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Analysis of genetic diversity in the core collection of red clover (*Trifolium pratense*) with isozyme and RAPD markers

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ABSTRACT – *The genetic diversity in the red clover USA core collection was measured by RAPD and isozyme markers, comparing different approaches of isozyme data analysis and comparing the results of RAPD with those of isozymes. A total of 15 isozyme loci and 114 RAPD fragments were analyzed. Genetic diversity measured with RAPD and isozymes was high. The analyses of isozymes as binary data was highly correlated with the allele-frequency approach. There was no correlation between the distance matrices of different markers. Isozyme data grouped the accessions of red clover in four groups. The variation evidenced by AMOVA was higher among and within groups in the populations than at the intra-population level. Results showed the high diversity in the red clover core collection and indicated some populations that could be used in breeding programs of the crop in Brazil.*

Key words: Red clover, isozymes, RAPD, genetic diversity, core collection.

INTRODUCTION

Red clover (*Trifolium pratense*) is a short-lived herbaceous forage crop thought to be originated in southeastern Europe and Asia Minor near the Mediterranean Sea (Taylor and Quesenberry 1996). Widely used in the world agriculture, red clover is second to alfalfa in economic importance among forage legumes, and is grown for hay, silage, forage and as a soil conditioner (Greene et al. 2004). Nevertheless, red clover cultivars are not adapted to areas far from where they were developed (Taylor and Smith 1995). In Brazil, red clover is used to alleviate the lack of forage during the critical fall-winter period, especially in the state of Rio Grande do Sul, in the south of Brazil. However, due to the low persistency of the historically used cultivars in Brazil (Kenland, Quiñequeli, Estanzuela) red clover yields are rather low. A breeding program aimed at the creation of regionally adapted cultivars and the development of local seed production was therefore initiated at the Federal University of Rio Grande do Sul (UFRGS). Many studies using morphologic (Kouamé

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and Quesenberry 1993, Christie and Choo 1991, Bulinska-Radomska 2000), molecular (Kongkiatngam et al. 1995, Campos-de-Quiroz and Ortega-Klose 2001, Kölliker et al. 2003, Greene et al. 2004, Herrmann et al. 2005, Sato et al. 2006) and biochemical traits (Hagen and Hamrick 1998, Yu et al. 2001, Mosjidis and Klingler 2006) have demonstrated high genetic diversity, both among and within red clover populations. In view of the current interest in sustainable agriculture, red clover seems promising, since cultivated legumes play an important role in atmospheric N fixation and air and water pollution reduction (Rochon et al. 2004). The industrial use of red clover is expanding and diversifying (Humphreys 2005, Morris and Greene 2001) and future breeding activities have to be able to exploit a wider range of diversity in red clover (Greene et al. 2004) making new varieties available to supply the demand of this new market. The red clover genebank of the National Plant Germplasm System of the United States Department of Agricultural (NPGS-USDA) contains 800 accessions from 41 different countries (Kouamé and Quesenberry 1993). Brown (1989) induced the concept of "core collections" to facilitate the use of germplasm in breeding programs, which resulted in the establishment of a number of core collections in diverse cultivated plant species (Souza and Sorrells 1991, Steiner et al. 2001). A core collection was developed for red clover based on morphological and physiological descriptors (Kouamé and Quesenberry 1993). However, the collection was investigated by only few studies using morphologic (Bortnem and Boe 2002, Bortnem and Boe 2003) or biochemical traits (Mosjidis and Klingler 2006). Knowledge of the amount and distribution of genetic variability within a species is vital to plant breeders when selecting germplasm to be included in a breeding program (Yu et al. 2001). But when only one or few traits are used to characterize collections, there is little opportunity to understand relationships among different traits within collections (Brown 1989).

