CYTOGENETICAL STUDY IN SOME ALFALFA CULTIVARS OF IRAN

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Abstract

A cytogenetical study on 13 alfalfa *(Medicago sativa)* cultivars available in Iranian Forest and Rangelands Genebank was performed in order to obtain basic information on the variability in chiasma frequency/distribution, chromosome association and segregation. Such cytogenetic information is useful for planning hybridization programs. Cluster analysis of meiotic data and ordination of cultivars based on the first two principal component axes (PCA) grouped the genotypes with similar meiotic characteristics. PCA analysis of meiotic data revealed that the mean total chiasma, mean terminal chiasma, ring and rod bivalents are the most variable meiotic characteristics among the genotypes studied. Pollen fertility was studied in all genotypes that showed a significant positive correlation with the frequency of terminal and total chiasma as well as proper segregation of chromosomes during anaphase and telophase stages. B-chromosomes were observed in some of the cultivars affecting the chiasma frequency/distribution as well as chromosome association.

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Introduction

Medicago sativa is one of the most important forage crops in Iran growing in various regions of the country. Southwest Asia and possibly northern Iran are considered to be the place of its origin [18]. Cytogenetical research on alfalfa lagged far behind other crops mainly because: alfalfa chromosomes are very small, the chromosomes are morphologically very similar, cultivated alfalfa has relatively high number of chromosomes (2n = 32), and alfalfa is an autotetraploid [1].

Bolton [2] described difficulties involved in the meiotic study of *Medicago sativa* due to its small chromosomes and chromosome stickiness however, other authors believe that *M sativa* meiosis can be studied successfully [4].

There are also some reports on the occurrence of 2n pollen grains in meiosis of some diploid *Medicago* species. Such a phenomenon not only brings about more genetic variation, but also has been used in transferring useful agronomic characteristics from the diploid species of *M. falcata* to tetraploid cultivars of *M. sativa* [14].

Although there are several cytogenetical reports in *Medicago* species/cultivars from different regions of the world [12, 13, 14, 15 and 23], there are only few karyotypic reports from Iran [21]. The present study

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deals with meiotic analysis of *Medicago sativa* cultivars in order to provide basic information about their meiotic behavior, pollen fertility and occurrence of *2n* pollen if at all present. This information is useful in planning hybridization programs of *Medicago* cultivars.

Materials and Methods

Plant materials

Thirteen *Medicago sativa* cultivars/entries available in the Iranian Forest and Rangelands Genebank were studied (Table 1). Plants were grown according to a completely randomized block design under uniform conditions in the experimental farm of the Genebank located in Karaj during spring 1997.

Cytological methods

Meiotic studies were performed on ten randomly selected plants from each cultivar. Several flower buds were collected randomly from each plant and fixed in glacial acetic acid: 70% ethanol (1:3) for 24 h, then washed with water and preserved in 70% ethanol. The first time produced flowers were used for meiotic studies.

The squash technique was used for cytological preparation using 2% acetocarmine for staining. Pollen stainability as a measure of fertility was checked by staining with 2% acetocarmine: 50% glycerin (1:1) for 1/2 h. Round/completely stained pollen grains were considered fertile and unstained/ shrunken grains as infertile grains [19]. Meiotic characteristics such as chiasma number and distribution (terminal and intercalary) as well as chromosome associations were studied from at least 50 diakinesis/metaphase cells. Data with regard to laggard chromosomes in anaphase-I and II as well as telophase-I and II were obtained from at least 100 cells in each case [18].

Statistical Analysis

For grouping the cultivars with similar meiotic behavior, cluster analysis (single linkage and WARD methods) and an ordination method based on principal component analysis (PCA) were performed. Standardized meiotic characteristics (mean = 0, variance = 1) were used for multivariate statistical analysis [21]. The Euclidean/squared Euclidean distance was used as a measure of similarity for single linkage and WARD cluster analysis. Ordination of the cultivars was performed on the first two principal component (PCA) axes [23].

In order to identify the most variable meiotic characters among the cultivars studied, factor analysis based on PCA was performed. Pearson coefficient of correlation was calculated to determine the relationships between pollen fertility and the meiotic characteristics studied. SPSS version 6.1 (1993) software was used for statistical analysis.

