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Chromosome Study of Six Populations of *Trifolium tumens* Stev. ex M. B. in IRAN

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Abstract

Trifolium tumens Stev. ex M. B is a perennial legume with long-lived and deep rooting from the *Vesicaria* section in *Trifolium* genus. It is distributed in Iran. The base chromosome number (x = 8) in the population, confirmed the views of the previous researchers. In this study, the number of chromosomes in the mitotic metaphase stage in the meristematic cells of the root ends were counted in six populations of *Trifolium tumens*. Also, chloroplast count of stomata guard cells, morphological studies, pollen grain size and flowcytometry were studied in six accessions. Our results showed that this species has ploidy level (2n=16, 2n=32). and the tetraploid state in the species *T. tumens* is reported. Through flowcytometry, chloroplast count of stomata guard cells and compare the pollen grain, different characters were showed between diploid and tetraploid populations, too. Based on the results, this new cytotype is reported from Iran.
Keywords: Chromosome, Stomata, Ploidy level, *Trifolium*, Iran.

1. Introduction

The Fabaceae is one of the largest family of flowering plants. It has 727 genera and more than 1932500 species [1]. There are 42 tribes in this family. One of the tribes. *Trifolieae*, has four genus in Iran, and the clover genus, *Trifolium* L., is one of the largest genera of this tribe with about 255 species [2-4]. After Turkey, Iran is one of the richest variety centers of this genus with nearly 49 annual and perennial species in six sections. *Trifolium* has species with different chromosome numbers and ploidy levels [5]. The ancestral chromosome number in *Trifolium* species is considered to be diploid, with 2n = 16 [4] but some species forming aneuploid series (x = 7, 6, 5) and more than nine species have both aneuploid and diploid or polyploid counts [6].

Trifolium tumens Stev ex M. B. is an perennial species with deep roots. It is one of the species in *Vesicaria* section besides six other species. After 2008, it is introduced as one of the commerical cultivars from *Trifolium* genus [7-10]. The large deep roots of *T. tumens* gives it a high degree of tolerance to drought and grazing tolerance.

Hall, *et al.* [8], studied the habitat and plant diversity of this species in Tasmania, Australia and showed that *T. tumens* was found growing alongside a diverse range of companion species in heavily grazed lowland pastures up to lightly grazed alpine meadows [8]. It has been found that this plant shows good growth in dry areas with low rainfall, and for this reason, it has been considered as a valuable fodder plant in dry areas in the last decade [11, 12].

On the other hand, polyploidy, has played a major role in the domestication of several crops such as Trifolium. In the last decades, improved cultivars of economically important species have been developed by inducing polyploidy.

Chromosome counts and ploidy levels in Iranian species of *Trifolium* was studied and showed that some species have ploidy levels [13]. Also, Karyology study of ten *Trifolium* species in Fars Province of Iran were investigated [14]. Their results indicated a significant quantitative change in the amount of chromatin in *Trifolium* species diversification. On the other hand, *Trifolium* species have small chromosomes so determining the ploidy level is

difficult. Ghanavati et al. studied the relationship between the ploidy level and chloroplast number in stomatal guard cells of *Medicago* species and showed that the ploidy level was highly correlated with the number of chloroplasts in stomatal guard cells of lower surface of the leaf [15]. In a similar study, the relationship between ploidy level and the number of stomatal guard cells in diploid and amphidiploid *Brassia* species was studied. Their results confirmed these correlated [16].

The present study revises the chromosome and chloroplast counts in the mitotic metaphase in the six Iranian populations of *Trifolium tumens*.

Also the pollen grains of populations are compared in different ploidy level. Moreover, the efficiency of cytogenetic features in the separation and identification of the cytotypes of this species, is studied too.

2. Material and Methods

2.1. Plant Materials

Six populations of *T. tumens* were gathered from the natural growing habitats of Iran (Table 1). From each population, three repetitions were placed in Petri dishes. After germinated, the seedlings were planted at the greenhouse of the National Iranian Plant Gene Bank in plastic pots. When the seedlings reached the height of about 20 cm, they were used for the chloroplast count.