The use of many traits therefore extends the possibilities of evidence of broad-range genetic differences. Molecular markers such as RAPD (random amplified polymorphic DNAs), have been used to analyze genetic diversity in red clover (Kongkiatngam et al. 1996, Campos-de-Quiroz and Ortega-Klose 2001, Greene et al. 2004, Ulloa et al. 2003) and in other forage crops such as alfalfa (Mengoni et al. 2000), Birdsfoot trefoil (Steiner and los Santos 2001) and white clover (Dolanska and Curn 2004). However, RAPD markers are largely limited in the analysis of population structure since a complete genotypic determination is hampered by their dominant nature (Lynch and Milligan 1994). In contrast, isozymes as codominant markers are useful to estimate the allelic frequency and population genetic parameters and have proved reliable in genetic studies with red clover natural populations (Hagen and Hamrick 1998, Lange and Schifino-Wittmann 2000, Semerikov et al. 2002, Mosjidis et al. 2003) and cultivars (Kongkiatngam et al. 1995, Yu et al. 2001). Different aspects of the diversity can be analyzed by the use of molecular and biochemical markers. The purpose of this study was a first evaluation of the genetic diversity in the core collection of the USDA red clover genebank, based on isozyme and RAPD markers together. Results may be useful for breeding purposes, particularly to support the development of cultivars better adapted to Brazilian conditions.

MATERIAL AND METHODS

Plant Material

Seeds of 78 red clover accessions from the core collection of the National Plant Germplasm System (NPGS) of the United States Department of Agriculture (USDA) were obtained for analysis from the USDA Agricultural Research Service (ARS) Plant Introduction Station in Washington and one population cultivated in Brazil, totaling 79 populations (Table 1). The diverse accessions of the core collection had been classified by the NPGS as cultivars, landraces or wild populations, according to the breeding status, as far as data were available (Mosjidis and Klingler 2006).

Isozyme markers

Of each accession, 10 seeds were germinated in Petri dishes with moist filter paper and transferred to plastic pots (750 mL) after 15 days, in a greenhouse in Porto Alegre, Rio Grande do Sul, Brazil (30° 01'59" S, 51°13'48" W and 10 m asl). Each plant was inoculated with 1 mL of a solution of *Rhizobium leguminosarum bv. trifolii* (SEMIA 222 and SEMIA 2082), as recommended by the Laboratório de Fixação Biológica de Nitrogênio of the Fundação Estadual de Pesquisa Agropecuária (FEPAGRO) in aqueous solution (10^9 cel mL^{-1}). Five to ten plants per accession, totaling 535 individuals were examined for isozyme patterns. The

enzyme system selected was esterase α and β (EST, EC, 3.1.1.-). Isozymes (esterase α and β) were analyzed in about 100mg young leaves taken from mature plants ground in 170 µL extraction buffer (Collins et al. 1984). One *Adesmia bicolor* plant was used as control in all gels. Electrophoresis was conducted in 10% polyacrylamide gel in horizontal migration at a constant current of 40mA. Migration was performed at approximately 4°C and interrupted when the front line reached a distance of 8 cm from the application point, which occurred after 2h30. Gels were stained with specific EST staining solution (Scandalios 1969). The gels were fixed in Ayala solution, photographed and analyzed. All monomorphic and polymorphic anodic and cathodic bands were analyzed. Bands were characterized by their migration rates as well as by their color (α dark brown to blackish and β light to dark reddish). The migration rate of each band was compared with the migration rate of the front line of the band used as control.

