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Assessment of genetic diversity in naturally growing 29 *Trifolium* L. taxa from Bolu Province using RAPD and SSR markers

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Abstract: Two molecular marker technologies, RAPD and SSR, were used to determine the genetic diversity of 29 taxa of *Trifolium* L. collected from Bolu Province. The analysis was carried out using 11 RAPD and 5 SSR markers. A total of 446 and 331 fragments were produced by RAPD and SSR markers, respectively. The amplification products were 300–3500 bp with RAPD and 125–3000 bp with SSR markers. These bands were scored as absence and presence for all taxa. Data obtained were used to estimate genetic similarity using Jaccard's coefficient, and dendrograms were constructed by the UPGMA method using either RAPD and SSR markers individually or as combined sets of data. The highest genetic similarity values obtained were 0.33 between *T. hirtum* All. and *T. constantinapolitanum* Ser. for RAPD and 0.49 between *T. medium* L. var. *medium* and var. *eriocalycinum* Hausskn. for SSR. The dendrograms produced from combined sets of RAPD and SSR data revealed 2 main clusters. One contained members of the sections *Chronosemium* Ser., *Lotoidea* L., and *Vesicaria* Ser., and the other had only the section *Trifolium*. The results suggest that the use of 2 different molecular markers gives the most reliable genetic data for *Trifolium* taxa.

Key words: *Trifolium*, Fabaceae, Bolu, Turkey, RAPD, SSR, genetic relationship

1. Introduction

The clover genus, *Trifolium* L., belongs to one of the largest groups in the family Fabaceae. It contains about 255 species (Zohary and Heller, 1984; Gillet and Taylor, 2001), and of those the highly cultured ones are especially agronomically important (Coombe, 1972; Zohary and Heller, 1984). It is particularly common in the northern hemisphere. Major centers are found in North America (60–65 species), Africa (25–30 species), and Eurasia (150–160 species) (Zohary and Heller, 1984).

In the classification of species based on morphology, the taxa were divided into 8 sections (Zohary and Heller, 1984). These sections were referred to as *Lotoidea, Paramesus* (C.Presl), *Mystillus* (C.Presl), *Vesicaria, Chronosemium, Trifolium, Tricocephalum* Koch, and *Involucrarium* Hooker. Six sections out of these 8 were restricted to the Old World or Eurasia and some extend to Africa. Only *Involucrarium* was distributed in the New World, in North and South America (Zohary and Heller, 1984; Steiner et al., 1997).

Molecular methods are emphasized in research on plant diversity as direct agents of genetic information (Ghariani et al., 2003; Poyraz et al., 2012; Taşkın et al., 2012). Molecular techniques are usually preferred to explain the genetic status of taxa and also for phylogenetic and biogeographical distributions. The polymerase chain reaction (PCR) method is used to solve taxonomic problems caused by classification based on morphological data (Mullis and Falcona, 1987; Hillis et al., 1996; Soltis et al., 1998; Wolfe and Liston, 1998). In this respect, random-amplified polymorphic DNA (RAPD) markers have been applied to species of *Trifolium* (Bullitta and Hayward, 1996; Kongkiatngam et al., 1996) in order to study the relationships of taxa. Genetic diversity was shown with amplified fragment length polymorphism (AFLP) markers in 2 *Trifolium* species from Italy (Bennett and Mathews, 2003). Another study was done with 31 *Trifolium* species using internal transcribed spacer (ITS) markers (Vizintin et al., 2006). Chloroplast (cp) DNA and *mat*K markers were used for a phylogenetic study of 23 *Trifolium* species (Steele and Wojciechowski, 2003). The genetic diversity of white clover was also shown with SSR markers (George et al., 2006). Both RAPD and SSR markers were used to show the genetic relationships of *Medicago sativa* plants (Mengoni et al., 2000).

Some molecular phylogenetic studies have shown that *Trifolium* presents monophyly (Steele and Wojciechowski, 2003; Ellison et al., 2006). Although Watson et al. (2000)

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performed the first molecular phylogenetic study, their results did not show that many sections of *Trifolium* exhibit monophyly. Ellison et al. (2006) studied 218 *Trifolium* species with 2 loci: one nuclear, ITS, and the other chloroplast *trn*L. Their results revealed that the genus *Trifolium* shows monophyly. They classified the genus *Trifolium* with 2 subgenera *Trifolium* L. and *Chronosemium* (Ser.) Reichenb.

Turkey is a Mediterranean country containing 7 out of the 8 sections, with more than 100 *Trifolium* species (Cocks, 1993; Benett et al., 1998). Naturally growing *Trifolium* taxa around Bolu Province are represented by 4 of the 7 sections. Therefore, it gives the opportunity to obtain extensive information on *Trifolium* taxa in this area. In this preliminary study, the genetic relationships of *Trifolium* taxa from Bolu Province were obtained using 7 microsatellites and 14 RAPD markers. Some of the varieties such as *T. medium* var. *eriocalycinum* Hausskn., *T. repens* var. *macrorhizum* Boiss., and *T. hybridum* var. *anatolicum* Boiss. and var. *elegans* Savi Boiss. were used for the first time in molecular studies.

2. Materials and methods

2.1. Plant materials

Trifolium taxa were collected in and around Bolu Province. The number of species was about twice that which had been reported in Turkish flora (Zohary, 1970). Twenty-six species and 3 varieties, a total of 29 taxa (Table 1), were sampled during field studies in 2010 and 2011. Samples were identified using the delta intkey program (delta-intkey.com) and *Flora of Turkey* (Zohary, 1970).

Table 1. List of sampled *Trifolium* taxa and their distributions.

