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Original Article

Cytogenetic Characterization and Comparative Karyotype Analysis of Iranian Ecotypes of *Zataria multiflora* Boiss.

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ABSTRACT

Zataria multiflora Boiss. is a medicinally important plant endemic to Iran and neighboring regions. Despite its pharmacological significance, cytogenetic characteristics have remained unknown for this monotypic genus. This study presents the first comprehensive karyotype analysis of *Z. multiflora*, examining six ecotypes collected from different geographical regions of Iran (Arsanjan, Kavar, Darab, Sarvestan, Tabas, and Chupanan). Chromosome measurements were performed on four well-spread metaphase plates per ecotype. All ecotypes exhibited a consistent diploid chromosome number of 2n = 2x = 30, consistent with reports in closely related *Thymus* species. Although all ecotypes were classified within Stebbins' highly symmetrical 1A category, significant differences in chromosomal morphometric parameters and quantitative asymmetry indices revealed measurable intraspecific variation in chromosome morphology and centromere position among the ecotypes. Principal component analysis (PCA) and cluster analysis based on chromosome morphometric parameters and karyotype asymmetry indices revealed clear patterns of cytogenetic differentiation among ecotypes. PCA indicated that variables associated with intrachromosomal asymmetry contributed more strongly to karyotypic variation in *Z. multiflora* than interchromosomal size variation. Cluster analysis grouped ecotypes into two major clusters, separating more symmetrical ecotypes (Kavar and Sarvestan) from relatively asymmetric ones. This study establishes the first cytogenetic characterization of *Z. multiflora* and lays a foundation for future studies of chromosome variation, genome organization, and evolutionary relationships within the species.

Keywords: Chromosome number; Cytogenetics; Ecotypic variation; Intrachromosomal asymmetry; Lamiaceae; *Zataria multiflora*

INTRODUCTION

Zataria multiflora Boiss. is a perennial medicinal and aromatic shrub belonging to the Lamiaceae family, naturally growing on the rugged slopes of arid regions in central and southern Iran, as well as parts of Afghanistan and Pakistan [1]. The species exhibits notable chemical and pharmacological similarities to *Thymus vulgaris*, a well-characterized medicinal plant [2]. Owing to these shared properties, *Z. multiflora* is commonly known as *Avishan-e-Shirazi* in Persian, where "Avishan" denotes thyme and "Shirazi" refers to the city of Shiraz in southern Iran [3]. This species reaches 60–90 cm in height, characterized by woody, leafless mature branches and young branches densely covered with glandular, spreading pilose indumentum. The leaves are small (5–10 mm^2), orbicular to ovate, and densely covered with glandular trichomes, along with densely hairy, spherical buds in the leaf axils. Flowering stems are typically unbranched, occasionally with short lateral branches, bearing small, white, sub-sessile flowers that are often male sterile [4, 5]. The essential oil of *Z. multiflora* contains substantial concentrations of thymol and carvacrol, two monoterpenoid phenols widely recognized for their antimicrobial and antifungal properties [6, 7]. Furthermore, experimental studies have demonstrated that *Z. multiflora* exhibits a range of therapeutic activities, including antinociceptive, antimicrobial, spasmolytic, antioxidative, and anti-inflammatory effects [3, 8-10], highlighting the need for further biological characterization of this species. Karyological studies, which examine chromosome number, structure, and behavior, provide fundamental insight into plant systematics, genetics, evolution, and breeding. Chromosome number changes, structural chromosome rearrangements, polyploidization, and dysploidy are recognized as major mechanisms driving diversification and adaptation in plants [11, 12]. Consequently, detailed karyotype characterization is an important tool for understanding variation in genome organization and evolutionary relationships within plant lineages [11, 13].

