TÜBİTAK

Turkish Journal of Botany

http://journals.tubitak.gov.tr/botany/

Research Article

Turk J Bot
(2022) 46: 50-61

doi:10.3906/bot-2105-22

Karyomorphology, ploidy analysis, and flow cytometric genome size estimation of *Medicago monantha* populations

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Received: 17.05.2021 • **Accepted/Published Online:** 20.10.2021 • **Final Version:** 31.01.2022

Abstract: Karayomorphological analysis and monoploid genome size (2Cx DNA) of 12 Iranian *Medicago monantha* populations are reported. Flow cytometric analysis were conducted on freshly collected seeds, using *Solanum lycopersicum* cv. Stupicke (2C DNA = 1.96 pg), as the internal reference standard. All populations were diploid with variable degrees of mixoploidy and two different chromosome numbers within each population. Totally, six chromosome numbers of 22, 28, 30, 32, 36, and 40 were identified. Two-typed chromosomes ("m", "sm") with a mean length of 1.65 µm (0.98-2.99 µm) formed seven different karyotype formulas. Karyotypes were mostly symmetrical, implying the evolutionary effect of natural and unnatural selection. The UPGMA phenogram categorized the populations into four major clusters. The mean 2Cx DNA value of all assessed populations was 4.10 pg (2.88–4.56 pg). Using the matrix of karyotype similarities, cluster analysis was carried out and the dendrogram classified the populations into four major clusters. Our results can be employed in the taxonomic and phylogenetic consideration of the genus and toward improved breeding programs.

Key words: *Medicago monantha*, mixoploidy, chromosome, 2C DNA, genome size

1. Introduction

Medicago monantha (C.A.Mey.) Trautv. (Fabaceae) is an aromatic herbaceous plant widely cultivated in Mediterranean countries and Asia (Ahmadiani et al., 2001). In Iranian traditional medicine, the seeds of *M. monantha* and its hardly distinguishable relative, *Trigonella foenum-graecum* L., were used as tonic and blood sugar lowering medicine (Hajimehdipoor et al., 2010). Other therapeutic effects like anti-cancer (Kamran et al., 2018), aphrodisiac (Ullah et al., 2014), antibacterial (Sharma et al., 1990), and anti-inflammatory effects (Ahmadiani et al., 2004) have also been reported for *M. monantha*. Different parts of the plant are reported to contain several pharmaceutically important constituents such as alkaloids, proteins, flavonoids as well as saponins. Furthermore, trigonelline has been reported as a major active component of *M. monantha* extract with considerable antiseptic, antimigraine, antitumor, and osmoregulator properties (Barnes et al., 2002).

Current breeding strategies have not been able to generate improved *Medicago* populations with favorable characteristics. This may eventually result in reduced rates of diversification and even a crippled evolutionary path, due to the many adverse effects of the modern world. Thus, there is an urgent demand for identifying unknown species and their cytological characteristics. Karyotypic studies on the evolutionary relationships among populations, which originate from different habitats, and establishing novel breeding strategies based on the obtained genotypic data may lead to improved productivity, variability, and survival aptitude of the species. Mixoploidy, simply defined as coexistence of cells with different chromosome numbers in a single tissue, is known as the common cytogenetic feature in plants. Mixoploid plants could be found naturally in many plant populations. It was suggested that the natural mixoploidy is more prevalent in species with small chromosome numbers (Kunakh et al., 2008). It is believed to associate with a promoted ability of resistance against environmental stresses and to adopt extreme conditions during plant growth and development. Meanwhile, though mixoploidy is seen mainly in somatic tissues, it may be associated with an increased rate of gametic cells with varied chromosome numbers, potentially leading to elevated incidence of natural polyploid events (Maletskii, 2005). Additionally, the natural and artificially induced mixoploid events are

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regarded as valuable sources of genetic material in ploidy breeding programs and pharmaceutical applications, particularly in plant species that are expected to produce high mixoploidy rates during polyploidy induction works (Tavan et al., 2015; Javadian et al., 2017; Tarkesh Esfahani et al., 2020). In the current study, we studied karyomorphological traits and the range of variation in some important karyomorphological parameters and 2Cx DNA values in 12 Iranian *M. monantha* populations. Also, the role of these parameters in species diversification was investigated.

2. Materials and methods

Seeds of 12 Iranian native populations of *Medicago monantha* were obtained from the germplasm collection of the Iranian Biological Resource Center (IBRC), Tehran, Iran. The gene bank codes as well as the geographical coordinates (latitude, longitude, and altitude) and climatic specifications (mean annual temperature and rainfall) of the collection site for studied populations are listed in Table 1. Since the seed dormancy is known to cause a very low germination capacity in wild *Medicago*, in the current study, different treatments and procedures such as soaking with running water, chilling at low temperature, embryo culture as well as gibberellic acid (GA_3) treatment (Geneve, 1998) were examined to break the seeds dormancy. Briefly, after scarification of the seeds with blades, we used 20 µL of GA_3 (1000 ppm) on each seed. Scarified seeds were placed in petri dishes, containing GA_3 and incubated at 24 °C for a 16/8 h (light/dark) photoperiod. Germination occurred after four to seven days.