Taxon name	Taxon code	Locality	Coordinate	Alt. (m)
T. resupinatum L.	RES	Karacasu	40°40.920′N 31°36.325′E	740
T. physodes Stev.	PHY	Campus*	40°42.375′N 31°31.119′E	836
T. fragiferum L.	FRA	Campus*	40°42.524′N 31°30.490′E	870
T. repens L. var. *macrorhizum* Boiss.	REPM	Kartalkaya	40°35.412′N 31°48.208′E	2050
T. repens L. var. *repens*	REPR	Karacasu	40°40.920′N 31°36.325′E	740
T. hybridum L. var. *anatolicum* (Boiss.) Boiss.	HYBA	Campus*	40°42.789′N 31°30.890′E	865
T. hybridum L. var. *elegans* (Savi) Boiss.	HYBE	Kızılcahamam	40°39.120′N 32°27.779′E	1425
T. nigrescens Viv. subsp. *petrisavi* Clem.	NIG	Kıbrıscık	40°27.107′N 31°44.099′E	1350
T. retusum L.	RET	Kıbrıscık	40°28.672′N 31°42.472′E	1385
T. dubium Sibth.	DUB	Kartalkaya	40°35.489′N 31°48.195′E	2010
T. micranthum Viv.	MIC	Kartalkaya	40°35.489′N 31°48.195′E	2010
T. spadiceum L.	SPA	Bolu-Seben	40°26.768′N 31°43.765′E	1300
T. aureum Poll.	AUR	Bolu-Seben	40°26.768′N 31°43.765′E	1300
T. campestre Schreb.	CAM	Campus*	40°42.858′N 31°30.673′E	861
T. patens Schreb.	PAT	Kartalkaya	40°35.823′N 31°38.643′E	1332
T. pratens L. var. *pratens*	PRA	Campus*	40°42.789′N 31°30.890′E	865
T. pannonicum Jacq. subsp. *elongatum* Willd.	PAN	Campus*	40°42.983′N 31°31.067′E	880
T. scabrum L.	SCA	Campus*	40°42.510′N 31°31.062′E	860
T. striatum L.	STR	Kaynaşlı	40°46.415′N 31°20.829′E	363
T. hirtum All.	HIR	Kaynaşlı	40°46.415′N 31°20.829′E	363
T. constantinapolitanum Ser.	CON	Kaynaşlı	40°46.415′N 31°20.829′E	363
T. echinatum M.Bieb.	ECH	Campus*	40°43.122′N 31°31.620′E	814
T. pallidum Waldst. & Kit.	PAL	Mudurnu	40°38.523′N 31°27.388′E	880
T. medium L. var. *eriocalycinum* Hausskn.	MEDE	Campus*	40°42.789′N 31°30.890′E	865
T. medium L. var. *medium* L.	MEDM	Gerede	40°39.195′N 32° 22.048′E	1300
T. ochroleucum Huds.	OCH	Gerede	40°39.195′N 32° 22.048′E	1300
T. bocconei Savi.	BOC	Gerede	40°39.195′N 32° 22.048′E	1300
T. diffusum Ehrh.	DIF	Kıbrıscık	40°28.672′N 31° 42.472′E	1385
T. arvense L.	ARV	Gerede	40°39.195′N 32° 22.048′E	1300

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2.2. DNA extraction and RAPD PCR analysis

Fresh leaf material was dried in silica gel for DNA extraction. Total genomic DNA was isolated from these dried samples using the hexadecyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1990). A total of 14 RAPD primers were tested but 11 of them gave polymorphism and reproducible banding patterns for all studied taxa. Their names and sequences are given in Table 2. The amplification of these primers with *Trifolium* taxa DNA was carried out by means of a PCR. DNA amplification was performed in a Progene Thermal Cycler (Techne-Promega). The RAPD reaction was as follows: 25 ng of genomic DNA, 2.0 mM $MgCl_2$, 1.0 mM of each dNTP, 1.0 µM of each primer, 1.0 U of *Taq* polymerase, and 1X PCR buffer ($(NH_4)SO_4$) in a final volume of 25 µL. The PCR cycle program was as follows: 5 min at 93 °C, and then 45 cycles at 92 °C for 1 min, 35 °C for 2 min, and 72 °C for 3 min, and a final elongation at 72 °C for 10 min. Blank samples containing water instead of DNA were used for the negative control. Each reaction was repeated to ensure the reproducibility of amplified products. The PCR products were separated by electrophoresis in 1.5% agarose gel containing 0.5 µg/mL ethidium bromide. Images were photographed with a gel documentation system (Ultra-lum-Teknis). Sizes of the PCR products were estimated using a 0.25–10 kb DNA ladder (Promega).

2.3. SSR analysis

Seven SSR markers were selected from those previously used for *Trifolium* DNA amplification studies (Kölliker et al., 2001). About 5 of them produced polymorphic band patterns for all the studied taxa. These and their sequences are presented in Table 3. Reactions were carried out with a 25 µL final volume solution containing 1X buffer ($(NH_4)SO_4$), 1.0 mM of each dNTP, 1.0 µM of each SSR primer (forward and reverse), 2.0 mM of $MgCl_2$, 1.0 U of *Taq* polymerase, and 50 ng of genomic DNA. The reaction program of the SSR markers was as follows: initial denaturation at 95 °C for 4 min, then 30 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C, followed further by 10 of the same cycles with annealing temperature 53 °C and 10 min at 72 °C as the final extension (Herrmann et al., 2006). PCR products were separated on 8% polyacrylamide gel with a 1X TBE buffer. Electrophoresis was performed at 70 W for approximately 3 hours on a 16 × 14 cm gel apparatus (Thermo Scientific-OWL P9DS) and was revealed through ethidium bromide staining. The amplification of the total number of taxa was performed in 2 gels that ran at the same time and in the same conditions. PCR products were estimated using 100 bp plus a DNA ladder (Fermentas). All SSR fragments for the 29 samples were scored manually.

2.4. Data analysis

The absence (0) or presence (1) of an amplified fragment was treated as an independent character. Faint and unreliable bands were not considered for the analysis. The genetic similarity between the *Trifolium* taxa was calculated according to Jaccard's coefficient (Jaccard, 1908). UPGMA dendrograms were constructed using the FreeTree program (Hampl et al., 2001). RAPD and SSR marker data were evaluated separately and finally combined for analysis.

3. Results

3.1. RAPD PCR results

In order to determine the genetic relationships of 29 *Trifolium* taxa from Bolu, 14 primers were initially tested. Only 11 primers out of the 14 were selected based on the

Table 2. RAPD primer details and their products.

Primer	Sequence (5'-3')	Total number of bands	Percentage of polymorphic bands (PBS%)
OPAL-12	CCCAGGCTAC	38	83.93
OPAB-14	CTGATCGCGG	37	83.86
OPAR-19	AACCCTTCCC	38	83.48
OPH-02	TCGGACGTGA	45	86.13
OPH-07	CTGCATCGTG	32	85.45
OPH-12	ACGCGCATGT	45	82.92
OPH-15	AATGGCGCAG	46	85.31
OPAV-08	AAGTGCGACC	38	84.66
OPBA-07	GGGTCGCATC	46	82.75
OPAQ-09	AGTCCCCCTC	41	81.91
OPB14	TCCGCTCTGG	40	86.98
Total		446	mean = 84.30

Table 3. SSR primer details and their products.