Cytogenetic investigations across the Lamiaceae family have revealed substantial chromosomal diversity, with polyploidy, dysploidy, and structural chromosome rearrangements contributing to genome evolution in several genera [14-16]. In *Salvia*, for example, dysploidy and polyploidy have been identified as major drivers of chromosome evolution [17], whereas karyotype studies of *Origanum* [18], *Nepeta* [19],

and *Satureja* [20] have demonstrated variation in chromosome morphology and karyotype asymmetry among species and populations, indicating that structural chromosomal rearrangements have contributed to diversification within these genera. The genus *Thymus*, which is phylogenetically and phytochemically closely related to *Z. multiflora* [21], exhibits considerable chromosomal diversity, primarily characterized by diploid and tetraploid ploidy levels. Species of *Thymus* possess multiple basic chromosome numbers (x = 7, 14, and 15) and a broad range of somatic chromosome counts (2n = 28, 30, 42, 54, 56, 58, 60), reflecting extensive polyploidy and dysploidy within the genus [22-24]. For instance, *Thymus kotschyanus* possesses a basic chromosome number of x = 15 with both diploid (2n = 30) and tetraploid (2n = 60) cytotypes, while *T. vulgaris* and *T. praecox* exhibit multiple chromosome numbers associated with different ploidy levels [25].

Despite advances in karyotype analysis of several Lamiaceae genera, no information is currently available regarding chromosome number, karyotype structure, or cytogenetic variation in *Z. multiflora.* This lack of cytogenetic knowledge represents a significant gap in understanding genome organization, chromosomal evolution, and cytogenetic diversity within this monotypic genus. Given the extensive chromosomal diversity reported in related genera of the Lamiaceae [14, 17, 18, 24], we investigated whether geographically diverse ecotypes of *Z. multiflora* exhibit differences in chromosome number and karyotype characteristics, reflected in chromosome morphology, karyotype structure, and asymmetry parameters. Therefore, the objectives of this study were to determine the somatic chromosome number of *Z. multiflora* for the first time, characterize the karyotype structure of six Iranian ecotypes, and evaluate patterns of cytogenetic differentiation. To achieve this, conventional karyotype descriptors and asymmetry indices were integrated with multivariate statistical approaches, including principal component analysis (PCA) and cluster analysis, enabling a comprehensive assessment of chromosomal variation among ecotypes. This study provides the first cytogenetic framework for *Z. multiflora* and contributes to a better understanding of genome organization and cytogenetic diversity within this medicinally important species.

MATERIALS AND METHODS

Plant Materials

In this study, seeds of six ecotypes of Shirazi thyme (*Z. multiflora* Boiss.) were collected from natural habitats in different regions of Iran (Fig. 1). The selected ecotypes represent geographically and environmentally diverse regions within the natural distribution range of the species, including the semi-arid regions of Fars Province in southern Iran (Arsanjan, Kavar, Darab, and Sarvestan) and the arid desert regions of central-eastern Iran (Chupanan and Tabas). These locations differ in altitude, temperature regime, precipitation, soil characteristics, and degree of aridity, thereby providing a representative assessment of cytogenetic variation within the species. Voucher specimens representing each ecotype were deposited in the Herbarium of the Fars Agricultural and Natural Resources Research and Education Center, Shiraz, Iran, under the following accession numbers: Chupanan (22094), Darab (22095), Arsanjan (22096), Tabas (22097), Sarvestan (22098), and Kavar (22099).

