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Comparison of morphological and RAPD markers in evaluation of red clover (*Trifolium pratense* L.) changes caused by natural selection

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Abstract

Background and Purpose: *Accurate assessment of the levels and patterns of genetic variability and identification of differences resulting from natural selection can be invaluable in red clover breeding for diverse applications. The study was conducted to compare the efficiency of morphological and random amplified polymorphic DNA (RAPD) markers in evaluation of changes in red clover* (Trifolium pratense L.) *cultivars caused by natural selection.*

Materials and Methods: *Three red clover cultivars and their selections, which had survived for three years growing in an upland region, provided the basic material. Morphological and RAPD data were analyzed by NTSYS-pc software. The number of markers detected, values and ranges of distances calculated and 2D Principal Coordinate Analyses (2D PCoA) based on both marker techniques were compared.*

Results: *Although both marker methods indicated differences among cultivars and selections, parameters differed in values. The number of loci detected by RAPD markers was much higher than that determined with morphological traits. The values and ranges of morphological distances were higher and wider than those of molecular distances. The eigenvectors of 2D PCoA based on morphological data accounted for more of the total variation observed in the whole data set.*

Conclusions: *Each of the data sets has its own strengths and constraints. For reasonably accurate estimates of genetic variability and its changes under natural selection, adequate attention has to be devoted to both marker methods.*

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INTRODUCTION

Accurate assessment of the levels and patterns of genetic variability and identification of differences resulting from natural selection can be invaluable in plant breeding for diverse applications *(1)*. Populations with more genetic variation are less vulnerable to any change in their environment and give a breeder better opportunity to find useful genes.

The range of genetic variation and differences among and within populations are traditionally estimated by morphological and agronomic traits. However, the use of these traits to estimate genetic varia-

tion is hampered by the lack of informative and stable markers. Consequently, such estimates may be unreliable. As molecular and biochemical markers avoid many of the environmental effects acting upon characters by directly observing variation controlled by genes or by observing the genetic material *(2)*, estimates of genetic variation are increasingly being based upon information at the DNA level *(3)*. Plant DNA can be analyzed by various methods. Random amplified polymorphic DNA (RAPD) method provides a powerful tool for the investigation of genetic variation *(4)* in various plant species.

Although some problems with reproducibility and reliability have been reported *(5, 6)*, RAPD-based studies are still on the increase *(7)*. Since no sequence information for the target species is required, RAPD markers are suited to situations where little or no molecular genetic research has been conducted previously *(8)*.

Despite the importance of red clover (*Trifolium pratense* L.) as a livestock feed crop and soil improver *(9)*, little is known about patterns, extents, and changes of genetic variation in red clover under natural selection. For example, it has not been determined if natural and/or artificial selection are responsible for variation among populations in monogenic morphological characters such as leaf marking descriptor *(10)*. The species is described as diploid (2n=14) with exception of some induced tetraploid cultivars (2n=28). Self-incompatible alleles and insect pollination ensure cross-pollination *(11)*. Thus, populations are characterized by extensive heterozygosity and a high level of phenotypic variation and assessment of genetic variability and identification of differences resulting from natural selection by using phenotypic markers are invariably long. The most recently developed class of molecular markers promises to make red clover breeding easier. An enhanced understanding of strengths and constraints of the morphological and RAPD markers may provide useful information for considering how to continue conventional red clover breeding and complement it with molecular breeding opportunities. This study was conducted to compare the efficiency of morphological and RAPD markers in analysis and interpretation of changes in red clover cultivars caused by natural selection.

