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KARYOTYPIC STUDY OF *TRIFOLIUM* SPECIES AND CULTIVARS IN IRAN

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Abstract

Trifolium species and cultivars are considered important forage crops in Iran, growing throughout the country. The present article considered the karyotypic details of *Trifolium* taxa using multivariate statistical analysis. Taxa studied included: *Trifolium resupinatum* (six populations: Shazand, Soriyan, Kordestan, Harati, Enaj and Lordegan), *T. repense*, *T. fragiferum*, *T. pratense* and *T. alexandrinum*. Somatic chromosome numbers from 2n=14 in *T. pratense* to 2n=32 in *T. repense*. Most of the chromosomes were of m type with a few M and sm. Only in *T. pratense* was a single subtelocentric (st) chromosome observed. Karl Pearson coefficient of correlation for karyological data showed occurrence of structural changes in chromosomes. Statistical analysis of data based on a factorial experiment revealed a significant difference in the size of chromosomes in taxa studied. Factor analysis followed by Varimax rotation showed that chromosomes 2,5 and 6 are the chromosomes which undergo extensive changes during genomic differentiation in the *Trifolium* taxa studied. Cluster analysis using UPGMA and WARD methods as well as ordination of the taxa on the first two principal component axes produced five distinct clusters indicating genomic differences among taxa studied. Data obtained can be used in further breeding programs.

Introduction

The genus *Trifolium* L. comprises 240 species of which no less than 10 are of agricultural importance [5,1]. Its centers of diversity are the Mediterranean region, Europe and the mountainous regions of Africa and Central, South and North America [21].

Trifolium taxa in Iran are considered important forage plants which grow throughout the country. In order to identify the genetic variations present in the species and the populations of these taxa, as well as to provide basic information about their genomes, cytogenetical studies were performed.

The present article considers karyotypic details of *Trifolium* taxa available in Iran with the emphasis on local cultivars. This is the first report from Iran.

Keywords: Cluster analysis; Karyotype; *Trifolium*

Materials and Methods

Seeds of the *Trifolium* species and cultivars were obtained from the Forest and Range Land Gene Bank. The species studied are: *Trifolium resupinatum* (six

populations: Shazand, Soriyan, Kordestan, Harati, Enaj and Lordegan), *T. repense*, *T. fragiferum*, *T. pratense* and *T. alexandrinum*. Seeds were germinated on Petri dishes and fresh root tips were collected for karyotypic studies. Different pretreatments were used and the best result was obtained from 0.002 M 8-hydroxyquinolin (2 hours). Other steps in cytological preparations followed the methods reported earlier [13, 14], using 2% aceto-orcein. A minimum of five well-prepared metaphase plates were analyzed.

Chromosomes were identified according to Levan *et al.* [8]. Karyotypes were compared using total form percentage (TF%) [6] and total volume [4]. TF% was estimated as follows: TF% = Total length of all short arms/Total length of chromosomes × 100. Total volume was estimated by adding up volumes of each chromosome and was determined as: $V=\pi r^2 h$; in which V is chromosome volume, π is 3.13, r is chromosome radius and h the chromosome length.

Pearson coefficient of correlation was determined for total length of chromosomes, length of the long arms, length of the short arms and L/S ratio [20,13,14]. A factorial experiment based on a completely randomized design, with species and chromosomes as the two factors, was used to analyze the data on size of chromosomes; means were compared by the least significant difference test (LSD) [4,15].

Cluster analysis using UPGMA and WARD methods was performed on standardized karyological data [16,3,2]. Fit of the clusters to the original data was checked using cophenetic correlation [11,17].

In order to determine the most variable chromosomes/ chromosome arms among the species, factor analysis based on principal component analysis (PCA) was performed on standardized karyological data. Varimax rotation was carried out after Kaiser normalization [3,14]. Ordination of the species was performed on the first two principal component axes [9,16,17]. Statistical analysis was performed using SPSS ver. 3.0 [10] and NTSYS ver. 1.4 [11] software.