Primer	Forward primers (F) (5'-3') Reverse primers (R) (5'-3')	Total number of bands	Percentage of polymorphic bands (PBS%)
TRSSRA**TS054** (F)	GACACCGATTATGTGCAAGA	83	83.42
(R)	AATCACGACGAGCGACAAC		
TRSSRA**TS055** (F)	CAATACAATCACCGCACCAG	62	75.80
(R)	TCTCTGCTTCGCGTCTTCTC		
TRSSRA**XX31** (F)	TCTGTTTTGTTGGCCATGC	56	83.50
(R)	TTGCAAAGTGTTTGGAAGGA		
TRSSRA**02C03** (F)	TATGCTGGTAGATAAACTTAAA	67	83.47
(R)	TGCTCTGGAGATTGATGG		
TRSSRA**02C02** (F)	AAATAAAAACCACAAGTAACTAG	63	83.02
(R)	TATAGGTGATTTGAAATGGC		
Total		331	mean = 81.84

quality and reliability of their amplification with all taxa. The number of PCR products according to the primers varied from 32 to 46 and presented molecular weights between 300 and 3500 bp (Table 2). An example of a RAPD banding pattern with the OPH2 primer is given in Figure 1. The most common band was 1250 bp, found in 12 of 29 taxa in this primer. A total of 446 bands were observed within 11 primers. Amplified polymorphic DNA fragments were scored for Jaccard's similarity coefficient calculations (Table 4). The highest genetic similarity (0.33)

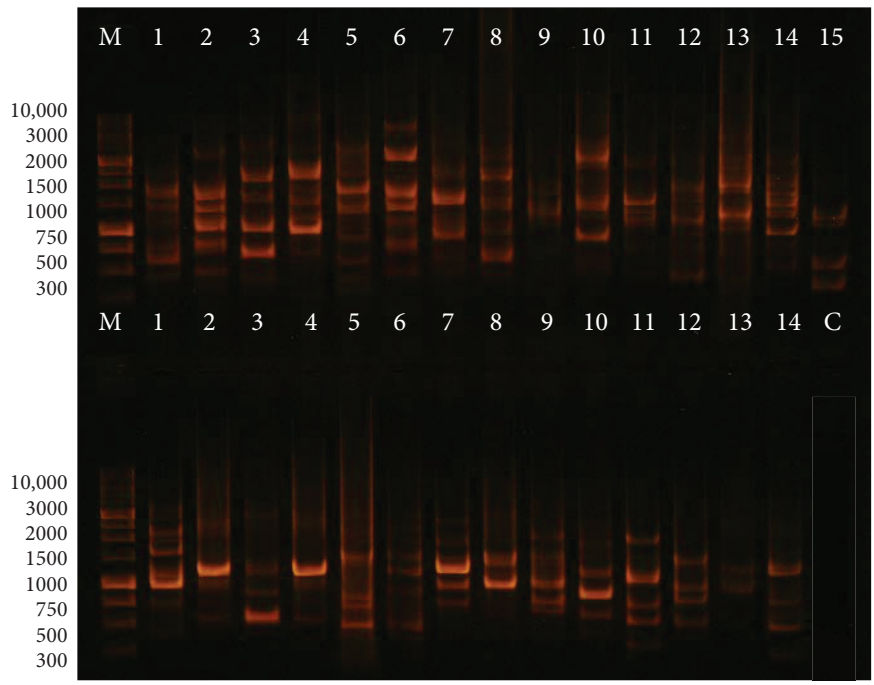

Figure 1. RAPD banding patterns of *Trifolium* taxa revealed by OPH2 primer. Upper part M: Marker (0.25–10 kb DNA ladder, Promega) 1: RES, 2: PHY, 3: FRA, 4: REPM, 5: REPR, 6: HYBA, 7: HYBE, 8: NIG, 9: RET, 10: DUB, 11: MIC, 12: SPA, 13: AUR, 14: CAM, 15: PAT, Lower part M: marker 1: PRA, 2: PAN, 3: SCA, 4: STR, 5: HIR, 6: CON, 7: ECH, 8: PAL, 9: MEDE, 10: MEDM, 11: OCH, 12: BOC, 13: DIF, 14: ARV C: control.

was found between *T. hirtum* and *T. constantinapolitanum,* and the least similarity (0.06) was observed between *T. aureum* and *T. pallidum*. Those species with high genetic similarity belonged to the section *Trifolium*. In contrast, those species which had the least similarity, *T. aureum* and *T. pallidum,* were in the sections *Chronosemium* and *Trifolium*, respectively. The mean percentage of polymorphism for 11 RAPD primers was 84% (Table 2).

A phenogram using the UPGMA algorithm based upon Jaccard's coefficient similarity was constructed (Figure 2). The 29 *Trifolium* taxa formed 2 main clusters. One contained all members of the section *Trifolium* and the other contained members of 3 sections: *Chronosemium*, *Lotoidea*, and *Vesicaria*. However, all members of these sections were grouped as separate subclusters under the main cluster. Members of the section *Vesicaria* and the subcluster *Lotoidea* were the most similar groups. *Chronosemium* members were also separated as *T. patens, T. aureum*, and *T. campestre* in one small group and *T. dubium*, *T. micranthum*, and *T. spadiceum* in another small group. Members of the section *Trifolium* were first separated into 2 clusters and then these clusters were divided into small clusters (Figure 2).

3.2. SSR results

Initially, 7 SSR markers were selected for the testing of polymorphism with 29 *Trifolium* taxa. Two of them, TSSRA02B08 and TSSRA01H11, did not produce any fragments in taxa. Therefore, 5 of the 7 SSR markers gave a total of 331 fragments among 29 *Trifolium* taxa (Table 3). These markers amplified high numbers of fragments compared to RAPD markers. Between 56 and 83 fragments were amplified in each marker and their sizes varied from 125 to 3000 bp (Table 3). The mean percentage of polymorphism with SSR markers was 81%. An example of an SSR banding pattern is given in Figure 3. In this figure the most common bands were 370 and 400 bp fragments, found in 19 taxa of 29. The similarity matrix of SSR scored bands was calculated by Jaccard's similarity coefficient (Table 5). According to the SSR banding scored results, the highest similarity (0.49) was found between *T. medium* var. *eriocalycinum* and *T. medium* var. *medium,* and the least similarity (0.03) was shared between *T. ochroleucum* and *T. aureum*, and between *T. aureum* and *T. resupinatum*. An UPGMA dendrogram was drawn using Jaccard's similarity coefficient (Figure 4). There were 2 main clusters, with one containing all members of the section *Trifolium*, and the other presenting the remaining members of the 3 sections. In this regard, the SSR marker results showed similar results with those of RAPD markers (Figures 2 and 4).