Results and Discussion

Meiosis-I prophase

We could not observe cells with leptotene or zygotene stage in prophase-I. The first meiosis-I substage observed as synezetic knot in which thin chromatin strands surround the nucleolus till covering it totally (Fig. 1. 1,2). Later on, paired chromosomes (now thick strands) unraveled from the knot entering the pachytene stage (Fig. 1. 3,4). End to end attachment of chromosomes in pachytene stage is a feature reported in taxa with synezetic knot [8,9]. Diplotene began normally, but later on, despiralization of chromosomes leads to start the diffuse stage (Fig. 1. 5,6). This stage has been reported in several plant species [24]. There are two types of diffuse stage**,** complete and partial. In complete diffuse all the chromosomes (genome) undergo despiralization. In partial diffuse, only some parts of the genome despiralize. The present study indicates occurrence of partial diffuse in *Medicago.* Various reasons have been suggested for its occurrence in other plants. It has been associated with high synthetic activity, analogous to the lampbrush stage in amphibians [11]; shedding of lateral elements in the synaptonemal complex [8]; post pachytene elimination or modification of histone proteins which permits more progressive condensation of chromosomes throughout later stages of meiosis [3] and as a meiotic arrest and adaptation to withstand adverse environmental conditions [16].

Although the reason for occurrence of diffuse stage in *Medicago* species is not known, Owens and Molder [16] explanation might be considered. *Medicago* species grow throughout the rangelands of Iran and face different environmental conditions; hence a diffuse stage may be a genomic adaptation.

Chromosome paring and chiasma distribution

Data on chiasma number and distribution as well as on chromosome associations (univalents & bivalents) are presented in Table 1. Representative meiotic cells of the studied cultivars are presented in Figures 2 and 3. All the cultivars possessed n = 2x = 16, hence they are ancestral tetraploid in which diplodization has occurred throughout generations. The highest value of total and terminal chiasma occurred in cultivars/entry number 522057 (31.870 and 31.340, respectively) and the lowest in entry number 0312 (26.08 and 22.460) and 2755 (26.860 and 22.290). Intercalary chiasma value ranged from 0.380 in entry number 2585 to 4.570 in entry number 2755.

In cultivars 22057 and 2585 the highest value of total and terminal chiasmata as well as the lowest value for intercalary chiasmata occurred. Therefore the genetic rearrangements mainly occur among the genes present at the end of chromosome arms. The reverse situation is

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Table 1. Meiotic characters and pollen fertility in *Medicago sativa* cultivars in Iran. All cultivars possess 2n = 2x = 16

	Cultivar												
	20364 KH	2585 US	22057 KH	2198 USS	2564 USS	2755 AUS	20312 K	2122 TU	20246 T	2199 USS	2568 F	20321 KO	2421 AUS
TX	25.540	30.940	31.340	27.540	27.080	22.290	22.460	28.670	30.430	28.000	27.850	30.780	26.500
IX	3.000	0.380	0.530	1.850	1.080	4.570	3.620	1.750	0.780	1.810	1.690	0.610	2.500
TO	28.540	31.320	31.870	29.390	28.160	26.860	26.080	30.420	31.210	29.810	29.540	31.390	29.000
RII	13.670	15.680	15.430	14.080	14.000	10.600	12.780	15.000	15.430	14.650	14.300	15.660	14.300
DII	1.390	0.130	0.570	1.460	1.300	4.500	2.340	1.000	0.570	0.910	1.240	0.240	1.430
I	1.240	0.380	0.000	0.920	1.400	0.900	1.720	0.000	0.000	0.880	0.920	0.000	0.800
L1	18.200	3.200	26.000	10.300	14.300	7.700	80.100	10.000	17.400	70.600	3.100	23.300	49.600
L2	7.700	0.000	12.000	8.700	26.100	7.400	65.400	4.300	12.000	68.000	6.300	18.500	26.400
L3	0.000	0.000	11.700	5.000	8.000	0.000	0.000	0.000	0.000	33.000	3.000	8.000	3.000
L4	5.000	0.000	3.700	0.000	0.000	0.000	30.100	3.700	0.000	20.000	0.000	0.000	7.200
N1	0.273	0.032	0.348	0.138	0.238	0.078	0.700	0.150	0.348	1.060	0.032	0.300	0.666
N2	0.120	0.000	0.120	0.087	0.522	0.074	0.910	0.043	0.292	1.950	0.094	0.296	0.315
N3	0.000	0.000	0.160	0.050	0.080	0.000	0.000	0.000	0.000	0.330	0.030	0.080	0.028
N4	0.050	0.000	0.037	0.000	0.000	0.000	0.849	0.069	0.000	0.433	0.000	0.000	0.500
XB	1.856	1.978	2.033	1.937	1.926	1.719	1.800	1.901	1.950	2.029	1.918	1.962	1.842
PF	98.760	99.700	99.010	97.330	98.250	94.130	92.110	97.230	99.030	94.770	98.250	98.880	94.430