2.1. Karyomorphological analysis

For the karyomorphological preparations, actively growing root tips (1 cm length) were selected and placed in 0.2% (w/v) colchicine at 4 °C for 4 h in darkness to induce metaphase arrest. Then, the root samples were washed with dsH_2O at room temperature (RT; 22 °C) for 3 times, 5 min each followed by fixing in fresh Carnoy's fixative solution (absolute ethanol:glacial acetic acid; 3:1 (v/v)) at 4 °C for 24 h. The samples were kept in 70% (v/v) aqueous ethanol solution at 4 °C. Then, the fixed roots were washed in dsH_2O, followed by hydrolyzing in 1M HC1 at 60 °C for 14 min in a water bath, and staining in 2% (w/v) aceto-orcein at RT for 8 h. Five well-spread monolayer metaphase plates from different individuals were examined per population. High-resolution microscopic digital photographs (Super High Quality; SHQ; Tiff format images) were acquired, using an Olympus BX50 (Olympus Optical Co., Ltd., Tokyo, Japan) microscope equipped with an Olympus DP12 digital camera. Nine chromosomal parameters, including long arm (L) and short arm (S) length, chromosome length (CL = L + S), arm ratio (AR = L/S), r-value (S/L), form percentage (F% = S/ΣCL), total chromosome volume (TCV = πr^2 CL, where r = average chromosome radius), relative length of chromosome (RL = L/ΣCL), and centromeric index (CI = S/CL) were measured. Idiograms were drawn from mean values, and chromosome types were determined using Levan et al. (1964) formula. For karyotypic analysis, five parameters, including karyotype total form percentage (TF% = ΣS/ΣCL × 100), coefficient of variation of total chromosome length (CV_{CL}% = (total CL standard deviation/total CL mean) × 100) (Paszko, 2006), mean centromeric asymmetry (M_{CA} = A × 100) (Paszko, 2006; Peruzzi and Eroğlu, 2013), where the degree of karyotype asymmetry (A = [S(L-S/L+S(/n] × 100(, Stebbins (1971)

Table 1. Locality characteristics of Iranian native *Medicago monantha* populations.

Population code	Local collection locations	Latitude (N)	Longitude (E)	Altitude (m)	Mean Temp (°C)	Mean rainfall (mm)
P1	Hamedan, Iran	34° 57' 11.0"	48° 08' 15.2"	2087	13.69	31.83
P2	Hamedan, Iran	34° 41' 46.9"	48° 13' 57.8"	1696	12.38	23.52
P3	Hamedan, Iran	34° 41' 46.9"	48° 13' 57.8"	1696	12.38	23.52
P4	Hamedan, Iran	34° 58' 25.1"	48° 10' 26.9"	1956	13.44	29.54
P5	Hamedan, Iran	34° 48' 15.5"	47° 54' 55.7"	1505	13.16	24.32
P6	Kermanshah, Iran	34° 27' 52.8"	47° 24' 49.0"	1347	16.54	23.08
P7	Kermanshah, Iran	34° 28' 38.5"	47° 09' 25.8"	2007	15.42	29.08
P8	Kermanshah, Iran	34° 26' 23.6"	47° 26' 30.4"	1347	18.44	28.32
P9	Tehran, Iran	35° 48' 35.9	51° 35' 24.3	1735	17.74	15.08
P10	Tehran, Iran	35° 56' 06.4"	51° 37' 44.3"	1722	20.54	17.08
P11	Karaj, Iran	35° 54' 20.1"	50° 53' 11.0"	1474	16.56	13.45
P12	Karaj, Iran	35° 53' 26.1"	50° 53' 12.0"	1474	14.66	16.18

asymmetry categories (ST), and Romero-Zarco (1986) indices, which are intrachromosomal asymmetry index (A1) and interchromosomal asymmetry index (A2), were measured.

2.2. Flow cytometric analysis

The monoploid genome size (2Cx DNA) of each *Medicago monantha* population was estimated, using flow cytometry technique and propidium iodide (PI) staining method. Flow cytometry showed variation in chromosome number (mixoploidy). To prepare nuclear suspensions, 8–10 seeds (Sliwinska et al., 2005; Sliwinska, 2006; Jedrzejczyk and Sliwinska, 2010) of each *M. monantha* sample along with the healthy fresh young leaves of *Solanum lycopersicum* cv. Stupicke (2C DNA = 1.96 pg; Doležel et al., 2007) as the internal reference standard plant were chopped with a sharp razor blade in ice-cold woody plant buffer (WPB, Loureiro et al., 2007). The suspension was filtered through a 30 µm Partec (Partec, Münster, Germany) green nylon mesh. Then, a mixture of 1 mL staining buffer, 50 µg/mL Propidium Iodide (PI, Fluka) solution, and 50 µg/mL RNase (Sigma-Aldrich Corporation, MO, USA) stock solution was added to each sample. Flow cytometry was performed by BD FACSCanto ll flow cytometer (BD Biosciences, Bedford, MA, USA), and BD FACSDiva™ Software. Data were then transferred to Flowing Software version 2.5.0 (Cell Imaging Core, Turku Centre for Biotechnology) to make them editable in Partec FloMax ver. 2.4e software (Partec, Münster, Germany). The relative fluorescence intensity of stained nuclei was measured on a linear scale. At least 5000 nuclei were typically analyzed for each sample. It is worthwhile to note that, in FloMax software, advanced gating was performed to select only the G1-2C DNA peaks of either the *M. monantha* seed embryo samples (ignoring the G1-3C DNA peaks of seed endosperm) or the internal standard leaf samples. The absolute DNA amount of a sample was calculated based on the values of the G1 peak means (Doležel et al., 2003, 2007; Doležel and Bartoš, 2005; Mahdavi and Karimzadeh, 2010) as follows:

Sample 2CX DNA (pg) = (Sample G1 peak mean/ Standard G1 peak mean) × Standard $2C_X$ DNA (pg)

Monoploid genome size in the form of base-pair was calculated based on the formula proposed by Doležel et al. (2003) when 1 pg of DNA represents 978 mega base pairs (Mbp). Monoploid genome size was considered as the amount of DNA of one chromosome set, 1CX-value, with chromosome base number *x*, and holoploid genome size as the amount of DNA of the whole chromosome complement, 1C-value, with chromosome number *n*, irrespective of the degree of generative polyploidy (Greilhuber et al., 2005; Mahdavi and Karimzadeh, 2010; Karimzadeh et al., 2011; Abedi et al., 2015; Tavan et al., 2015).

2.3. Statistical analysis

The karyotypic and flow cytometric data were first tested for normality and then analyzed based on a completely randomized design (CRD) with 5 and 3 replications, respectively. Mean comparisons were performed by the least significant difference (LSD) method. Multivariate statistical analysis was carried out in the Minitab software package (Minitab ver. 16.1.0, Minitab Ltd.). A cluster analysis on chromosomal parameters was performed using the Ward's method, and the Euclidean distance was applied to evaluate variations and similarities among the populations. The analysis of variance and the mean comparison analysis was accomplished on normalized data using the nested ANOVA procedure of SAS program (Statistical analysis system, SAS Institute Inc., 2002).