Results and Discussion

The species studied and details of their karyotypes are presented in Table 1. Somatic chromosome numbers ranged from 2n=14 in *T. pratense* to 2n= 32 in *T. repense* (Figs. 1 and 2), supporting the reports of other workers [19,1]. As is evident, two different basic chromosome numbers are present in *Trifolium* (X=7 and 8). In about 80% of the *Trifolium* taxa studied X=8 has been reported and is considered to be the primitive basic number by Zohary and Heller [21] and Senn [12], from which X=7,6 and 5 have been derived. However, Goldblatt [7], studying a large number of *Trifolium* species with worldwide distribution, considered X=7 as the ancestral basic chromosome number. Therefore, species having 2n=14 and 16 are diploid and *T. repense* having 2n=32 is tetraploid.

Among the genotypes having 2n=16, the highest total chromatin length was observed in *T. resupinatum* (Soriyan) with 27.68 µm and the lowest in *T. resupinatum* (Enaj) with 18.64 µm. Due to its lower chromosome number (2n=14), *T. pratense* possessed the lowest value of total chromatin length (13.05 µm).

The highest value for size of the longest chromosome occurred in *T. resupinatum* Soriyan (4.77 µm) and *T. resupinatum* Lordegan (4.34 µm). The lowest value was observed in *T. pratense* (2.08 µm). The low value for the size of the longest chromosome in tetraploid *T. repense* (4. 16 µm) indicates that the occurrence of polyploidy and the increase in chromosome number have been accompanied by some sort of chromatin redistribution (structural changes of chromosomes) and possibly chromatin loss, as will be supported by statistical analysis later in the text.

The size of the shortest chromosome in genotypes with 2n=16 varied from 1.62 µm in *T. resupinatum* (Enaj) to 2.53 µm in *T. resupinatum* (Lordegan). The size of the shortest chromosome in tetraploid *T. pratense* was 1.48 µm, much lower than those of the diploid species.

Ratio of the longest to shortest chromosome (L/S) is an indicator of variation among the chromosomes. *T. resupinatum* (Harati), with 2.26 L/S ratio, possessed the highest variation among its chromosomes while *T. resupinatum* (Soriyan) possessed the lowest (1.37).

With regard to karyotype asymmetry, *T. fragiferum* and *T. resupinatum* (Soriyan) with highest value of TF% (68.07 and 57.79) possess the most symmetrical karyotype.

Karyotypic formulae are presented in Table 1. Chromosomes were mostly of m type (centromere in median region). In a few cases, M (centromere at median point) and sm (centromere in summedian region) chromosomes were present and only in *T. pratense* was a single subtelocentric (st = centromere in subterminal region) chromosome identified. As is evident from Table 1, both species and populations studied vary in their karyotypic formula indicating their genomic differences. Even the species having single sm chromosomes differed in their chromosomes having sm morphology. For example, in *T. resupinatum* (Shazand) chromosome number 4 is sm, while in *T. resupinatum* (Harati) and *T. alexandrinum* chromosome number 7 and 5 are sm. This indicates changes in chromosome structure, as evidenced from Karl Pearson coefficient of correlation computed for karyological parameters.

A high r value (>0.90) was obtained for total chromatin length, indicating homogeneity of the group and supporting