Abbreviations: KH = Khozestan , US = USA, USS = Former USSR, AUS = Australia, K = Karaj, TU = Turkey, T = Tabriz, F = FAO, KO = Kordestan, TX = Terminal chiasma, IX = Intercalary chiasma, TO = Total chiasma, RII = Ring bivalent, DII = Rod bivalent, I = Univalent, L1-L4 = Percentage of the cells having laggard chromosomes in anaphase-I, II and telophase-I and II respectively, N1-N4 = Number of laggard chromosomes in anaphase-I, II and telophase-I and II respectively. XB = Mean chiasma/bivalent, PF = Pollen fertility (values other than % are the means)

present in 2755 and 20312 possessing a low value for total and terminal chiasma but the highest value for intercalary chiasma; therefore genes present in the internal regions of chromosome arms are mainly affected by crossover. The other cultivars show intermediate values.

Data on chromosome associations and anaphase/ telophase segregation are presented in Table 1. Although the studied cultivars are autotetraploid and are expected to form quadrivalents, they formed bivalents and univalents showing diplontic behavior, a known behavior in alfalfa [22].

The highest number of ring bivalents occurred in cultivar 2585 and 20321, while the lowest occurred in 2755. The highest value of rod bivalents occurred in 2755 and the lowest in 2585.

Cultivars 20312 and 2199 possessed the highest value for anaphase-1 and II as well as telophase-I and II laggards while the lowest value occurred in 2585 and 2568.

Heritable adjustment in the frequency and distribution of chiasmata and recombination as well as their effects on the variability of progenies and populations is established in both experimental and natural populations [17]. Differences observed in chiasma frequency and distribution as well as bivalents/ quadrivalents among the studied cultivars may reflect partly their genomic differences as these plants were grown under uniform conditions in the experimental field.

Pearson coefficients of correlation determined among meiotic characteristics and pollen fertility are presented in Table 2. Pollen fertility showed significant positive correlations with total chiasma (r = 0.78), terminal chiasma (r = 0.75) as well as ring bivalents (r = 0.67) and significant negative correlations with intercalary chiasma (r = -0.77), rod bivalents (r = -0.65) and univalents (r = -0.15).

Investigations of autotetraploid rye and *Lolium* varieties selected for improvement in fertility showed that an increase in the number of quadrivalents as a direct result of increase in chiasma frequency as well as