Table-1. *T. tumens populations* and their habitat

Populations	Locality
T.tumens 1	Tehran: Polur, 1200m, Salimpour, 160
T.tumens 2	Mazendaran: Veresk, 1700m, Salimpour, 167
T.tumens 3	W.Azerbaigan: Mergovar, 1800m, Salimpour, 162
T.tumens 4	W.Azeerbaigan, Pyranshahr, 1500m, Salimpour, 163
T.tumens 5	Golestan: Radkan, 2100m, Salimpour, 151
T. tumens 6	Gilan:Asalem to Khalkal, 1050m, Salimpour 152

2.2. Chloroplast Count

In the early hours of the day, three samples from each population were randomly selected from the young middle leaves and quickly transferred to the lab. The epidermis of the lower surface of the leaf was removed and after preparation, the specimen was observed under a microscope with magnifications of 100x, 400x and 1000x and the number of chloroplasts in 20 pairs of stomata cells were counted under a light and SEM microscope.

2.3. Chromosome Counting in root tips

Chromosome counting was done using 5 plants of each population. After seeds germinated, the rootlets were kept in a pre-treatment solution (alfa-monobromonaphthalene) for three hours at room temperature. Draining off with distilled water. Keeping the roots in Lewitsky fixator solution for 30 hours in the refrigerator. After being washed, hydrolysis in NAOH N at 60 °C for 12-15 minutes. Rising the roots for 3 times with distilled water. Staining with hematoxylin for 18 hours and then draining off. Prepared samples were squashed. At least 10 metaphase states were studied for each population.

2. 4. Flow Cytometry

To release the nuclei and prepare a cellular suspension, 1 cm² of young fresh leaf was placed in a Petri dish containing 1m of cold Lysis buffer (Tris buffer) after being cut up by a scalpel and then crushed. After completely mixing the buffer with plant materials using a pipette, the filtration of the suspension was conducted using 20-30µm filters so as to separate small single-celled or double-celled pieces. After rinsing with Tris's buffer and 10 minutes of centrifugation, Florence PI color was added. Then, the solution was incubated at 37° C for 30 minutes. The DNA contents of the separated nuclei were measured using the flow cytometer and the DNA histograms were created.

3. Results

The number of chromosomes in mitotic metaphase of apical root meristem and chloroplast number in stomata guard cells of six populations were counted according to Table 2.

Table-2. Chromosome number and chloroplast number in populations of *T. tumens*

Populations	Ploid level	Karyotype formula	Chloroplast number (Min-Max)
T.tumens 1	16	2M+4M+2Sm	6-8
T.tumens 2	16	3M+5m	12-13
T.tumens 3	32	10M+3Sm+ 3St	12-13
T.tumens 4	32	12M+2Sm+2St	6-7
T.tumens 5	16	2M+4m+1SM+1St	6-8
T.tumens 6	32	10M+ 4Sm+2St	16-18

This species had three diploid (2n=16) and three tetraploid (2n=32) populations (Table 2, Figure1). Observing tetra ploidy in this species showed new cytotypes of this species for different ecological parts in Iran. For karyotype analysis, lengths of chromosomes were measured (Table 2).