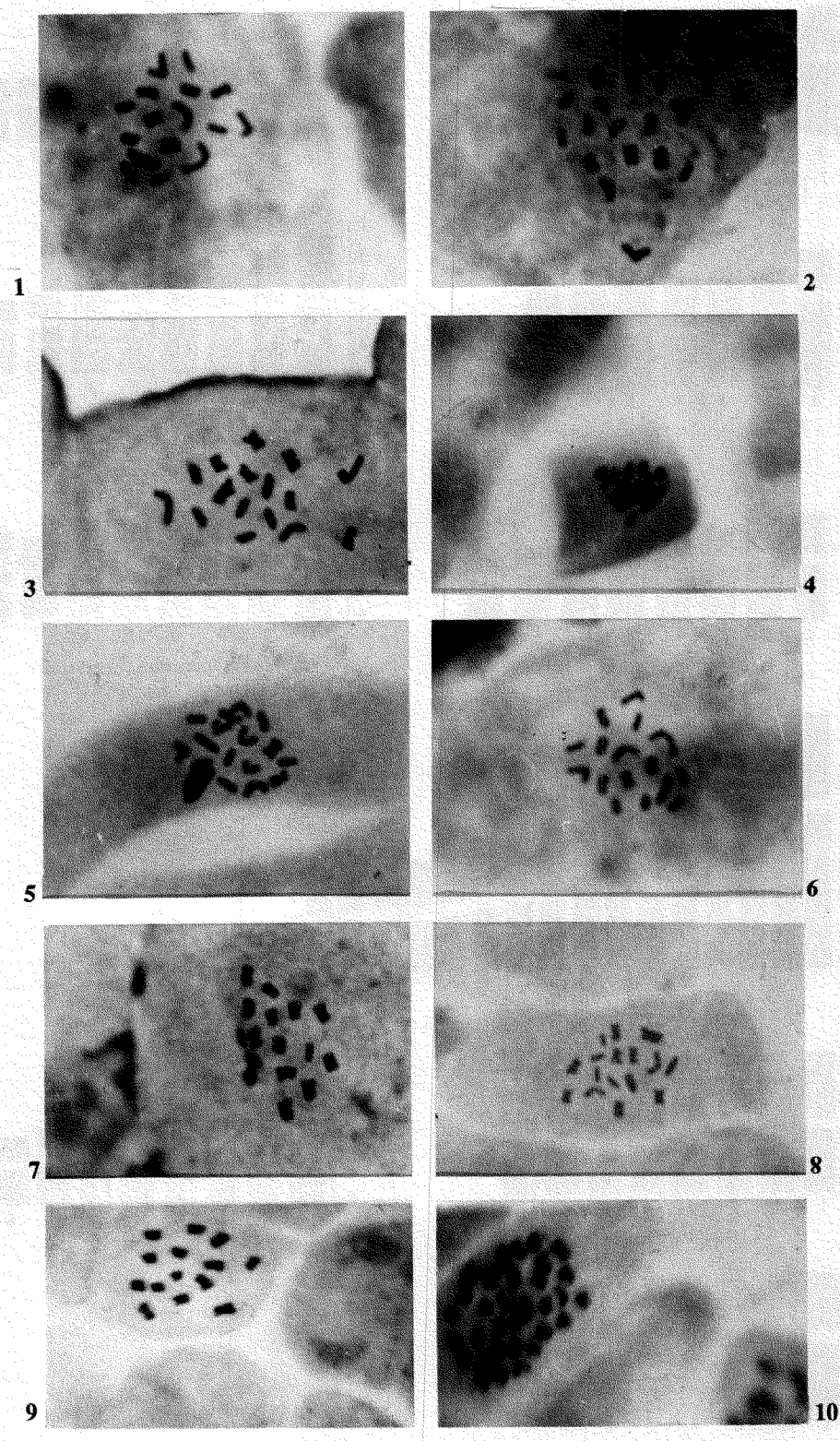

Figure 1. Somatic chromosome numbers in *Trifolium* taxa 1- *Trifolium resupinatum* (Shazand) 2- *T. resupinatum* (Enaj) 3- *T. resupinatum* (Kordestan) 4- *T. resupinatum* (Soriyan) 5- *T. resupinatum* (Harati) 6- *T. resupinatum* (Lordegan) 7. *T. alexandrinum* 8- *T. fragiferum* 9- *T. pratense* 10- *T. repense*

