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Karyology Study of Ten *Trifolium* Species in Fars Province

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ABSTRACT

Chromosome information is an important key for taxonomy, phylogeny and genetic in *Trifolium* plants. Thus, ten *Trifolium* species namely *T. grandiflorum, T. resupinatum, T. dasyurum, T. campestre, T. tomentosum, T. hirtum, T. scabrum, T. lappaceum, T. stellatum,* and *T. repens* were collected from their habitats in Fars province based on their morphological characteristics to investigate their karyotypes. To analyze the karyology of samples, the fresh grown root tips were used. Then α-bromonaphtaline, formaldehyde and chromium trioxide (1:1), 1N NaOH and hematoxylin were used for pre-treatment, fixative, hydrolyzer and chromosome staining agent, respectively. We found the three usual basic chromosome numbers in the genus as x=5, x=7 and x=8. Nine species were diploid and *T. repense* was only tetraploid species (2n=4x=32). The detailed correlation coefficient was estimated for all paired combinations of the karyotypic characteristics to investigate their inter-relationships. Duncan's test applied to the chromosome morphometric traits showed a highly significant difference among all examined species belongs to different sections.

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Introduction

The genus *Trifolium* belongs to the *Papilionaceae* family. The taxon includes about 255 annual and perennial species (Gillett and Taylor, 2001) distributed all over the world. Its centers of diversity are the Mediterranean region, Europe and the mountainous regions of Africa and Central, South and North America (Sheidai *et al.*, 1998). *Trifolium* is an important rangeland plant species in Iran that grows throughout the country and has approximately 46 species in six sections such as *Lotoidae*, *Versicaria*, *Mistyllus*, *Trichocephallum*, *Trifolium* and *Chronosemium*. Some of these Taxa are important forage and pasture plants. At least 184 species of *Trifolium* have shown their chromosome numbers (Taylor *et al.*, 1979; Goldblatt and Johnson, 2003; Salimpour *et al.,* 2008) that about 80% of *Trifolium* species are diploid with 2n=16 and the most common base number is x=8 (Goldblatt, 1981). About 31 species of the genus show aneuploidy with the basic number of x=5, 6 and 7 (Ellison *et al.,* 2006; Emel, 2012). Polyploidy is present in 20% of *Trifolium* species (4x, 6x and 12x) (Majumdar *et al.,* 2004; Ellison *et al.,* 2006). Salimpour *et al.,* (2008) investigated karyotype of 140 accessions in 37 species of *Trifolium* in six sections in Iran. The result showed that the sections of *Versicaria*, *Mistyllus,* and *Lotoidae* were x=8 (2n=16 and 32) and the basic chromosome number found in *T. fragiferum* and *T. tumens* of the section *Versicaria*. In addition, the species of *T. ambigum* and *T. montanum* of the section *Lotoidae* showed tetraploidy for the first time. Also, four basic chromosome numbers of x=5, 6, 7 and 8 were found in section *Trifolium* (2n=10, 12, 14 and 16) (Salimpour *et al.,* 2008). Sheidai *et al.,* (1998) studied five species of *Trifolium* and reported somatic chromosome numbers ranged from 2n=14 in *T. pratense* to 2n=32 in *T. repense*. Hesamzadeh and Ziaei Nasab (2006) investigated karyotype of 19 genotypes from 10 species suggesting that the genotypes of the species studied differed in their karyotypic characteristics and ploidy levels (x=5,7 and 8). Emel (2012) investigated the karyology of

nine *Trifolium* taxa in three sections in Turkey and indicated that all taxa chromosome number were 2n=16 except for *T. striatum* (2n=14), *T. leucanthum* (2n=14) and *T. phleoides* (2n=12). Thus, chromosome data is the main key for taxonomy, phylogeny and genetic studies of *Trifolium* plants. Here, the aim of this study was to determine the chromosome numbers, ploidy levels and to compare the karyotypic traits of some species of *Trifolium* genus in Fars province.

Materials and Methods

Ten *Trifolium* species including *T. dasyurum* C. Presl*, T. hirtum* All*., T. scabrum* L*., T. lappaceum* L., and *T. stellatum* L. belong to *Trifolium* section, *T. campestre* Schreb., and *T. grandiflorum* Schreb. belong to *the Chronosemium* section*, T. resupinatum* L. and *T. tomentosum* L. belong to *the Vesicaria* section and *T. repens* L. belongs to the *Lotoidea* section were collected from different areas of nature in Fars province. Vouchers were deposited in the Herbarium of Fars Agricultural and Natural Resources Research and Education Center (Table 1).

