BIOLOGIJA. 2008. Vol. 54. No. 2. P. 56–59
DOI: 10.2478/V10054-008-0011-y
© Lietuvos mokslų akademija, 2008
© Lietuvos mokslų akademijos leidykla, 2008

# Genetic variability determination using ISSR–PCR markers in red clover varieties

**Vanda Paplauskienė,**

**Giedrė Dabkevičienė**

*Lithuanian Institute of Agriculture, Instituto 1, LT-58344 Akademija, Kėdainiai distr., Lithuania*
*E-mail: vanda@lzi.lt, giedre@lzi.lt*

Studies on DNA polymorphism are of great relevance in plant breeding since they give a deeper insight into genetic diversity. DNA profiles of individual plants of red clover varieties produced using the simple-sequence repeat primers UBC825, UBC827, UBC857, 77H, 78H, 104H and GO4 were assessed in this work. In the DNA profiles, in total 39–43 fragments were amplified, of which 52.5–60.4% were polymorphic. The highest number of fragments was amplified by the UBC827 primer, moreover, these fragments were found to be most polymorphic (90.5%). Slightly fewer polymorphic fragments were generated by the primers 77H and 78H. The primers 104H and GO4, when used in PCR, produced in the DNA profiles only one fragment each, which was identical for all individuals. Comparison of the varieties revealed the lowest number of fragments in the individuals of the variety 'Vyliai' (39), of which 57.4% were polymorphic. In the DNA profiles of the individuals of the varieties 'Liepsna' and 'Arimaičiai' 43 fragments were identified.

Research on the intravarietal genetic diversity suggests that plants of the variety 'Liepsna' are characterised by the greatest polymorphism (0.313), while 'Arimaičiai' has been found to be the most homogeneous variety (0.241).

The genetic distance between the varieties varied from 0.010 to 0.458. According to Neïs coefficient value, the greatest genetic distance was noted between the varieties 'Vyliai' and 'Arimaičiai' (0.177) and between 'Liepsna' and 'Arimaičiai' (0.181). Variety-specific DNA fragments were identified in some red clover varieties. Amplification done with the primer 78H produced a fragment of 1400 bp which is variety-specific to 'Arimaičiai'. The 800 bp fragment, generated by the UBC857 primer, was specific to individuals of the varieties 'Liepsna' and 'Vyliai'.

**Key words:** red clover, varieties, DNA, ISSR fingerprinting

## INTRODUCTION

Red clover (*Trifolium pratense* L.) in Lithuania is valued as an excellent forage plant. However, it has some drawbacks, such as insufficient seed production, medium winterhardiness, inadequate disease and pest resistance [1]. As a result, the central task for clover breeders is to search for new possibilities to improve these qualities. The basis for red clover breeding schemes is the diversity of the initial breeding material (germplasm) [2]. Intensive search for the sources of more diverse initial breeding material is currently being conducted with a special emphasis on the phenomenon of polymorphism within a species and within a variety [3, 4].

Recently, biochemical-molecular techniques, including DNA fingerprint analysis, have been extensively used to reveal plant diversity. There are different molecular methods for germplasm evaluation using simple and effective markers based on the polymerase chain reaction (PCR) [5–7]. For germplasm variability evaluation, identification of cultivars and genetic distance estimation, dominant markers such as amplified fragment length polymorphisms (AFLPs), random amplified polymorphic DNAs (RAPDs) and inter-simple sequence repeats (ISSR) have been widely utilized during the past 15 years [8]. Research on DNA polymorphism is of great relevance in breeding since it indicates genetic differences that are not necessarily observed phenotypically. This method is effective in selection of plants for hybridization, estimation of relatidness, and variety identification. DNA markers are being widely used in diversity tests of cereals and industrial crops, whereas experimental evidence on legumes is less comprehensive. In the legume family, genomics and molecular genetics have rapidly advanced for the last several years with the central focus on two model legume species, *Lotus japonicus* L. and *Medicago truncatula* Gaertn. [9]. There is a limited number of studies conducted with molecular markers in red clover as compared with other forage species. In *Trifolium*, primarily RAPDs (dominant markers) have been utilized for the evaluation of genetic diversity in *T. pratense* [10–13]. The genetic diversity of red clover has been intensively studied using amplified fragment length polymorphism (AFLP) markers [14, 15]. The advantage of the use of ISSR as a dominant marker compared to RAPD has been the reproducibility of ISSR methodology reported for several species [5, 13].

The objective of the present study was to estimate DNA diversity of the Lithuanian red clover varieties using the ISSR method and to carry out a search for markers suitable for variety identification.