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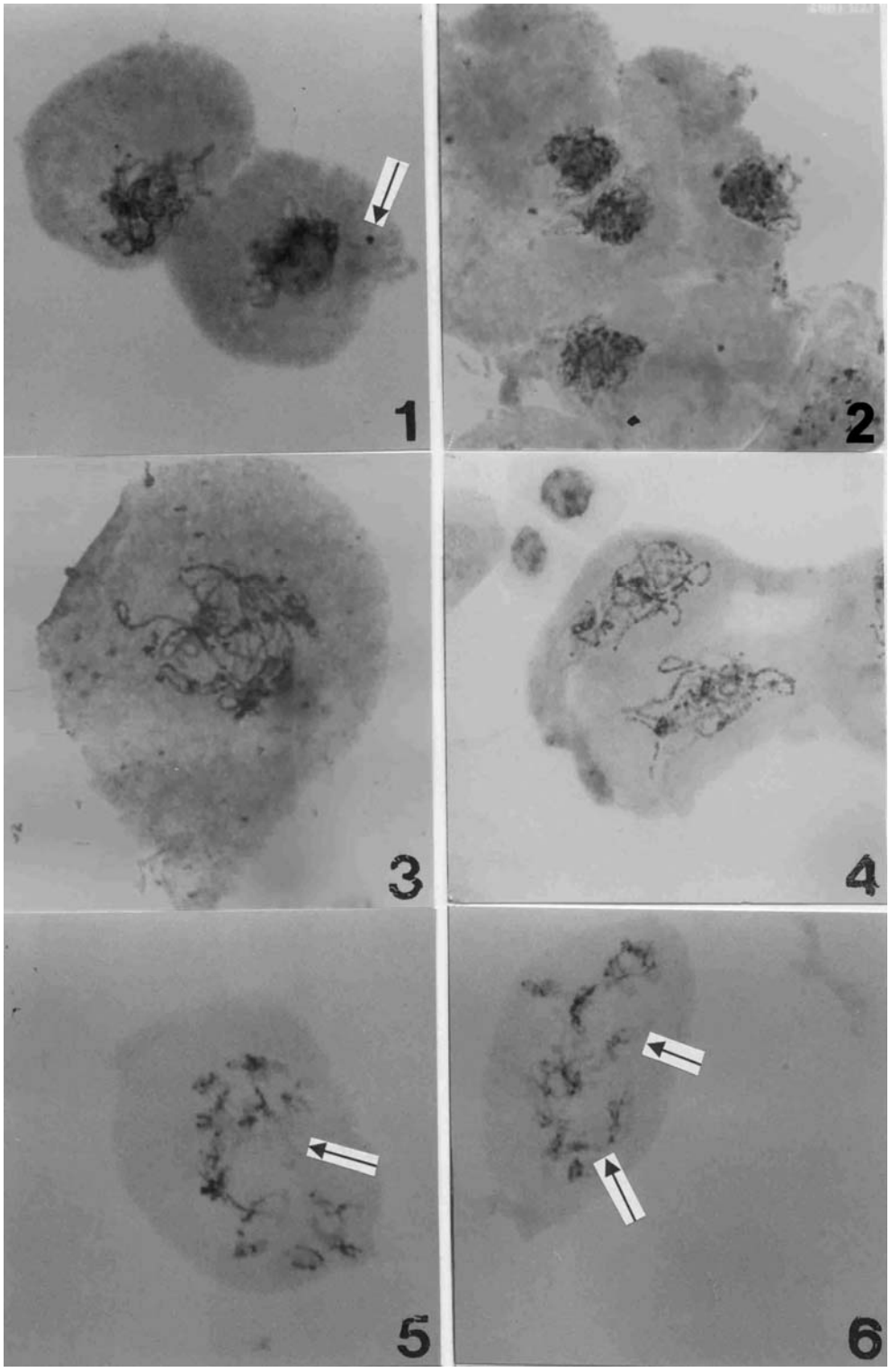

Figure 1. (1) Prophase-I cell in 20312 showing early synezetic knot stage (thin chromatin strands surround the nucleolus, arrow indicates the B-chromosome); (2) Synezetic knot stage in 2755; (3) Unraveling of the knot in 2755, paired chromosomes come out of the knot; (4) Pachytene stage in 2564, end to end attachment of chromosome is evident; (5 & 6) Diffuse stage in 20312 and 2421 respectively, arrows indicate desparalized regions of the genome (less stained chromatin regions).