Table 1. Summary of isozyme variation for 79 populations of *Trifolium pratense*

N°	USDA code	USDA name	Origin	P(0.95%)	A	H_o	H_e
1	PI 237705	Hinderupgaard III	Denmark	1.000	2.00	0.500	0.516
2	PI 196424	N° 9500	Denmark	0.333	1.33	0.111	0.101
3	PI 217507	Early otofte III	Denmark	0.500	1.50	0.500	0.263
4	PI 237714	Otofte III	Denmark	0.333	1.33	0.056	0.177
5	PI 314840	JRIPO F81	Norway	0.333	1.33	0.333	0.175
6	PI 188905	Merkur	Sweden	0.333	1.33	0.333	0.175
7	PI 235847	HEBY	Sweden	0.333	1.33	0.300	0.174
8	PI 235854	Labberud	Sweden	0.200	1.20	0.200	0.111
9	PI 235870	Svanvik	Sweden	0.500	1.50	0.250	0.212
10	PI 229799	Tammisto	Finland	0.667	1.67	0.533	0.344
11	PI 236455	Mynskyla	Finland	0.667	1.67	0.333	0.351
12	PI 310459	Dettenbuhl	Switzerland	1.000	2.00	0.458	0.486
13	PI 310465	G15485	Switzerland	1.000	2.00	0.313	0.392
14	PI 179146	Perennial Bernois	Switzerland	0.500	1.50	0.292	0.178
15	PI 234925	N° 16213	Switzerland	0.500	1.50	0.250	0.206
16	PI 239696	N 150	Switzerland	0.750	1.75	0.213	0.384
17	PI 266047	Wielkolistna	Poland	0.250	1.25	0.250	0.132
18	PI 632214	-	G 21245	0.500	1.50	0.458	0.269
19	PI 293591	NSL 060492	Poland	0.250	1.25	0.250	0.143
20	PI 384058	Viola	Poland	1.000	2.00	1.000	0.526
21	PI 255894	Hruszowska	Poland	0.500	1.50	0.500	0.278
22	PI 225119	NSL 060146	Germany	0.333	1.33	0.067	0.063
23	PI 187008	N° 1687	Germany	1.000	2.00	0.000	0.394
24	PI 234836	N° 8	Germany	1.000	2.00	0.000	0.394
25	PI 294481	reichersbersger	Austria	1.000	2.00	0.389	0.406
26	PI 318887	Bakonyi	Hungary	1.000	2.00	0.905	0.524
27	PI 318888	Cigandi	Hungary	1.000	2.00	0.905	0.524
28	PI 315522	G 17210	Italy	1.000	2.00	0.250	0.417
29	PI 418889	296	Italy	1.000	2.00	0.467	0.456
30	PI 249870	N 17109	Greece	0.333	1.33	0.148	0.122
31	PI 419294	122	Greece	0.667	1.67	0.222	0.202
32	PI 220856	NSL 060141	Portugal	0.667	1.67	0.200	0.328
33	PI 311492	M-193	Spain	0.333	1.33	0.200	0.156
34	PI 307948	N° 942	Spain	0.333	1.33	0.000	0.063
35	PI 253583	K 2086	Spain	0.667	1.67	0.067	0.211

to be continued

N°	USDA code	USDA name	Origin	P(0.95%)	A	H_o	H_e
36	PI 188680	G 13945	France	0.500	1.50	0.500	0.263
37	PI 207972	NSL 060134	France	0.500	1.50	0.500	0.263
38	PI 189174	Groninger	Netherlands	0.333	1.33	0.200	0.156
39	PI 201191	NSL 060106	Netherlands	0.333	1.33	0.074	0.122
40	PI 187224	Oudenaerdse	Belgium	0.333	1.33	0.200	0.156
41	PI 234448	N° 16104	Belgium	0.333	1.33	0.067	0.067
42	PI 205313	N 642a	Turkey	0.333	1.33	0.200	0.156
43	PI 120105	BN9466-59	Turkey	0.333	1.33	0.000	0.119
44	PI 171870	N° 6948	Turkey	0.333	1.33	0.111	0.101
45	PI 204506	N° 292	Turkey	0.333	1.33	0.333	0.185
46	PI 204507	N° 382	Turkey	0.333	1.33	0.333	0.182
47	PI 314487	N° 188	Georgia	0.667	1.67	0.267	0.296
48	PI 371959	SOFIA 52	Bulgaria	0.333	1.33	0.333	0.182
49	PI 294797	ARSAN	Bulgaria	0.333	1.33	0.167	0.136
50	PI 315533	Sofia 66	Bulgaria	0.333	1.33	0.167	0.143
51	PI 251564	19660	Yugoslavia	0.500	1.50	0.357	0.269
52	PI 207520	N° 12623	Afghanistan	0.333	1.33	0.083	0.083
53	PI 228365	N 14849	Iran	0.333	1.33	0.100	0.090
54	PI 250899	K 1689	Iran	0.333	1.33	0.000	0.147
55	PI 401469	Perena	Romania	0.500	1.50	0.250	0.214
56	PI 232941	Fertodi	Hungary	0.500	1.50	0.125	0.125
57	PI 345673	Gloria mestnaja	Ukraine	0.250	1.25	0.250	0.143
58	PI 228160	Zazersk	Russia	0.250	1.25	0.000	0.121
59	PI 440737	D-1600	Russia	0.333	1.33	0.333	0.191
60	PI 345675	Kirovskij 159	Russia	0.667	1.67	0.500	0.318
61	PI 419565	SV-0116	Japan	0.667	1.67	0.667	0.381
62	PI 419550	TENDA-Selection	Japan	0.667	1.67	0.267	0.252
63	PI 184960	NSL 060086	Australia	0.500	1.50	0.250	0.212
64	PI 376880	Grasslands Hamua	New Zealand	0.250	1.25	0.125	0.102
65	PI 187284	Aberystwyth s 151	UK	0.333	1.33	0.333	0.180
66	PI 306188	Drewitts	UK	0.667	1.67	0.571	0.344
67	PI 306185	Berrys	UK	0.667	1.67	0.583	0.360
68	PI 315534	Memmos	Canada	0.333	1.33	0.333	0.185
69	PI 286116	Redon	Canada	0.250	1.25	0.100	0.089
70	PI 286222	CAN 5027	Canada	0.500	1.50	0.100	0.178
71	PI 295355	Rahn Redclover	USA	0.500	1.50	0.250	0.279
72	PI 230229	N° 55-7	USA	0.250	1.25	0.250	0.136
73	PI 306677	N° A-1847	Ecuador	1.000	2.00	0.333	0.448
74	PI 302421	Trebol rojo	Colombia	0.250	1.25	0.000	0.133
75	PI 304842	Quinekeli	Chile	0.250	1.25	0.083	0.076
76	PI 449326	Trebol rosado	Chile	1.000	2.00	0.208	0.375
77	PI 271627	N° 1214	India	1.000	2.00	0.875	0.525
78	PI 226952	N° 363	Ethiopia	0.500	1.50	0.200	0.178
79	Brazil	-	Brazil	0.250	1.25	0.125	0.102
Mean				0.523	1.523	0.283	0.236
SD				0.261	0.261	0.217	0.130