Finally, RAPD and SSR data were combined to observe the genetic relationships of *Trifolium* taxa. A phenogram was generated using Jaccard's similarity coefficients (Figure 5; Table 6). According to this result, the highest genetic similarity (0.40) was found between *T. micranthum* and *T. spadiceum* (Table 6). Both taxa were members of the section *Chronosemium*. On the other hand, the least similarity (0.07) was detected between *T. patens* and *T. bocconei*. *Trifolium patens,* a member of the section *Chronosemium*, was grouped in the first main cluster (Figure 5) and *T. bocconei* appeared together with the members of the section *Trifolium* in the second main cluster (Figure 5).

4. Discussion

Determinations of the genetic similarity of 29 *Trifolium* taxa involved the use of SSR and RAPD markers. Both results formed 2 clusters. One of the clusters contained members of the section *Trifolium* and the other cluster had 3 different sections: *Vesicaria*, *Lotoidea*, and *Chronosemium* members. These findings showed congruence with the results of RAPD and SSR markers (Figures 2 and 4). According to molecular phylogenetic studies, *Trifolium* taxa were grouped under 2 subgenera: *Trifolium* and *Chronosemium* (Ellison et al., 2006). In another phylogenetic study (Steele and Wojciechowski, 2003) on the tribes *Trifolieae* and *Vicieae*, results similar to those of Ellison et al. (2006) were obtained. The subgenus *Chronosemium* contained all the members of the section *Chronosemium* (Zohary and Heller, 1984) and the subgenus *Trifolium* covered all the remaining sections' members (*Glycyrrhizum* Bertol., *Paramesus* (C.Presl) Berchtold and J.Presl, *Lupinaster* (Fabricius) Ser., *Trifolium*, *Trichocephalum* Koch, *Vesicastrum* Ser, *Trifoliastrum* S.F.Gray, and *Involucrarium* Hooker). In our study, the results showed some similarities and contradictions to the findings mentioned above. Our results also presented 2 main groups, but only 1 of these groups showed congruence with the subgenus *Trifolium*. The other group contained the members of the subgenus *Chronosemium* and the members of the sections *Vesicaria* and *Lotoidea*. *Lotoidea* and *Vesicaria* section members joined the section *Chronosemium* instead of the section *Trifolium*. In other words, it could be concluded that section *Trifolium* members were distantly related to members of the remaining sections in the genus. The study showed a reliable result, since this genetic relationship was supported by both RAPD and SSR markers.

In the comparisons between the RAPD and SSR results, although the main groupings (2 clusters) were the same in both figures (Figures 2 and 4), there were some differences in the subgroupings of the sections *Chronosemium*, *Lotoidea*, and *Trifolium*. In the RAPD marker results, *Chronosemium* members *T. patens*, *T. aureum* and *T. campestre* separated as a first subcluster and the remaining 3 taxa, *T. dubium*, *T. micranthum*, and *T. spadiceum*, were in a second subcluster (Figure 2). By contrast, the SSR results gave 2 subclusters. One contained 4 members, *T.*

Table 4. Jaccard's coefficient of similarity matrix, based on RAPD data.

	RES	PHY	FRA	REPM	REPR	HYBA	HYBE	NIG	RET	DUB	MIC	SPA
RES												
PHY	0.293											
FRA	0.279	0.295										
REPM	0.206	0.180	0.220									
REPR	0.199	0.131	0.193	0.243								
HYBA	0.194	0.207	0.157	0.298	0.303							
HYBE	0.129	0.084	0.154	0.182	0.232	0.231						
NIG	0.134	0.109	0.104	0.187	0.138	0.172	0.224					
RET	0.147	0.133	0.127	0.240	0.229	0.239	0.174	0.190				
DUB	0.180	0.161	0.202	0.135	0.182	0.156	0.106	0.139	0.212			
MIC	0.180	0.149	0.171	0.160	0.191	0.205	0.130	0.137	0.234	0.290		
SPA	0.169	0.116	0.129	0.139	0.154	0.142	0.117	0.155	0.191	0.307	0.416	
AUR	0.115	0.106	0.149	0.149	0.128	0.112	0.127	0.085	0.180	0.200	0.167	0.176
CAM	0.129	0.115	0.134	0.127	0.140	0.128	0.142	0.104	0.169	0.205	0.215	0.175
PAT	0.159	0.145	0.211	0.120	0.119	0.130	0.116	0.076	0.128	0.167	0.187	0.198
PRA	0.166	0.122	0.157	0.104	0.160	0.111	0.099	0.082	0.125	0.156	0.154	0.119
PAN	0.105	0.141	0.116	0.070	0.065	0.101	0.096	0.077	0.083	0.074	0.094	0.081
SCA	0.131	0.161	0.145	0.170	0.150	0.156	0.163	0.150	0.172	0.135	0.160	0.149
STR	0.142	0.137	0.130	0.148	0.129	0.132	0.128	0.089	0.112	0.138	0.175	0.133
HIR	0.180	0.170	0.132	0.179	0.147	0.174	0.102	0.099	0.142	0.113	0.158	0.125
CON	0.152	0.091	0.122	0.168	0.154	0.133	0.083	0.136	0.160	0.103	0.127	0.124
ECH	0.158	0.110	0.114	0.165	0.099	0.142	0.120	0.135	0.140	0.138	0.164	0.153
PAL	0.168	0.118	0.130	0.157	0.162	0.161	0.168	0.144	0.178	0.111	0.136	0.103
MEDE	0.178	0.119	0.113	0.123	0.113	0.115	0.101	0.107	0.179	0.130	0.127	0.155
MEDM	0.139	0.134	0.154	0.129	0.112	0.138	0.109	0.081	0.190	0.119	0.142	0.103
OCH	0.126	0.110	0.114	0.157	0.129	0.160	0.148	0.144	0.176	0.113	0.193	0.106
BOC	0.176	0.155	0.102	0.130	0.120	0.160	0.118	0.124	0.157	0.128	0.116	0.084
DIF	0.105	0.093	0.108	0.120	0.101	0.142	0.126	0.102	0.183	0.117	0.093	0.120
ARV	0.168	0.099	0.121	0.131	0.162	0.181	0.100	0.071	0.168	0.197	0.186	0.143