## MATERIALS AND METHODS

For DNA analyses, clover (20 plants per variety) was grown in pots in a greenhouse at 20–24 °C, with a 16-hour photoperiod. The clover varieties 'Liepsna', 'Vyliai', 'Vyčiai' and 'Arimaičiai' were included.

Genomic DNA was extracted from a fresh young actively growing leaves of a five-week-old seedling. Approximately 500 mg of leaf tissue was frozen in liquid nitrogen and ground into a fine powder with a mortar and pestle. The pulverized material was transferred to a tube and extraction solution was added. DNA was extracted by the CTAB method following the procedure described by Doyle and Doyle [16]. DNA concentration was determined with an Eppendorf biophotometer. Polymerase chain reactions (PCR) were carried out in 25 µl volume in an Eppendorf Master Cycler Gradient thermocycler under the following conditions: 1 cycle of 95 °C for 2 min, 50 °C for 1 min, 72 °C for 30 s; 39 cycles of 95 °C for 30 s, 50 °C for 1 min, 72 °C for 30 s; 1 cycle of 72 °C for 6 min. The amplification products were separated by gel electrophoresis on 1.5% agarose gel in 1 × TAE buffer and stained with etidium bromide. GeneRuler™ DNA Ladder Mix (Fermentas) was used as the DNA fragment size marker. The simple-sequence primers used in this study UBC825-$(AC)_8$T, UBC857-$(AC)_8$CG, UBC827-$(AC)_8$G, UBC847$(CT)_8$RG, GO4-$(GACA)_4$TC were obtained from Fermentas (Lithuania) and 77H-$(AGAC)_4$GC, 78H-AC$(GACA)_4$, 104H-$(GACA)_4$GT from Metabion (Germany).

The genetic Nei's distance between the varieties was calculated using NTSYSpc2.20M N software.

## RESULTS AND DISCUSSION

To reveal DNA diversity, we used analysis of inter-SSR fingerprinting (ISSR). PCR was done with di- and tetra-nucleotide motif primers. With the UBC825 primer we amplified 7–8 fragments in the varieties whose size varied within 450–1250 bp (Table 1). In *Olea europaea* L. variety tests, PCR done with the above-mentioned primer gave rise to 8 fragments of which 88% were polymorphic [17]. This primer generated one common 650 bp fragment in the DNA fingerprints of all individuals of the varieties, the other fragments being polymorphic. When other AC motif primers possessing different anchors had been used in the PCR, a higher number of fragments were amplified in the DNA profiles of many individuals. The highest number of fragments (10) generated with the UBC857 primer was found for the individuals of var. 'Liepsna'. Fewer fragments (7) were identified in the profiles of plants of var. 'Vyliai'. The size of the fragments produced varied within a wider 350–2000 bp range. The fragment 1000 bp was found to be common for the plants of all varieties tested. The UBC827 primer generated 10–11 fragments in the DNA profiles of the varieties, but only one fragment of 750 bp was common. The primers UBC825, UBC827 and UBC857 were also successfully used in the genetic diversity tests of *Arabidopsis thaliana* L. [18].

Slightly fewer fragments were amplified with tetranucleotide motif primers. Part of the fragments amplified with the 77H primer (2000 and 2200 bp) were common, and the individuals of var. 'Arimaičiai' had another variety-specific fragment – 1000 bp. Using this primer in white clover genetic diversity studies, DNA profiles were also found to possess a low number (5) of fragments [19].

The 78H primer generated 6–7 fragments of which 57.1–83.3% were polymorphic. This primer was successfully used to reveal DNA polymorphism in white clover, meadow fescue, and Italian ryegrass [19, 20]. In the GACA nucleotide sequence, on replacing the anchor AC by TC or CG, i. e. using 104H or GO4 primers, each of them generated only one monomorphic fragment in clover DNA profiles. Analysis of ryegrass and fescue DNA profiles showed that the change of anchor in the GACA nucleotide sequence also had some effect on the number of fragments produced and on their size [20]. Although 104H primer did not reveal red clover polymorphism, it suited for identification of interspecific hybrids *T. pratense* L. × *T. diffusum* Ehrh. [21], while in research on *Pheonix dactylifera* L. genetic resources no fragments were amplified by GACA motif primers [22].

Amplification of clover DNA with the UBC847 primer did not produce any fragments. In the genus *Oryza*, this primer did not generate distinct fragments, either [23]. In the studies of red clover genetic variability by the RAPD method, out of the 55 primers used only 21 provided reproducible results. [11]. Of the simple-sequence repeat primers used in our study, UBC827 produced the greatest number of fragments, moreover, these fragments were most polymorphic (90.5%).