Preparation and Staining of Chromosomes

For each ecotype, 20 seeds were placed on moist filter paper in Petri dishes and incubated in a germinator at 20–23 °C. Of these, 10-15 seedlings produced root tips suitable for cytological analysis. Once the roots reached 1–1.5 cm in length, the root tips were excised for chromosome staining. Root tips were pretreated in a 2 mM solution of 8-hydroxyquinoline at 4 °C for 3 h, followed by rinsing in distilled water for 30 min. Subsequently, the roots were dehydrated on filter paper and immediately fixed in Carnoy's solution (absolute ethanol: glacial acetic acid, 3:1 v/v) at 4 °C for 20 h. After fixation, roots were treated with 1N hydrochloric acid at 60 °C for 15 min, stained with aceto-iron-hematoxylin for 1-2 h, and finally squashed in a droplet of 45% acetic acid and lactic acid mixture (10:1 v/v) [26]. Prepared slides were examined under a light microscope (Olympus BH2) equipped with a digital camera. All ecotypes were processed using the same pretreatment, fixation, hydrolysis, staining, and squashing procedures. Slide preparation and chromosome measurements were performed by the same operator to minimize technical variation. For each ecotype, approximately 25–30 metaphase cells were initially examined. Four well-spread metaphase plates exhibiting complete chromosome complements, minimal overlap, clear centromere positions, and uniform chromosome condensation were selected for detailed karyotype analysis. Because the chromosomes of *Z. multiflora* are relatively small, high-quality metaphases suitable for accurate measurements were limited. For each ecotype, chromosome measurements were performed independently on the four selected metaphase plates obtained from different root tips originating from different seedlings.

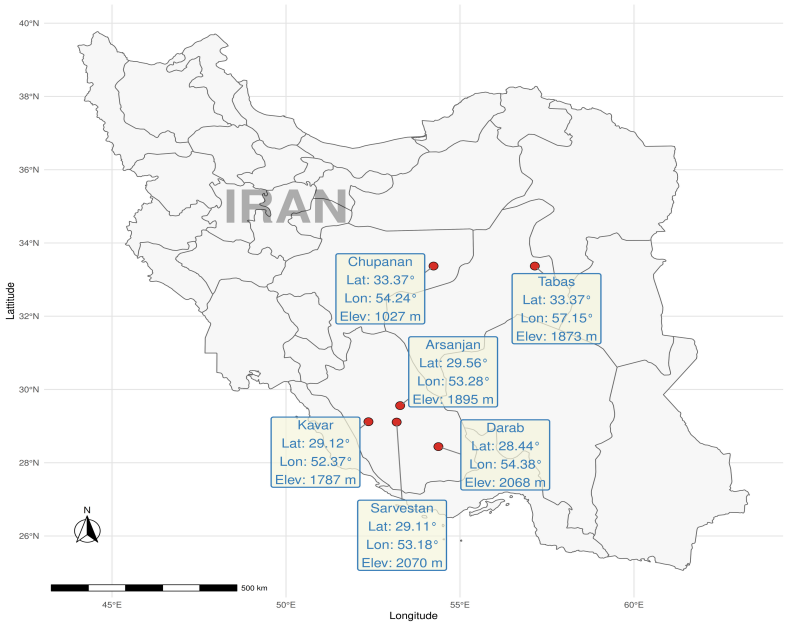


Fig. 1 Geographical regions of Iran where seeds of the *Zataria multiflora* ecotypes were collected.

Chromosome Measurements and Karyotype Analysis

Chromosome measurements were carried out using Micromeasure version 3.3 [27]. To numerically characterize the karyotypes, the parameters including long arm length (LA), short arm length (SA), total chromosome length (CL), relative length percentage (RL%), arm ratio (AR), and centromeric index (CI) were measured for each chromosome following [28]. Chromosomes were morphologically classified based on centromere positions following Levan *et al.* (1964) [29], and karyotype formulas were established accordingly. Karyograms were constructed for each ecotype by arranging chromosomes in descending order of size. Karyotype asymmetry was assessed using various indices, listed in **Error! Not a valid bookmark self-reference.**, such as value of relative chromatin (VRC), total form percentage (TF%), difference of relative length (DRL) [28], and also the intra-chromosomal (A_1) and interchromosomal (A_2) asymmetry indices proposed by Zarco (1986) [30]. Karyotypic evolution was further analyzed based on Stebbins' symmetry classes [31].

