladder Mix” (“Fermentas”, Lithuania) was used as the DNA fragment size marker. The gels were analysed in UV light by staining with ethidium bromide.

Data analysis. The coefficients of variation of morphological and quality traits, correlations between molecular markers and morphological and quality characters were computed using ANOVA. The quantitative morphological and quality data were standardized and the Euclidean distance matrix was calculated. ISSR profiles were scored as presence (1) or absence (0) of a band, and the data obtained were used in a rectangular matrix. The data matrix was then used to generate a genetic distance index (Nei, 1972). Cluster analysis was carried out based on genetic distance, using unweighted pair-group method using arithmetic averages (UPGMA). The resulting clusters were represented as dendrograms. These computations were performed with NTSYSpc v. 2.2 analysis software.

After DNA extraction, the plants were transplanted to the field trial nursery with 50 × 50 cm nutritional area, 20 plants per variety. The plants were assessed for the following morphological characters: height, stem and inflorescence number, fresh mass and dry matter content, seed yield, beginning of flowering, characters of feeding value (crude protein, water soluble carbohydrates, crude fibre, and dry matter digestibility).

Results and discussion

Agromorphological assessments of Trifolium hybridum. Assessment of individual plants for agromorphological characters revealed a high polymorphism within varieties. Nearly all agromorphological characters tested varied within a wide range (Table 1). The coefficient of variation (CV) for the plants of ‘Daubiai’ ranged from 0.6% to 49.9%, for ‘Lomiaiai’ – from 5.0% to 71.9%, for ‘Poliaiai’ from 5.4% to 62.1%, for ‘Skriveru’ from 5.4% to 48.9%.

Table 1. Variation of morphological and quality traits of *Trifolium hybridum* varieties (for individual plants)

Morphological and quality traits	Variety							
	‘Daubiai’		‘Lomiaiai’		‘Poliaiai’		‘Skriveru’	
	Average value of trait	CV %	Average value of trait	CV %	Average value of trait	CV %	Average value of trait	CV %
Morphological traits								
Number of flowers	231.1 ± 26.02	39.0	318.2 ± 45.40	51.4	240.2 ± 30.05	43.3	242.7 ± 34.26	48.9
Seed yield g	3.3 ± 0.34	38.7	3.0 ± 0.57	71.9	0.6 ± 0.10	62.1	0.8 ± 0.10	47.1
Number of stems	41.3 ± 5.96	49.9	45.6 ± 4.31	34.1	41.0 ± 2.71	22.9	44.7 ± 4.21	32.6
Height cm	56.2 ± 2.73	16.8	62.5 ± 2.76	15.9	66.2 ± 3.95	20.7	61.6 ± 2.07	11.6
Fresh mass g	478.3 ± 49.13	35.6	493.1 ± 48.36	35.4	624.2 ± 58.68	32.6	570.0 ± 62.57	38.0
Dry matter g	129.1 ± 13.22	35.5	128.5 ± 1.61	45.2	164.7 ± 1.74	36.6	153.8 ± 1.70	38.2
Quality traits								
Crude protein g kg ⁻¹	160.3 ± 0.71	0.6	146.9 ± 3.62	8.8	139.4 ± 0.26	6.5	144.9 ± 0.29	7.0
Water soluble carbohydrates g kg ⁻¹	100.0 ± 10.02	14.1	120.5 ± 5.54	16.6	139.1 ± 0.54	13.5	135.6 ± 0.56	14.4
Dry matter digestibility g kg ⁻¹	613.7 ± 28.12	6.5	643.4 ± 8.93	5.0	647.5 ± 1.04	5.6	661.3 ± 1.03	5.4
Crude fibre DM g kg ⁻¹	306.8 ± 6.04	2.9	298.4 ± 4.45	5.4	290.5 ± 0.45	5.4	280.5 ± 0.47	5.8
Total		239.6		289.6		248.9		249.0
Average		23.6		29.0		24.9		25.0