Isozyme Data Analysis

Two types of analysis were performed to compare the accuracy of estimation methods. Firstly the mobility values of each band of all gels were evaluated and these bands were scored as binary characteristics (1 for band presence and 0 for bans absence for each individual). Data were transformed into a binary matrix and Jaccard's similarity index was calculated, using software NTSYS-PC 2.10 (Rohlf 2000) for data processing. Secondly, the number of alleles for each isozyme locus was scored and population genetic parameters were calculated. The genetic parameters percentage of polymorphic loci (P), mean number of alleles per locus (A), observed heterozygosity (H_o) and expected heterozygosity (H_e) based on Nei's unbiased estimate were calculated using Genetix version 4.05 (Belkhir 2004), and the fixation index (F_{is}) was estimated according to Weir and Cockerham 1984. The deviations from the Hardy Weinberg Equilibrium (HWE) were tested at the individual locus level and for all loci by the Markov chain method, with default parameters using GENEPOP software (Raymond and Rousset 1995). Rogers´ distance between the 79 populations was calculated using software NTSYSPC 2.10 (Rohlf 2000). To infer the structure and genetic relationships among the 79 populations, the dataset was analyzed using Structure version 2.1 (Pritchard and Donnelly 2003), in search of the appropriate group number. The no-admixture ancestry model was chosen because it was cited as adequate for very discrete populations (Falush et al. 2003) and may be more successful in detecting subtle population structures. Using the correlated allele frequency option, multiple runs of Structure were performed (K from 1 to 10 groups). The burn-in time and replication number was set at 10.000 and 100.000 respectively (Pritchard and Donnelly 2003) and each run was replicated ten times. For each run, individuals were assigned to the group were the participation (Q) was highest. Analysis of molecular variance (AMOVA) was performed using Arlequin version 3.1 (Excoffier and Schneider 2005) based on the allele frequencies to test the parts of among and within-population diversities. Finally the distance matrices generated by binary data and allelic frequencies data were compared computing the correlation based on the product moment correlations (r) derived from the normalized Mantel Z. Matrices were compared using NTSYS-PC (Rohlf 2000).