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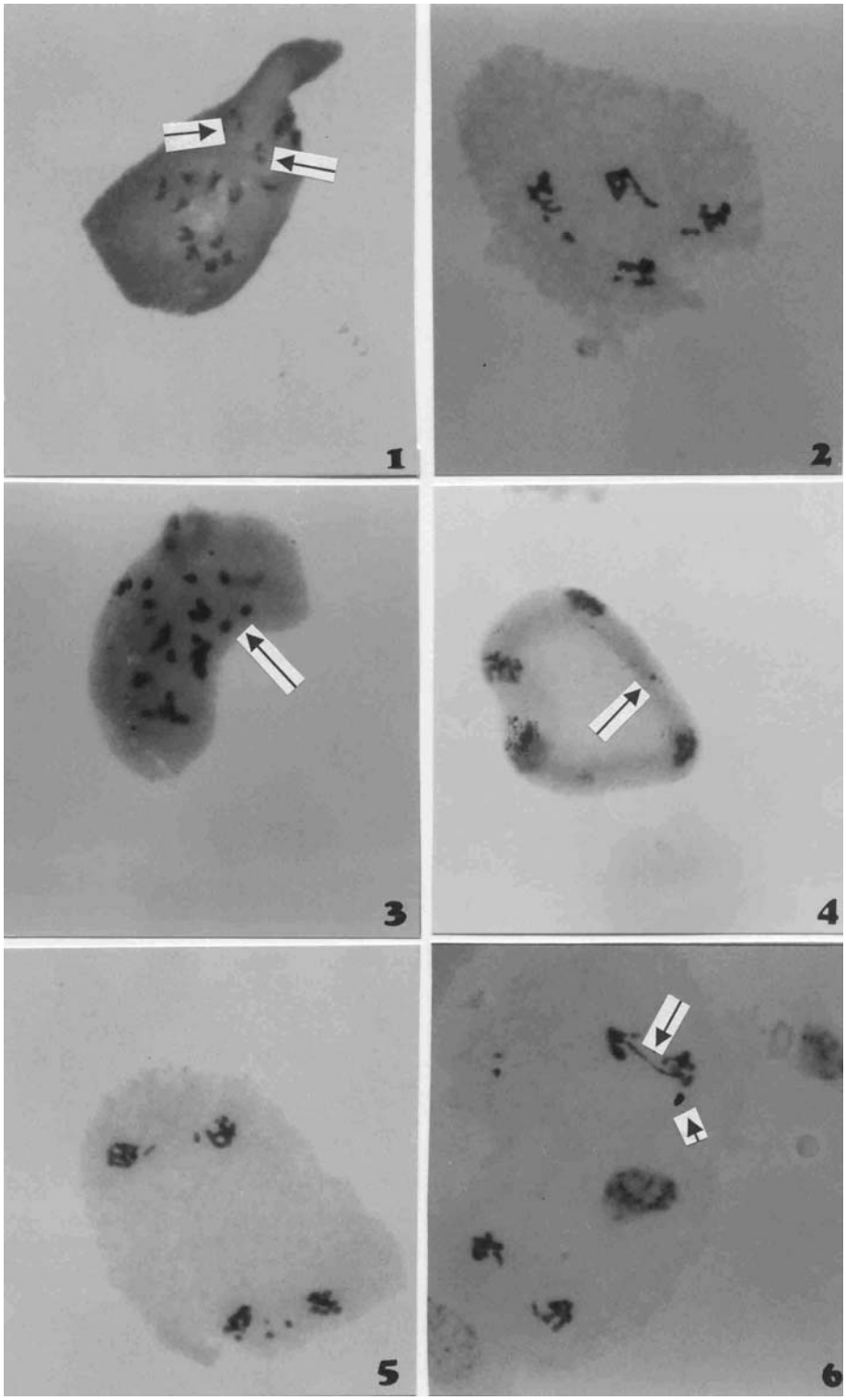

Figure 2. (1) Metaphase cell in 20321, arrows indicate univalents; (2) Metaphase cell in 20312, showing chromosome stickiness; (3) Metaphase cell in 20311, arrow indicates univalents; (4) Anaphase-II in 2564, arrow indicates laggard chromosome; (5) Anaphase-II in 20321, showing laggard chromosomes; (6) Anaphase-I in 20312, arrow (inversion bridge), arrow head (acentric fragment), two B-chromosomes are on the other side of the cell.

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Table 2. Correlation among pollen fertility and meiotic characterticsis. Abbreviations as in Table 1. R >0.60 = significant at 0.05

	TX	IX	TO	RII	DII	IV	I	L1	L2	L3	L4	N1	N2	N3	N4	XB	PF
TX	1.00																
IX	-.96	1.00															
TO	.98	-.87	1.00														
RII	.93	-.92	.89	1.00													
DII	-.87	.89	-.81	-.98	1.00												
IV	-.47	.60	-.35	-.56	.50	1.00											
I	-.80	.62	-.88	-.64	.51	.16	1.00										
L1	-.33	.29	-.35	-.11	.04	-.12	.38	1.00									
L2	-.35	.25	-.41	-.15	.09	-.22	.49	.94	1.00								
L3	.22	-.23	.20	.22	-.23	-.26	-.01	.47	.59	1.00							
L4	-.46	.40	-.47	-.22	.16	-.17	.50	.93	.90	.33	1.00						
N1	-.16	.14	-.16	.04	-.09	-.12	.24	.94	.90	.66	.79	1.00					
N2	-.16	.09	-.20	-.02	-.02	-.25	.33	.80	.92	.81	.74	.88	1.00				
N3	.26	-.26	.25	.25	-.25	-.27	-.05	.45	.56	.99	.32	.64	.76	1.00			
N4	-.49	.43	-.51	-.23	.16	-.12	.50	.93	.84	.19	.93	.78	.62	.16	1.00		
XB	.87	-.89	.82	.86	-.84	-.62	-.50	-.04	.02	.59	-.14	.15	.24	.62	-.27	1.00	
PF	.78	-.77	.75	.67	-.65	-.15	-.55	-.72	-.69	-.12	-.74	-.58	-.50	-.09	-.82	.60	1.00

bivalent formation against trivalent and univalents was effective [17]. It may be suggested that selection practices with the aim to increase chiasma frequency/ring bivalents and reducing intercalary chiasma/univalents may improve fertility in *Medicago* cultivars studied.

