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Molecular methods employed in the studies of genetic diversity of clover

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Studies of the genetic diversity of clover grown in the Lithuanian Institute of Agriculture collection involved the use of two molecular methods: analyses of electrophoretic spectra of seed proteins and enzymes and DNA fingerprints.

Analysis of protein electrophoretic spectra confirmed the hybrid origin of *T. pratense* × *T. diffusum* F_1 individuals and partly of B_1 individuals, but did not reveal their genetic diversity. Interspecific *T. pratense* × *T. diffusum* F_1 and B_1 hybrids were found to possess two types of superoxide dismutase (SOD) spectra and six types of peroxidase spectra, which give a significant evidence of their genetic diversity. DNA polymorphism of *T. pratense* × *T. diffusum* F_1 hybrids was revealed by using $(GACA)_4GT$, $(CAA)_5CG$ primers for PCR, while $(CAA)_5CG$ and $(TC)_8G$ primers were better suited for revealing the genetic diversity of B_1 hybrids. Using $(TCC)_5GT$, $(GA)_8CT$, $(TC)_8G$ primers of microsatellite sequence hybridity was confirmed for 84.2–97.5% of *T. ambiquum* × *T. hybridum* individuals, and DNA polymorphism was identified using $(GA)_8CT$, $(TC)_8G$ primers.

Key words: molecular methods, clover, interspecific hybrids

INTRODUCTION

A lot of efforts have been made recently to conserve the genetic resources of perennial forage grasses. Increasingly more countries are launching research into various legume species (*Trifolium* L, *Medicago* L., *Lotus* L., *Galega* Lam., etc.). Non-traditional uses of legumes are being searched for. Research publications report attempts to domesticate wild clover, use it as green manure or 'live mulch'. It is recommended to use some clover species in pastures not only to add variety to forage but also for the conservation of eroded soils [1–3]. An interest in annual clover species characterised by some exceptional traits, such as high seed yield, tolerance of dry, acid or saline soils, has been noted [4–6].

Red, white and alsike clover are the three species most common in Lithuania. They are very valuable, protein-rich plants used as livestock forage. A vast clover collection has been accrued at the Lithuanian Institute of Agriculture. It is composed of various species of wild ecotypes, populations, breeding lines, Lithuanian and foreign varieties. The collection has been recently supplemented by the populations of interspecific hybrids.

Analysis of molecular markers (DNA fingerprints, isozymes, electrophoretic spectra, etc.) has been widely used in the world plant diversity research practice. However, in grasses and legumes, clover in particular, this area has been scarcely investigated, only sporadic research being known. In foreign scientific press one can find scanty publications on the use of molecular markers (mostly analysis of electrophoretic spectra of storage proteins and isozymes) for the studies of polymorphism and for identification of clover species, varieties, breeding lines, and genotypes. The first attempts of to use DNA markers for marking the relevant traits of legume plants have been described [7–10].

The objective of the present study was to investigate the electrophoretic profiles of clover seed proteins, enzymes and nucleic acids and to estimate their suitability for the discrimination of interspecific hybrids and evaluation of genetic diversity.

MATERIAL AND METHODS

The following clover species and populations of interspecific hybrids were studied:

1. *Trifolium pratense* L., (high forage yield, medium seed setting, susceptible to diseases), varieties 'Liepsna' (2n = 2x = 14) and 'Vyliai' (2n = 4x = 28); **2**. *T. diffusum* Ehrh., 2n = 2x = 16 (wild form, self-pollinator, low forage yield, disease resistant, annual); **3**. *T. ambiquum* Bieb., 2n = 2x, 4x, 6x = 16, 32, 48 (wild, rhizomatous, persistent, disease resistant). **4**. *T. hyb-*

ridum L., (high forage yield, medium seed setting, susceptible to diseases, tolerant of acid soils). The variety 'Daubiai' (2n = 2x = 16). **5**. Interspecific hybrids F_1 between *T. pratense* and *T. diffusum* and backcross population: B_1F_2(♀*T. pratense*); **6**. Interspecific hybrids F_1 between *T. ambiquum* and *T. hybridum*.

Production of interspecific hybrids. Clover species and their hybrids were grown in a greenhouse at 22–25 C°, 13,000 lux, 16 h photoperiod. Interspecific hybrids between *T. pratense* × *T. diffusum* and *T. ambiquum* × *T. hybridum* were obtained using embryo culture. Fertility of the hybrids between *T. pratense* × *T. diffusum* was restored by colchicine treatment [11]. Backcross hybrids were produced by backcrossing with tetraploid red clover (variety 'Vyliai'): *T. pratense* × $F_1 \rightarrow B_1F_2$(♀*T. pratense*)

Protein isolation and electrophoresis. Protein isolation and electrophoresis in SDS – Tris-Glycine pH 8.3 buffer were performed following the Gardiner and Forde protocol [12].