Table 1. The origin of materials used in chromosome studies of *Trifolium*

Species (population)	Origin	Altitude	Herbarium code
T. campestre	Fars, Mamasani, Galgoon	1000 m	15248
T. dasyurum	Fars, Mamasani, Galgoon	1000 m	15546
T. tomentosum	Fars, Sarab Bahram	1000 m	15251
T. grandiflorum	Fars, Mamasani, Galgoon	1000 m	15255
T. stellatum	Fars, Mamasani, Galgoon	1000 m	15233
T. lappaceum	Fars, Sarab Bahram	1000 m	15517
T. scabrum	Fars, Mamasani, Galgoon	1000 m	15004
T. resupinatum	Fars, Sarab Bahram	1000 m	15529
T. hirtum	Fars, Mamasani, Galgoon	1000 m	15023
T. repens	Fars, Eghlid	2300 m	16536

Cytogenetic studies were performed on these species in order to specify their karyotypic characteristics. Preparations were made using fresh grown root tips for the karyotypic studies. The root tips meristems were treated with 0.5% saturated α-Bromo naphthalene at 4°C for 4 h. Then, it was fixed in 10% formaldehyde and chromium trioxide (1:1) for 24 h at 4°C. After that, the root tips were rinsed for 3 h in distilled water. Hydrolysis was carried out with 1 N NaOH at 60ºC for 10 min and used hematoxylin-iron for chromosome staining for 2-3 h at room temperature. Root tips were squashed in a droplet of 45% acetic acid and lactic acid (10:1) (Wittmann, 1965). The preparations were observed with an optical microscope (BH2 Olympus supplemented Digital color video camera) at a magnification of 2000x. Chromosomal recounts were done in at least five complete metaphases cells and were used to prepare the karyotype by Adobe Photoshop 7.0 software and measured by Micro Measure 3.3 software for each genotype (Reeves and Tear, 2000).

In each mitotic metaphase (at least 5 plates), cytogenetic parameters were calculated as long arm (LA), short arm (SA), total length (TL), the percent of relative length of each chromosome (RL%), arm ratio (AR), centromeric index (CI), value of relative chromatin (VRC). Karyotype asymmetry was estimated by four different methods namely: total form percentage (TF%), the difference of relative length (DRL), intra-chromosomal asymmetry index (A_1) and inter-chromosomal asymmetry index (A_2). Both indices A_1 and A_2 (Romero Zarco 1986) are independent of chromosome number and size. Karyotype symmetry was determined according to Stebbins (SC) (Stebbins, 1971). Chromosomes were identified according to Levan (Levan *et al.,* 1964). For each species, karyograms and haploid ideograms were drawn based on the mean centromeric index and arranged in order to size decrease. In order to determine the variation between species, one-way analysis of variance (ANOVA) was performed to compare the chromosomes pair in each population by Duncan's test. Factor analysis based on principal components analysis (PCA) was performed on standardized karyological data of species. Cluster analysis using Ward's method was performed after the calculation of the Cophenetic correlation coefficient (*r*) to examine karyotype similarity among species. Statistical analyses were performed using SAS ver. 6.12 (1996), JMP ver. 3.1.2 (1995) and Statisti XL ver 1.7 (2007) softwares.

Results

The results showed that the basic chromosome number was varied between x=5, x=7, and x=8. The somatic chromosome numbers (2n), karyotype formulae and parameters for the studied species are summarized in Table 2. *T. repens* was the only tetraploid (2n=4x=32) and other species were diploid with 2n=2x=10, 2n=2x=14 and 2n=2x=16 and four species (*T. scabrum*, *T. stellatum*, *T. hirtum*, and *T. tomentosum*) had one pair of visible small satellites (Fig. 1). The maximum length of the satellite was observed in *T. tomentosum* (2.34 µm) and the minimum length was observed in *T. hirtum* (1.40 µm) species.