The CV within all cultivars was low or moderate only for plant height (16.8, 15.9, 20.7, and 11.6 %, respectively) and for nutritive value characters (0.6–16.6%). Seed yield was found to be the most variable character for all varieties tested. The coefficient of variation ranged from 38.7% for ‘Daubiai’ to 71.9% for ‘Lo-

miai’. The lowest seed yield per plant was 0.01 g, while the highest 6.6 g. The diversity of seed yield value per plant was largely determined by high number of stems and inflorescences. The CV values of these characters were 22.9–49.9% and 39.1–51.4%. Having summed the CV values of all traits it is obvious that the values

of agromorphological characters are rather similar for all varieties: 239.6–289.6%. The least variation of the values of these characters was recorded for ‘Daubiai’ plants (CV 0.6–49.9%), while the highest variation was noted for ‘Lomiaiai’ plants (CV 5.0–71.9%).

Our research findings agree with those obtained by other researchers. Several studies have shown that the genome of *Trifolium* spp. is extremely polymorphic due to its strongly self-incompatible fertilization. Diversity of traits enables division of varieties or populations into four clusters. In South Brazil, having estimated the values of 21 morphological traits, 85 *T. pratense* populations were divided into groups that differed markedly in flowering earliness, persistency, growth habit and dry matter productivity (Dias et al., 2008). Comparison of biometric characteristics of *T. scabrum* L. wild populations allowed selection of populations suitable for cultivation on arid soils of Algeria (Issolach, Abdelguerfi, 1999). Many researchers suggest that *T. pratense* populations have been noted for high variation (CV% > 20.0) of fresh mass, dry matter content and number of flowers per inflorescence, while *T. scabrum* is characterised by high variation of seed yield value (Issolach, Abdelguerfi, 1999; Tucak et al., 2009). The variation of other parameters in the above mentioned species was moderate or weak.

Many research publications have reported that in *Fabaceae* plants intrapopulation genomic heterozygosity was higher than interpopulation heterozygosity (Bulinska-Radomska, 2000; Isobe et al., 2009). Analysis of the seed yield of 11 *Medicago sativa* L. populations showed that the among-population variance accounted for 5% to 31% of the total genetic variance for seed yield components, while the within-population variance explained 69% to 95% (Bolanos-Aguilar et al., 2000). The study on morphological characters’ diversity of *V-*

cia villosa Roth. indicated the highest polymorphism for the number of stem branchings, inflorescences per plant, number of pods, stem height at the beginning of flowering (Sliesaravičius et al., 2004). The Turkish researchers investigated 11 clones of *Medicago sativa* and established moderate variance for the following parameters: plant height, leaf width and length, green and dry herbage (Basbag, Demirel, 2009).

ISSR marker analysis. Five inter-simple sequence repeat, di- and tetranucleotide motif primers were used to study the genetic diversity of *T. hybridum*. The efficacy of the chosen ISSR primers slightly differed (Table 2). The highest number of fragments was amplified with the (AC)₈CG primer, while the lowest number was amplified with AC(GACA)₄ – 10 and 5 fragments, respectively. One di-nucleotide motif primer produced on average 8.3 fragments. The fragment size amplified with these primers was similar and ranged from 450 to 1500 bp, the generated DNA fragments were characterised by high (79.6%) polymorphism. Compared with other micro-satellites, di-nucleotide repeats are more common in plants (Varshney et al., 2000). Di-nucleotidic AC motif primers generated distinct and polymorphic fragments in oilseed rape varieties (Martins-Lopes et al., 2007) and some clover species (Dalla Rizza et al., 2007). Tetranucleotide motif primers generated slightly fewer fragments 5–7 and their polymorphism was lower. Although the AC(GACA)₄ primer generated fewer fragments, they were more polymorphic. In a clover species study, the GACA motif primer without an anchor amplified 4–10 fragments in *T. pratense* and as many as 16 fragments in *T. riograndense*. The attractiveness of the ISSR method is especially enhanced by the fact that replacement of just one anchor nucleotide by another can produce new fragments (Dalla Rizza et al., 2007).