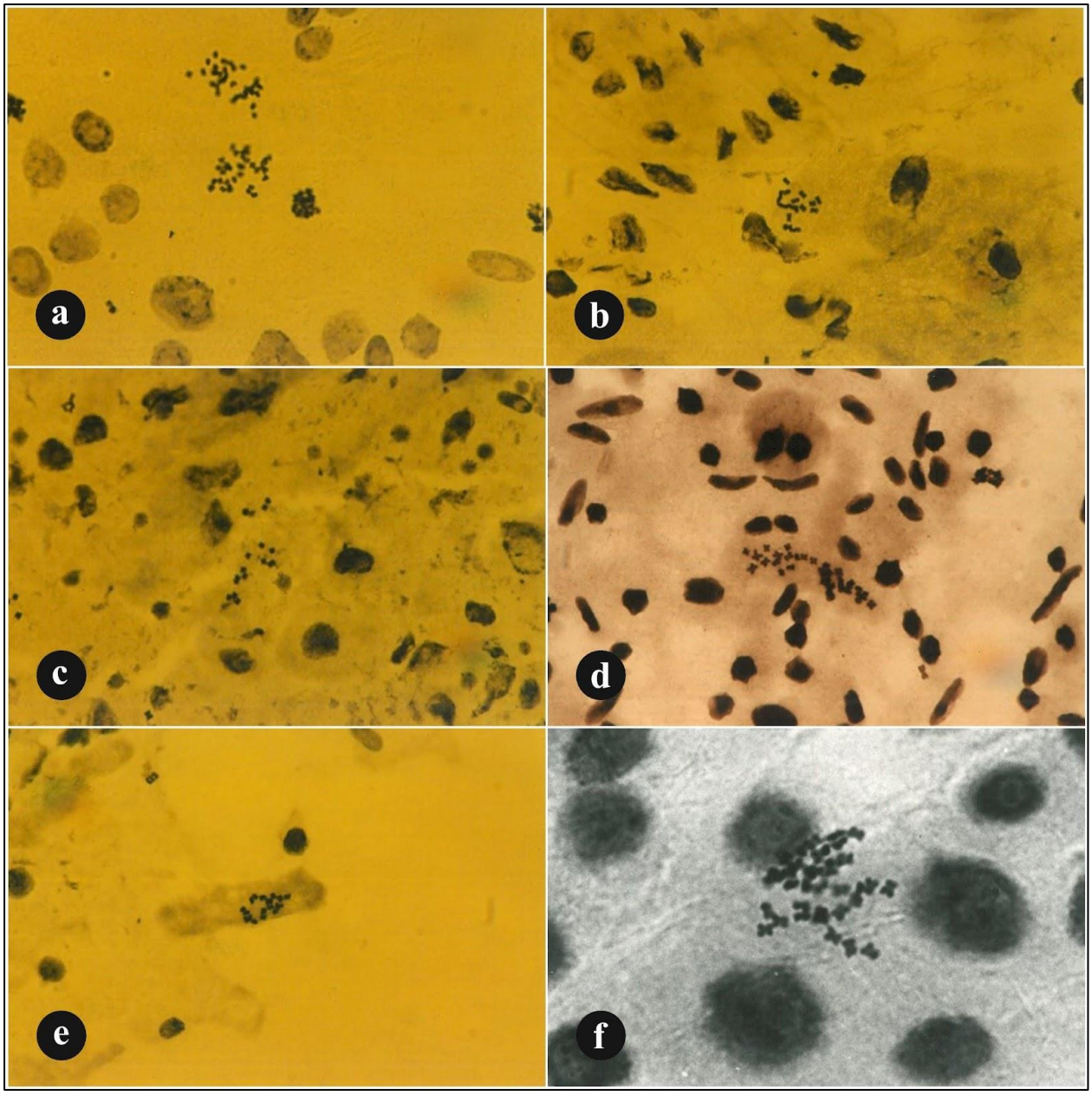

Figure-1. Chromosome numbers in *T. tumens* (diploid b,c,e) and Tetra Ploidy (a,d,f) populations

Tetraploid and diploid populations resulting from the chromosome count studies underwent flow cytometry. Based on the results, the DNA content of the nuclei was determined in the diploid population (mean Gl=28.9), which almost two-fold in the tetraploid population (mean Gi=60.6) (Table 3, Figure 3) only one result is shown. Table 3 demonstrates the interphase of the cellular cycle in 2n and 4n populations of *T.tumens*.

Table-3. Flow cytometry results in two selected populations of *T.tumens*

Populations	Chromosome number	G_1%	Mean G_1	G_2%	Mean G_2	%S	G_1/G_2
T.tumens 1	16	5.17	28/9	6/2	57	43	1/97
T.tumens 3	32	80/3	60/6	1 /2	129/2	18/5	2/134