When comparing individual varieties it was found that the fewest fragments (39, of which 57.4% were polymorphic) were

Table 1. **ISSR products generated by 5 primers in Lithuanian red clover varieties**

| Primer code | Oligonucleotide sequence | Size range of amplified bands, bp | 'Liepsna' | | 'Vyliai' | | 'Vyčiai' | | 'Arimaičiai' | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 |
| UBC825 | (AC)8T | 450–1250 | 7 | 85.7 | 7 | 85.7 | 7 | 85.7 | 8 | 87.5 |
| UBC857 | (AC)8CG | 350–2000 | 10 | 90.0 | 7 | 71.4 | 8 | 75 | 8 | 75 |
| UBC827 | (AC)8G | 400–1100 | 10 | 90.0 | 10 | 90 | 11 | 90.9 | 11 | 90.9 |
| 77H | (AGAC)4GC | 450–2200 | 7 | 85.7 | 7 | 71.4 | 7 | 71.4 | 7 | 57.1 |
| 78H | AC(GACA)4 | 350–2500 | 7 | 71.4 | 6 | 83.3 | 7 | 71.4 | 7 | 57.1 |
| 104H | (GACA)4GT | 1050 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 |
| GO4 | (GACA)4TC | 600 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 |
| Total | | 350–2500 | 43 | 60.4 | 39 | 57.4 | 42 | 56.3 | 43 | 52.5 |

1 – number of bands, 2 – polymorphic bands, %.

amplified in the individuals of var. 'Vyliai'. In the DNA profiles of var. 'Liepsna' and 'Arimaičiai' there were identified 43 fragments, however, the individuals of var. 'Arimaičiai' had more common fragments.

Table 2. **Genetic diversity within red clover varieties**

| Primer code | 'Liepsna' | 'Vyliai' | 'Vyčiai' | 'Arimaičiai' |
|---|---|---|---|---|
| UBC825 | 0.357 | 0.278 | 0.221 | 0.250 |
| UB857 | 0.285 | 0.193 | 0.168 | 0.143 |
| UB827 | 0.375 | 0.345 | 0.378 | 0.371 |
| 77H | 0.357 | 0.278 | 0.342 | 0.210 |
| 78H | 0.191 | 0.325 | 0.257 | 0.230 |
| Mean | 0.313 | 0.284 | 0.273 | 0.241 |

The UBC827 primer was found to be best suited for revealing clover intra-varietal genetic diversity (Table 2). In all varieties tested this primer produced the highest number of fragments and showed the greatest DNA diversity. Slightly lesser genetic diversity was determined using UBC825 and 77H primers. Different primers demonstrated a varying polymorphism within varieties. The value of the genetic diversity ranged from 0.143 ('Arimaičiai' – UBC857) to 0.378 ('Vyčiai' – UBC827). When comparing individual varieties according to average genetic diversity, the highest polymorphism (0.313) was identified for the individuals of var. 'Liepsna'. This clover variety has been grown in Lithuania for the longest time (registered in 1957) and apparantely outcrossing with other red clover populations has not been prevented. The least genetic diversity value (0.241) was identified for var. 'Arimaičiai' characterised by resistance to clover rot and persistence. Lower levels of genetic variability were detected in a subset of parents selected for resistance to stem nematode [11].

Our experimental evidence suggests that within varieties there exist individuals distinguished by the presence or absence of individual DNA fragments. Similar results were obtained by Hagen and Hamrick [24] while testing red clover enzyme systems. High intra-varietal variation was observed in two cultivars of red clover, 'Essi' from Europe and 'Ottawa' from Canada, using isozyme and RAPD markers [25].

To estimate varietal similarities, their genetic distance was calculated. Using different primers, the value of Nei's coefficient for separate varieties varied from 0.010 to 0.458 (Table 3). Similar data were obtained by American researchers investigating white clover collections and cultivars [12]. The genetic distance of RAPD markers for red clover between 20 populations and varieties varied from 0.19 to 0.67 [8]. In red clover DNA tests by the RAPD method, genetic distance between 20 populations and varieties varied from 0.19 to 0.67 [8]. In our research, the greatest genetic distance was determined between 'Liepsna' and 'Arimaičiai' (0.181) and 'Vyliai' and 'Arimaičiai' (0.177). The variety 'Arimaičiai' is a complex synthetic population combining in its genome characteristics of 80 various clover varieties that survived the long-term *Sclerotinia trifoliorum* Eriksson. infection.

