Identification of white clover (*Trifolium repens* L.) cultivars using molecular markers

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ABSTRACT

The different molecular analysis for specification of white clover (*Trifolium repens* L.) populations was studied between 2002 and 2003. RAPD, SSR (microsatellites), rDNA and PCR-RFLP markers were used for this study. The high genetic variation was detected among the cultivars but also within the cultivars by RAPD markers. For this reason RAPD markers were not found as a suitable marker system for determination of white clover cultivars. The distribution of low genetic variation of rDNA and PCR-RFLP markers was not able to differentiate cultivars. SSR and rDNA markers did not show variability of patterns within one cultivar. The different sizes of PCR fragments were obtained after amplification with microsatellite primers. SSR markers are therefore suggested as the suitable markers for the identification of different *T. repens* cultivars.

Keywords: *Trifolium repens* L.; identification of cultivars; RAPD; microsatellites; rDNA; PCR-RFLP

Trifolium repens L. (white clover, $2n = 4x = 32$) is one of the most important and widely used forage legumes in temperate regions of the world. White clover is a perennial species but stands often decline significantly in the second or third year of growth due to susceptibility to a number of stress factors including drought, viruses, nematodes and root-chewing insects (Williams 1987). *T. repens* is a significant forage legume for sheep and beef cattle. Therefore, there are continuous requirements for breeding cultivars of higher quality better adapted to a harsh environment (Marshall et al. 1995).

A number of PCR-based DNA markers have been developed in recent years to evaluate genetic variation at the intraspecific and interspecific levels (Wolfe and Liston 1998). Molecular markers allow the selection of desired traits based on genotype and can therefore complete and accelerate plant breeding programs. They can be also used for the early selection of traits, which are not expressed during the juvenile phase such as persistence, competitive ability and seed yield (Kölliker et al. 2001).

RAPD (random amplified polymorphic DNA) technology is a reliable method for characterizing variation within and among species and populations (Gustine and Huff 1999). Phylogenetically conserved and individual-specific products abundant in plant genomes are generated in RAPD reaction (Williams et al. 1990, Echt et al. 1992). It is assumed that primer-target sites are randomly distributed along the template genome and cover both the conserved and hypervariable regions (Caetano-Anollés et al. 1991), thereby some RAPD-amplified fragments are polymorphic.

Microsatellites or simple sequence repeats (SSRs, Sweeney and Dannenberger 1995), other genetic markers based on the PCR (polymerase chain reaction) which are short tandem repeat units of between 1 and 6 bp in length (Tautz 1989). Di-, tri- and tetranucleotide repeats are the most common and are widely distributed throughout the genomes of plants and animals (Jarne and Lagoda 1996). The use of SSRs as genetic markers results from their inherent variability. The hypervariability of SSR loci is a consequence of unusually high mutation rates for these nucleotide sequences (Weber and Wong 1993).

During the past decade, analysis of the molecular diversity of the rDNA genes (5S and 18S-5.8S-26S rDNA) have already been shown to be suitable for establishing taxonomic and phylogenetic analyses (Kollipara et al. 1997) and even for cultivar identification (Kolchinski et al. 1991, Baum and Johnson 1998). Eucaryotic ribosomal RNA genes (known as ribosomal DNA or rDNA) are found as parts of repeat units that are arranged in tandem arrays. Each repeat unit consists of a transcribed region (having genes for 18S, 5.8S and 26S rRNAs and the external transcribed spacers i.e. ETS1 and ETS2) and a non-transcribed spacer (NTS) region. In the transcribed region, internal transcribed spacers (ITS)

Supported by the Ministry of Agriculture of the Czech Republic, Grants No. 1074, MSM J06/98 122200002, MSM J06/98 122200004.

are found on either side of 5.8S rRNA gene and are described as ITS1 and ITS2. Internal transcribed spacer region varying in size and sequence was found to be useful for DNA analysis.

PCR-RFLP (PCR/restriction fragment length polymorphism) was developed and employed to detect DNA variation. This method uses a combination of PCR and restriction digests of PCR products, especially after amplification of low number of fragments without differentiation in their length. This approach is able to detect higher levels of polymorphism. PCR-RFLP is suitable for rapid identification of origin and specific parentage of hybrids or alloploid species (Chen and Sun 1998).

The objective of this study was to evaluate and compare genetic variation among white clover cultivars using different DNA markers (RAPD, SSR, rDNA and PCR-RFLP). The results should be used for determination of white clover cultivars and evaluation of the persistence of these cultivars in recently established grasslands in Šumava Mountain Range.