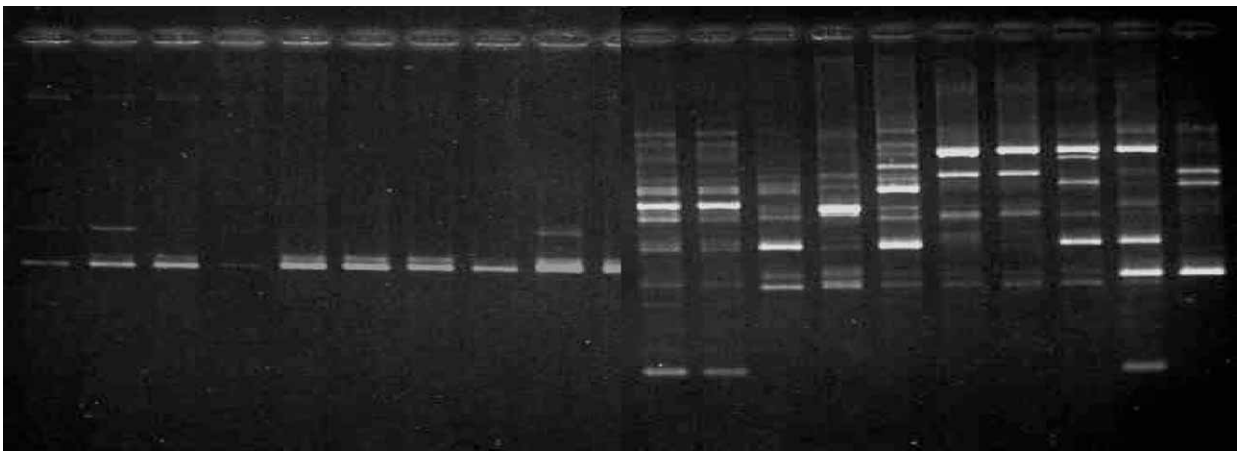
MATERIALS AND METHODS

Sources and derivation of genetic stocks. Three red clover cultivars, Reichersberger, K-17, and Croatia provided the basic material for the study. Reichersberger (C1) originated from Austria (Linz, lat 48°14'N, long 14°17'E), K-17 (C2) originated from Serbia and Montenegro (Krusevac, lat 43°58'N, long 21°32'E), and Croatia (C3) originated from Croatia (Botinec near Zagreb, lat 45°45'N, long 15°56'E). C1 and C2 seed was provided by commercial distributors and breeder's seed was used for C3. These three cultivars were planted in an upland region (Medvednica near Zagreb, altitude 650 m, lat 45°15'N, long 15°30'E) in 1995. After three years of natural selection in this upland region, 50 of the surviving clones selected at random from each of the three cultivars were transplanted at a lowland location (Zagreb, altitude 106 m, lat 45°44'N, long 16°04'E) and allowed to open pollinate in three isolated plots. The three groups of surviving clones from C1, C2, and C3 are referred to as selections and designated SC1, SC2, and SC3, respectively. Seed for the subsequent field trial was harvested from each clone separately in 1998.

Field trial of cultivars and selections. Seeds of cultivars (C1, C2, and C3) and the three selections (SC1, SC2, and SC3) were germinated in peat pellets and the seedlings were transplanted to the field at a lowland location (Osijek, altitude 90 m, lat 45°32'N, long 18°44'E) in 1999. The trial was arranged in a randomized complete block design (RCBD) with three replications. Each experimental plot included 200 spaced plants (0.50 × 0.30 m). For each of the three selections, each plot included four progenies of each of the 50 clones. No specific cultural conditions such as irrigation, fertilizer application, etc, were present at either the upland or the lowland locations.

Morphological trait analyses. Each plant of the cultivars and their selections was described morphologically mostly complying with the UPOV guidelines for tests for distinctness, homogeneity and stability *(12)* and the US protocol of Plant Variety Protection Office for red clover variety description *(13)*.