Electrophoretic seed protein testing was carried out only for *T. pratense* and *T. diffusum* species and their hybrids. F_1 hybrids between *T. ambiquum* and *T. hybridum* even after colchicine treatment procedures remained sterile (did not set seed), therefore it was impossible to carry out their testing.

Methods for the analysis of enzymes. Enzymes were extracted from 4–6-week-old leaves of clover. Peroxidase (PO) and superoxide dismutase (SOD) electrophoresis was performed in 7.5% polyacrilamide gel, Tris-Glycine, pH 8.3 buffer. PO enzymes were developed following the methods of V Safonov and M. Safonov [13] and SOD following Pasteur et al. [14]. Electrophoresis of the enzymes glutamate oxalatetransferase (GOT), leucine aminopeptidase (LAP), phospho glucoisomerase (PGI) was done in 11% starch gel Tris-Citrate Borate, pH 8.3 buffer. Spectra for each enzyme were developed according to corresponding methods [14].

DNA isolation and analysis methods. DNA was isolated from young leaves of clover by a micro-method following the DNA extraction protocol of Doyle and Doyle [15]. Primers of simple repeat sequences (microsatellites) were used in polymerase chain reaction (PCR). PCR was performed in an Eppendorf Gradient Mastercycler. Amplification products were analysed on 1.5% agarose gel in 1 × TAE buffer. GeneRuler™ DNR DNA Ladder Mix (Fermentas) was used as the DNA fragment size marker. The gels were analysed in UV light by staining with ethidium bromide.

RESULTS AND DISCUSSION

Seed protein testing. Electrophoretic spectra of seed storage proteins of *T. pratense* and *T. diffusum* species had some fragments which were common to both species, however, two species-specific components were identified in the area of higher molecular weight (97–45 KDa) components. Similar differences in protein component composition were also noted for the other clover species: *T. repens*, *T. pratense, T. suteraneum* [12]. It has been also stated that analysis of protein electrophoresis is a reliable method for identification of genera and species of legume plants [16, 17]. Electrophoretic spectra of interspecific F_1 hybrids have common and species-specific components (Table 1). Protein electrophoretic spectra for all F_1 hybrids tested were found to be identical, whereas in backcross population B_1, two types of protein spectra were discriminated: I – identical with *T. pratense* spectrum, although it differed in the staining intensity of individual fragments, III – *T. pratense* spectrum, supplemented with a novel high-molecular weight Rf 0.16 component, which is not present in protein profiles of parental forms.

Analyses of enzymes. The spectra of SOD and PO enzymes of *T. pratense* and *T. diffusum* species were found to have components specific to each spe-

Table 1. **Electrophoretic spectra and their frequency (%) of proteins and SOD and PO enzymes of *T. pratense, T. diffusum* and their interspecific hybrids**

Population	Types of electrophoretic spectra						
	T. pratense components	*T. pratense* and *T. diffusum* components	*T. pratense* and novel components		*T. pratense, T. diffusum* and novel components		
	I	II	III	IV	V	VI	VII
Protein							
F_1	0	100	0	0	0	0	0
B_1	33.3	0	66.7	0	0	0	0
SOD							
F_1	0	0	21.4	0	78.6	0	0
B_1	60.5	0	8.6	0	30.9	0	0
PO							
F_1	3.3	59.0	13.1	0	4.9	11.5	8.2
B_1	33.0	9.9	16.5	12.0	4.4	2.2	22.0

cies (Rf 0.61 and 0.67, respectively). SOD spectrum of interspecific hybrids between *T. pratense* × *T. diffusum* was supplemented with a novel component Rf 0.63, which is not specific to the parental species. F_1 hybrids had SOD spectra of two types: III – *T. pratense*, spectrum supplemented with a novel component, V – electrophoretic spectrum components of both parental clover species supplemented with a novel component. SOD spectra of most backcross hybrids (60.5%) were identical with *T. pratense* species; the other hybrids had the same spectra as F_1 hybrids.