Infertility in polyploids is not solely due to the production of aneuploid gametes formed by improper segregation of chromosomes during anaphase/telophase stages; genic factors may bring about infertility as evidenced in different tetraploid strains of rye [5]. Although these strains displayed similar pattern of chromosome pairing and segregation producing much the same proportion of aneuploid gametes, they varied considerably in seed set.

The studied *Medicago* cultivars showed significant negative correlation between pollen fertility and laggard chromosomes (Table 2). Moreover telophase-II laggards showed significant positive correlation with univalents (anaphase-I, II and telophase-I showed positive but not significant correlation); hence cultivars with higher chiasma frequency and ring bivalents possess proper chromosome segregation as well as high pollen fertility. Therefore we may suggest that pollen infertility in studied *Medicago* cultivars may be related to chromosomal factors.

Cluster analysis of meiotic characteristics using single linkage and WARD methods produced the same results (Fig. 4). In general five main clusters are formed at 4-linkage distance. The first cluster is made up of the cultivars 2198, 20364, 2568, 2564, and 2421.

The second main cluster is comprised of the cultivars 20246, 20321, 2585, 2122 and 2057. Cultivars 2755, 20312 and 2199 stand in separate clusters. Ordination of the cultivars based on the first two PCA axes supported the clustering results (Fig. 5).

Cultivars 2755, 20312 and 2564 and 2421 possessed B-chromosomes (see bellow). These cultivars stand separate from the other cultivars in cluster analysis; therefore presence of B- chromosomes may have played a major role in causing variations in the meiosis of these cultivars. It is also interesting to mention that cluster analysis of karyotypic features of these cultivars produced the same results [21] i.e. cultivars 2755, 20312 and 2564 stand in separate clusters while the members of the other two clusters remain the same. Therefore meiotic as well as karyotypic differences observed in the studied cultivars may partly indicate their genomic differences and if combined with other morphological, agronomic, etc. characteristics may be used in planning hybridization programs. However with the present findings of clustering and ordination (Figs. 4 and 5), we may tentatively suggest hybridization plan excluding cultivars 2755, 20312 and 2199. These cultivars possess the lowest value of pollen fertility (Table 1) and also due to differences in their meiotic characteristics stand in separate clusters far from the other cultivars.

PCA of meiotic data showed that the first three components comprise about 93 % of the total variance, hence those characteristics having high correlation with these components are the most variable meiotic characteristics among the studied genotypes. The mean of terminal chiasma, total chiasma, ring bivalents and

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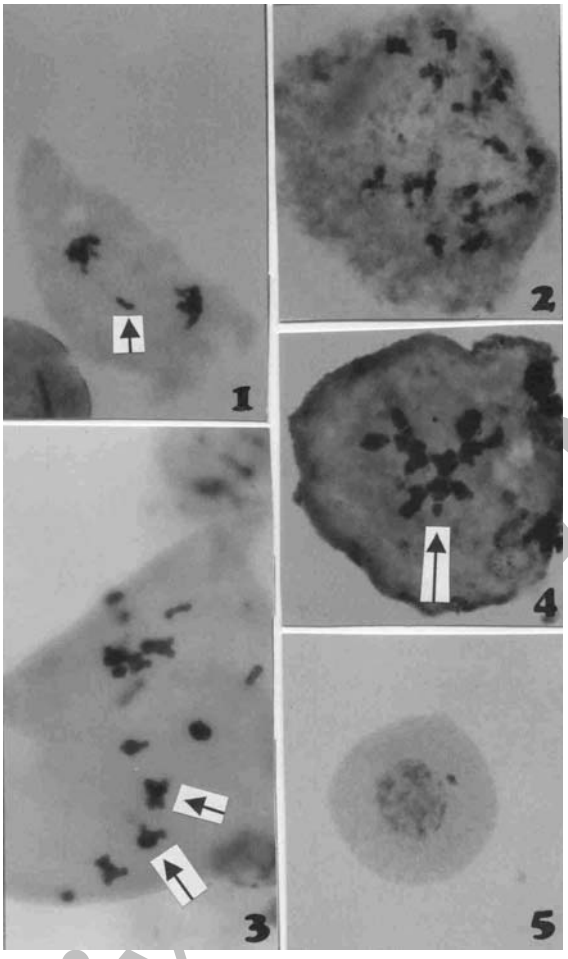