3. Results

All 12 populations of *Medicago monantha* were diploid with varying degrees of mixoploidy and two different chromosome number within each root meristematic tissues of each population. The *ANOVA* results for chromosomal parameters of *M. monantha* indicated three moods: a) between populations, b) between similar types in populations, and c) between different types within each population; *ANOVA* verified significant differences in some parameters (Table 2). Totally, six different chromosome numbers (22, 28, 30, 32, 36, 40) were detected (Table 3). Since most of the populations (11 out of 12 populations, 92%) contained 40 chromosomes, all statistical analyses were carried out based on this chromosome number. The only exception was detected in the P2 population (Table 3). Analyzed karyotypes of somatic complement and the idiograms of the haploid complement of *M. monantha* populations are depicted in Figures 1 and 2, respectively. The mean value of chromosome length (CL) was 1.65 µm, ranging from 0.98 µm (P11) to 2.99 µm (P4). The mean TCV was 1.36 μm^3, varied from 0.94 μm^3 (P11) to 2.29 μm^3 (P1). The mean CI of the complement was 40.1%, ranging from 37.82% (P5) to 41.84% (P9).

According to the tested karyotypic symmetrical indices, the studied *M. monantha* populations were known to exhibit different chromosomal symmetry properties (Table 3). For instance, the highest value of total form percentage of karyotype (TF%) was reported in P11 (45.94%; the most asymmetric), and the least value was detected in P1 (34.81%; the most symmetric). The highest and the least values of coefficient of variation for the total chromosome lengths (CV_{CL}%) were observed in P11 (20.01%; the most asymmetric) and P10 (12.20%; the most symmetric), respectively. The types of chromosomes were determined as "m" (centromere at medium region) and "sm" (centromere at sub medium region) in all populations, using Levan et al. (1964) chromosome nomenclature,

Table 2. *ANOVA* for chromosomal parameters of *Medicago monantha*: a) between populations, b) between similar types in populations, and c) between different types within each population.

	S.O.V.	Df	S	L	CL	AR	r-value	F%	TCV	CI
	Population	11	43.00^{**}	42.92^{**}	47.43^{**}	14.72^{ns}	15.07^{ns}	25.07^{ns}	27.39^{ns}	15.14^{ns}
a	Population×Type	12	5.72	6.74	5.79	9.17	9.10	35.35	11.09	9.16
	Type×Repeat	24	3.53	2.61	3.26	1.55	1.42	3.50	33.85	1.40
	Repeat×Chromosome	2077	0.72	0.72	0.70	0.87	0.87	0.64	0.42	0.87
	Similar Type	5	15.75^{ns}	7.42^{ns}	11.55^{ns}	8.09^{*}	8.23^{*}	132.53^{**}	11.11^{ns}	8.40^{*}
b	Similar Type×Repeat×Population	114	7.16	7.55	8.04	3.44	3.46	1.34	12.52	3.45
	Repeat×Chromosome	2005	0.61	0.61	0.57	0.84	0.84	0.65	0.32	0.84
	Different Type	1	2.98^{ns}	21.49^{**}	5.40^{ns}	67.32^{**}	64.76^{*}	80.47^{**}	21.34^{ns}	65.47^{*}
c	Different Type×Repeat	8	2.87	1.14	1.18	5.95	6.47	2.64	6.55	6.51
	Repeat×Chromosome	165	0.63	0.55	0.53	0.80	0.82	0.66	0.22	0.83

$^{ns, *, **}$: Non significant ($p > 0.05$), significant differences ($p < 0.05$) and ($p < 0.01$), respectively.

Table 3. Karyotypic symmetry analysis of *Medicago monantha* populations.

Population	Chromosome No.	Stebbin's category	Karyotype formula	CV_{CL}%	CL	Range	TF%	CI	Asymmetry Indices		M_{CA}
									A_1	A_2	
P1	30	1A	30m	18.14	1.79	1.27-2.45	41.67	0.41	0.29	0.18	17.51
	40	2A	4m+36sm	16.49	1.95	1.35-2.64	34.81	0.36	0.44	0.16	29.29
P2	22	1A	22m	14.62	1.73	1.34-2.22	42.06	0.42	0.27	0.15	16.09
	30	1A	30m	13.08	1.69	1.31-2.09	41.13	0.41	0.30	0.13	17.98
P3	32	1A	32m	18.26	1.84	1.29-2.58	42.02	0.41	0.27	0.18	18.31
	40	1A	40m	18.29	1.87	1.29-2.51	41.04	0.40	0.30	0.18	16.09
P4	30	1A	28m+2sm	17.15	2.25	1.68-3.08	40.82	0.40	0.31	0.07	17.24
	40	1A	38m+2sm	15.11	2.12	1.67-2.99	41.37	0.41	0.29	0.15	18.59
P5	28	1A	24m+4sm	15.30	1.61	1.22-2.10	40.24	0.40	0.31	0.15	24.17
	40	1A	32m+8sm	16.33	1.50	1.08-2.10	37.93	0.37	0.38	0.16	19.28
P6	36	1A	30m+6sm	15.36	1.58	1.17-2.11	39.14	0.38	0.36	0.15	19.57
	40	1A	36m+4sm	16.34	1.54	1.10-2.08	40.40	0.40	0.32	0.16	22.25
P7	32	1A	32m	17.11	1.47	1.00-1.99	39.92	0.39	0.33	0.17	26.66
	40	3A	18m+22sm	16.45	1.48	1.06-2.06	36.92	0.36	0.41	0.16	20.14
P8	36	1A	34m+2sm	19.65	1.73	1.23-2.56	41.37	0.41	0.30	0.20	18.06
	40	2A	34m+6sm	18.27	1.57	1.07-2.31	40.33	0.40	0.32	0.19	19.57
P9	32	1A	32m	15.98	1.97	1.44-2.66	42.65	0.38	0.25	0.16	16.35
	40	1A	40m	19.55	1.62	1.13-2.35	42.00	0.41	0.27	0.20	14.95
P10	36	1A	34m+2sm	12.20	1.60	1.24-1.98	40.67	0.41	0.29	0.12	17.17
	40	1A	38m+2sm	14.63	1.42	1.07-1.93	41.39	0.41	0.29	0.15	17.57
P11	36	1A	36m	19.51	1.57	1.06-2.18	45.94	0.41	0.29	0.20	19.00
	40	1A	38m+2sm	20.01	1.45	0.98-2.12	40.64	0.40	0.31	0.20	17.44
P12	30	1A	30m	17.75	1.54	1.11-2.18	40.29	0.40	0.31	0.18	18.67
	40	1A	40m	17.96	1.55	1.07-2.12	41.28	0.41	0.29	0.18	17.46