MATERIAL AND METHODS

Plant material and DNA extraction

Three Danish cultivars *T. repens* Milka (2), Rivendel (3) and Nanouk (4) and one breeding material DP 85087 (1), seven Czech cultivars Vysočan (5), Ovčák (6), Klement (7), Král (8), Jordán (9), Jura (10) and Hájek (11) and *T. pratense*, *T. hybridum* and *T. incarnatum* were analysed. Original seed of cultivars was obtained from the Breeding Station Hladké Životice and Research Institute for Fodder Plants Troubsko, Czech Republic.

Genomic DNA of white clover cultivars was extracted from 8-week-old seedlings' leaves by bulking method from 30 plants using Invisorb Spin Plant Kit (INVITEK). Genomic DNA of cv. Hájek was extracted from 30 plants individually (30 templates) and three samples were extracted for cultivars Milka, Nanouk, Vysočan and Jordán by bulking method for evaluation of genetic variation within cultivars.

RAPD protocol

Each reaction was prepared on ice with a volume of 25 µl containing 10mM Tris-HCl (pH 8.3), 50mM KCl, 2mM $MgCl_2$, 1% Triton X-100, 100µM of each dATP, dCTP, dGTP and dTTP, 1 U TaKaRa *Taq* DNA polymerase, 10pM primer (Operon Technologies, Inc., Alameda, CA) – from kits OPA, OPB and OPF and 25 ng of template DNA. Amplifications were performed using a Thermocycler PROGENE (Techne-Cambridge, Ltd.) programmed for 93°C (5 min) for starting separation of DNA strands, followed by 45 cycles of 1 min at 92°C, 2 min at 35°C and 3 min at 72°C. After all cycles were completed, the reactions were held at 72°C for 10 min and slowly cooled to 4°C. The amplified DNA fragments were analysed by electrophoresis on 1.5% agarose gels running in 1 × TBE buffer (Sambrook et al. 1989) at 70 V for 3 h. The gels were visualised by ethidium bromide staining and photographed under UV light.

Microsatellite (SSR) protocol

Microsatellite (SSR) loci were amplified using 5 primer pairs:
TRSSRA01H11 (5'AGAAAGGTGAATGATGAAA, 3'TCTAATTCTTCCAATAGGG)
TRSSRA02B08 (5'TTTTGCTAATAAGTAATGCTGC, 3'GGACATTATGCAATGGTGAG),
TRSSRA02C02 (5'AAATAAAAACCACAAGTAACTAG, 3'TATAGGTGATTTGAAATGGC),
TRSSRA02C03 (5'TATGCTGGTAGATAAACTTAAA, 3'TGCTCTGGAGATTGATGG),
TRSSRAXX31 (5'TCTGTTTTGTTGGCCATGC, 3'TTGCAAAGTGTTTGGAAGGA)
(Kölliker et al. 2001) under the following conditions: 50 ng DNA template, 1 × buffer (10mM Tris-HCl, pH 8.3, 50mM KCl, 2mM $MgCl_2$, 1% Triton X-100), 100µM dNTP, 10pM of primer, 1 U TaKaRa *Taq* DNA polymerase. 30 PCR cycles were performed, with 30 s of denaturation at 94°C, 30 s of annealing at 53°C, and 1 min of polymerisation at 72°C. PCR products were visualised by ethidium bromide staining after electrophoresis on 10% polyacrylamide gels in TBE buffer.

rDNA protocol

The content of reaction mixture is consistent with microsatellite protocol. Reaction conditions were following: 33 PCR cycles were performed with 30 s of denaturation at 94°C, 1 min of annealing at 53°C, and 1 min of polymerisation at 72°C and final elongation temperature 72°C – 7 min. PCR products were visualised by ethidium bromide staining after electrophoresis on 10% polyacrylamide gels in TBE buffer.

White clover was analysed by universal primers for:
ITS1-5.8S-ITS2
[ITS(1) 5'TCCGTAGGTGAACCTGCGG,
ITS(4) 3'TCCTCCGCTTATTGATATGC]
and for 5.8S-ITS2
[ITS(3)5'GCATCGATGAAGAACGCAGC,
ITS(4), White et al. 1990].

PCR-RFLP

The amplified DNA fragments after SSR or rDNA analysis were digested with restriction endonuclease – 15 µl of PCR product was mixed with 2 µl RE buffer, 0.5 µl (5 U) restriction endonuclease (Takara) and 2.5 µl H_2O to give a final volume of 20 µl. The mixture was incubated by instruction (mostly at 37°C for 4 h), and subjected to electrophoresis on 10% polyacrylamide gel in 1 × TBE buffer at 150 V for 6 hours and detected by staining with ethidium bromide.