Figure 3. (1) Anaphase-I laggard (arrow) in 20312; (2) Metaphase cell in 2564; (3) Metaphase cell in 20312, showing bivalents with intercalary chiasma (arrows); (4) Metaphase cell in 2755, arrow indicates B- chromosome; (5) Microspore with micronuclei in 20312.

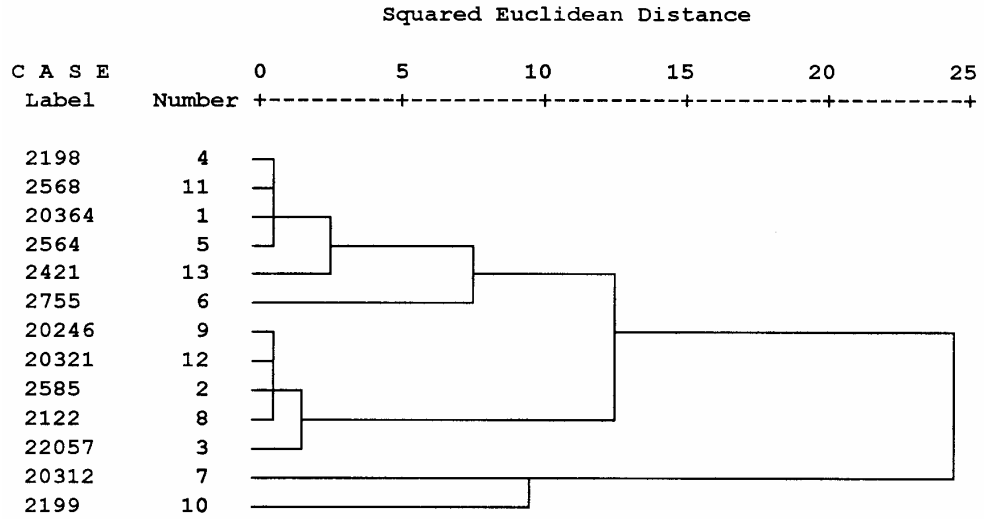

Figure 4. WARD cluster analysis of meiotic data of the Table 1.

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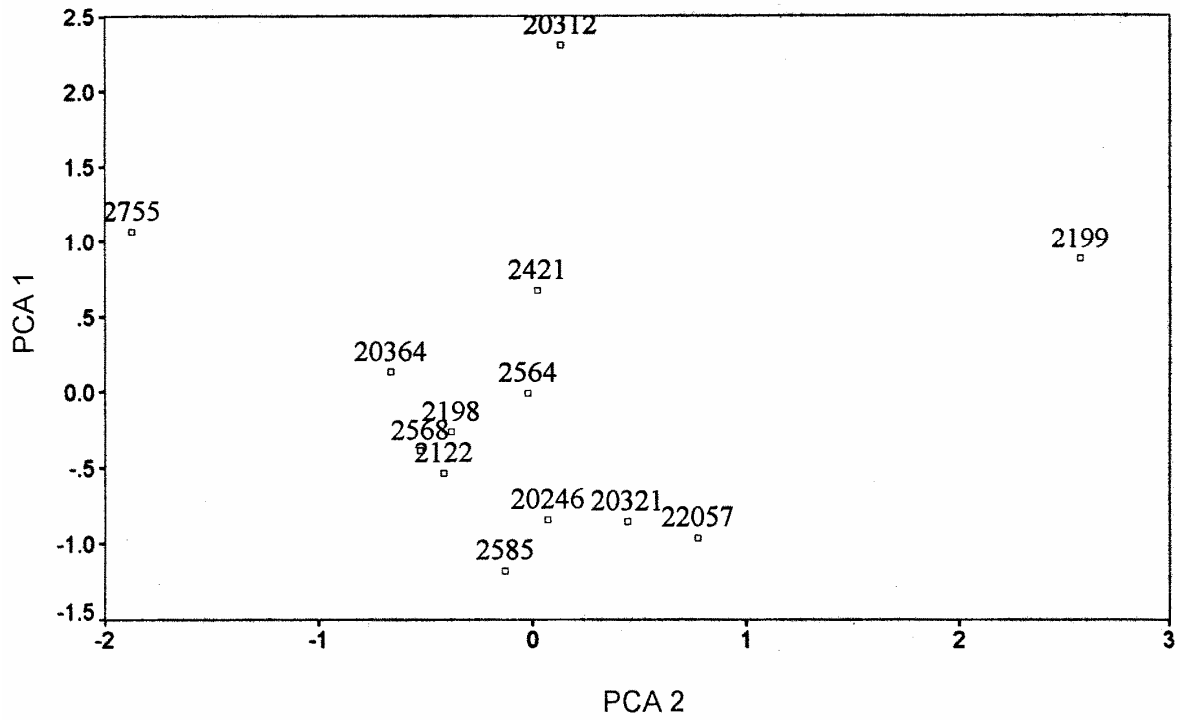