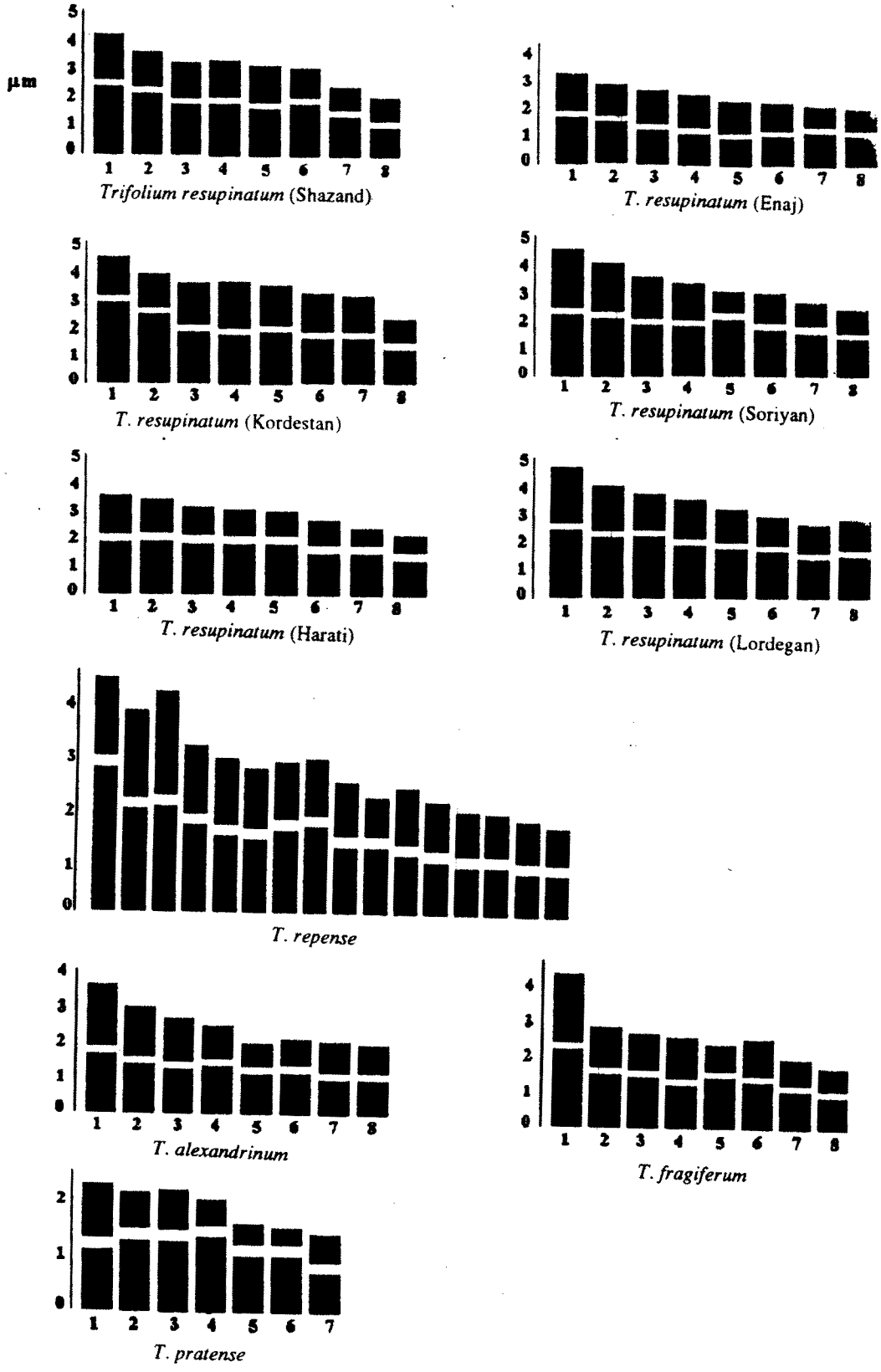

Figure 2. Ideograms of *Trifolium* taxa

Table 1. Karyotypic details of *Trifolium* taxa. Abbreviations: TL = Total chromatin length, L = Longest chromosome, S = Shortest chromosome, L/S = Longest/shortest chromosome, TF% = Total form percentage, TV = Total volume