RAPD markers

From the same 79 populations 20 seeds per population were bulked and used directly for DNA extraction based on the method of Doyle and Doyle (1987). DNA was quantified by comparison with the ethidium-bromide-stained standard concentrations in 1% agarose gels. DNA was diluted in TE (10mM Tris-HCl, pH 8.0, 1mM EDTA) to a final concentration of 3ng µL. A total of eight random primers (Operon Technologies, Alameda, CA, USA) were used for RAPD analysis (Table 2). The amplifications reactions were performed according to Ferreira and Grattapaglia (1998). The final reaction volume was 15 µL containing 15ng genomic DNA, 15ng primer, 2.5mM of each dNTP, 50mM $MgCl_2$, 1x PCR Buffer (20mM Tris-HCl (pH 8.4), 50mM KCl) and 1U Taq Polymerase (Invitrogen Inc.). The DNA amplification was conducted on a PTC-100 (Programmable Thermal Controller MJ Research, INC.) thermal cycler programmed for 40 cycles of: 1 min at 93°C, 1 min at 36°C, 2 min at 72°C and a final extension step of 5 min at 72°C. Reaction products were separated by electrophoresis in 1.5% agarose gel using a Tris-Borate-EDTA (TBE) buffer system. Gels were stained

Table 2. Primers, sequences and summary of RAPD markers used to characterize red clover populations

Primers	Sequence 5' to 3'	N° of markers	Size (bp) min-max
OPA01	CAGGCCTTC	13	520-1800
OPA09	GGTCCCTGAC	10	350-1280
OPA18	AGGTGACCGT	17	196-2000
UBC2	CCTGGGCTTC	13	230-1600
UBC4	CCTGGGCTGG	13	220-1870
OPB14	TCCGCTCTGG	15	260-1850
OPG04	AGCGTGTCTG	17	270-1800
OPG06	GTGCCTAACC	16	280-1784
	Total	114	

with ethidium bromide, visualized under UV light and photographed with a Kodak DC 290 digital camera. Gel images were analyzed with Kodak 1D Image Analysis software (Version 2.0.2, Eastman Kodak Co., Scientific Imaging Systems, Rochester, N.Y.).

RAPD data analysis and comparison with Isozymes

RAPD data were transformed into a binary matrix and Jaccard's similarity index was calculated. The Jaccard's distance matrices generated by RAPD and isozyme binary data were compared by the Mantel test of correlation using NTSYS-PC 2.10 (Rohlf 2000).

RESULTS AND DISCUSSION

Isozyme binary data

A total of 14 EST bands were detected (eight α and six β). The mean Jaccard's similarity in the 79 populations was 0.30 and ranged from 0 to 1. The number of bands and levels of genetic diversity found here were in agreement with previous studies where esterase isozyme patterns were also scored as binary data. Lange and Schifino-Wittmann (2000) found 17 bands in four red clover populations of commercial cultivars, locally-developed cultivars and naturalized populations and Malaviya et al. (2005) reported 18 esterase bands in six different species of *Trifolium*. Similar genetic diversity levels for esterase loci were found by Lange and Schifino-Wittmann (2000) ranging from 0.24 to 0.96 (mean of 0.30) when analyzing eight *Trifolium* species with four enzymatic systems also based on isozymes markers as binary data.