Molecular analyses. Young leaves from 20 plants of different clones selected at random of each cultivar and selection (120 samples in total) were harvested, lyophilized and ground. DNA was extracted according to the CTAB method *(14)* combined with the method according to Schweizer *et al. (15)*; 40 mg of each sample were incubated in 1 ml of extraction buffer (2% CTAB, 1 M Tris-HCl pH 8, 0.5 M Na2EDTA pH 8, 5 M NaCl, 0.2% 2-mercaptoethanol) at 65°C for 45 min. 670 µl of chloroform-isoamyl alcohol (24:1) were added and samples were mixed for 30 min and then centrifuged at 14000 ϕ/min for 8 min. The liquid phase was treated with 160 mg/µl RNAse A (Ribonuclease A type II-A, Sigma) for 30 min. DNA was precipitated by the addition of 650 µl of 0.7 V of cold isopropanol (–20°C) for 1 h and centrifuged at 11000 ϕ/min for 1 min. Pellets were first washed with 500 µl of 0.2 mM sodium acetate in 76% ethanol for 30 min and centrifuged at 11000 ϕ/min for 1 min and then washed with 500 µl of 10 mM of ammonium acetate in 76% ethanol for 10 min and centrifuged for 1 min at 11000 ϕ/min. Pellets were resuspended in 100 µl of 1X TE buffer (10 mM TRIS-HCl pH 8, 1 mM Na2EDTA pH 8). DNA concentration was quantified by comparing its intensity with those of DNA standard (λ DNA conc. 10, 50, 100, 200 ng/µl) on ethidium bromide stained 0.8% (w/v) agarose gel in 0.5X TBE buffer. Thirty-seven of the oligonucleotide primers, which according to Kongkiatngam *et al. (16, 17)* and Campos-de-Quiroz and Ortega-Klose *(18)* amplify polymorphic RAPD markers in red clover, were initially screened (Figure 1). Four of them (Table 1) with clear and consistent amplified products were used. PCR reactions were performed in volumes of 25 µl under final conditions of 1X PCR buffer



a) 5'-TGTAGCTGGG-3' b) 5'-GTGCCCCACT-3'

Figure 1. *Initial screening of oligonucleotide primers on agarose gels. Products of PCR amplification with primers rejected (a) and used (b) for further screening.*

TABLE 1

Nucleotide sequences of the decamer primers used and the number of polymorphic markers detected within each cultivar and selection.

Nucleotide sequence	Number of polymorphic markers						
	C1	SC1	C2	SC2	C3	SC3	Average
5'-GGC TCA TGT G-3'	25	24	20	25	23	24	25
5'-GTG CCC CAC T-3'	24	24	23	24	21	23	24
5'-ACC CTC GGA C-3'	21	20	20	19	19	19	22
5'-AAG TGC GAC C-3'	21	21	21	21	20	21	21
Total	91	89	84	89	83	87	92

(10 mM Tris-HCl pH 8.3; 50 mM KCl), 1.5 mM MgCl2, 0.2 mM each of dATP, dCTP, dGTP and dTTP (Sigma), 0.2 μM of a single primer (Metabion GmbH), 1 U of Taq polymerase and approx. 18 ng of genomic DNA. PCR was performed in MJ PTC-100 (MJ Research, Waltham, Mass., USA) thermocycler. The cycling regime for the reaction was 92 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min, repeated over 40 cycles. Amplified DNA products were separated by electrophoresis in 1.4% (w/v) agarose gels with 0.5X TBE buffer, stained with ethidium bromide, visualized by UV transilluminator and photographed by GelCam camera, Polaroid. To determine band size, a 100 base-pair ladder (Amersham Biosciences) was used in each electrophoretic run as a standard.

Data analyses. To generate a binary matrix for morphological data, the presence or absence of a character class for the 20 individuals of each of the cultivar/selection plants used in the RAPD analyses was marked as 1 or 0, respectively. Morphological variables were standardized and Euclidean distances were calculated following the procedure used by Roldan Ruiz *et al. (19)*. The size and frequency of polymorphic RAPD markers were determined with reference to the standard used. The presence or absence of a band was recorded as 1 or 0, respectively, in a binary matrix. The frequency of RAPD markers was assessed *(3)*. For the RAPD data, modified Roger's distances were calculated as $(\sum (p_{ia}-p_{ij})^2)^{1/2})/A$, where p_{ia} and p_{ij} specify the frequency of band a in the cultivars/selections i and j, respectively, and A specifies the number of bands revealed. Morphological and molecular distance matrices were used to construct phenograms using 2D Principal Coordinate Analyses (PCoA) *(20)* by the NTSYS-pc software package, version 1.6 *(21)*. The same software was also used to calculate the correspondence between the morphological and molecular matrices by the Mantel matrix correspondence test *(22)*. Significance of the Z value was assessed by comparing the observed Z value to the critical Z value obtained from the permutation distribution of the former value *(23)*.