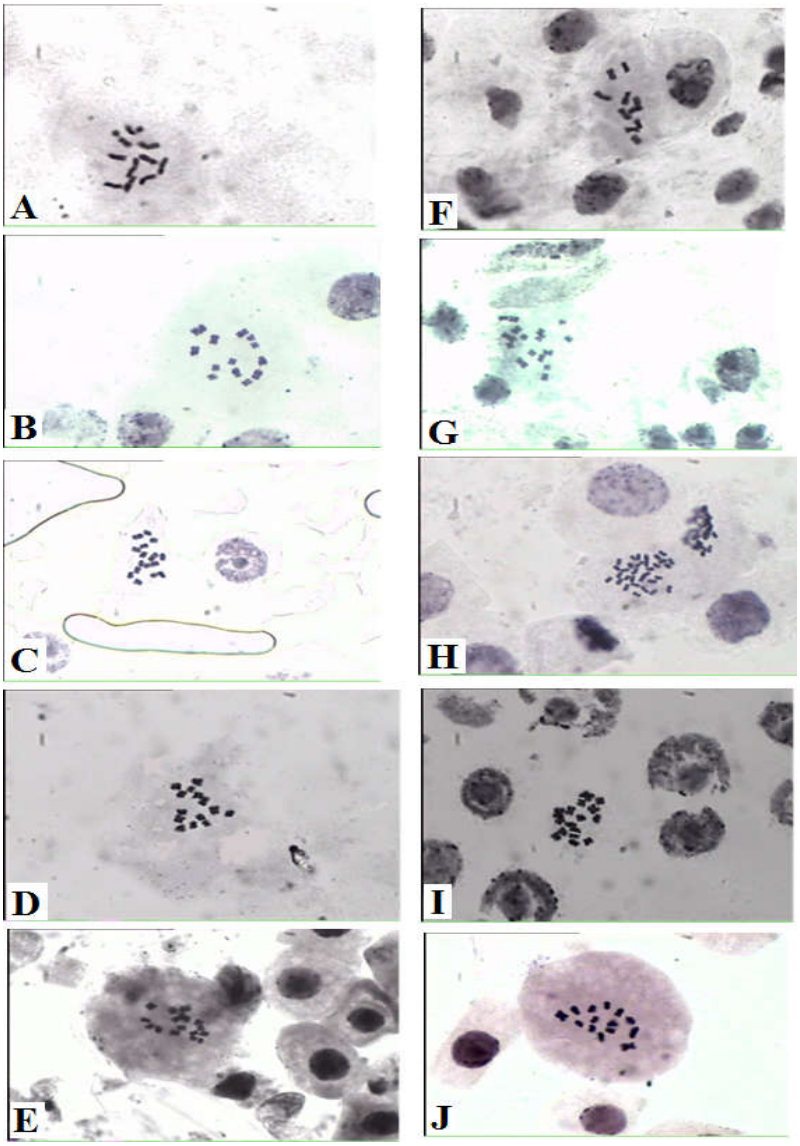

Fig. 1. Representative mitotic plates of *Trifolium* species: A) *T. hirtum*; B) *T. scabrum*; C) *T. stellatum*; D) *T. grandiflorum*; E) *T. lappaceum*; F) *T. repens*; G) *T. campestre*; H) *T. resupinatum*; I) *T. tomentosum*; J) *T. dasyurum*.

The chromosomes were mostly metacentric (m) in all species (Table 2). It meant that there was karyotypic symmetry among them. According to the Stebbin's bilateral table, *T. stellatum* included the highest value regarding the intra-chromosomal asymmetry index (0.27 µm) and was classified as group 1A and *T. grandiflorum, T. hirtum* and *T. repens* included the lowest value regarding the intrachromosomal asymmetry index (0.11 µm) and were classified as group 1A.

Table 2. Karyotypic characters of different *Trifolium* species

Species	x	2n	A1	A2	%TF	SC	DRL	VRC	K.F.
T.campestre	7	2x=14	0.12	0.10	46.71	1A	4.10	1.99	14m
T.dasyurum.	7	2x=14	0.25	0.21	42.07	1A	8.42	2.83	14m
T. tomentosum	8	2x=16	0.20	0.14	44.59	1A	5.71	2.33	16m
T. grandiflorum	8	2x=16	0.11	0.19	46.88	1A	7.67	1.86	16m
T. stellatum	7	2x=14	0.27	0.21	41.31	1A	8.27	2.56	12m+2sm
T. lappaceum	8	2x=16	0.18	0.20	45.05	1A	7.91	1.79	16m
T. scabrum	5	2x=10	0.20	0.14	44.00	1A	7.12	2.73	10m
T. resupinatum	8	2x=16	0.25	0.13	46.46	1A	5.37	2.01	16m
T. hirtum	5	2x=10	0.11	0.08	46.99	1A	4.13	3.49	10m
T. repens	8	4x=32	0.11	0.16	47.05	1A	3.73	1.78	32m

2n=Diploid chromosome numbers; A_1=Intrachromosome asymmetry index; A_2=Interchromosome asymmetry index; TF%= total form percentage; DRL=difference of relative length; VRC=value of relative chromatin; SC=symmetry classes of Stebbins; K.F.=karyotype formula.