Table 2. ISSR products generated by 5 primers in Lithuanian *Trifolium hybridum* varieties

Primer code	Oligonucleotide sequence	Size range of amplified bands bp	<i>Trifolium hybridum</i> varieties							
			‘Lomiaiai’		‘Poliaiai’		‘Daubiai’		‘Skriveru’	
			1	2	1	2	1	2	1	2
UBC825	(AC) ₈ T	550–1200	8	87.5	8	87.5	7	85.7	6	66.7
UBC857	(AC) ₈ CG	450–1300	10	70.0	9	88.9	8	50.0	8	37.5
UBC827	(AC) ₈ G	500–1500	6	66.7	6	66.7	7	71.4	7	57.1
77H	(AGAC) ₄ GC	600–2000	6	50.0	6	33.3	7	42.8	7	42.8
78H	AC(GACA) ₄	1000–2600	5	60.0	5	60.0	5	60.0	5	40.0
Total		450–2600	35	68.6	34	70.6	34	61.9	33	48.8

1 – number of fragments, 2 – polymorphism %

A total of 37 fragments were generated having used 5 primers for the DNA amplification of four varieties. Polymorphic fragments accounted for 62.2%. According to the number of fragments, the varieties were rather similar (33–35 fragments), the level of polymorphism also varied little: from 48.5% (‘Skriveru’) to 70.6% (‘Poliaiai’). The relatedness between the individuals of *T. hybridum* varieties is shown in the dendrogram (Fig. 1).

The dendrogram made according to the DNA markers did not split the plants into distinct clusters according to varieties, yet, it can be maintained that plants of the tetraploid varieties ‘Poliaiai’ and ‘Skriveru’ split into two sub-clusters. The highest genetic distance was established between ‘Daubiai’ and ‘Skriveru’.

Classification according to morphological traits and quality characters distinguished four bigger sub-clusters (Fig. 2). The first one included most of the plants

of ‘Lomiaiai’ and a few plants of ‘Daubiai’. Both varieties are diploid. The second and third clusters were mostly composed of the plants of the tetraploid varieties. Many of the plants of ‘Daubiai’ fell within the fourth cluster. The comparison of Euclidean distances of morphological traits and quality parameters with genetic distances revealed a very weak correlation $r = 0.15$.

Other researchers have also indicated similar trends. Dias et al. (2008) studied polymorphism of 57 populations of *T. pratense* at the level of morphological traits and molecular marker. Although both methods generated 5 clusters, there was no coincidence between the composition of groups with morphological and molecular data. Comparison of SSR against morphological traits in lucerne populations did not reveal any correlations. Regression analysis showed no relationship between genetic distance and phenotypic distance (Li et al., 2009). Similar

trends have been noted for the plants of other families. In the *Rubus idaeus* populations, having compared morphological Euclidean distances against RAPD primers only very weak correlation was established (Patamsytė et al.,

2008). The results of *Triticum aestivum* study revealed that the genotypes differed for morphological characters and SSR markers (Salem et al., 2008).