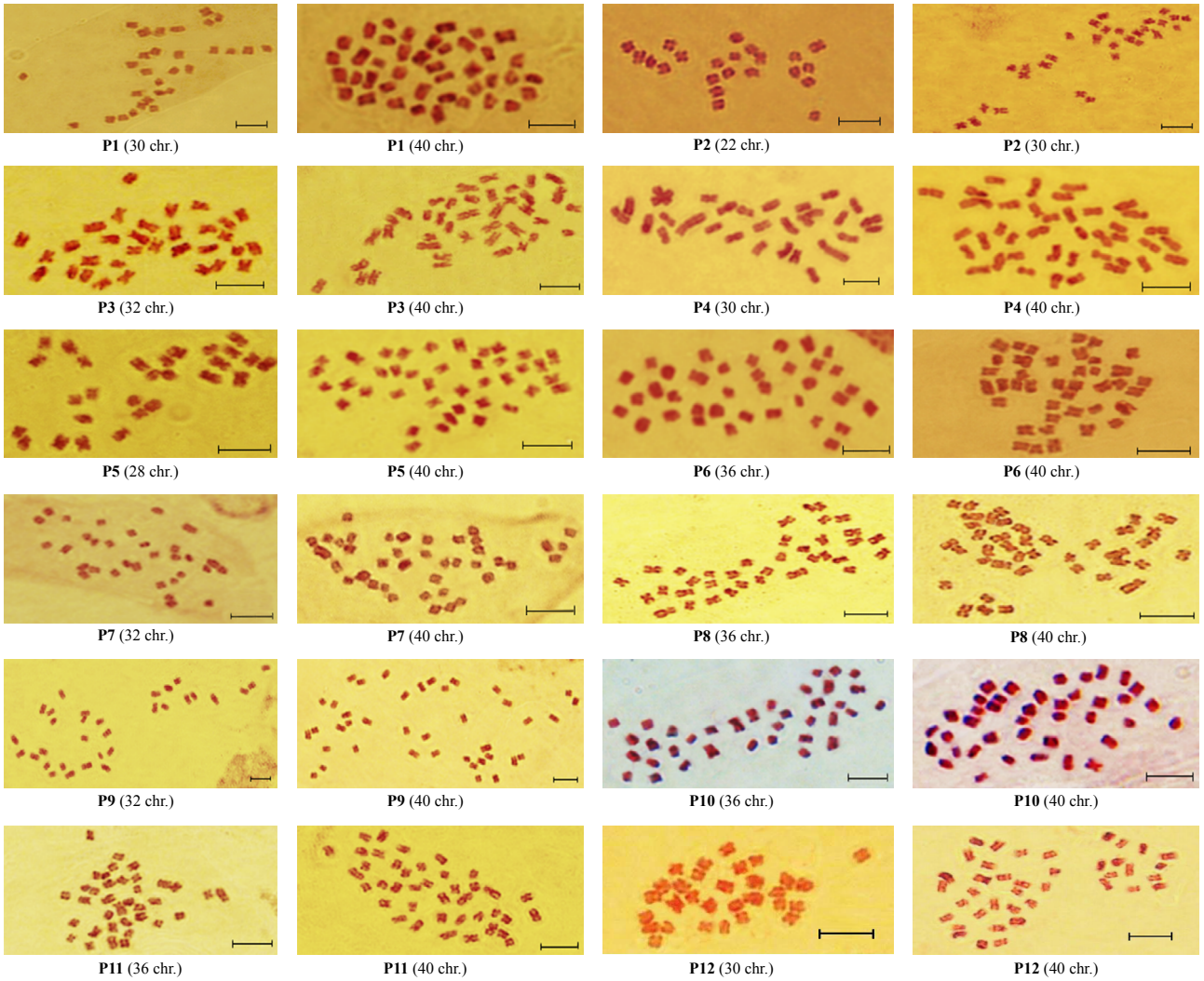

Figure 1. Karyotypes of somatic chromosomes of *Medicago monantha* populations. Scale bars = 5 μm. (Chr = Chromosome)

which was in line with the findings on the *Medicago* genus. Seven different karyotypic formula were revealed; 40m for three populations (P3, P9, P12), 38m+2sm for three populations (P4, P10, P11), 36m+4sm for P6, 34m+6sm for P8, 32m+8sm for P5, 18m+22sm for P7, and 4m+36sm for P1 (Table 3). Karyotypes of all populations were classified in the 1A class based on Stebbins classification method (Stebbins, 1971), except for P1 and P8, which both belonged to the 2A, and P7 belonged to the 3A class. For detailed study on asymmetry status, Romero-Zarco (1986) indices of A_1 and A_2 were also measured. The scatter diagram of these indices showed three groups of populations (Figure 3a). Based on the A_1 index, the highest (0.44; the most asymmetric) and the least (0.27; the most symmetric) values were obtained for P1 and P9, respectively. The results of the A_2 index indicated that P11 (0.20) and P10 (0.15) have the most asymmetric and the most symmetric karyotypes, respectively. The grouping pattern observed in A_1-A_2 scatter diagram was mostly supported by the scatter diagram of the CV_{CL} and M_{CA} indices (Figure 3b).