	AUR	CAM	PAT	PRA	PAN	SCA	STR	HIR	CON	ECH	PAL	MEDE	MEDM	OCH	BOC	DIF	ARV
AUR																	
CAM	0.248																
PAT	0.210	0.224															
PRA	0.110	0.178	0.109														
PAN	0.073	0.124	0.136	0.188													
SCA	0.112	0.127	0.094	0.156	0.178												
STR	0.133	0.164	0.112	0.128	0.195	0.185											
HIR	0.135	0.185	0.124	0.172	0.130	0.179	0.252										
CON	0.176	0.156	0.133	0.188	0.129	0.132	0.144	**0.330**									
ECH	0.124	0.130	0.096	0.144	0.120	0.157	0.161	0.183	0.153								
PAL	**0.067**	0.146	0.103	0.178	0.128	0.167	0.153	0.145	0.125	0.134							
MEDE	0.124	0.138	0.143	0.121	0.085	0.168	0.125	0.108	0.145	0.092	0.216						
MEDM	0.086	0.111	0.129	0.111	0.109	0.153	0.131	0.098	0.106	0.183	0.167	0.159					
OCH	0.106	0.130	0.132	0.136	0.173	0.192	0.125	0.118	0.153	0.094	0.125	0.144	0.148				
BOC	0.093	0.136	0.074	0.143	0.118	0.165	0.151	0.184	0.124	0.133	0.161	0.124	0.156	0.142			
DIF	0.131	0.127	0.108	0.144	0.157	0.120	0.132	0.073	0.167	0.123	0.154	0.133	0.120	0.153	0.163		
ARV	0.133	0.164	0.152	0.144	0.128	0.122	0.106	0.165	0.164	0.152	0.162	0.116	0.185	0.210	0.212	0.224	

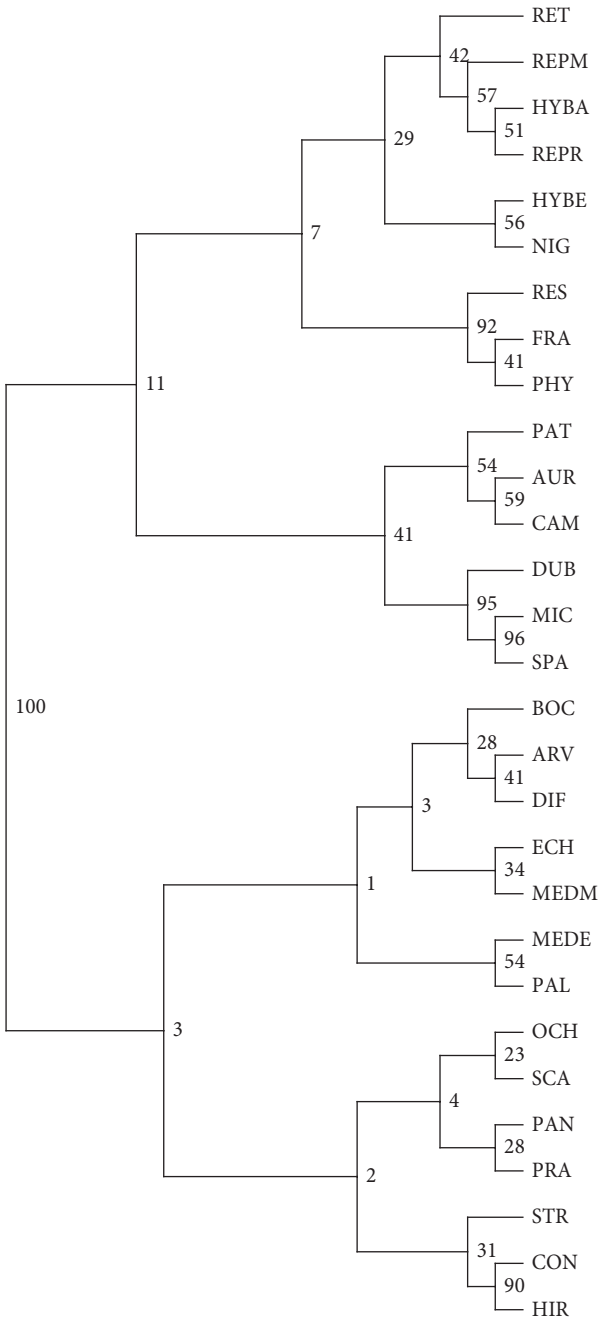

Figure 2. UPGMA dendrogram showing the genetic relationships among 29 *Trifolium* taxa, based on RAPD data.

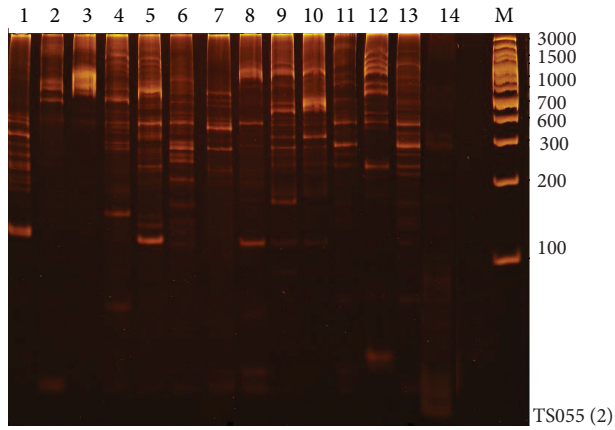

Figure 3. SSR banding patterns of *Trifolium* taxa revealed by TS055 primer. 1: PRA, 2: PAN, 3: SCA, 4: STR, 5: HIR, 6: CON, 7: ECH, 8: PAL, 9: MEDE, 10: MEDM, 11: OCH, 2: BOC, 13: DIF, 14: ARV, M: 100 bp DNA ladder plus marker (Fermentas).

patens, *T. campestre*, *T. micranthum*, and *T. spadiceum* and the other contained only *T. aureum* and *T. dubium* (Figure 4). The combined result (Figure 5) also gave the same groupings as the RAPD marker results, with a high genetic similarity rate (86%). *T. campestre* and *T. dubium* from the section *Chronosemium* were found to be closer in another study (Ellison et al., 2006). In similar research, *T. aureum, T. campestre*, and *T. dubium* were also found to be very close to each other (Vizintin et al., 2006). Our results also supported these findings.