Data analysis

RAPD, SSR, rDNA and PCR-RFLP products were digitalised and analysed by *Bioprofil 1D+* software (Vilber Lourmat, France) with manual correction. Dendrograms were constructed by the UPGMA (Unweighted Pair Group Mean Average) method using STATISTICA 6 software package (Statsoft).

RESULTS AND DISCUSSION

This paper is intent on the completed methodology of new cultivars testing in compound cenoses. Polymorphism of arbitrary primers was tested by RAPD analysis of various species of *Trifolium* (*T. pratense*, *T. repens*, *T. hybridum*, *T. incarnatum*). Among 60 tested primers (OPA 1-20, OPB 1-20, OPF 1-20) 6 primers OPA-02, OPA-20, OPB-04, OPB-15, OPF-03 and OPF-14 gave polymorphic amplification patterns. RAPD analysis showed high interspecies polymorphism. RAPD analysis of 30 plants (templates DNA) of cv. Hájek was performed for better evaluation of the extent and patterns of distribution of RAPD diversity in *T. repens* as outcrossing species. A high level of polymorphism has been detected with primers OPB-04 and OPF-14 (Figure 1).

Variability of RAPD markers was tested within cultivars as well. The distribution of genetic variation was high among the cultivars but also within the cultivars. Figure 2 presents RAPD pattern of four cultivars, Milka (2), Nanouk (4), Vysočan (5) and Jordán (9) when three samples of each cultivar were amplificated with RAPD primer OPA-02. Persson et al. (2001) used RAPD markers to quantify the amount and distribution of genetic variation within and between accessions of rye. Most of the RAPD variation was found within rather than between the accessions, which is consistent with the pattern expected for a cross-pollinated crop.

On the contrary, SSR and rDNA markers did not show variability within one cultivar. These markers could be therefore more suitable for ge-

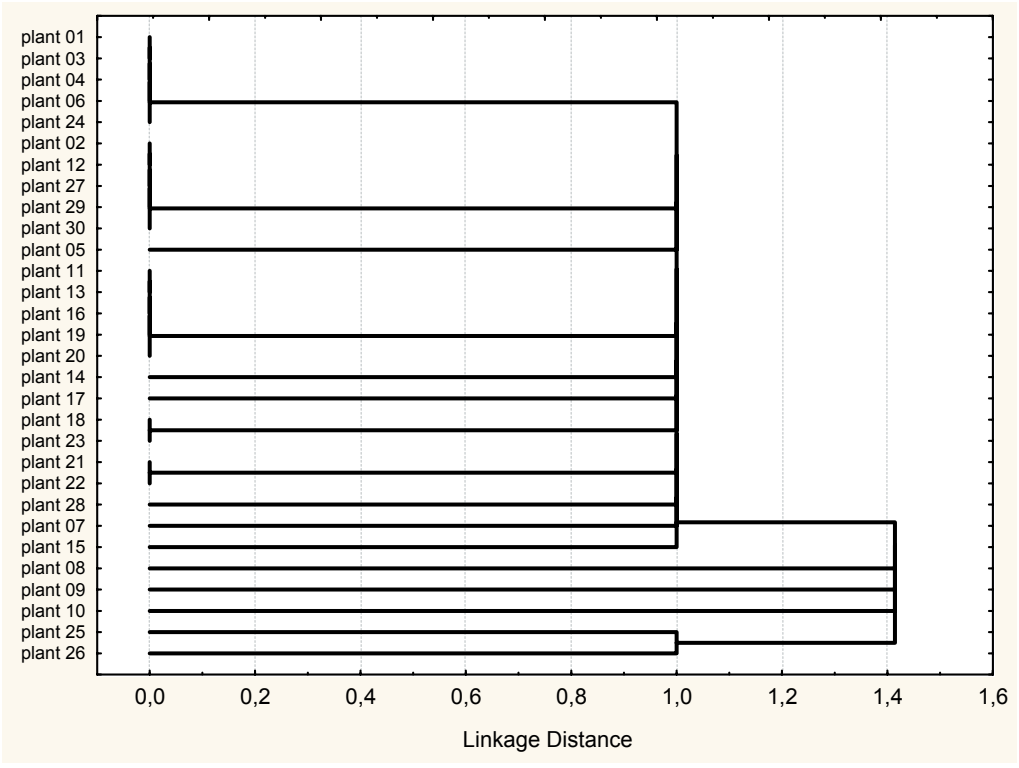

Figure 1. Dendrogram for RAPD analysis – 30 *Trifolium repens* plants, cultivar Hájek (primer OPF-14)