The correlation between studied chromosomal parameters was evaluated by using Pearson correlation analysis. The highest level of correlation was observed between r-value and F%. There was a significant positive correlation between F% and CI. A similar linear relationship was also revealed between short arm length (S) and chromosome length (CL). To determine total variation in populations and parameters quota, principal component analysis (PCA) was performed, showing that the first three principal components account for 97% of the cumulative variation. The first two components were projected in a 2-dimensional graphic (Figure 4). The results showed that the parameters of r-value (–0.46), AR (0.45), F% (–0.45), and CI (–0.45) had stronger associations with the first component, which accounted for 54% of the variations in

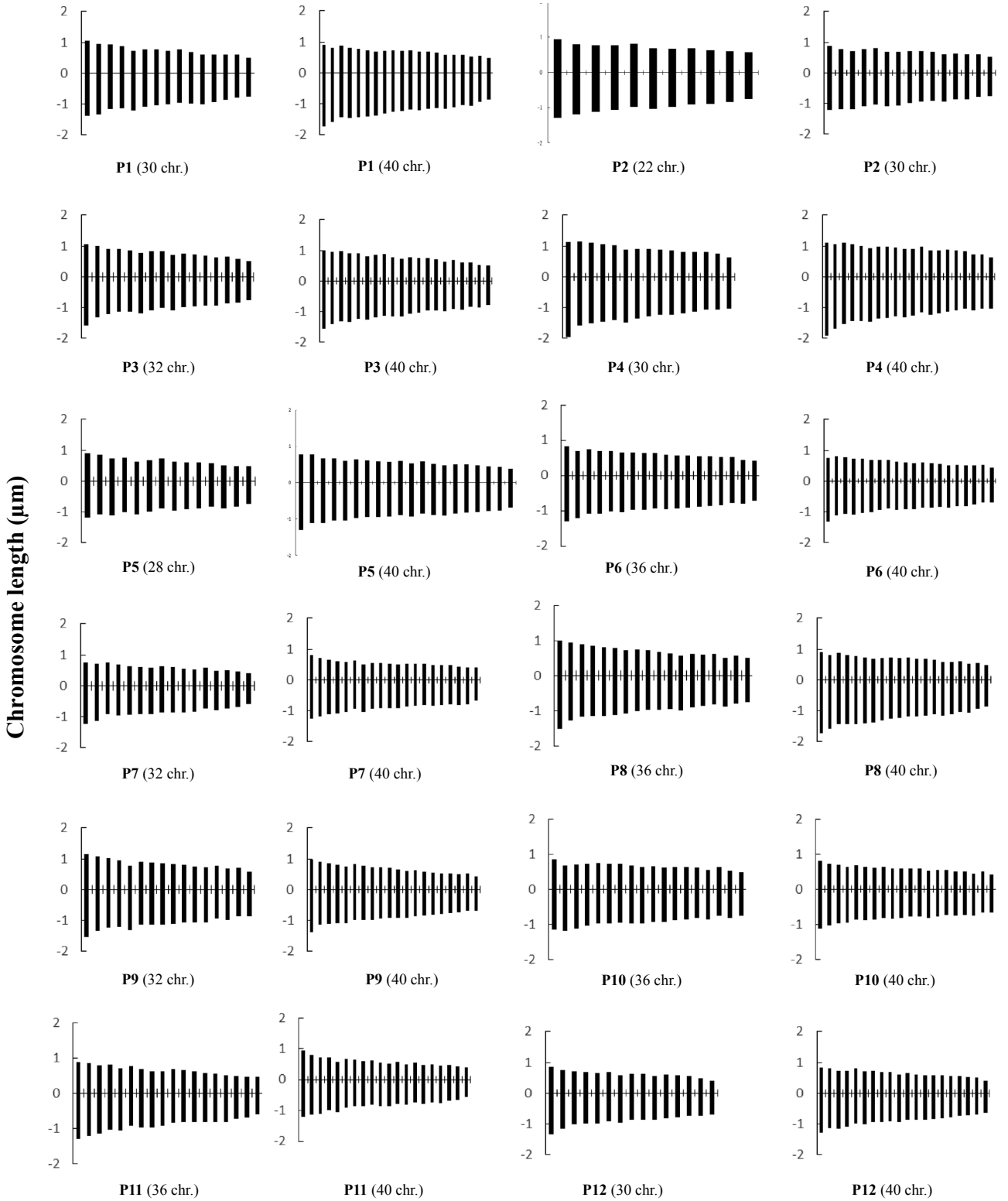

Figure 2. Haploid chromosome idiograms of 12 Iranian *Medicago monantha* populations. (Chr = Chromosome).

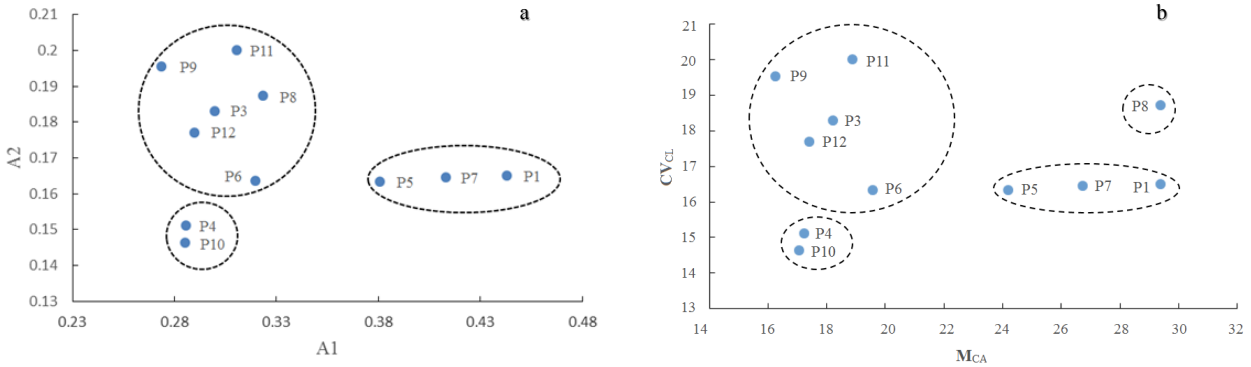

Figure 3. The scatter diagram of 12 Iranian *Medicago monantha* populations: Romero-Zarco asymmetry indices (a), CV_{CL} and M_{CA} (b).