Species/population formula	2n	TL	L	S	L/S	TF%	TV	Karyotype
T. pratense	14	13.05	2.08	1.25	1.67	29.58	10.54	3m+3sm+1st
T. resupinatum (Sh)	16	25.05	4.02	1.87	2.14	38.60	62.21	7m+1sm
T. resupinatum (So)	16	27.68	4.77	2.27	1.37	57.79	20.26	6m+2M
T. resupinatum (K)	16	25.82	4.52	2.02	2.24	43.03	10.98	8m
T. resupinatum (H)	16	23.03	3.70	1.64	2.26	42.25	12.73	7m+1sm
T. resupinatum (L)	16	25.39	4.34	2.53	1.71	41.67	12.17	7m+1M
T. resupinatum (E)	16	18.64	3.08	1.62	1.90	43.62	8.83	8m
T. fragiferum	16	22.76	4.15	1.90	2.18	68.07	42.81	6m+2sm
T. alexandrinum	16	20.17	3.44	1.85	1.86	41.99	10.54	7m+1sm
T. repense	32	39.91	4.16	1.48	3.11	45.98	38.14	14m+1sm+1M

the inclusion of all taxa studied in the genus *Trifolium*. However, a lower r value was obtained for length of the long arms (0.65-0.97), length of the short arms (-.02-0.85) and L/S ratio (-0.04-0.67), indicating the occurrence of structural changes in the chromosomes [15,16].

Analysis of variance for the size of chromosomes revealed a significant difference for species/populations and chromosomes (Table 2). Furthermore, the least significant test (LSD) performed on pairs of the genotypes showed that the main differences appear between *T. resupinatum* (Enaj) and the other species as well as between *T. resupinatum* (Soriyan) and the other *T. resupinatum* populations indicating that, along with a change in karyotype symmetry, a significant increase or decrease in the size of the chromatin has occurred.

It has been suggested that a change in karyotype through deletion of heterochromatin segments occurs due to adaptation to environmental conditions. Stebbins [18] states that a decrease in chromosome size is not always accompanied by a change in morphological characters. However, comparison of morphological characters among the genotypes studied (unpublished) showed significant

Table 2. Results of analysis of variance for the size of chromosomes

Source of variation	Sum of squares	Degrees of freedom	Mean square	F	P
Genotypes	28.61	5	5.72	20.78	>0.01
Chromosomes	92.99	7	13.28	48.25	>0.01
GenotypesxChromosomes	4.44	35	0.12	0.46	>0.01
Residual	52.85	192	0.27		
Total	178.91	239	0.74		

Table 3. Factor analysis of karyological data after Varimax rotation. Abbrevations: TL = Total chromatin length, L = Long arm, S = Short arm, S/L = Short arm/Long arm, V =Volume

CHARACTER	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5
TL1	.77933	.04061	.57041	.15831	.02617
L1	.78561	.42627	.24426	-.06344	.24789
S1	.47140	-.22786	.79433	.12557	.15015
L/S1	-.65666	.42010	.35172	.42400	-.00595
V1	.06258	.98570	.10447	.03576	-.06585
TL2	.90158	-.01660	.33768	-.04347	.15583
L2	.95092	-.02193	.14886	-.12764	.16179
S2	.62466	-.16489	.44570	.00243	.45474
L/S2	.01082	.95675	.12447	.13322	-.11851
V2	.08194	.99214	.02838	.06069	-.04239
TL3	.94483	-.02232	.29485	.08539	-.04682
L3	.77188	-.46949	.33816	-.10226	.01402
S3	.54086	.79514	.01804	.21872	.09740
L/S3	-.21401	.86069	.30161	-.33312	-.07784
V3	.25543	.87060	.37760	.07123	-.08151
TL4	.94043	.11687	.27978	.04832	.03669
L4	.84152	-.33540	.33535	.00125	.03368
S4	.85088	.31504	-.01068	-.13282	.37181
L/S4	-.21120	.96146	-.06295	-.02754	.10097
V4	.09625	.97865	-.08053	.01723	.09802
TL5	.96158	-.06965	.14825	.02325	.14934
L5	.96217	.21750	-.00106	.10005	-.01224
S5	.92572	-.16359	.06481	.00653	.29321
L/S5	.09459	.94718	-.24436	-.00032	.04309
V5	.16131	.95495	-.16004	-.07649	-.02128
TL6	.78937	-.14514	.50100	-.30473	.02335
L6	.93608	.21660	.14421	.03844	-.19965
S6	.85147	.26048	.15343	.34740	.12186
L/S6	-.12772	.96697	-.11023	.09384	-.13640
V6	-.03354	.99297	-.07298	.01640	-.07555
TL7	.88911	-.02536	.35698	.19032	.12592
L7	.91619	.06753	-.05399	.23061	-.10718
S7	.51759	-.09092	.21259	.24021	.76214
L/S7	-.07693	.97248	-.13230	.07593	.12189
V7	.07327	.98890	.04931	.02911	.06228
TL8	.39911	.10829	.79602	.31798	.05616
L8	.41084	.01382	.71564	-.41384	.34118
S8	.35361	.00220	.90908	.01003	-.05584
L/S8	-.01897	.86130	-.00763	.41146	-.02226
V8	.39282	.35288	.24422	.73065	.25673