RESULTS

Analysis of morphological markers. Four morphological loci were polymorphic in cultivars and selections. A total of 14 markers were scored. Stem habit, growth type of crown, pubescence, and leaf marks were classified as erect, semi-erect, semi-prostrate and prostrate stem habit; compact, moderately compact, moderately incompact and incompact growth type of crown; high density or

TABLE 2

Values of distances among cultivars and selections computed using morphological (above) and RAPD (below) data.

	C1	SC1	C2	SC2	C3	SC3
C1	–	0.32	0.45	0.39	0.43	0.37
SC1	0.30	–	0.31	0.36	0.35	0.30
C2	0.33	0.32	–	0.09	0.30	0.28
SC2	0.30	0.30	0.26	–	0.34	0.23
C3	0.31	0.30	0.23	0.27	–	0.21
SC3	0.33	0.34	0.25	0.27	0.24	–

medium density pubescence, or glabrous stem; and leaf with completely shaped marks, incompletely shaped marks and unmarked. The number of markers per class within each character varied from 0 to 20, dependening on character and population.

Analysis of RAPDs. All of the four selected decamer primers generated a polymorphic banding pattern for the cultivars and selections investigated. Individuals were characterized by up to 17 RAPD markers for the each primer used. Cultivar/selection specific markers were not identified. A total of 92 polymorphic RAPD markers were scored, ranging in size from 300 to 2500 base pairs.

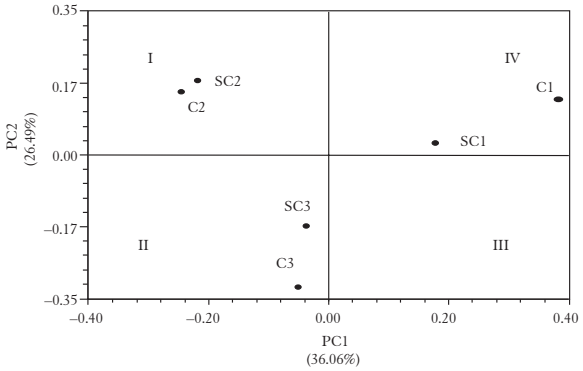


Figure 2. *2D Principal Coordinate analysis of cultivars (C1, C2, C3) and selections (SC1, SC2, SC3) based on morphological data.*

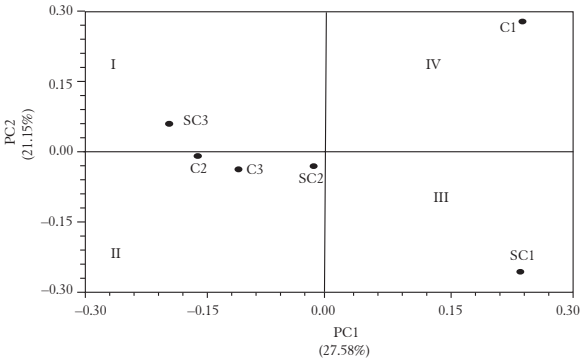


Figure 3. *2D Principal Coordinate analysis of cultivars (C1, C2, C3) and selections (SC1, SC2, SC3) based on RAPD data.*

The number of polymorphic markers within cultivars/selections ranged from 83 (C3) to 91 (C1) (Table 1). The number of polymorphic markers per primer ranged from 21 to 25 with an average of 23.

Morphological vs. RAPD markers. The range of morphologic distances (from 0.09 to 0.45) across cultivars and selections was wider than the range of RAPD marker distances (from 0.23 to 0.34) (Table 2). Also, the calculated average value of morphological distance (0.32) was higher than the average value of molecular distance (0.29). The highest value of distance between a cultivar and its selection occurred between C1 and SC1. The correspondence (Z) between pairs of matrices based on the morphological and molecular data was significant (p=0.95). The estimated correlation value was 0.57.