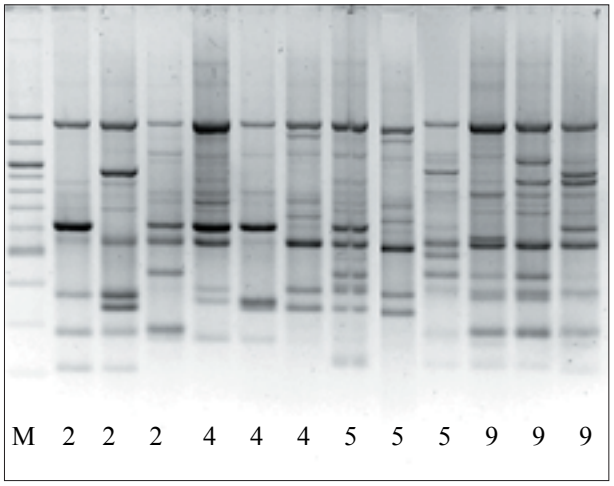

Figure 2. RAPD analysis of four cultivars of *Trifolium repens*: Milka (2), Nanouk (4), Vysočan (5) and Jordán (9) – primer OPA-02 was provided for comparison of variability RAPD patterns within cultivar, M-100 bp DNA ladder

nomic analysis of *T. repens* cultivars and as well for resolution of cultivated and wild form. SSR markers developed for *T. repens* (Kölliker et al. 2001) yielded very low percentage of polymorphic SSR loci. Among the 87 bands that appeared using 5 primer pairs, 25 were polymorphic, on average by five polymorphic bands on one primer pair. The highest polymorphism of amplification products was detected for SSR locus TRSSRA02C02. Genetic similarity value based on Nei-Li coefficient using *BioProfil 1D+* software was 90% for Danish population DP 8508 and cultivar Milka. The high similarity between these two samples was found also by RAPD analysis. Other cultivars had genetic similarity to others by 70–80% and the highest polymorphism was detected across species of *Trifolium* genera (*T. repens*, *T. pratense*, *T. hybridum* and *T. incarnatum*). The size of amplification products ranged from 142 bp to 305 bp (Figure 3). Kölliker et al. (2001) presented analogous the size of amplification products for SSR locus TRSSRA01H11 (Tr1) across different seven *T. repens* genotypes (84–312 bp). However, there was very low variability after amplification with the same primer pair for eleven *T. repens* populations tested.

PCR amplification of the 5.8S-ITS2 produced the banding pattern shown in Figure 4. The same sizes of rDNA repeats were observed for all species of *Trifolium* genera. Even if it was not possible to use restriction digestion of PCR products, since the restriction site was not found for eight restriction endonucleases, which were disposed at our laboratory. Different sizes of rDNA repeats were observed after PCR amplification of the ITS1-5.8S-ITS2 and restriction digest with endonuclease *Msp* I (Figure 5). Additional bands were observed only for three cultivars (Rivendel 3, Klement 7 and Hájek 11). The specific patterns obtained for different species of *Trifolium* genera confirmed that rDNA analysis was suitable for phylogenetic study (Zoldos et al. 1999). Successful rDNA analysis was found for identifying cultivated and wild forms of *T. repens*, while SSR markers were presented as ambiguous (Dolanská 2002).

Evaluation of genetic variation for outbreeding forage species is important for the process of cultivar identification and seed purity analysis, the ecological analyses of pasture populations, for selection of the genetically divergent parents for genetic mapping studies and for practise breeding. The outbreeding species are genetically heterogenous. Strategies for genetic diversity analysis based on DNA profiles must address this issue and allow the quantification of variation within and among species or cultivars.

RAPD analysis was an unsatisfactory method due to high polymorphism within one population. The RAPD profiling can characterise a genotype but not a population. Only by SSRs (microsatellite) analysis was it possible to identify different *T. repens* populations, even if this method expects to test a number of primer pairs and select one or several primer pairs that generate binding patterns representative for each population. The study of variability of ITS of rDNA cistron – genetically conserved source of interspecific variability is a suitable method for distinguishing different species, ecotypes and pathotypes (Vejl et al. 2002). PCR-RFLP method can improve DNA analysis, but