Figure 5. Ordination of the cultivars on the first two PCA axes. Numbers representing cultivars as in Figure 4.

chiasma/bivalent possessed high positive correlation (> 0.85) with the first component while intercalary chiasma and rod bivalents possessed negative correlation (> -090). In the second component, laggard chromosomes in anaphase-I, II and telophase-II and in the third component, laggard chromosomes in telophase-I are important characteristics.

Meiotic analysis of the studied cultivars did not show any metaphase cell possessing double (2n) chromosome number, or any restitution nucleus to be formed at the end of telophase. Therefore the cultivars studied do not form 2n pollens.

B-chromosomes

B-chromosomes (Bs) are accessory chromosomes found in some species, varying in number in different cells of a tissue, different tissues of an individual and different individuals of a population. When present in high numbers, they affect negatively the growth and vigor of the plants [10], while in low numbers may benefit the plant possessing them [6]. Bs affect the frequency and distribution of chiasma as well as chromosome association [20,26]. It has been suggested that B-chromosomes affect meiotic behavior either directly or by affecting the genes controlling meiosis present on the A-chromosomes [25].

The occurrence of Bs in somatic cells of roots in *Medicago sativa* ssp. *falcata* has been reported recently by Hossain & Bucham [7], however the plants grown by them did not survive so the meiotic behavior of B-chromosomes could not be worked out. Therefore the present study is the first report on meiotic behavior of B-chromosomes and their effect on chiasma frequency/distribution in *Medicago.*

Bs were observed in cultivars 20312, 2755, 2421 and 2564. Their number varied from 0-2 in different cells. Bs did not form any association with A-chromosomes and could move independently to the anaphase poles. However in some of cells they were seen as laggards too. This may work as a limiting factor against B-chromosomes accumulation, since large number of Bs may have negative effects on the plants bearing them.

In order to determine the effect of B-chromosomes, chiasma frequency and distribution as well as chromosomes association was compared among the cells having Bs and those devoid of Bs in cultivars 20312, 2755, 2421 and 2564. Fifty metaphase/ diakinesis cells were analyzed for each case. T-test analysis (Table 3) revealed significant reduction in the terminal and total chiasma as well as ring bivalents in the cells possessing Bs in the studied cultivars, while the mean intercalary chiasma increased significantly in cultivar 2421. Change in chiasma distribution may bring about more variability in the progenies. This is the first report on the effect of B-chromosomes in *Medicago sativa* cultivars.

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Table 3. T-test analysis of meiotic characters in cells possessing Bs and devoid of Bs (abbreviations as in Table 1)

Cultivar	TX	IX	TO	RII	DII
20312	25.33-19.67	3.17-4.00	28.50-23.50	13.83-10.17	2.00-2.67
P	0.05	0.25	0.05	0.02	0.08
2564	30.86-22.40	1.13-2.40	31.93-24.80	15.43-11.60	2.18-1.22
P	0.01	0.40	0.01	0.05	0.63
2421	22.92-2125	1.33-4.50	30.56-25.75	15.00-13.00	1.11-2.25
P	0.01	0.05	0.01	0.05	0.43
2755	25.50-17.20	3.00-6.60	28.50-26.80	12.75-8.60	3.00-6.40
P	0.11	0.07	0.05	0.06	0.19

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