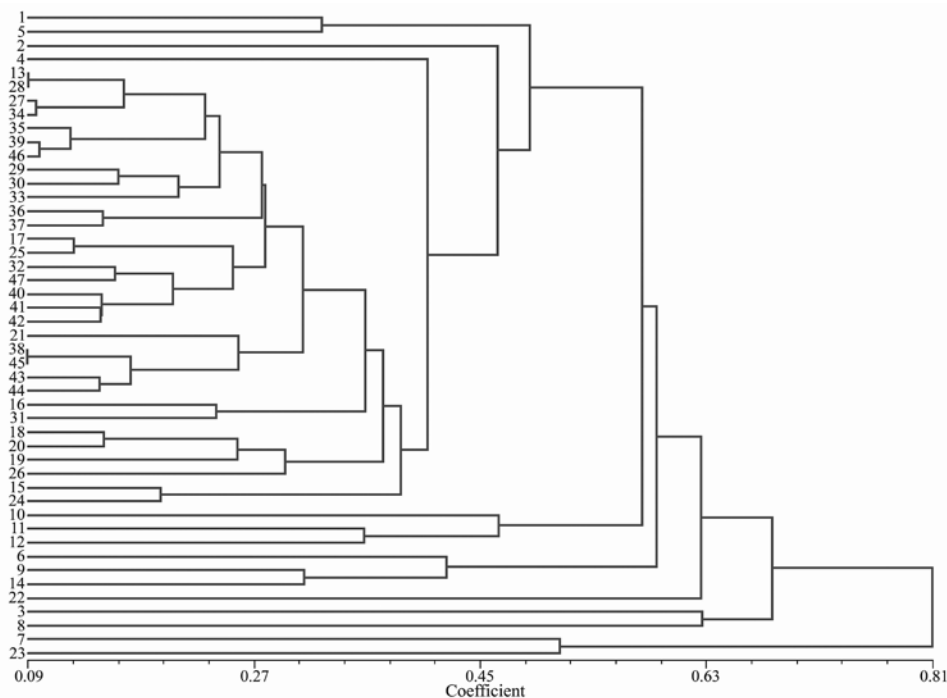


Figure 1. The dendrogram of genetic distances (Nei's coefficient) of individual plants of *Trifolium hybridum* varieties: 1–11 'Lomia', 12–23 'Daubiai', 24–34 'Poliai', 35–47 'Skriveru'

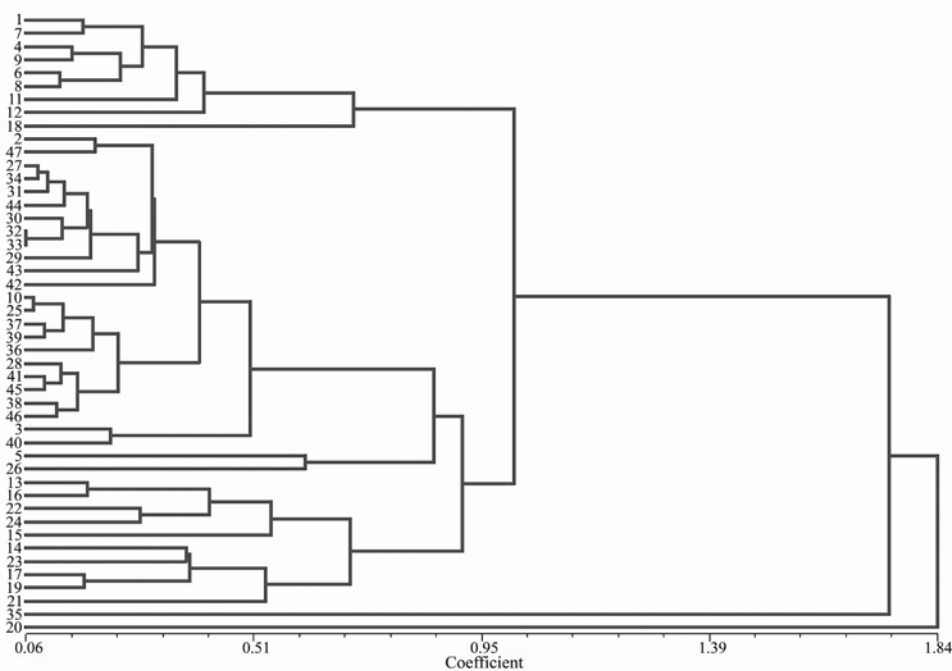


Figure 2. The dendrogram of morphological and quality characters (Euclidean distances) of *Trifolium hybridum* varieties: 1–11 'Lomia', 12–23 'Daubiai', 24–34 'Poliai', 35–47 'Skriveru'