variation in most of the characters, indicating that changes of the karyotypes have led to a change in the morphological characters. Thus, changes in heterochromatin segments have affected the genes controlling morphological characters, which in turn means that the segments which underwent change possessed active genes.

Factor analysis followed by Varimax rotation was performed on karyological data in order to identify the most variable chromosome/chromosome parts among *Trifolium* species, and the populations were studied (Table 3). Results showed that the first four factors comprise more than 90% of the total variation. In the first factor, total length of chromosomes 2-7, long arm of 2,5,6 and 7 and short arm of 5 are the most variable characters (having high loading >0.90), and in the second factor volume of chromosomes 1-7 and L/S ratio of chromosome 2,3,4,5,6 and 8 are the most variable characters. Thus, chromosomes 2,5 and 6 have undergone extensive changes during genomic differentiation in *Trifolium* taxa studied.

In order to group similar genotypes (2n=16), cluster analysis using UPGMA and WARD methods as well as ordination of the taxa on the first two principal component axes (PCA) were performed (Figs. 3 and 4). Both methods of cluster analysis produced the same results indicating distinctness of the clusters [11]. Five distinct clusters were recognized. *T. resupinatum* populations of Shazand, Lordegan Charmahal, Kordestan and Enaj form the first cluster. Populations of Harati and Soriyan stand separate from the others indicating their genomic differences. Species of *T. alexandrinum* and *T. fragiferum* form the fourth and fifth clusters in which *T. fragiferum* stands in a far distant cluster due to its genomic difference from the others. It is interesting to note that cluster analysis based on seed storage proteins (unpublished) also grouped the genotypes similarly. The high cophenetic value (r=>0.80) obtained for cluster analysis supports the fit of the clusters to the original data [11,17]. Ordination of the genotypes on the first two PCA axes also grouped them similarly (Fig. 4).

In short, the results obtained from the present study revealed karyotypic details of the *Trifolium* taxa cultivated in Iran for the first time, indicating their genomic differences. Such data can be used in further breeding programs. Morphological variations observed in the genotypes studied (unpublished) further support genetic differences in the species and cultivars studied. Genomic difference in the different populations of *T. resupinatum* can be used in hybridization programs and further evaluation of these taxa against cold and diseases.

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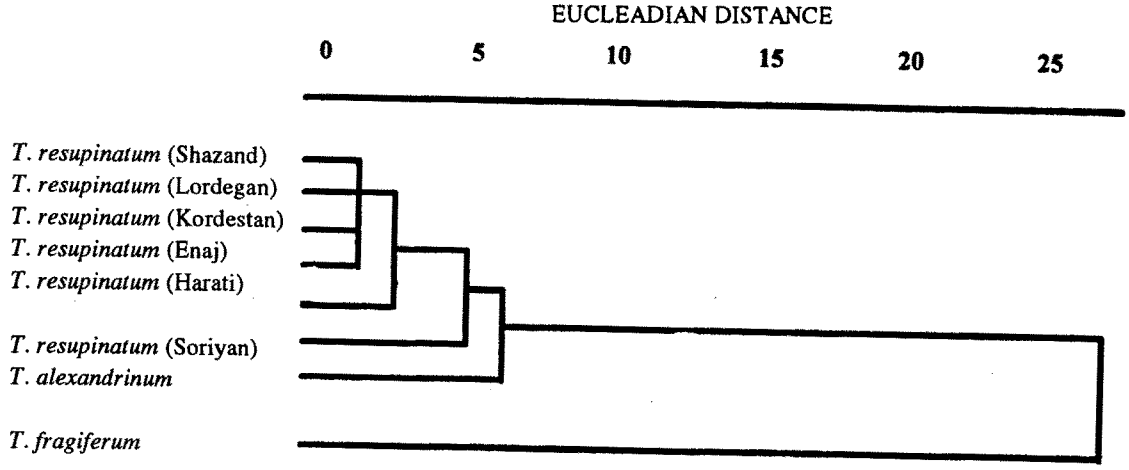