The mean value of the chromosome's long arm was varied from 1.85 µm in *T. hirtum* to 0.94 µm in *T. repens*. Averages of chromosome's short arm were different from 1.64 µm in *T. hirtum* to 0.80 µm in *T. lappaceum*. The total length of the chromosome was varied from 3.49 µm in *T. hirtum* to 1.78 µm in *T. repens* and the mean value of the chromosome's arm ratio was in a range from 1.41 in *T. stellatum* to 1.12 in *T. repens* (Table 3). Symmetry type of Stebbins (1971) and asymmetry indices of Romero-Zarco (1986) are given in (Table 2).

Table 3. Mean of chromosomes analysis of *Trifolium species*

Populations	SA	LA	TL	AR	CI	A1	A2	DRL	%TF
T. campestre	0.93	1.06	1.99	1.14	0.47	0.12	0.10	4.10	46.71
T. dasyurum	1.19	1.64	2.83	1.36	0.43	0.25	0.21	8.42	42.07
T. tomentosum	1.04	1.29	2.33	1.26	0.44	0.20	0.14	5.71	44.59
T. grandiflorum	0.87	0.99	1.86	1.13	0.47	0.11	0.19	7.67	46.88
T. stellatum	1.05	1.50	2.56	1.41	0.42	0.27	0.21	8.27	41.31
T. lappaceum	0.80	0.98	1.79	1.23	0.45	0.18	0.20	7.91	45.05
T. scabrum	1.20	1.53	2.73	1.27	0.44	0.20	0.14	7.12	44.00
T. resupinatum	0.93	1.26	2.01	1.34	0.46	0.25	0.13	5.37	46.46
T. hirtum	1.64	1.85	3.49	1.13	0.47	0.11	0.08	4.13	46.99
T. repens	0.84	0.94	1.78	1.12	0.47	0.11	0.16	3.73	47.05

TL= total length of chromosome; LA= long arm; SA= short arm; AR= arm ratio; CI= centromeric index; DRL= difference of relative length; TF%= total form percentage; A_1= intra-chromosome asymmetry index; A_2= inter-chromosome asymmetry index.

The results showed that the highest VRC amongst all populations was obtained for *T. hirtum* (3.49 µm) and the lowest was obtained for *T. repens* (1.78 µm). Based on intra-chromosomal asymmetry, some species had the most asymmetrical and evolutionary karyotype. According to inter-chromosomal asymmetry, *T. dasyurum* and *T. stellatum* had the most asymmetrical karyotype in all of the populations (Table 2).

The difference in the relative length percentage (DRL) of the highest and the smallest chromosomes varied from 8.42 µm in *T. dasyurum* to 3.73 µm in *T. repens*. According to table 1, *T. stellatum* was placed in 1A and had the highest values of the intra-asymmetry chromosomal index. It had the lowest of TF%. TF% and A_1 values had an inverse ratio (Table 2).

The ratio of long arm /short arm chromosomes (AR) showed a highly significant difference among some species belong to different sections, while other species are not clearly distinct (Table 3). Diploid species of *T. repens* for instance, had the lowest AR value (1.12), the highest TF% value (47.05) and the lowest A_1 value (0.11), exhibiting the most symmetrically karyotypes, while *T. stellatum* with the highest AR value (1.41), the lowest TF% value (41.31) and the highest A1 value (0.27) was introduced as the most asymmetrical karyotypes (Table 3). The pattern of variation of A_1 and A_2 values has been compared with the pattern of Stebbins' system in this study. In view of the fact that fewer DRL values illustrated more symmetry of karyotype, *T. dasyurum,* and *T. repens* respectively with DRL 8.42 and 3.73 values

had the most symmetric and asymmetric karyotypes. Similarly, high DRL value leads to more changes in the construction of chromosomes.

The principal component analysis (PCA), of the karyotypic parameter, shows that the first two principal components account for 0.887% of the total variance. Component one (0.546%) put emphasized on the A_1, A_2, and DRL While component two (0.342%) accentuates, chromosome total length, long arm length, short arm length and TF% values which had the highest coefficients of eigen vectors (Table 4).