The first eigenvector (PC1) of 2D PCoA (Figure 2) based on morphological data accounted for 36.06% of the total variation observed in the whole data set. The second eigenvector (PC2) accounted for 26.49% of morphological variation. Cultivars and selections were separated into groups according to their origin. While C1 and C2 were separated from their selections by eigenvector 1, the distance between C3 and SC3 was mainly due to eigenvector 2. The separation between C1 and SC1, and C3 and SC3 by eigenvector 2 was higher than separation between C2 and SC2. 2D Principal Coordinate analyses based on RAPD data separated cultivars and selections into six groups. The first eigenvector (PC1) accounted for 27.58% of the total RAPD variation among groups (Figure 3). The second eigenvector (PC2) explained 21.15 % of the variation. C1 and SC1 were clearly separated from the other two cultivars and their selections by eigenvector 1. C1 and SC1 were also separated by eigenvector 2. The separations between C2 and SC2, and C3 and SC3 by eigenvector 1 were similar. The separation between C3 and SC3 by eigenvector 2 was higher than separation between C2 and SC2.

DISCUSSION

Both marker methods indicated the changes in red clover cultivars caused by natural selection. Morphological data as well as RAPD data grouped the 120 samples into six distinct groups. The correspondence between pairs of matrices based on the morphological and molec-

ular data suggested that both marker methods classified the samples in the similar manner. This result indicated that cultivars and selections were in genetic equilibrium with the environment *(24)*. The changes generally corresponded to the site of cultivar origin. Less evident changes were recorded for the cultivar that originated from western Croatia and was most likely bred in environmental conditions similar to the conditions in which plants of this study survived after three years of natural selection. More evident changes were related to the more distant site of cultivar origin. However, for the Austrian cultivar, whose origin was geographically close to the site where the selections were collected, changes were higher than expected, possibly due to the influence of ecological conditions on genotypes. This result was in agreement with previous analysis carried out in *Lotus corniculatus* L. *(25)* in which, even when the sites of origin where geographically close, genotypes were determined by ecological conditions in which they were grown.

The number of polymorphic loci detected by RAPD markers was much higher than that determined with morphological traits. Conversely, the values and ranges of morphological distances were higher and wider than those of molecular distances. The eigenvectors of 2D PCoA based on morphological data accounted for more of the total variation observed in the whole data set. This discrepancy could be ascribed to a difference in the number of the polymorphic loci used. Because only a small number of morphological loci were examined, estimates obtained with morphological traits may be overestimated *(16)*. Discrepancy could also be ascribed to dominant nature of RAPD markers *(16)* and/or to morphological trait expression controlled by single or few genes and not detected by RAPDs *(25)*. In addition, discrepancy may be explained by differences in evolutionary rates between morphological characters and the characters originating from selectively neutral, non-coding DNA *(26)* and/or by selective response of morphological markers which may be more effective at understanding adaptive variation than markers that are selectively neutral *(27)*.

However, 2D PCo analyses based on both, morphological and molecular data, separated cultivars and selections into respective groups. In addition, while cultivars C1 and SC1 were clearly separated from other accessions by eigenvector 1, C2 and C3 as well as their selections showed less clear distinction. The latter cultivars may have common elements in their pedigree. Lakhanpaul *et al. (28)* found close genetic similarity between the cultivars of *Vicia radiata* L. due to the high degree of commonness in their pedigrees. Gustine *et al. (29)* reported genetic similarities among eight white clover populations, which might have indicated a common European origin.

This study showed that although calculated parameters differed in values, both methods exhibited comparable accuracy in evaluation of red clover changes caused by natural selection. Each of the data sets has its own strengths and constraints. Morphological data indicated morphological traits that could be related to adaptive mechanisms in red clover plants. Still, other studies that examined correspondence among distances based upon different markers suggested that the estimates of genetic variation are less reliable when a small number of polymorphic loci are sampled *(1)*. RAPD data have given valuable information on the genetic structure of cultivars and provided a description that determines heterogeneity within cultivars and selections. However, additional costs associated with the molecular marker evaluation and expected return of analysis should be taken into account *(30)*. Use of the two different marker methods provided more information, thus, for reasonably accurate estimates of genetic variability and its changes under natural selection, adequate attention has to be devoted to both marker methods.

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