A subcluster of *Lotoidea* members also showed differences between the 2 marker results (Figures 2 and 4), but the dendrogram based on the integration of data of both markers provided the highest genetic similarity rate (56%) in comparison to the results of those in separate markers (Figures 4 and 5). In this dendrogram, there were 2 subclusters. One contained both *T. hybridum* var. *elegans* and var. *anatolicum*, as well as both varieties of *T. repens*, var. *repens* and var. *macrorhizum*. The second subcluster consisted of only *T. nigrescens* and *T. retusum* (Figure 5). Similar to our results, in another study (Watson et al., 2000), *T. hybridum* and *T. nigrescens* were also found to be quite distant from each other. Our findings were more similar to those of Ellison et al. (2006). Although section *Trifolium* members presented a separate main cluster in both marker results (Figures 2 and 4) and both contained the same number of subgroupings, their members in these subgroups were different according to RAPD and SSR markers. The dendrogram based on the integration of both data markers was closer to the dendrogram of the SSR markers (Figures 4 and 5). *T. bocconei*, *T. arvense*, *T. diffusum*, and *T. ochroleucum* were distantly joined to the other subclusters of the section *Trifolium* in both SSR and the integrated data dendrograms (Figures 4 and 5). In the SSR dendrogram, both varieties of *T. medium* were separated from the second subcluster, but, in the dendrogram of the integrated data, *T. pallidum* was also found in the same subcluster as *T. medium*. The third subcluster of section *Trifolium* members contained *T. scabrum*, *T. pannonicum*, and *T. striatum* and the last subcluster covered *T. hirtum*, *T. constantinapolitanum*, *T. echinatum*, and *T. pratense* as the remaining members of the section *Trifolium* (Figure

Table 5. Jaccard's coefficient of similarity matrix, based on SSR data.

	RES	PHY	FRA	REPM	REPR	HYBA	HYBE	NIG	RET	DUB	MIC
RES											
PHY	0.270										
FRA	0.282	0.231									
REPM	0.165	0.219	0.278								
REPR	0.157	0.123	0.190	0.267							
HYBA	0.087	0.161	0.165	0.211	0.165						
HYBE	0.162	0.196	0.250	0.238	0.210	0.272					
NIG	0.065	0.136	0.167	0.152	0.129	0.225	0.214				
RET	0.186	0.144	0.196	0.206	0.174	0.184	0.235	0.290			
DUB	0.087	0.098	0.111	0.162	0.218	0.222	0.226	0.145	0.154		
MIC	0.175	0.173	0.196	0.303	0.250	0.186	0.219	0.122	0.268	0.291	
SPA	0.157	0.118	0.194	0.229	0.160	0.182	0.165	0.125	0.208	0.247	0.382
AUR	**0.037**	0.104	0.095	0.138	0.200	0.220	0.130	0.092	0.121	0.333	0.229
CAM	0.149	0.086	0.154	0.186	0.191	0.086	0.198	0.101	0.216	0.189	0.291
PAT	0.155	0.077	0.136	0.179	0.272	0.164	0.190	0.105	0.130	0.232	0.320
PRA	0.170	0.138	0.189	0.187	0.132	0.133	0.207	0.180	0.198	0.155	0.188
PAN	0.094	0.104	0.106	0.139	0.173	0.106	0.167	0.096	0.140	0.143	0.146
SCA	0.088	0.120	0.120	0.167	0.114	0.138	0.140	0.126	0.135	0.165	0.177
STR	0.116	0.133	0.131	0.112	0.119	0.119	0.131	0.129	0.136	0.109	0.141
HIR	0.103	0.102	0.172	0.187	0.114	0.087	0.109	0.123	0.167	0.104	0.188
CON	0.120	0.100	0.119	0.159	0.079	0.063	0.107	0.103	0.152	0.082	0.137
ECH	0.165	0.089	0.167	0.164	0.102	0.151	0.161	0.142	0.181	0.140	0.172
PAL	0.123	0.093	0.130	0.144	0.098	0.084	0.074	0.075	0.113	0.094	0.150
MEDE	0.081	0.090	0.102	0.144	0.077	0.091	0.089	0.115	0.146	0.102	0.140
MEDM	0.130	0.098	0.109	0.114	0.058	0.058	0.050	0.069	0.109	0.038	0.088
OCH	0.143	0.119	0.109	0.095	0.083	0.064	0.106	0.125	0.121	0.064	0.107
BOC	0.085	0.115	0.107	0.121	0.071	0.108	0.124	0.097	0.105	0.096	0.125
DIF	0.138	0.096	0.116	0.086	0.066	0.098	0.134	0.088	0.096	0.077	0.115
ARV	0.072	0.105	0.107	0.112	0.100	0.122	0.126	0.123	0.068	0.095	0.082

	SPA	AUR	CAM	PAT	PRA	PAN	SCA	STR	HIR	CON	ECH	PAL	MEDE	MEDM	OCH	BOC	DIF
SPA																	
AUR	0.316																
CAM	0.300	0.180															
PAT	0.257	0.278	0.288														
PRA	0.162	0.084	0.167	0.149													
PAN	0.103	0.086	0.130	0.122	0.286												
SCA	0.109	0.153	0.138	0.157	0.250	0.292											
STR	0.116	0.074	0.109	0.132	0.336	0.310	0.288										
HIR	0.146	0.080	0.141	0.116	0.280	0.232	0.221	0.273									
CON	0.140	0.073	0.082	0.126	0.271	0.174	0.275	0.235	0.331								
ECH	0.202	0.104	0.119	0.144	0.319	0.243	0.243	0.339	0.313	0.404							
PAL	0.155	0.099	0.149	0.130	0.336	0.257	0.323	0.311	0.328	0.313	0.376						
MEDE	0.133	0.095	0.091	0.129	0.230	0.240	0.202	0.267	0.295	0.230	0.268	0.333					
MEDM	0.108	0.068	0.068	0.138	0.237	0.178	0.222	0.273	0.280	0.248	0.220	0.340	**0.494**				
OCH	0.096	**0.037**	0.064	0.090	0.224	0.184	0.181	0.252	0.230	0.272	0.229	0.208	0.154	0.208			
BOC	0.082	0.048	0.084	0.064	0.229	0.178	0.200	0.167	0.205	0.207	0.191	0.224	0.268	0.214	0.278		
DIF	0.117	0.055	0.067	0.112	0.222	0.164	0.230	0.182	0.246	0.265	0.207	0.284	0.222	0.241	0.409	0.366	
ARV	0.092	0.086	0.070	0.099	0.139	0.161	0.170	0.193	0.127	0.171	0.189	0.188	0.143	0.175	0.284	0.230	0.247