The PO spectra of *T. pratense* and *T. diffusum* plants differed only in the position of two components (Rf 0.41 and 0.64, respectively). Assessment of PO spectra of the hybrids revealed a greater diversity in the location of novel components. According to their location five types of PO spectra were identified in F_1 hybrids and six types in backcross individuals. Appearance of novel components in PO spectra of interspecific hybrids was also noticed while investigating other crossing combinations. Hybrids between *T. repense* and *T. hybridum* possessed in their electrophoretic spectrum not only components specific to both parents but also novel components [18, 19]. In our experiments, part of F_1 and B_1 hybrids had PO spectra identical with those of female *T. pratense* species. Similar data were obtained while investigating interspecific hybrids between *T. alexandrinum* and *T. apertum* [19]. PO spectra confirming the hybrid origin of *T. pratense* × *T. diffusum* F_1 and B_1 plants were characteristic of 96.7% and 77.0% of individuals, respectively.

In our experiments, SOD and PO spectra of *T. ambiquum* and *T. hybridum* were identical, therefore we used analysis of other enzymes, PGI, GOT, LAP, for the investigation of these species. PGI spectra of these clover species were found to have components specific to each species. PGI spectra of part of hybrids (39.2%) were identical with female *T. ambiquum* species, and the spectra of other hybrids contained components of both parental species, according to the position of which two types of PGI spectra were identified. However, British researchers determined that PGI spectra of F_1 hybrids between *T. repens* and *T. nigrescens* were identical with those of male *T. nigrescens* species [20]. Electrophoretic spectra analysis of GOT and LAP of *T. ambiquum* and *T. hybridum* revealed specific components to mark these species. However, the spectral composition of GOT and LAP of F_1 hybrids was identical with that of female species. Similar results were obtained while investigating electrophoretic spectra of LAP of *T. repens* × *T. nigrescens* hybrids, where the spectra of this enzyme for the larger part of the hybrids was identical with those of female *T. repens* L. species [20].

Analysis of DNA fingerprints. For DNA fingerprint analysis of *T. pratense, T diffusum* and their F_1 interspecific hybrids 18 microsatellite tetra- tri- and dinucleotide repeats were used as primers, of which 12 generated different DNA fingerprints and were suitable for hybridity confirmation. Most of the primers used generated DNA profiles of one or two types in interspecific hybrids. The primers $(GACA)_4GT$, $(TC)_8G$, $(CAA)_5CG$ were shown to have a high potential not only for confirmation of hybridity but also for revealing DNA polymorphism within F_1 and B_1 hybrids. DNA profiles of interspecific hybrids were divided into five groups (Table 2). Profiles of F_1 hybrids amplified by $(GACA)_4GT$ primers and belonging to groups III and V differed by the location of extra fragments: profiles of two and five types were identified in them. Also, when using $(CAA)_5CG$ primer, hybrid profiles of group V were of four types. However, $(TC)_8G$ primer in F_1 hybrids generated DNA fingerprints identical with those of *T. diffusum* species.

When $(GACA)_4GT$ primer was used for DNA amplification of B_1 hybrids, the profiles obtained were identical with those of *T. pratense* plants. DNA profiles of part of B_1 individuals (60.0%) amplified by $(CAA)_5CG$ primer were also identical with those of the female form, and DNA fingerprints of the other individuals were different.

Although PCR performed with $(TC)_8G$ primer F_1 hybrids had identical profiles, different (groups IV

Table 2. **Groups of DNA profiles and their frequency (%) in interspecific hybrids between *T. pratense* × *T. diffusum***

Primers	Population	Profile identical to *T. pratense*	Profile identical to *T. diffusum*	*T. diffusum* and novel fragments	*T. pratense* and *T. diffusum* fragments	*T. pratense* *T. diffusum* and novel fragments
		I	II	III	IV	V
$(GACA)_4GT$	F_1	0	0	13.2	40.0	46.8
	B_1	100	0	0	0	0
$(GAA)_5CG$	F_1	0	6.7	0	0	93.3
	B_1	60.0	0	16.7	23.3	0
$(TC)_8G$	F_1	0	100	0	0	0
	B_1	0	0	0	73.3	26.7

and V) DNA profiles were characteristic of B_1 hybrids.

Primers used for DNA analysis of *T. ambiquum* and *T. hybridum* induced formation of species-specific fragments, but in as many as 82.5% of cases the profiles of hybrids were identical with those of female clover species. Using $(TCC)_5GT$, $(TC)_8G$, $(GA)_8CT$ primers for PCR, hybridity of 84.2–97.5% of the individuals was confirmed. DNA profiles of interspecific hybrids amplified by $(TCC)_5GT$ primer were identical. DNA polymorphism was revealed by using $(GA)_8CT$ and $(TC)_8G$ primers: fingerprints of two and three types, respectively, were determined.