Table 4. The results of variance analysis for karyotypic data based on CRD design

		Mean of squares								
S.O.V	**DF**	**SA**	**LA**	**TL**	**AR**	**CI**	**A_1**	**A_2**	**DRL**	**%TF**
Populations	9	1.614**	3.258**	9.177**	0.696*	0.016**	0.162**	0.072**	4.53*	445.91**
Error	34	0.485	1.821	3.003	1.176	0.013	0.012	0.017	6.862	137.985
%C.V.	43	2.099	5.079	12.180	1.872	0.030	0.173	0.090	11.392	583.891

**=Significant at 1%; *=Significant at 5%. S.O. V= Sources of variation; D. F= Degrees of freedom

The statistical comparison based on a completely randomized design demonstrates that there are significant differences among species for all the measured traits (P≥%1) (Table 5).

The diagram of the species dispersion, based on two first components showed the species separated into four groups, which completely fits with the results obtained through the average grouping analysis method (Fig. 2).

The changes of A_1 and TF% parameters showed that there was a negative and reverse relation between them and also the changes of A_2 and DRL parameters showed that there was a positive and direct relation between them (Fig. 3; Fig. 4).

Table 5. Specific values of variance percentage and coefficients of specific vectors in analyzing main components

Name of traits	First component	Second component
SA	0.162	0.973
LA	0.513	0.852
TL	0.366	0.916
AR	0.922	-0.086
CI	-0.963	0.052
A1	0.894	-0.109
A2	0.608	-0.646
DRL	0.783	-0.349
TF	-0.964	0.038
Specific values	4.962	3.076
Percentage of Variance	0.546	0.342
Cum Percentage of Variance	0.546	0.887

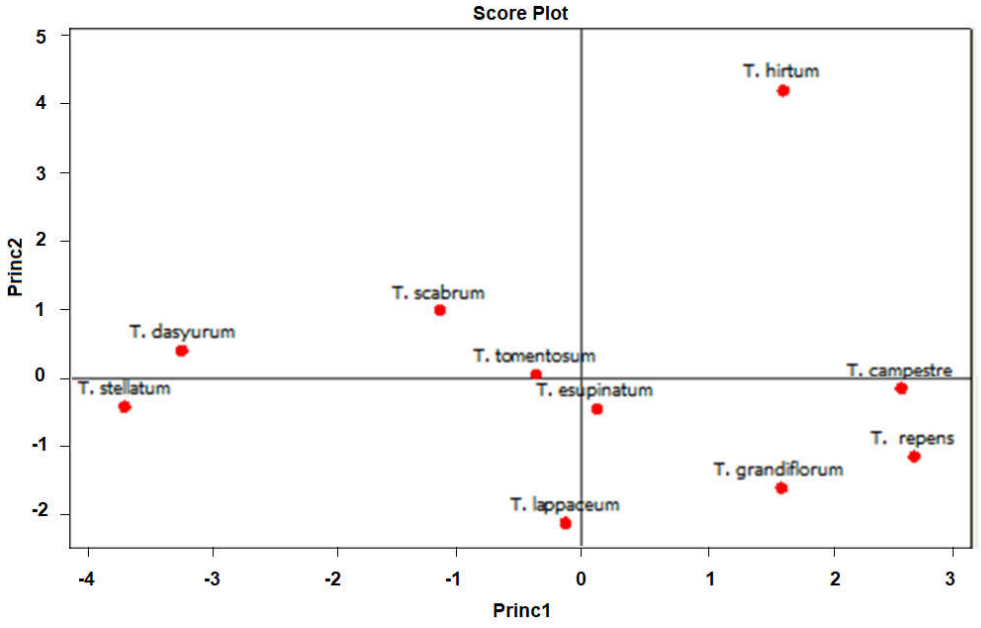

Fig. 2. Scatter plot of 10 species.

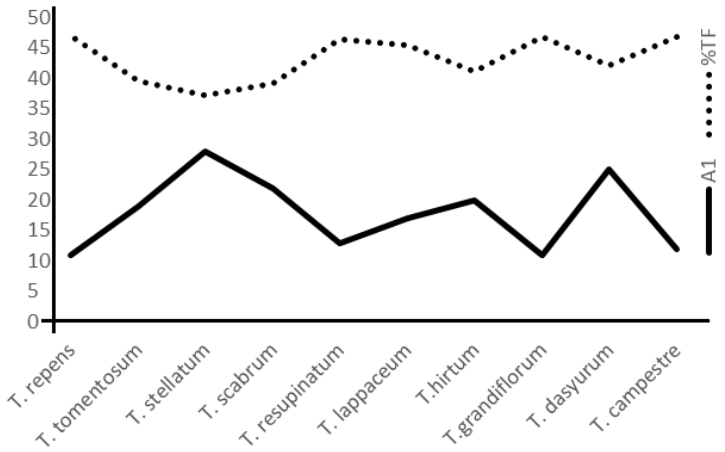

Fig. 3. Intrachromosome asymmetry index and total form percentage trend in different *Trifolium* species.