DNA markers are widely used in the studies on many important agricultural crops. Genetic diversity of clover species was explored using RAPD (Ulloa et al., 2003), AFLP (Jones et al., 2003), ISJ (Samei et al., 2008), SSR (Dolanska, Čurn, 2004), ISSR (Dalla Rizza et al., 2007) and other DNA assay methods. In recent years, various countries' researchers have been searching for DNA markers suitable for identifying important traits

(e.g., pest resistance, productivity, feeding value and others) (Abberton, 2007). *T. hybridum* has been little researched in this respect.

We calculated the relationship between individual DNA markers and agromorphological characters as well as quality characters. The UBC825 primer generated three markers correlating with seed yield, crude fibre and water soluble carbohydrates contents (Table 3).

Two primers (550 and 1200 bp) were linked to seed yield. Negative relationship was established between the presence of 550 bp marker in the DNA profile and seed yield, whereas positive relationship was found between the 1200 bp marker and seed yield character. The plants with only 550 bp marker present in the DNA profile produced an average seed yield of 2.20 g per plant, and those whose

DNA profile was supplemented with the 1200 bp fragment produced a seed yield of 2.98 g. The 550 bp marker correlated with crude fibre content. Plants with this fragment absent tended to accumulate significantly higher crude fibre level. The 780 bp marker was found to be specific to plants with low water soluble carbohydrates contents.

Table 3. Correlation coefficients between ISSR markers and morphological and quality traits of *Trifolium hybridum* varieties

Primer (size of DNA fragment, bp)	Morphological and quality traits	Coefficient of variation	Mean trait values		Fisher's criterion
			fragment present	fragment absent	
UBC 825 (550)	Seed yield g	-0.405**	2.20	3.42	2.69*
	Crude fibre g kg ⁻¹	-0.393**	289.3	307.0	7.937**
UBC 825 (780)	Water soluble carbohydrates g kg ⁻¹	-0.293*	116.7	130.9	4.225*
UBC825 (1200)	Seed yield g	0.448**	2.98	1.40	11.317**
UBC 827 (850)	Seed yield g	-0.446**	1.17	2.44	11.654**
UBC 857 (500)	Seed yield g	-0.381**	1.32	2.57	7.964**
UBC 857 (650)	Number of stems	0.298*	54.7	42.1	4.575*
UBC 857 (980)	Plant height cm	0.286*	64.3	58.3	2.048*
78 H (1000)	Crude protein g kg ⁻¹	-0.364*	135.2	147.9	6.88*
	Crude protein g kg ⁻¹	-0.355*	138.1	148.4	6.491*
78 H (1200)	Water soluble carbohydrates g kg ⁻¹	0.324*	141.3	123.1	5.273*

* - $P < 0.005$, ** - $P < 0.001$

Clover seed yield is one of the most variable parameters, which were linked to two markers, generated with the AC motif primers UBC827 (850 bp) and UBC857 (500 bp). Plants with no 850 bp marker, generated with the UBC827 primer, produced significantly higher seed yield. Using the UBC857 primer, the plants with 500 bp marker present in the DNA profile were characterised by lower (1.32 g) seed yield, while when this marker was absent, the yield increased to 2.57 g. Weak correlation was established between the 650 bp marker and number of stems and between the 980 bp marker and plant height characters. The tetranucleotide motif primer 78H generated two markers associated with crude proteins and water soluble carbohydrates characters. Higher crude proteins contents were accumulated by the plants whose DNA profiles did not have 1000 bp and 1200 bp fragments amplified by the 78H primer. In the presence of one or both fragments, crude proteins content declined to 135.2–138.1 g kg⁻¹. Weak positive correlation was established between the 1200 bp marker and water soluble carbohydrates.