Figure 3. Cluster analysis (WARD) of *Trifolium* taxa

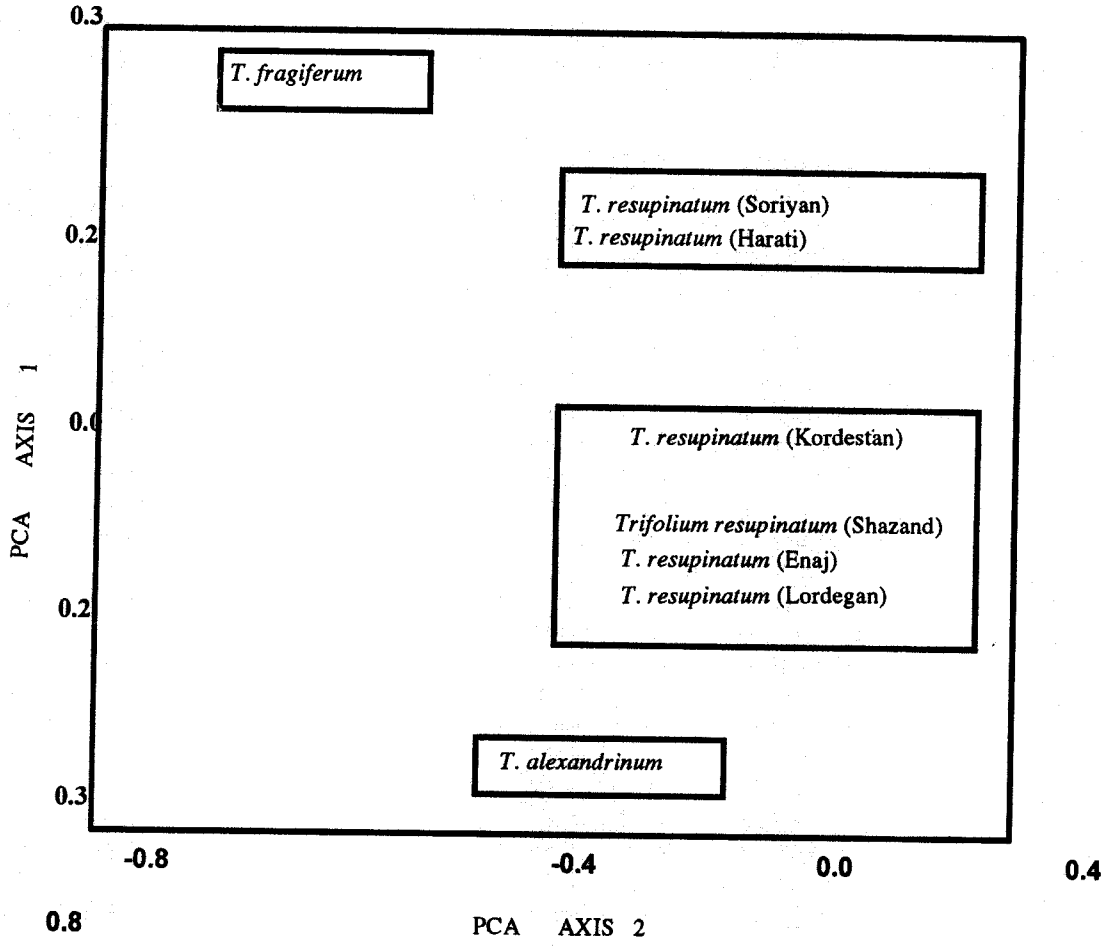

Figure 4. Ordination of *Trifolium* taxa on the first two PCA axes

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