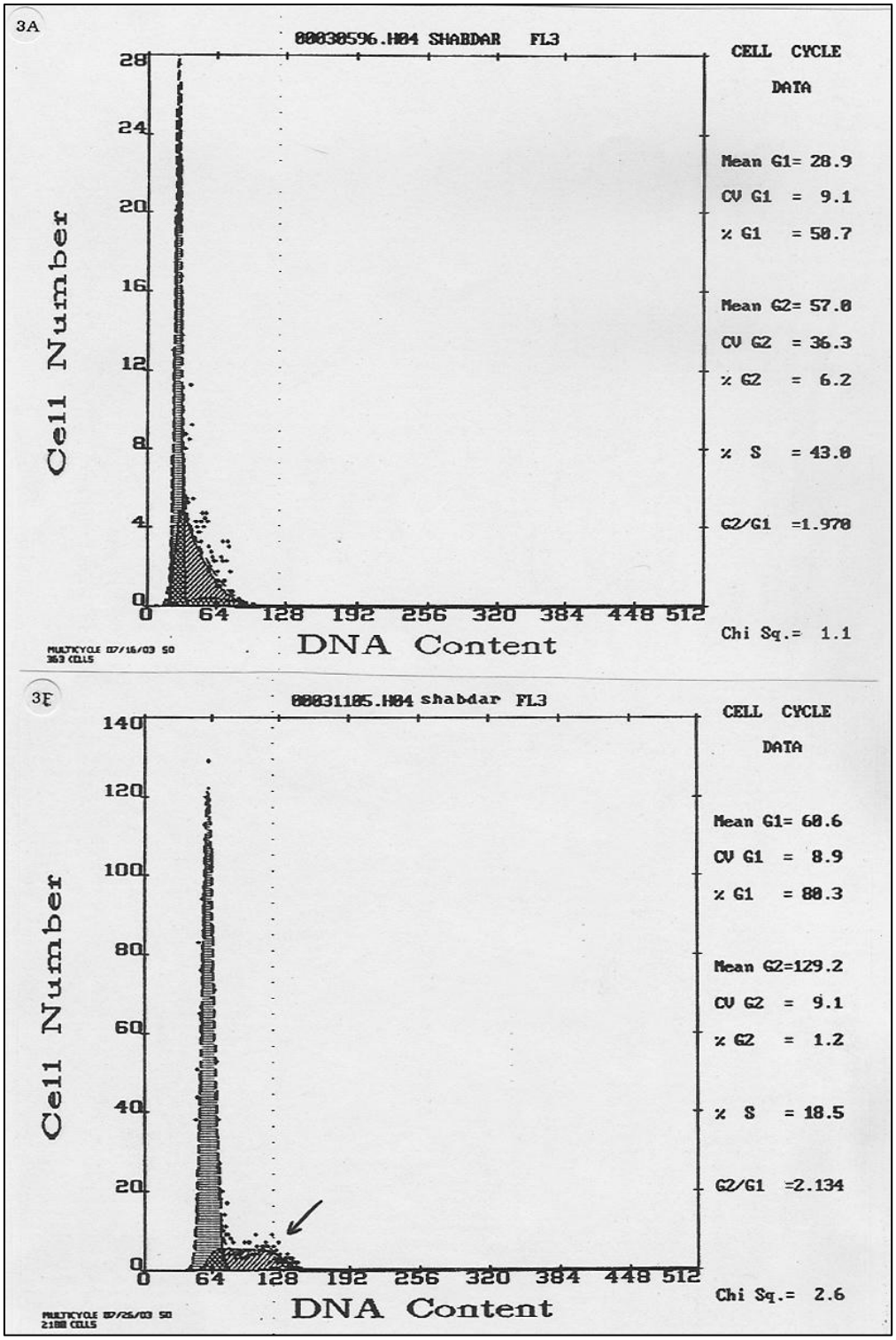

Figure-3. DNA content histogram in diploid and tetraploid cytotypes of *T.tumens*

The chloroplast count of the stomata guard cells also showed that the number of chloroplasts in the diploid populations of this species was 6-7 or 6-8, while in the tetraploid populations of this species, the number of chloroplasts increased to 12-13 or 16-18 with an increase in the ploidy level (Table 4).

Table-4. Chloroplast number, Pollen size in *T.tumens*

Populations	Chloroplast number	Pollen size
T.tumens 1	6-7	28-28/5
T.tumens 3	12-13	32-32/5

As can be seen in the Figure 2, the increase in the size of the pollen grain in tetraploid populations are evident (Figure 2).