Chromosome morphometric parameters and karyotype asymmetry indices were calculated using the R package idiogramFISH (v. 2.0.13) [32], which was also used to generate graphical representations of the karyotypes. For each ecotype, the parameters and indices were determined from four independently measured metaphase plates, which served as replicates in the statistical analyses. One-way Analysis of variance (ANOVA) was performed to test differences among the ecotypes for the measured parameters and indices. Mean comparisons were carried out using Tukey's HSD test ($P \leq 0.05$). Principal component analysis (PCA) was also performed to explore relationships among the karyotypic variables and the ecotypes of *Z. multiflora*. The analysis was conducted in the R statistical environment using the FactoMineR package for PCA computation [33] and factoextra for visualization [34]. Cluster analysis was conducted using Ward's method based on Euclidean distance to classify the ecotypes of *Z. multiflora* according to the karyotypic variables.

Table 1 Formulae of chromosomal parameters and karyotype asymmetry indices used in this study.

Karyotype asymmetry index	Formula	Karyotype asymmetry index	Formula
Intrachromosomal asymmetry index [30]	$A_1 = \frac{\sum_{i=1}^{n} 1 - \frac{SA_i}{LA_i}}{n}$	Interchromosomal asymmetry index [30]	$A_2 = \frac{s_{CL}}{X_{CL}}$
Total form percentage [28]	$TF = \left(\frac{\sum SA}{\sum CL}\right) * 100$	Difference of relative length [28]	$DRL = \max(RL\%) - \min(RL\%)$
Centromeric Index [28]	$CI = \frac{SA}{CL}$	Asymmetry index [35]	$AI = \frac{CV_{CI}}{CV_{CL}} * 100$
Arano index of karyotype asymmetry [36]	$AsK\% = \left(\frac{\sum LA}{\sum CL}\right) * 100$	Symmetry index [37]	$S\% = \left(\frac{CL_{min}}{CL_{max}}\right) * 100$
Mean centromeric index [37]	$X_{CI} = \sum \frac{CL}{n}$	Degree of karyotype asymmetry [38]	$A = \frac{\sum_{i=1}^{n} \frac{LAi - SAi}{LAi + SAi}}{n}$
Mean centromeric asymmetry [37]	$X_{CA} = A * 100$	Coefficient of variation of centromeric index [35]	$CV_{CI} = \left(\frac{SA_{CI}}{X_{CI}}\right) * 100$
Total chromosome length of the haploid complement [37]	$HCL = \sum CL$	Mean arm ratio [28]	$r = \frac{\sum AR}{n}$

LA: length of long arm; SA: length of short arm; CL: chromosome length (SA+LA); i: Homologous group number; s: Standard deviation; X: mean; n: Haploid chromosome number, RL: Relative length, AR: Arm ratio

RESULTS

The karyotype analysis of six *Z. multiflora* ecotypes from different regions of Iran revealed a consistent diploid chromosome number of 2n = 30 across all samples (

Fig. **3**). Despite this conserved diploid number, analysis of variance uncovered significant inter-ecotypic differences in chromosome morphometric parameters, and all measured karyotype asymmetry indices ($P < 0.01$ and $P < 0.05$; Table 2), indicating considerable karyotype diversification among the *Z. multiflora* ecotypes. Based on the chromosome classification system of Levan *et al.* (1964), the chromosome complements were predominantly composed of metacentric (m) and submetacentric (sm) chromosomes, suggesting relatively symmetrical karyotypes. However, the karyotype formulas varied among the ecotypes (

Fig. **3**). The Darab ecotype exhibited the most asymmetric karyotype formula, comprising a mixture of metacentric and submetacentric chromosomes (8m+7sm). In contrast, the Kavar and Sarvestan ecotypes exhibited the most symmetrical karyotypes, consisting exclusively of metacentric chromosomes (15m). Moreover, the Arsanjan (14m+1m-sm), Tabas (13m + 2sm), and Chupanan (14m + 1sm) ecotypes displayed intermediate karyotype formulas with a predominance of metacentric chromosomes. Consistent with these patterns, the mean centromeric asymmetry (X_{CA}) varied significantly among the ecotypes (Table 1). Darab showed the highest X_{CA} value (24.70), indicating a greater tendency toward submetacentric chromosomes, whereas Sarvestan (7.43) and Kavar (10.31) had the lowest X_{CA} values, reflecting a more metacentric tendency. Furthermore, the highest coefficients of variation of centromeric index (CV_{CI}) were observed in the Chupanan and Arsanjan ecotypes (9.50% and 8.84%, respectively), indicating greater diversity in centromere positions among chromosomes, while the Sarvestan ecotype showed the lowest CV_{CI} (4.41%), supporting its more uniform karyotype (Table 1).