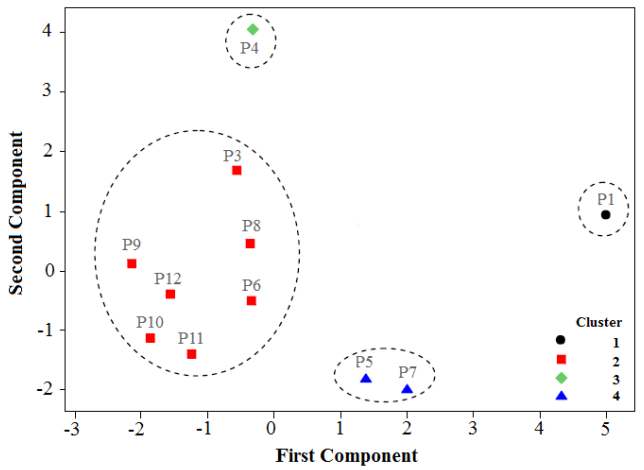

Figure 4. The scatter diagram of 12 Iranian *Medicago monantha* populations based on the first two components of principal component analysis (PCA) of the chromosomal parameters.

the computed data. Respectively, S (0.56), CL (0.53), and L (0.45) played the most important roles in the second component, explaining 39% of the total variation. Finally, the third component was mainly associated with L (0.68) and accounted for 4% of the variation. These results were also confirmed by comparing correlation and determination coefficients of the analyzed parameters (data not shown). Moreover, cluster analysis was performed, using the matrix of karyotype similarities, the Euclidean distance to determine the distance between populations, and the UPGMA method for agglomeration of the clusters (Figure 5). A high cophenetic correlation coefficient (r = 0.92) was obtained. The dendrogram divided the populations into four major clusters. The first cluster contained only P1, while seven populations (P3, P6, P8-P12) formed the second cluster. The third cluster contained a single population (P4) and the fourth included P5 and P7 populations.

The nuclear suspensions resulted in DNA histograms with G0/G1 peaks showing the lowest coefficient of variation. Thus, the isolation and staining procedures provided suspensions, containing isolated and intact stained nuclei. Using the histograms, the mean monoploid 2Cx nuclear DNA content of *Medicago* populations was measured for the first time. The results of flow cytometric data were tested for normality and analyzed according to a completely randomized design (CRD) with three replicate cells. ANOVA reported significant differences ($P < 0.01$) in nuclear 2Cx DNA value between examined populations, where the highest (4.56 pg) and the lowest (2.88 pg) values were detected in P8 and P2, respectively, and the total mean value was 4.10 pg (Table 4). The histograms obtained for nuclear DNA content contained two peaks (Figure 6): the left peak in each histogram refers to the *Solanum lycopersicum* (2C DNA = 1.96 pg), which was used as the reference standard, and the right peak belongs to the *M. monantha* sample. Analysis of the correlation between 2Cx DNA values and geographical parameters of the twelve populations revealed no significant correlation between genome size and latitude, longitude, altitude, mean annual temperature, and mean annual rain in the collection site.

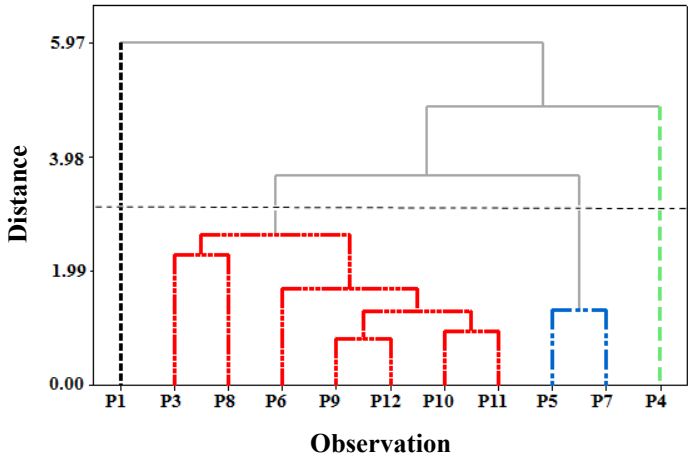

Figure 5. Dendrogram showing the phenetic relationship among the 12 Iranian *Medicago monantha* populations.

4. Discussion

In Iran, there are 48 identified taxa of *Medicago*, including 12 sections localized in different phytogeographical regions of which 15 (32%) taxa are native to the country. Thus, Iran can be regarded as the center of origin of the genus (Ranjbar and Hajmoradi, 2016). Leaves of the *Medicago* genus are a rich source of calcium, iron, β-carotene, and other vitamins, and so, they are used in herbal medicine in many regions of the world. Furthermore, the seeds are recognized for their carminative, tonic, aphrodisiac, antidiabetic, curative gastric antiulcer, and hypocholesterolaemic effects (Al-Habori and Raman, 1998; Srinivasan, 2006). Despite many known and unknown nutritional and medical benefits, this annual plant still suffers from the problems that hinder it from reaching its full potential. Lack of advanced breeding methods, inadequate genetic variability as well as limited range of research on its genetic characteristics have dwarfed the potential applicability of this genus (Petropoulos, 2002).

In the current study, we studied 12 *Medicago monantha* populations of Iranian origin. The chromosome numbers for all tested populations were diploid, revealing mixoploidy. Two different chromosome number values within root meristem tissues of each population and a total of six chromosome numbers (22, 28, 30, 32, 36, 40) were detected. The existence of polyploidy in the same populations, or within one (mixopolyploidy) may indicate the autopolyploid origin of the tetraploid cytotypes and suggests recurrent origins (Esra et al., 2008). Several studies indicate that mixoploidy can be frequently seen in meristem tissues, particularly in plant species, that reveal high levels of apomixis and those with intrinsic vegetative reproduction. It is also highly prevalent in naturally occurring hybrids and polyploids (Snowdon, 2007).

Esra et al. (2008) reported *M. monantha* as a tetraploid species consisting of only metacentric chromosome pairs.

Table 4. Flow cytometric assessment of monoploid genome size (2Cx DNA) of *Medicago monantha* populations.