Isozyme allelic frequency data

A total of 15 isozyme loci with 30 alleles were detected based on the esterase (EST) enzyme system in the 79 red clover populations. All loci were polymorphic in at least one population. The percentage of polymorphic loci (P) within populations ranged from 0.20 to 1.00 (Table 1). The average percentage of polymorphic loci (P) was 0.52 for all populations. The effective number of alleles per locus (A) ranged from 1.2 to 2.0 and the average within populations was 1.52. The expected heterozygosity (H_e) ranged from 0.063 in PI 307948 and PI 225119 to 0.526 in PI 304058 and the mean value for the populations was H_e = 0.236. Considering the 15 loci evaluated, F_{is} was not significantly different from zero suggesting that the populations as a whole were in Hardy-Weinberg equilibrium. Furthermore, the mean observed heterozygosity within accessions (H_o = 0.283) was close to H_e, 0.236 (Table 1). When isozymes were analyzed as allelic frequencies data, we also found high levels of genetic diversity, although the mean percent value of polymorphic loci found here (P= 0.52) was lower than in previous reports. These results agree with reports for wild as well as cultivated red clover populations and also indicated that the diversity in red clover is high. For naturalized red clover populations, Hagen and Hamrick (1998) found P = 0.68, A = 1.45 and H_e = 0.250. For wild red clover populations Semerikov et al. (2002) found H_e values that ranged from 0.128 to 0.196 and P ranging from 0.41 to 0.52 and Mosjidis et al. (2003) reported P= 74, A = 1.7 and H_e = 0.323. For cultivated red clover Kongkiatngam et al. (1995) reported H_e values ranging from 0.10 to 0.12 in a comparison of two different cultivars and for 34 cultivars of red clover Yu et al. (2001) observed mean values of P = 74, A = 1.59 and H_e = 0.285. For the same core collection Mosjidis and Klingler (2006) reported average mean of P = 77.5, A = 1.66 and H_e = 0.316. In this study we found a lower number of polymorphic loci than that reported by Mosjidis and Klingler (2006) but proportionally the same level of heterozygosity, since with one enzyme system we found the same number of alleles. Esterases are a complex and heterogeneous enzyme group with multiple substrate specificity (Scandalios 1969) and one of the enzymatic systems with highest polymorphism in plants (Weeden and Wendel 1990). The results based on isozymes found here agree with Mosjidis and Klingler (2006) and indicated that the genetic diversity in the core collection was high. The range in Rogers' genetic distance was high and varied from 0 to 0.26 and the mean Rogers' genetic distance was 0.14. Structure 2.1 assigned the 79 populations to four groups (Figure 1). Populations from the same country were grouped in the four groups, which possibly indicates low genetic variation for the loci examined in populations from the same country. The groups were not separated according to their geographic origin or population type (cultivar, wild or landrace). However, in group 1 there was a strong presence of European red clover cultivars from countries at high latitudes, such as Denmark, Finland, Sweden and Poland. Similar results were found by Dias et al. (2008) who analyzed the same core collection based on microsatellite and morphological markers and grouped

these populations as well. According to Taylor and Smith (1995), distinct plant types of red clover have evolved through natural selection. In Europe these types are largely distributed according to latitude and were classified as late-flowering, as also observed by Dias et al. (2008). Interestingly, some accessions found in group 1 (N° 29, 74, 75, 76, 77, 78) and group 4 (N° 34, 35 and 54) were also grouped by Dias et al. (2008) and classified, respectively, as highly persistent and high-yielding (dry matter) in Brazilian conditions. Since the populations in these groups are probably better adapted to Brazilian conditions and contain high genetic diversity, they could be used as a source of persistency and high yield in Southern Brazil.

The AMOVA results (Table 3) indicated highly significant genetic differences (P<0.000) between the four groups as well as between the red clover populations. Of the total genetic diversity, 44.11% was attributed to populations within groups, 40.77% to groups and only 15.12% to differences within populations. Many studies showed a high intrapopulational diversity in red clover (Mosjidis and Klingler 2006, Yu et al. 2001) analyzed with different types of isozyme markers. But other studies also detected an important fraction of the genetic diversity among groups and within groups by comparisons of natural populations and cultivars of red clover (Semerikov and Belyaev 1995, Semerikov et al. 2002). The low genetic diversity within populations found here may be explained by the fact that esterases are enzymes that use several different substrates and respond directly to the environmental diversity (Robinson 1998). Thus, populations from different geographic origins may be more differentiated than individuals in the same population.

Comparison between isozyme binary and allelic frequencies data

A comparison of the distance matrices generated by isozyme binary and allelic frequency data detected a high correlation (r = 0.70) between the two approaches. This type of information may make the interpretation of isozyme patterns less laborious, particularly when dealing with a high number of individuals, populations and enzymatic systems.

RAPD data

All eight primers used were polymorphic in all study populations, indicating a high degree of molecular variation. The total number of fragments detected was 114 and the number of fragments per primer ranged from 13 to 17 (Table 2). The fragments generated ranged from 196 to 1870bp (base pairs). Jaccard's distance based on 114 RAPD markers ranged from 0.00 to 0.68 and averaged 0.21. This high genetic diversity found with the RAPD markers agreed with previous reports when cultivated (Kongkiatngam et al. 1996, Campos-de-Quiroz and Ortega-Klose 2001, Ulloa Ortega and Campos 2003) or wild populations (Kölliker et al. 2003, Herrmann et al. 2005) of red clover were analyzed.

Table 3. Analysis of Molecular variance (AMOVA) for 79 populations of red clover

Source of variation	d.f.	SSD	MSD	% Total Variance	P-value*
Among groups	3	878.846	105.246	40.77	<0.00000
Among populations/Groups	76	1.182.251	113.868	44.11	<0.00000
Within populations	990	386.335	0.39024	15.12	<0.00000

*non-parametric randomization test (1000 permutations).