The obtained results enable us to maintain that the incorporation of marker technology for traits difficult to assess will represent a major advance in the genetic improvement of *T. hybridum*.

Conclusions

1. The characters of agromorphological traits varied within a wide range (CV 0.6–71.9%). Feeding value was the most stable character (CV 0.6–16.6%), while seed yield was the most variable indicator (CV 38.7–71.9%). According to the values of agromorphological traits and quality characters, the plants split into conditionally separate sub-clusters, encompassing different varieties.

2. Five primers used for *Trifolium hybridum* DNA amplification revealed its genetic diversity – a total of 37 fragments were generated, of which 62.2% were polymorphic. The greatest genetic distance was determined between the plants of 'Lomia' and 'Daubiai' varieties.

3. The following nine ISSR markers were found to correlate (or associate) with *Trifolium hybridum* agromorphological traits and quality characters: UBC 825 (550, 780, 1200 bp), UBC 827 (850 bp) and UBC 857 (500, 650, 980 bp) and 78H (1000, 1200 bp).

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Rausvojo dobilo (*Trifolium hybridum* L.) genetinė įvairovė pagal morfologinius požymius ir ISSR žymeklius

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Santrauka

Rausvojo dobilo (*Trifolium hybridum* L.) pašarinė vertė yra šiek tiek mažesnė nei raudonojo ir baltojo dobilų, tačiau rausvieji dobilai turi privalumų: ilgiau išsilaiko žolynuose, gerai dera rūgščiose ir drėgnose dirvose, tinka gaminti pašarui bei silosui, naudojami užarti kaip žalioji trąša, yra medingi. Dirvas mažiau kalkinant, dirvožemiai sparčiai rūgštėja, todėl didėja tikimybė, kad rausvųjų dobilų bus auginama daugiau.

Augalų įvairovei atskleisti pastaruoju metu ne tik atliekami agrobiologinių požymių tyrimai, bet ir taikomi biocheminiai bei molekuliniai metodai. Kartotinių paprastųjų sekų intarpai (ISSR) yra vienas iš DNR tyrimų metodų, plačiai taikomų atliekant augalų genetinės įvairovės tyrimus, tikslinant genetinę evoliuciją ir sistematiką, sudarant genolapius. Tyrimo tikslas – įvertinti rausvojo dobilo veislių atskirų individų morfologinių požymių, kokybės rodiklių ir genetinę įvairovę, nustatyti koreliacinius ryšius tarp ISSR žymeklių ir agrobiologinių požymių rodiklių.

Morfologinė įvairovė buvo vertinta pagal 10 požymių. Variacijos koeficientai plačiai įvairavo (CV 0,6–71,9 %). Stabiliausi buvo pašarinės vertės rodikliai (CV 0,6–16,6 %), o kintamiausias rodiklis – sėklų derlius (CV 38,7–71,9 %). Pagal agrobiologinių požymių reikšmes individai išsidėstė į sąlygiškai atskirus keturis subklasterius, sujungiančius skirtingas veisles.

Rausvojo dobilo DNR įvairovei atskleisti buvo panaudoti penki pradmenys. Iš viso pavyko amplifikuoti 37 fragmentus, kurių 62,2 % buvo polimorfiški. Genetiškai tolimiausi buvo veislių ‘Lomiai’ ir ‘Daubiai’ individai. Dendogramos, nubraižytos pagal agrobiologinių ir genetinių atstumų reikšmes, buvo nevienodos. Gauti ISSR žymekliai, koreliuojantys su rausvojo dobilo požymiais: sėklų derliumi, stiebų skaičiumi ir aukščiu, žalių baltymų ir vandenyje tirpių angliavandenių kiekiu.

Reikšminiai žodžiai: *Trifolium hybridum* veislės, genetinė įvairovė, ISSR žymekliai, morfologiniai požymiai.