Population	Mean 2Cx DNA (pg) ± Se	1Cx genome size (pg)	1Cx genome size (Mbp)
P1	4.22 ± 0.032^{bc}	2.11	2063.58
P2	2.88 ± 0.039^{d}	1.44	1408.32
P3	4.20 ± 0.018^{bc}	2.10	2048.91
P4	4.24 ± 0.030^{bc}	2.12	2068.47
P5	4.06 ± 0.068^{c}	2.03	1985.34
P6	4.38 ± 0.059^{ab}	2.19	2146.71
P7	4.16 ± 0.024^{c}	2.08	2034.24
P8	4.56 ± 0.055^{a}	2.28	2229.84
P9	4.10 ± 0.040^{c}	2.05	2004.90
P10	4.14 ± 0.066^{c}	2.07	2024.46
P11	4.26 ± 0.085^{bc}	2.13	2083.14
P12	4.08 ± 0.037^{c}	2.04	1990.23
Range	2.88–4.56	1.44–2.28	1408.32-2229.84

Means with the same symbol letter in the "Mean 2Cx DNA (pg)" column are not significantly different ($p > 0.01$), using the LSD test.

In another study on 13 populations of *M. monantha*, including four subspecies, all populations were identified as hexaploid with chromosome number 2n = 6*x* = 42, but one population of *M. monantha* and one population of *M. monantha* ssp. *noeana* (Boiss.) Greuter & Burdet. showed the chromosome number 2n = 4*x* = 28 and that represented a tetraploid. The base chromosome number of *M. monantha* was proposed to be *x* = 7. However, Bir and Kumari (1979) reported 2n = 44, and Khatoon and Ali (1991) reported 2n = 48 as the chromosome numbers

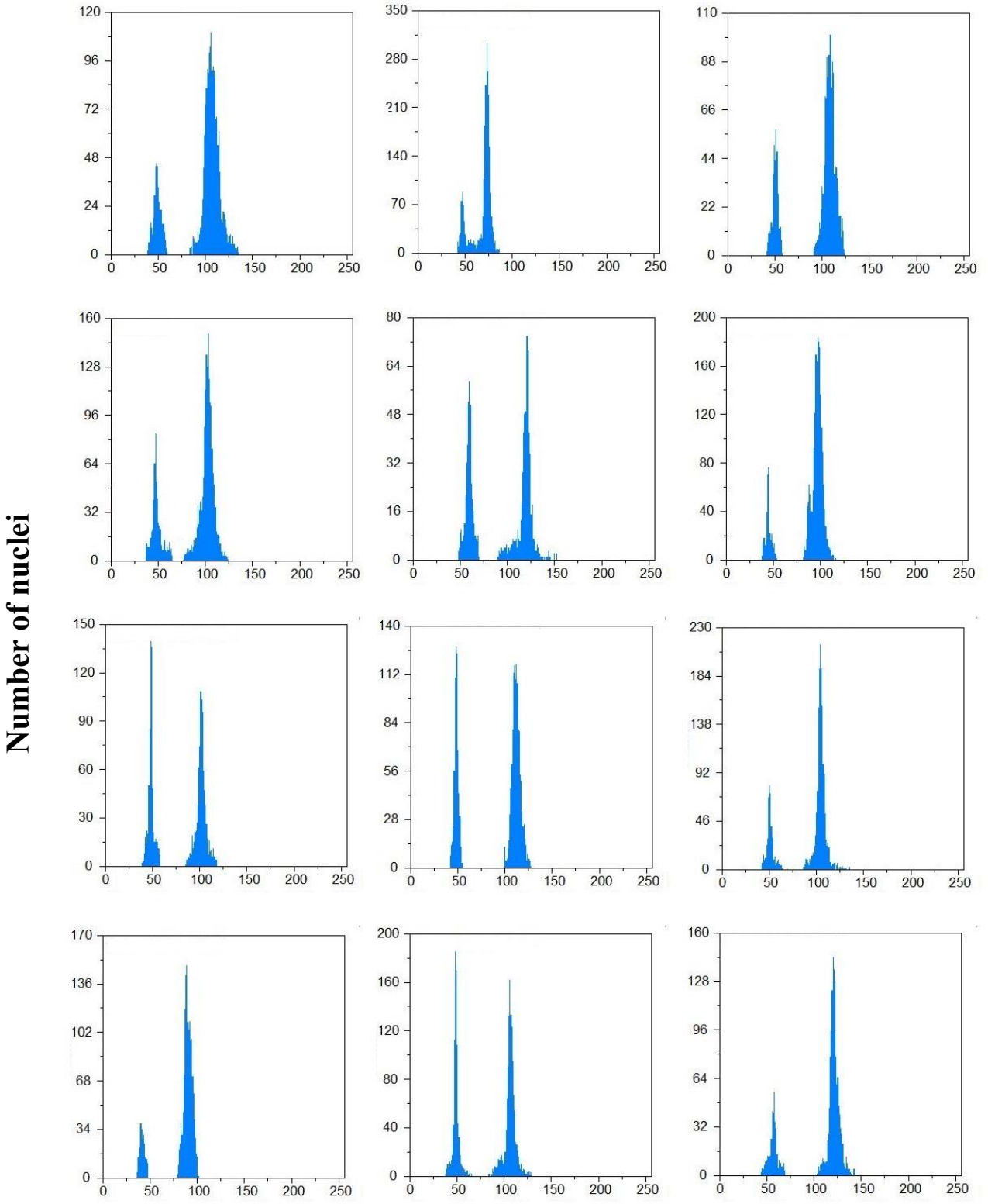

Figure 6. Flow cytometric histograms of 2Cx DNA content of 12 Iranian *Medicago monantha* populations. The left peaks refer to G1 of the standard *Solanum lycopersicum* (2C = 1.96 pg DNA) and the right peaks to G1 of the *Medicago monantha* samples.

for *Medicago monantha* subsp. *incisa* (Royle ex Benth.) Verloove & Lambinon. Based on our results, the base chromosome number for *M. monantha* populations may be 2n = 40 (the most abundant, 11 out of 12 populations, 92%). It is proposed that various recorded chromosome number values may be caused by ascending or descending aneuploidy (Ranjbar and Hajmoradi, 2016).