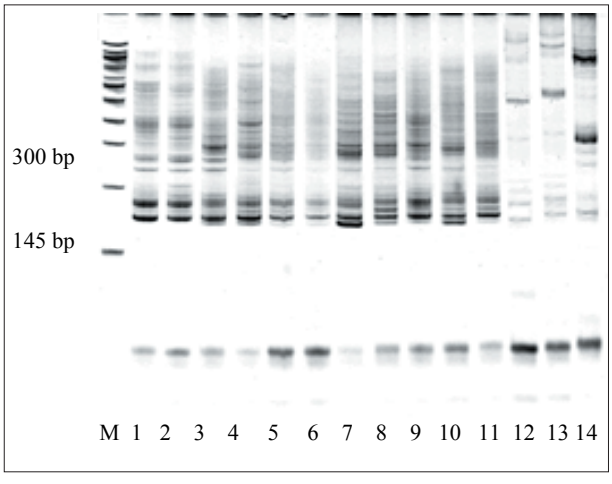

Figure 3. Amplification across eleven *Trifolium repens* genotypes (1 – DP 8508, 2 – Milka, 3 – Rivendel, 4 – Nanouk, 5 – Vysočan, 6 – Ovčák, 7 – Klement, 8 – Král, 9 – Jordán, 10 – Jura, 11 – Hájek) and *T. pratense* (12), *T. hybridum* (13), *T. incarnatum* (14) for SSR locus TRSSRA02C02 (Tr3), 100 bp DNA ladder

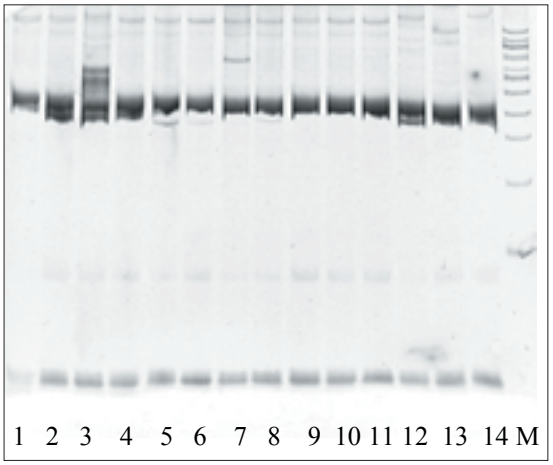

Figure 4. PCR ampification of the 5.8S-ITS2; *Trifolium repens* cultivars (1–11), *T. pratense* (12), *T. hybridum* (13), *T. incarnatum* (14), 100 bp DNA ladder

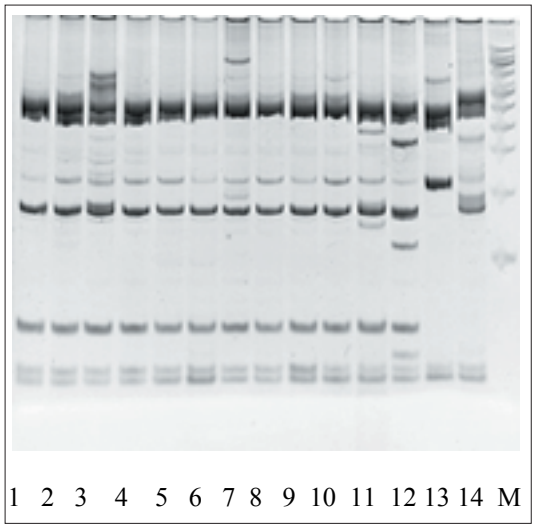

Figure 5. PCR amplification of the ITS1-5.8S-ITS2 and restriction digest with *Msp*I. *Trifolium repens* cultivars (1–11), *T. pratense* (12), *T. hybridum* (13), *T. incarnatum* (14), 100 bp DNA ladder

has limited use in breeding programmes, as it is relatively cumbersome to use, expensive and more time-consuming.

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Received on June 12, 2003

ABSTRAKT

Identifikace odrůd jetele plazivého (*Trifolium repens* L.) s využitím molekulárních markerů

V letech 2002–2003 byly studovány různé metody molekulární analýzy pro identifikaci vybraných populací jetele plazivého (*Trifolium repens* L.). Pro studium byly použity RAPD, SSR (mikrosatelity), rDNA a PCR-RFLP markery. RAPD analýzou byla detekována vysoká genetická variabilita mezi odrůdami, ale i uvnitř odrůdy. RAPD markery se ukázaly jako nejméně vhodný markerovací systém pro determinaci odrůd jetele plazivého. Nízká variabilita rDNA a PCR-RFLP markerů neumožnila rozlišení odrůd. Pomocí SSR a rDNA markerů nebyla zjištěna mezi rostlinami jedné odrůdy žádná genetická variabilita. Mikrosatelitovou analýzou byly amplifikovány rozdílné velikosti PCR fragmentů vhodné pro determinaci odrůd.

Klíčová slova: *Trifolium repens*; identifikace odrůdy; RAPD; mikrosatelity; rDNA; PCR-RFLP

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