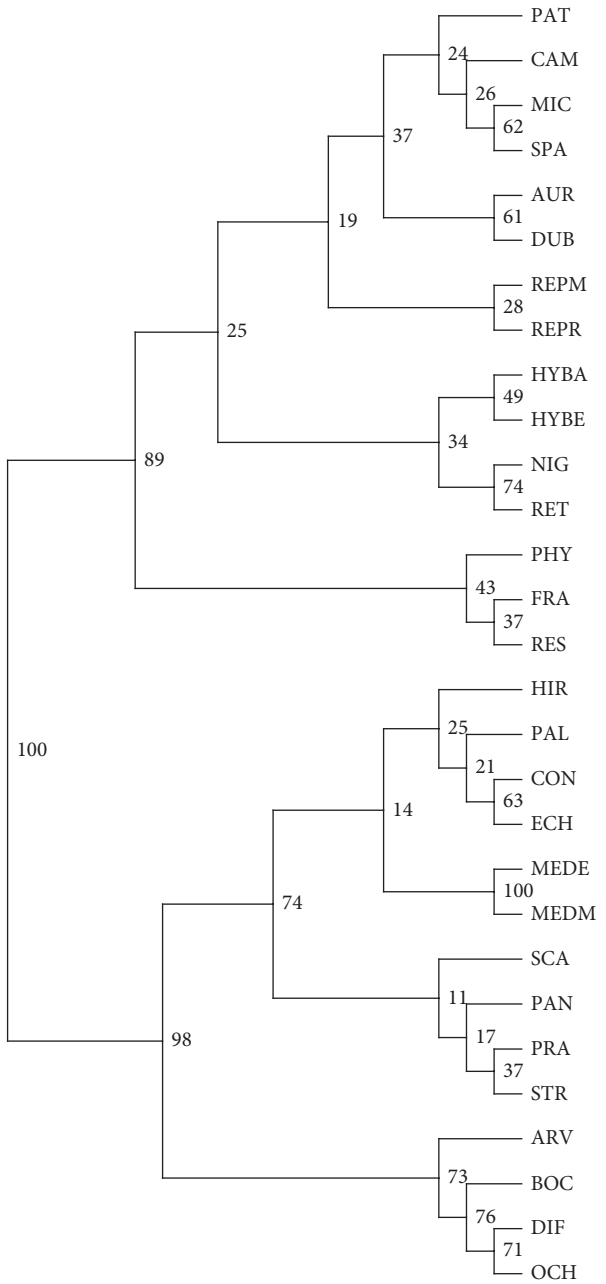

Figure 4. UPGMA dendrogram showing the genetic relationships among 29 *Trifolium* taxa, based on SSR data.

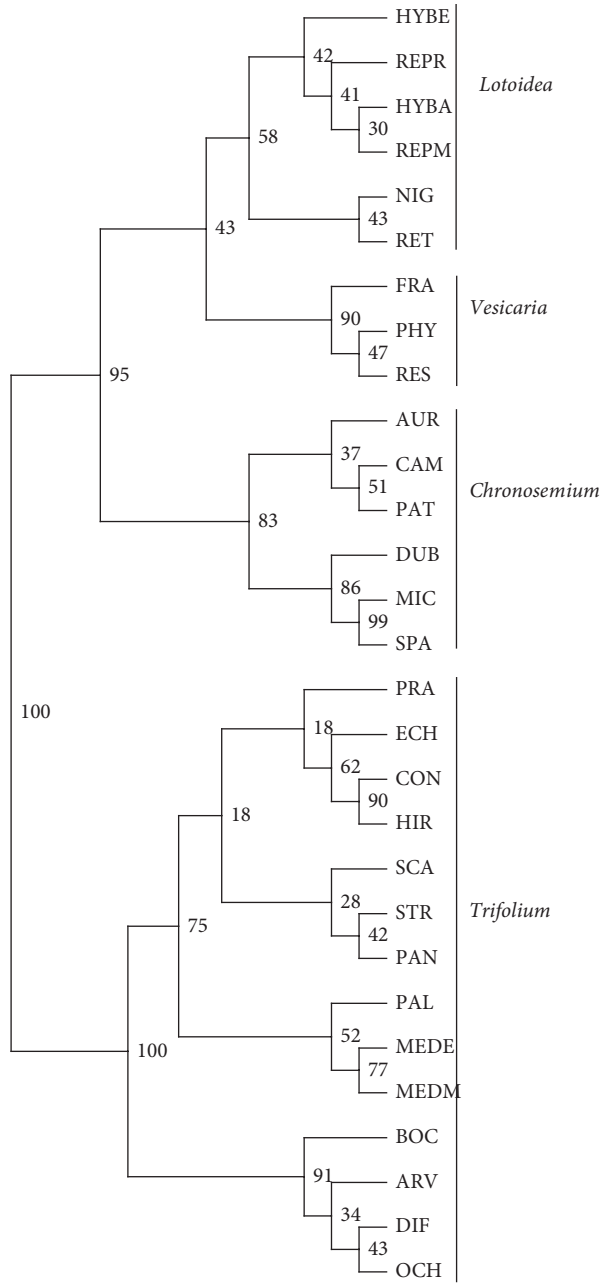

Figure 5. Dendrogram presenting the genetic relationships among 29 *Trifolium* taxa, obtained from UPGMA cluster analysis with the distance matrix based on RAPD and SSR marker combined results.

5). There were some similarities between the results of Ellison et al.'s (2006) groupings and our groupings, such as that *T. echinatum* and *T. constantinapolitanum* were found close to each other. Moreover, *T. arvense* and *T. bocconei* similarities were also supported in both results. The remaining subgroups of the section *Trifolium* in our study showed some different relationships than those from Ellison et al.'s (2006) groups. However, all the members of the section *Trifolium* remained under the main cluster in our study. In another study (Vizintin et al., 2006), the groups of the section *Trifolium* obtained also supported our results.

The last section *Vesicaria* members *T. resupinatum*, *T. physodes*, and *T. fragiferum* were found to be genetically close to each other and they were placed as a separate subgroup between the *Lotoidea* and *Chronosemium* section subclusters

Table 6. Jaccard's coefficient of similarity matrix, based on combined RAPD and SSR data.