DNA fragment distribution analysis has shown that some of DNA fragments or their compositions may be applied for varietal discrimination. Most of DNA profiles (75%) in 'Liepsna' and 'Vyliai' plants amplified by the UBC825 primer have a specific fragment, 800 bp. The fragment 450 bp amplified by this primer is specific only to some plants of var. 'Arimaičiai'. The DNA profiles of all individuals of var. 'Vyliai', generated with the primer C827, had the 1000 bp fragment, whereas in the individuals of other varieties this fragment is rare. The 800 bp fragment is specific to all individuals of var. 'Liepsna' and 'Vyliai', generated by the UBC857 primer. This fragment was not identified between the individuals of var. 'Arimaičiai'. The fragment was identified in discrete individuals of var. 'Vyčiai'. The 2000 bp fragment was specific to most individuals of 'Vyčiai' and 'Arimaičiai'. This fragment was not noted in the plants of 'Vyliai' and 'Liepsna'. The 'Arimaičiai'-specific 1400 bp fragment was produced with the 78H primer. The mentioned fragment was not identified in the individuals of other varieties.

Several DNA analysis methods were employed for white clover varietal identification, however, the great variety obtained by the RAPD method within varieties did not allow identification of varieties [26]. Studies on red clover isozyme systems also showed a great genetic diversity within varieties and therefore were suitable only for some variety identification [27]. In order to reduce intra-varietal polymorphism, bulked genomic DNA can be used for red clover variety identification. Bulked samples from twenty individuals were examined in order to minimize the variation within the cultivars. Cultivar-specific bands were observed with 13 primers. The amplification patterns obtained from two primers could distinguish all 15 red clover cultivars [10]. When the intravarietal DNA diversity is high, it is hard to generate variety-specific fragments. For a more accurate varietal identification, a higher number of primers should be used.

Table 3. **Genetic Nei's distance among red clover varieties**

| Primer code | 'Liepsna' x 'Vyliai' | 'Liepsna' x 'Vyčiai' | 'Liepsna' x 'Arimaičiai' | 'Vyliai' x 'Vyčiai' | 'Vyliai' x 'Arimaičiai' | 'Vyčiai' x 'Arimaičiai' |
|---|---|---|---|---|---|---|
| UBC825 | 0.029 | 0.048 | 0.126 | 0.060 | 0.126 | 0.062 |
| UB857 | 0.100 | 0.130 | 0.096 | 0.087 | 0.094 | 0.023 |
| UB827 | 0.034 | 0.016 | 0.027 | 0.010 | 0.052 | 0.046 |
| 77H | 0.034 | 0.458 | 0.069 | 0.304 | 0.044 | 0.208 |
| 78H | 0.078 | 0.119 | 0.278 | 0.136 | 0.270 | 0.049 |
| Mean | 0.068 | 0.086 | 0.181 | 0.076 | 0.177 | 0.080 |

Our experimental results showed that it is expedient to apply the ISSR method in red clover breeding schemes for revealing genetic diversity.

## CONCLUSIONS

1. Having used 7 primers in the PCR, 39–43 fragments were generated in the DNA profiles of the red clover varieties; 52.5–60.4% of the fragments were polymorphic.

2. The UBC825, UBC827, UBC857, 77H and 78H were found to be suitable to reveal varietal polymorphism.

3. The greatest intravarietal diversity was identified for individuals of var. 'Liepsna'.

4. The fragment 1400 bp generated with the 78H primer was variety-specific to the individuals of 'Arimaičiai'. The fragment 800 bp, specific to the individuals of var. 'Liepsna' and 'Vyliai', was amplified with the UBC857 primer.

## ACKNOWLEDGEMENTS

We gratefully acknowledge the support of this work by the Lithuanian Ministry of Education and Science programme 'Genefund'.