The mean chromosome morphometric parameters and karyotype asymmetry indices of the studied ecotypes are presented in Table 1. The total haploid chromosome length (HCL) differed significantly among the ecotypes, indicating substantial variation in chromosome size among the ecotypes despite the conservation of chromosome number. The highest HCL values were recorded in Tabas (11.24) and Darab (10.92), which were statistically comparable and significantly greater than those of the other ecotypes. In contrast, Kavar (7.80) and Chupanan (7.88) exhibited the lowest HCL values. Long arm (LA) and short arm (SA) lengths followed a similar pattern, being notably longer in Darab and Tabas, while Kavar and Chupanan exhibited the shortest LA and SA values, respectively.

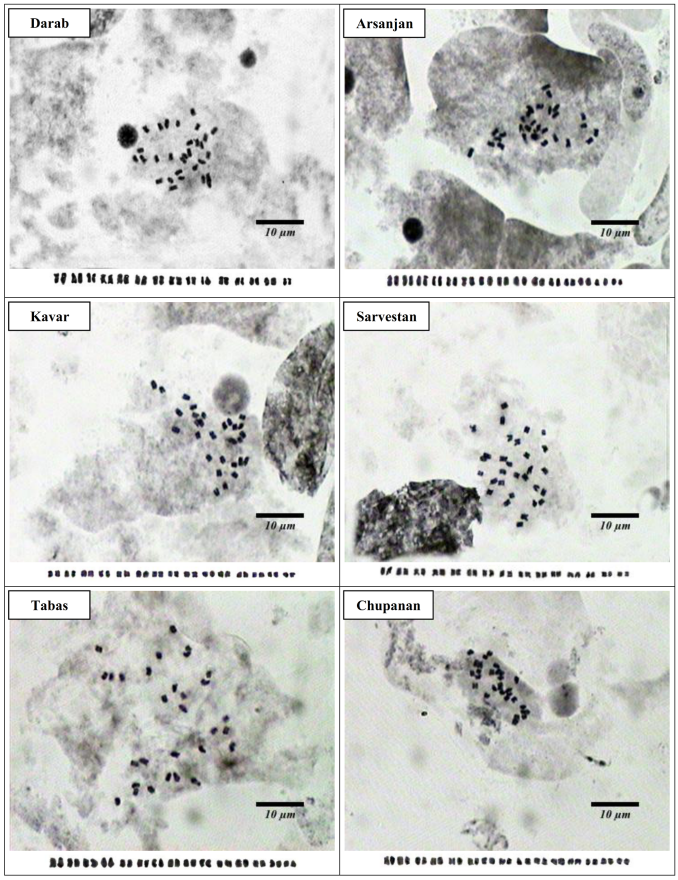
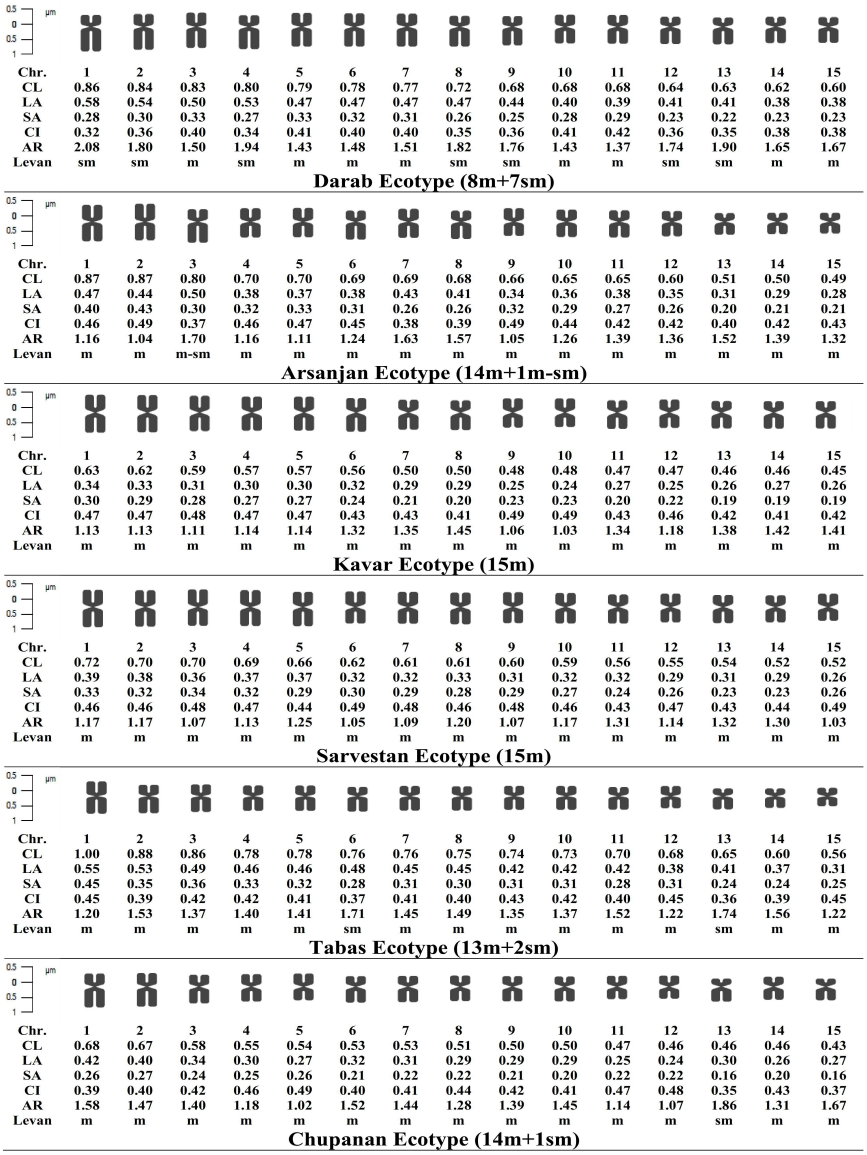