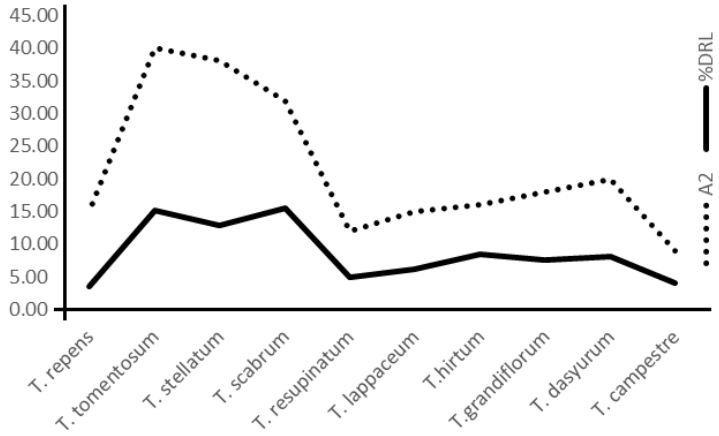

Fig. 4. Interchromosome asymmetry index and difference of relative length trend in different *Trifolium* species.

Discussion

This study revealed a detailed picture of the chromosome features in 10 *Trifolium* species of Iran. The knowledge of chromosome numbers, karyotype evolution, ploidy level, and genome size can provide additional information that not only gives further insight into the functioning of the genome but also have considerable predictive powers.

In this study, different chromosomal bases were observed, indicating variation among species. The results of this study showed great variations in the number of chromosomes (2n=10, 2n=14. 2n=16, and 2n=32) between *Trifolium* species. Three basic chromosomes (x=5, x=7, and x=8) were found in section *Trifolium*. The diploid chromosome number of *T. hirtum* and *T. scabrum* were 2n=10 (x=5). The same chromosome number was reported by Hesamzadeh and Ziaei Nasab (2006) and Salimpour *et al.,* (2008). In this study, the chromosome number of *T. dasyurum* and *T. stellatum* were determined as 2n=14 (x=7) but Salimpour *et al*. (2008) reported the chromosome number of *T. stellatum* 2n=16. *T. lappaceum* is another taxon studied from section *Trifolium*, and its chromosome number was 2n=16 (x=8). The same chromosome count was given by Salimpour *et al.,* (2008). Section *Vesicaria* was represented by *T. tomentosum* and *T. resupinatum* in this study, and they all had 2n=16 chromosome numbers. Our results were similar to the data reported by Salimpour *et al.,* (2008), Hesamzadeh and Ziaei Nasab (2006) and sheidaei *et al.,* (1998). In our study, *T. campestre* of the section *Chronosemium* displayed 2n=14 (x=7), while *T. grandiflorum* of the same section displayed 2n=16 (x=8). The same chromosome counts were reported by Salimpour *et al.,* (2008). *T. repens* was the single number studied from section *Lotoidea*, and it contained a 2n=32 chromosome number. This was in agreement with the results of an investigation recorded by

Salimpour *et al.,* (2008), Hesamzadeh and Ziaei Nasab (2006) and sheidaei *et al.,* (1998).

The Duncan's test applied to the chromosome morphometric traits (LA, SA, TL, AR, DRL, TF%, A_1 and A_2) showed a highly significant difference among all examined species belongs to different sections (Table 3). The study revealed cytogenetic differences ($P \geq 1\%$) in ANOVA for karyological date as well as the ratio of long arms too short arms among species. So these results indicated a significant quantitative change in the amount of chromatin in *Trifolium* species diversification (Tables 2 and 4).

In short, the grouping of the *Trifolium* species based on karyotypic data was partly in agreement with either the taxonomic treatment of the genus *Trifolium* or phylogenetic analysis of the same species based on morphological characters. It could be suggested to use the obtained data for further breeding purposes.

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Conflicts of interest

The authors have declared that no competing interests exist.

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