Received 24 January 2008
Accepted 16 April 2008

### References

1. Gudžinskas Z. Lietuvos induočiai augalai. Vilnius: Botanikos instituto leidykla, 1999: 147–8.
2. Barker EE, Kalton RR. Special publication, Crop Science Soc America. Madison, 1989: 5–19.
3. Lange O, Schifino-Wittman MT Ann Botany 2000; 86: 339–45.
4. Gataric D, Alibegovic-Grbic S, Lakic Z and Sveiko V. Grassland Science in Europe 2002; 7: 412–3.
5. Fernandez ME, Figueiras AM and Benito C. Theor Appl Genet 2002; 104: 845–51.
6. Barreto Dias PM, Dall'Agnol M and Schifino-Wittmann MT. J Plant Gen Res 2004; 2: 43–50.
7. Real D, Dalla Rizza M, Quesenberry KH et al. Crop Sci 2004; 44: 394–400.
8. Ulloa O, Ortega F and Campos H. Genome 2003; 46: 529–35.
9. Young ND, Cannon SB, Sato S et al. Plant Physiol 2005; 137: 1174–81.
10. Kongkiatngam P, Waterway MJ, Coulman BE et al. Euphytica 1996; 89: 355–61.
11. Campos-de-Quiroz H and Ortega-Klose F. Euphytica 2001; 122: 61–7.
12. Gustine DL, Voigt PW, Brummer EC et al. Crop Sci 2002; 42: 343–7.
13. Greene SL, Gritsenko M and Vandemark G. Genet Res Crop Evol 2004; 51: 643–53.
14. Kolliker R, Herrmann D, Boller B et al. Theor Appl Genet 2003; 107: 306–15.
15. Herrman D, Boller B, Widmer F et al. Genome 2005; 48: 474–86.
16. Doyle JJ, Doyle JL. Focus 1990; 12: 13–5.
17. Martins-Lopes P, Lima-Brito J, Gomes S et al. Genet Res Crop Evol 2007; 54: 117–28.
18. Barth S, Melchinger AE, Lubberstedt TH. Molecular Ecology 2002; 11: 495–505.
19. Paplauskienė V, Dabkevičienė G. VDU Botanikos sodo raštai 2006; XI: 106–14.
20. Pašakinskienė I, Griffiths CM, Bettany AJE et al. Theor Appl Genet 2000; 100: 384–90.
21. Paplauskienė V, Dabkevičienė G, Pašakinskienė I. Grassland Science in Europe 2005; 10: 281–4.
22. Zehdi S, Sakka H, Rhouma A.et al. African J Biotech 2004; 3(4): 215–9.
23. Joshi SP, Gupta VS, Aggarval RK et al. Theor Appl Genet 2000; 100: 311–20.
24. Hagen MJ, Hamrick JL. Heredity1998; 89: 178–81.
25. Kongkiatngam P, Waterway MJ, Fortin MG et al. Euphytica 1995; 84: 237–46.
26. Dolanska I, Čurn V. Plant Soil Environ 2004; 50(3): 95–100.
27. Yu J, Mosjidis JA, Klingler KA. Crop Sci 2001; 41: 1625–8.

**Vanda Paplauskienė, Giedrė Dabkevičienė**

## RAUDONŲJŲ DOBILŲ VEISLIŲ GENETINĖS ĮVAIROVĖS ĮVERTINIMAS ISSR METODU

*Santrauka*

DNR polimorfizmo tyrimai yra svarbūs selekcijai, nes labiau atskleidžia genetinius skirtumus. Šiame darbe įvertinti raudonųjų dobilų veislių atskirų individų DNR profiliai, gauti PCR naudojant paprastųjų pasikartojančių sekų pradmenis UBC825, UBC827, UBC857, 77H, 78H, 104H ir GO4. Dobilų DNR profiliuose iš viso amplifikuoti 39–43 fragmentai, kurių 52,5–60,4% buvo polimorfiški. Daugiausia fragmentų amplifikuota UBC827 pradmeniu, be to, šie fragmentai buvo polimorfiškiausi (90,5%). Kiek mažiau polimorfiškų fragmentų generavo 77H ir 78H pradmenys. PCR naudojant 104H ir GO4 pradmenis, dobilų DNR profiliuose gauta tik po vieną fragmentą, identišką visiems individams. Lyginant atskiras veisles, mažiausiai fragmentų buvo nutatyta veislės 'Vyliai' individuose – 39, kurių 57,4% buvo polimorfiški. Veislių 'Liepsna' ir 'Arimaičiai' individų DNR profiliuose nustatyta po 43 fragmentus.

Viduveislinės genetinės įvairovės tyrimai rodo, kad veislės 'Liepsna' augalai pasižymi didžiausiu polimorfiškumu (0,313), o 'Arimaičiai' – mažiausiai genetinių atmainų turinti veislė (0,241).

Genetinė distancija tarp atskirų veislių kito nuo 0,010 iki 0,458. Pagal Nei's koeficiento reikšmę labiausiai nutolusios viena nuo kitos yra veislės 'Vyliai' ir 'Arimaičiai' (0,177) bei 'Liepsna' ir 'Arimaičiai' (0,181). Kai kurioms raudonųjų dobilų veislėms buvo nustatyti jas specifiškai ženklinantys DNR fragmentai. Amplifikacijai naudojant 78H pradmenį, nustatytas 1400 bp fragmentas, specifiškai ženklinantis veislės 'Arimaičiai' individus. Visiems veislių 'Liepsna' ir 'Vyliai' individams buvo būdingas 800 bp fragmentas, generuotas UBC857 pradmeniu.