Our experimental findings show that investigation of interspecific clover hybrids by molecular methods has revealed their genetic polymorphism. Thus, further research work should be focused on the search for markers showing distinctive clover traits and characteristics.

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References

1. Fraser J, Cartney DMc, Najda H, Mir Z. Can J Plant Sci 2004; 84: 143–55.
2. Smith RR. Breeding for a Multifunction Agriculture. Swiss Federal Research Station For Agroecology and Agriculture. Zurich–Reckenholz, 1998: 87– 90.
3. lley JL, Garrett HE, McGraw RL et al. Agroforestry systems 1999; 44: 281–91.
4. Marshall AH, Williams TA, Powell HG et al. Grass & Forage Science 2002; 57(3): 232–8.
5. Lloveras J, Iglesias I. Grass and Forage Science 2001; 56(4): 395–404.
6. Stout DG, Brooke B, Hall JW and Thompson DJ. Grass and Forage Science 1997; 52(3): 298–308.
7. Arzani A, Samei K. Genetic Variation for Plant Breeding. Vienna 2004: 85–8.
8. Marshall AH, Abberton MT, Michaelson-Yeates TPT. Vortrage für Pflanzenzuchtung 2003; 59: 152–5.
9. Ansari HA, Ellison NW, Reader SM et al. Ann Botany 1999; 83: 199–206.
10. Marshall AH, Abberton MT, Williams TA et al. Grass & Forage Science 2003; 58(3): 295–301.
11. Dabkevièienë G. Sodininkystë ir darþininkystë 2000; 5–6[19(3)] – 1: 375–83.
12. Gardinier E, Forde MB. Biochemical Identification of Varieties. Leningrad, 1988: 65–72.
13. Ñàôîíîâ ÂÈ, Ñàôîíîâà ÌÍ. Áèîõèìè÷åñêèå ìåòîäû â ôèçèîëîãèè ðàñòåíèé. Ì îñêâà, Hayêa, 1971: 113–36.
14. Pasteur N, Pasteur G, Bonhomme F et al. Partical Isozyme Genetics. Paris, 1987: 215.
15. Doyle JJ, Doyle JL. Focus 1990; 12: 13–5.
16. Haider AS and El-Shanshoury AR. Biol Plant 2000; 43(2): 205–9.
17. Valizadeh M. J Agric Sci Technol 2001; 3: 287–92.
18. Przywara L, White DWR, Sanders PM et al. Ann Botany 1989; 64: 613–24.
19. Malaviya DR, Roy AK, Kaushal P et al. Plant Breeding 2004; 123: 536–42.
20. Marshall AH, Michaelson–Yeates TPT, Aluka P et al. Heredity 1995; 74: 136–45.

V. Paplauskienë, G. Dabkevièienë

DOBILØ GENETINËS ÁVAIROVËS TYRIMØ MOLEKULINIAI METODAI

Santrauka

Tiriant LÞI kolekcijos dobilø genetinæ ávairovæ taikyti ðie molekuliniai metodai: sëklø baltymø ir fermentø elektroforetiniø spektrø bei DNR atspaudø analizës. Baltymø elektroforetiniai spektrai patvirtino *T. pratense* × *T. diffusum* F_1 ir iš dalies B_1 individø hibridinæ prigimtá, bet neatskleidë jø genetinës ávairovës. Tarprûðiniuose *T. pratense* × *T. diffusum* F_1 ir B_1 hibriduose nustatyti du superoksidodismutaziø ir ðeði peroksidaziø spektrø tipai, árodantys jø genetiná ávairumà. *T. pratense* × *T. diffusum* F_1 DNR polimorfiðkumas iðryðkëjo PCR naudojant $(GACA)_4GT$, $(CAA)_5CG$ pradmenis, o B_1 hibridø genetinei ávairovei atskleisti labiau tinka $(CAA)_5CG$ ir $(TC)_8G$ pradmenys. Naudojant mikrosatelitiniø sekø $(TCC)_5GT$, $(GA)_8CT$, $(TC)_8G$ pradmenis 84,2–97,5% buvo patvirtintas tirtø *T. ambiquum* × *T. hybridum* individø hibridiðkumas, DNR polimorfiðkumas nustatytas pagal $(GA)_8CT$, $(TC)_8G$ pradmenis.