Previous cytogenetic studies on *M. monantha* summed up only the chromosome number and primary karyotype specifications. To the best of our knowledge, this is the first thorough study on karyological characteristics of native Iranian *M. monantha* populations. The CL values changed significantly among populations, as observed in species of *Medicago*, suggesting that randomly occurring changes in genome size may result in an unequally distributed variation in genome size among all chromosomes of the complement. Esra et al. (2008) reported a chromosome length range of 0.56 µm to 3.26 µm for the *Medicago* genus, while that of the *monantha* subspecies were in the range of 0.61 µm to 1.14 µm, which is slightly lower than those reported in the current study. The broader range of our results might be due to the variation in analyzed species especially *monantha* subspecies. The significant difference in karyological parameters among different populations is an indication of a dispersed pattern of evolution at molecular and sub-chromosomal levels, which can be interpreted as a result of considerable distance in the geographical origins of the *M. monantha* species. This, in turn, has led to the emergence of great diversity in the karyotype morphology of the species. Additionally, there were significant differences among genotypes in the long and short chromosome arms size, arm size ratio, r-value, total chromosome volume (TCV), and centromeric index (CI).

A low rate of karyological similarity in terms of chromosomal asymmetry may result in a failed intra-specific crossing and cause reproductive failure. According to the analyzed asymmetry indices, P11 was demonstrated as the most asymmetric population, while P4, P1, and P10 were among the most symmetric populations. It has been suggested that karyotypes with more asymmetry have a derived status in comparison to those with more symmetrical morphology (Lakshmi et al., 1984). For example, differences in chromosome length (CL) may indicate the occurrence of cyclic changes in genome size during the diversification of the genus. Thus, the study of asymmetry indices and variation in genome size is a valuable means for the establishment of the evolutionary relationship between the species and the origin of diversification of the population (Karimzadeh et al., 2011). Scatter diagrams of A1 and A2 asymmetry indicated three groups of genotypes. Almost all populations (P2-P6, P9-P12) were classified as 1A, which was considered as possessing rather primitive (symmetrical) karyotypes. Two populations (P1, P8) were recorded as 2A and one population (P7) as 3A class based on Stebbins classification method.

Principal component analysis (PCA) implied r-value, AR, F%, and CI to be the key karyotype characteristics and the major indications of karyological variation in assessed populations. The arrangement of populations based on PCA was fully agreed with the results of the cluster analysis. Variation in the karyotypic formula of the species and populations studied may infer the occurrence of chromosomes' structural changes like translocations. For over forty years, flow cytometry has been employed in plant science studies, mostly focusing on nuclear DNA content, ploidy, and cell cycle analysis. It is a powerful and reliable technique, commonly used for screening the DNA content of a large number of plant species (e.g., Doležel and Bartoš, 2005; Doležel et al., 2007; Loureiro et al., 2007; Karimzadeh et al., 2010, 2011; Tavan et al., 2015; Hamidi et al., 2018; Tarkesh Esfahani et al., 2020). Thanks to its practical feasibility and high reproducibility, the flow cytometry technique is useful to reveal differences between various plants. In the present study, the seeds materials (Sliwinska et al., 2005; Sliwinska, 2006; Jedrzejczyk and Sliwinska, 2010) were used for the flow cytometric analysis. The results indicated that *M. monantha* populations varied markedly in monoploid 2Cx nuclear DNA content, ranging from 2.88 pg to 4.56 pg. DNA histograms showed the total mean value is about 4.10 pg. Bidak and Brandham (1995) reported the genome size of *Medicago stellate* is 1.40 pg. In another study, the estimated 4C DNA content in root tip cells of ten *Medicago foenum-graecum* cultivars was ranged from 9.12 to 12.42 pg. There is limited data on 2C DNA content of the *Medicago* genus, yet the detected intraspecific variation in the stable DNA content might be attributed to the loss or addition of many repeats in the micro- and macro-environment of the genome during evolution (Das et al., 2002). One explanation for smaller genome sizes could be transfer of nuclear DNA fragments to the mitochondria and chloroplasts. Genome size is considered as a key factor through the evolution of plants. A negative correlation between DNA content and seed number has been proposed to reduce the rates of extinction of small-genome species and may also contribute to the increased dispersal into extreme habitats and therefore, the increased probability of allopatric speciation events (Karimzadeh et al., 2011).

PCA determined the key karyological characteristics that instigated genetic variation between the tested populations. Using the matrix of karyotype similarities,

cluster analysis was carried out and the dendrogram categorized the populations in four major clusters. Notably, populations originating from the same region were clustered in the same group. This may disprove the role of habitat in the genetic variability of *M. monantha* species, which can be due to genetic exchange between different vegetation zones of fenugreek and/or the effect of natural and unnatural selection. In general, cytological studies on *M. monantha* species growing in Iran indicated the role of chromosome structural changes and quantitative changes in the amount of DNA in species diversification, suggesting that such data may be exploited in the taxonomic and phylogenetic studies of the genus. Furthermore, this type of analysis can help breeders to choose populations with the most homology in chromosomal variation and karyomorphological characteristics for crossing in plant breeding programs with the purpose of improving productivity and choosing more heterotic parents for breeding programs aimed at variety improvement.

Acknowledgment
Authors gratefully acknowledge the support provided for this survey by the Tarbiat Modares University, Tehran, Iran. We also appreciate Dr João Loureiro, Centre for Functional Ecology, Department of Life Sciences, University of Coimbra, Calçada Martim de Freitas, 3000-456 Coimbra, Portugal for his expert advice and confirming the flow cytometric genome size data.

Author contribution
H. Zarabizadeh carried out the experiments and prepared the first draft of the manuscript under the supervision of Prof. G. Karimzadeh and the advisory of Assist. Prof. S. Rashidi Monfared. S. Tarkesh Esfahani revised the draft of the manuscript. All authors read and approved the final manuscript.

Conflict of interest
The authors have declared no conflict of interest.

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