	RES	PHY	FRA	REPM	REPR	HYBA	HYBE	NIG	RET	DUB	MIC
RES											
PHY	0.283										
FRA	0.280	0.263									
REPM	0.188	0.198	0.248								
REPR	0.181	0.128	0.191	0.253							
HYBA	0.149	0.187	0.161	0.258	0.244						
HYBE	0.143	0.134	0.200	0.208	0.222	0.249					
NIG	0.106	0.121	0.132	0.171	0.134	0.193	0.220				
RET	0.162	0.138	0.158	0.225	0.206	0.217	0.200	0.227			
DUB	0.142	0.133	0.159	0.147	0.196	0.180	0.155	0.141	0.189		
MIC	0.178	0.160	0.183	0.223	0.216	0.197	0.171	0.130	0.249	0.290	
SPA	0.164	0.117	0.160	0.179	0.156	0.158	0.139	0.142	0.198	0.282	**0.400**
AUR	0.085	0.105	0.124	0.144	0.154	0.149	0.128	0.088	0.158	0.244	0.191
CAM	0.137	0.103	0.143	0.151	0.159	0.112	0.164	0.103	0.186	0.199	0.245
PAT	0.157	0.114	0.175	0.145	0.176	0.144	0.148	0.087	0.129	0.191	0.242
PRA	0.167	0.129	0.172	0.141	0.148	0.120	0.147	0.122	0.155	0.156	0.169
PAN	0.100	0.123	0.111	0.101	0.109	0.103	0.128	0.085	0.106	0.102	0.117
SCA	0.113	0.143	0.133	0.169	0.135	0.149	0.152	0.140	0.157	0.147	0.167
STR	0.129	0.135	0.131	0.129	0.124	0.126	0.130	0.108	0.123	0.124	0.157
HIR	0.140	0.133	0.154	0.183	0.130	0.128	0.105	0.111	0.154	0.108	0.174
CON	0.137	0.095	0.120	0.163	0.118	0.099	0.095	0.120	0.156	0.093	0.132
ECH	0.161	0.100	0.140	0.165	0.101	0.146	0.140	0.138	0.158	0.139	0.168
PAL	0.147	0.105	0.130	0.151	0.132	0.124	0.119	0.111	0.147	0.103	0.143
MEDE	0.133	0.105	0.108	0.133	0.097	0.104	0.095	0.111	0.164	0.118	0.133
MEDM	0.135	0.117	0.132	0.122	0.088	0.103	0.081	0.076	0.154	0.083	0.116
OCH	0.133	0.114	0.112	0.128	0.109	0.119	0.128	0.136	0.153	0.093	0.153
BOC	0.135	0.136	0.104	0.126	0.098	0.137	0.121	0.112	0.134	0.114	0.120
DIF	0.120	0.095	0.113	0.102	0.084	0.120	0.130	0.095	0.139	0.098	0.104
ARV	0.127	0.102	0.114	0.122	0.136	0.157	0.112	0.092	0.126	0.154	0.137

	SPA	AUR	CAM	PAT	PRA	PAN	SCA	STR	HIR	CON	ECH	PAL	MEDE	MEDM	OCH	BOC	DIF
SPA																	
AUR	0.226																
CAM	0.221	0.224															
PAT	0.222	0.234	0.247														
PRA	0.138	0.100	0.173	0.126													
PAN	0.091	0.078	0.127	0.130	0.230												
SCA	0.131	0.127	0.131	0.118	0.195	0.225											
STR	0.124	0.104	0.138	0.122	0.224	0.251	0.234										
HIR	0.136	0.107	0.163	0.120	0.226	0.181	0.200	0.264									
CON	0.132	0.127	0.122	0.130	0.227	0.151	0.195	0.192	0.330								
ECH	0.176	0.115	0.125	0.118	0.221	0.176	0.195	0.250	0.251	0.270							
PAL	0.128	0.081	0.147	0.115	0.249	0.187	0.234	0.233	0.240	0.216	0.246						
MEDE	0.144	0.112	0.118	0.137	0.169	0.153	0.183	0.196	0.202	0.187	0.173	0.272					
MEDM	0.105	0.079	0.093	0.133	0.165	0.139	0.182	0.199	0.187	0.171	0.201	0.245	0.296				
OCH	0.101	0.078	0.103	0.114	0.173	0.178	0.188	0.185	0.173	0.207	0.153	0.162	0.148	0.174			
BOC	0.083	0.075	0.115	**0.070**	0.181	0.145	0.180	0.159	0.195	0.164	0.160	0.191	0.188	0.182	0.198		
DIF	0.118	0.095	0.099	0.110	0.182	0.161	0.171	0.160	0.167	0.219	0.166	0.221	0.178	0.178	0.266	0.259	
ARV	0.121	0.115	0.126	0.130	0.142	0.142	0.142	0.148	0.145	0.167	0.169	0.174	0.128	0.181	0.240	0.220	0.236

under the main cluster (Figure 5). However, in another study (Ellison et al., 2006), the members were found to be close to each other and were grouped under the section *Vesicastrum* of the subgenus *Trifolium*. Similar results were also found concerning members of the section *Vesicaria* (Vizintin et al., 2006).

About 11 RAPD primers were found to be highly polymorphic (0.84), and 5 SSR markers showed a 0.81 polymorphism rate (Tables 2 and 3) in this study. In similar studies with RAPD primers, the polymorphic rates were found to be 0.74 among red clover (*T. pratense*) (Ulloa et al., 2003) and 0.80 among clover genotypes in Iran (Arzani and Samei, 2004). The average polymorphism for 6 SSR markers was 0.88 among white clover (*T. repens*) (Kölliker et al., 2001).

In the comparison between RAPD and SSR results, there were different subgroup patterns obtained from the RAPD and SSR data in our study. This could be attributed to the different genomic constitution of the 2 markers (Ravi et al., 2003). However, the main groups (2 clusters) were the same in both marker results (Figures 2 and 4). Furthermore, the combined data results of RAPD and SSR markers provided complementary information for the genetic relationships of the studied *Trifolium* taxa.

In conclusion, the molecular marker results more or less supported the Zohary and Heller (1984) sectional relationships of *Trifolium* taxa. In our study, the members belonging to a section were not grouped under any other section. In addition, our results also supported Ellison et al.'s (2006) findings to some extent, such as that the section *Trifolium* was found to be quite distant from the remaining sections. The varieties of *T. medium* (*medium* and *eriocalycinum*) were found to be genetically (74%) close to each other. On the other hand, varieties of *T. hybridum* (*anatolicum* and *elegans*) and *T. repens* (*repens* and *macrorhizum*) were not genetically (39%) very close to each other. Although the results were similar in both markers, this study emphasized the necessity of the use of high numbers of markers and taxa for reliable results.

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