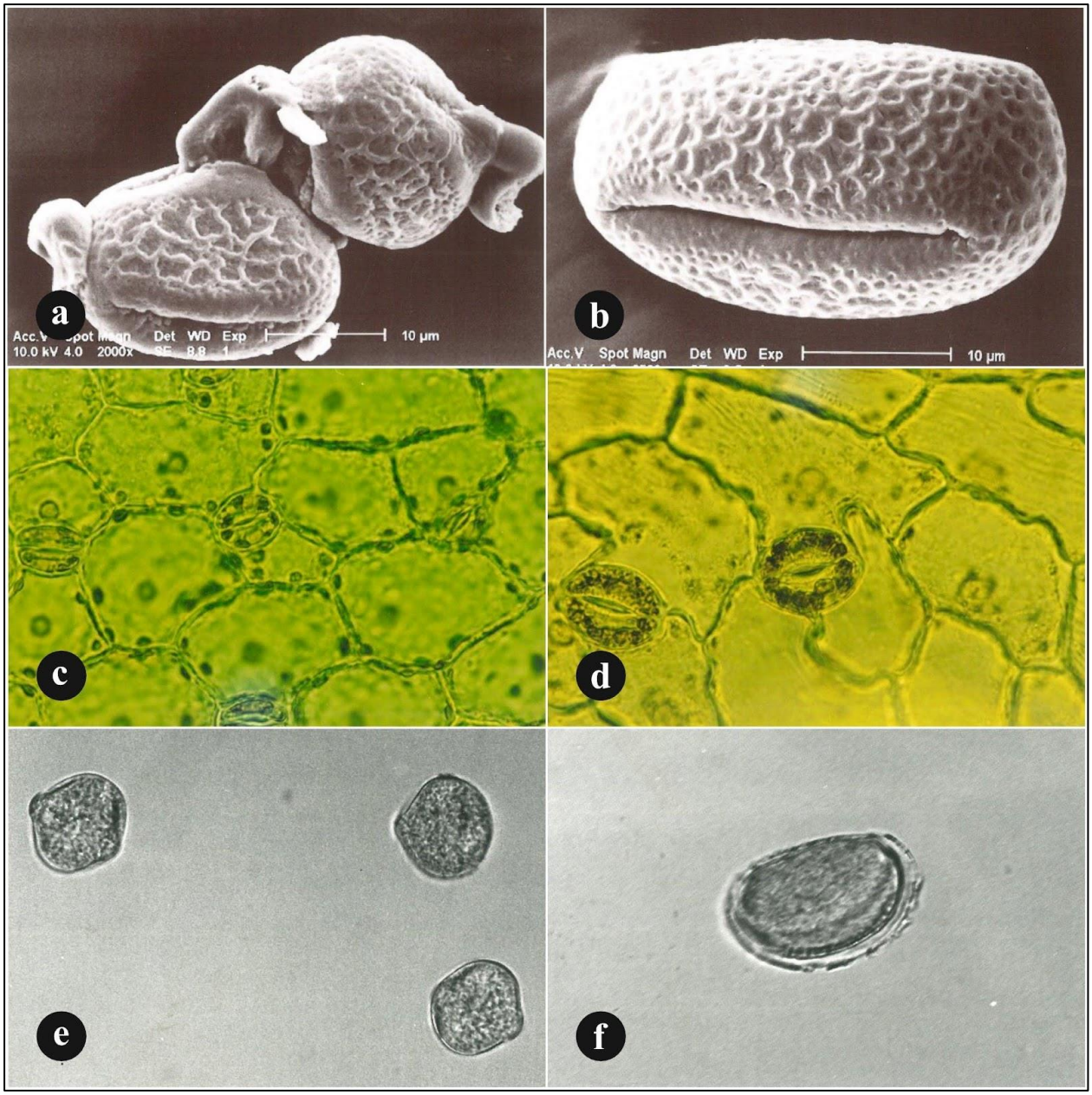

Figure-2. Pollen size (SEM), Chloroplast numbers and Pollen size (LM) in diploid and tetraploid cytotypes of *T. tumens* (diploid a-c-e) and Tetra Ploidy (b,d,f) populations

4. Discussion

The results of this study indicated that the ploidy level would be detected successfully by the four methods mentioned.to distinguish the ploidy level and the tetraploid plants from the diploid, indirect techniques were considered as an alternative to the classical method of chromosome counting. When the plantlets were young, DNA surveys using 1 cm² leaf samples could easily be employed to determine the ploidy level and flow cytometry was adapted for use on *Trifolium* crop specially in *T. tumens*. This study showed that chloroplast scoring methods could be used in *Trifolium* as in Brussel sprout Drore [17] , Sugerbeet Brown, *et al.* [18], *Medicago* Ghanavati, *et al.* [19].

In tetraploid plants, chloroplast number were found to be 12-13 and 16-18, respectively. Which in diploids they were about 6-7. Distinguishing the plants according to the morphological and pollen grain approach was also a useful. Therefore, based on all results of these observations, new cytotypes of *T.tumens* can be reported. Nevertheless, although the existing reports are indicative of the low level of ploidy in the genus *Trifolium*, it seems that in the *Vesicaria* section shows a considerable increase in ploidy level particularly in the species *T. tumens*. Evans believes that the occurrence of polyploidy increases the resistance of these species to a variety of diseases [20, 21]. Therefore, breeding should be conducted on the polyploid varieties. At the same time, Zohary believes that a decrease in the chromosome number shows that these species are in more advanced stages of evolution than the other species [5]. Another noteworthy point is that 75% of the polyploid species have the base chromosome number of x=8. Britten is of the belief that tetraploid species are considered as newer forms [22]. On the other hand, based on the existing sources and the results of the present study, the extreme smallness of the chromosomes is a big obstacle in the way of determining the karyotype in these species. Therefore, using other biosystematics methods, particularly DNA markers, is suggested for distinguished them.

5. Conclusion

Trifolium genus has smallest chromosomes. Determining the chloroplast number in stomata guard cells is a simple way to specify the ploidy level in species of this genus. Also, examining the DNA content by Flow cytometry method can be an effective help to breeders and taxonomists in the study of this genus.

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