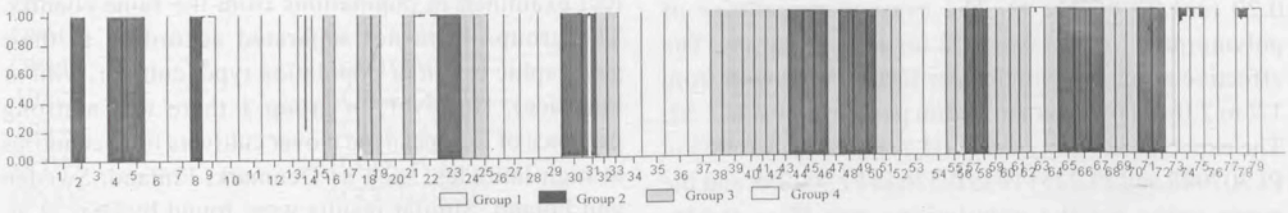

Figure 1. Clustering of 79 red clover populations into four groups. Each population is represented by a vertical line, portioned into four colored segments that represents the proportion of membership (Q) in the four groups established (See Table 1 for PI code information)

Comparison between RAPD and Isozyme distances

There was no correlation (r = 0.04) between Jaccard's matrix based on isozyme data and on RAPD marker data. Both markers indicated high genetic diversity in red clover, but genetic diversity detected by RAPD markers was higher (mean Jaccard similarity = 0.21) than by biochemical marker (mean Jaccard similarity = 0.30). Although the degree of molecular variation between the red clover populations with RAPD data was higher than with isozyme markers, the RAPD distance matrix was not correlated with the isozyme distance matrix. The isozyme markers detected less genetic diversity, but provided more in-depth information on its structure and allowed a clearer group formation than RAPD markers. Several reasons may explain this absence of correlation between the biochemical and molecular markers. Many studies that compared RAPD and isozyme markers in red clover and in other species established a correlation (Kongkiatngam et al. 1995) while in others no correlation was found (Jenczewski et al. 1999) between these two types of markers. The absence of correlation may be linked to the nature of the markers, since RAPD revealed highly neutral polymorphism from non-coding regions of the genome. The isozyme polymorphism was in fact estimated from coding regions that are possibly conserved to maintain the enzyme functions (Gottlieb 1982).

CONCLUSIONS

The high genetic diversity in the core collection of red clover was shown by biochemical and RAPD markers. The genetic diversity detected by the biochemical marker showed structuration that clearly separated the European populations from high latitudes within the core collection. The other groups had no differences for geographic origin nor for population type according to the classification of USDA-NPGS in cultivars, landraces or wild accessions of red clover. The molecular diversity measured for the first time in the core collection with the RAPD markers was high and in agreement with other studies. The information found here indicated the high diversity in the red clover core collection and detected some promising populations for breeding programs in Brazil.

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Análise da diversidade genética na coleção nuclear de trevo vermelho com marcadores RAPD e izoenzimaticos

RESUMO – *A diversidade genética da coleção nuclear (USA) de trevo vermelho foi medida com marcadores RAPD e isoenzimáticos, comparando diferentes métodos de análise das isoenzimas e comparando os resultados obtidos entre RAPD e isoenzimas. Um total de 15 locos isoenzimáticos e 114 fragmentos RAPD foram avaliados. A diversidade genética medida com os marcadores RAPD e isoenzimáticos foi alta. A análise das isoenzimas como dados binários foi altamente correlacionada com a análise de freqüência alélicas. Contudo, não houve correlação entre as matrizes de distância geradas pelos diferentes marcadores. Os dados de isoenzimas agruparam os acessos de trevo vermelho em quatro grupos. A AMOVA evidenciou maior variabilidade presente nas populações dentro dos grupos e nos grupos do que em nível intrapopulacional. A informação mostrada aqui evidenciou a alta variabilidade genética presente na coleção nuclear e apontou algumas populações que poderiam ser utilizadas em projetos de melhoramento de trevo vermelho no Brasil.*

Palavras-chave: Trevo vermelho, isoenzimas, RAPD, diversidade genética, coleção nuclear.

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