Fig. 2 Mitotic metaphase chromosomes and corresponding karyograms of six Iranian *Zataria multiflora* Boiss. ecotypes (Darab, Arsanjan, Kavar, Sarvestan, Tabas, Chupanan). Chromosomes are arranged in descending order of size according to centromere position. Scale bar = 10 µm



Darab Ecotype (8m+7sm)															
Chr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
CL	0.86	0.84	0.83	0.80	0.79	0.78	0.77	0.72	0.68	0.68	0.68	0.64	0.63	0.62	0.60
LA	0.58	0.54	0.50	0.53	0.47	0.47	0.47	0.47	0.44	0.40	0.39	0.41	0.41	0.38	0.38
SA	0.28	0.30	0.33	0.27	0.33	0.32	0.31	0.26	0.25	0.28	0.29	0.23	0.22	0.23	0.23
CI	0.32	0.36	0.40	0.34	0.41	0.40	0.40	0.35	0.36	0.41	0.42	0.36	0.35	0.38	0.38
AR	2.08	1.80	1.50	1.94	1.43	1.48	1.51	1.82	1.76	1.43	1.37	1.74	1.90	1.65	1.67
Levan	sm	sm	m	sm	m	m	m	sm	sm	m	m	sm	sm	m	m

Arsanjan Ecotype (14m+1m-sm)															
Chr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
CL	0.87	0.87	0.80	0.70	0.70	0.69	0.69	0.68	0.66	0.65	0.65	0.60	0.51	0.50	0.49
LA	0.47	0.44	0.50	0.38	0.37	0.38	0.43	0.41	0.34	0.36	0.38	0.35	0.31	0.29	0.28
SA	0.40	0.43	0.30	0.32	0.33	0.31	0.26	0.26	0.32	0.29	0.27	0.26	0.20	0.21	0.21
CI	0.46	0.49	0.37	0.46	0.47	0.45	0.38	0.39	0.49	0.44	0.42	0.42	0.40	0.42	0.43
AR	1.16	1.04	1.70	1.16	1.11	1.24	1.63	1.57	1.05	1.26	1.39	1.36	1.52	1.39	1.32
Levan	m	m	m-sm	m	m	m	m	m	m	m	m	m	m	m	m

Kavar Ecotype (15m)															
Chr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
CL	0.63	0.62	0.59	0.57	0.57	0.56	0.50	0.50	0.48	0.48	0.47	0.47	0.46	0.46	0.45
LA	0.34	0.33	0.31	0.30	0.30	0.32	0.29	0.29	0.25	0.24	0.27	0.25	0.26	0.27	0.26
SA	0.30	0.29	0.28	0.27	0.27	0.24	0.21	0.20	0.23	0.23	0.20	0.22	0.19	0.19	0.19
CI	0.47	0.47	0.48	0.47	0.47	0.43	0.43	0.41	0.49	0.49	0.43	0.46	0.42	0.41	0.42
AR	1.13	1.13	1.11	1.14	1.14	1.32	1.35	1.45	1.06	1.03	1.34	1.18	1.38	1.42	1.41
Levan	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m

Sarvestan Ecotype (15m)															
Chr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
CL	0.72	0.70	0.70	0.69	0.66	0.62	0.61	0.61	0.60	0.59	0.56	0.55	0.54	0.52	0.52
LA	0.39	0.38	0.36	0.37	0.37	0.32	0.32	0.33	0.31	0.32	0.32	0.29	0.31	0.29	0.26
SA	0.33	0.32	0.34	0.32	0.29	0.30	0.29	0.28	0.29	0.27	0.24	0.26	0.23	0.23	0.26
CI	0.46	0.46	0.48	0.47	0.44	0.49	0.48	0.46	0.48	0.46	0.43	0.47	0.43	0.44	0.49
AR	1.17	1.17	1.07	1.13	1.25	1.05	1.09	1.20	1.07	1.17	1.31	1.14	1.32	1.30	1.03
Levan	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m

Tabas Ecotype (13m+2sm)															
Chr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
CL	1.00	0.88	0.86	0.78	0.78	0.76	0.76	0.75	0.74	0.73	0.70	0.68	0.65	0.60	0.56
LA	0.55	0.53	0.49	0.46	0.46	0.48	0.45	0.45	0.42	0.42	0.42	0.38	0.41	0.37	0.31
SA	0.45	0.35	0.36	0.33	0.32	0.28	0.31	0.30	0.31	0.31	0.28	0.31	0.24	0.24	0.25
CI	0.45	0.39	0.42	0.42	0.41	0.37	0.41	0.40	0.43	0.42	0.40	0.45	0.36	0.39	0.45
AR	1.20	1.53	1.37	1.40	1.41	1.71	1.45	1.49	1.35	1.37	1.52	1.22	1.74	1.56	1.22
Levan	m	m	m	m	m	sm	m	m	m	m	m	m	sm	m	m

Chupanan Ecotype (14m+1sm)															
Chr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
CL	0.68	0.67	0.58	0.55	0.54	0.53	0.53	0.51	0.50	0.50	0.47	0.46	0.46	0.46	0.43
LA	0.42	0.40	0.34	0.30	0.27	0.32	0.31	0.29	0.29	0.29	0.25	0.24	0.30	0.26	0.27
SA	0.26	0.27	0.24	0.25	0.26	0.21	0.22	0.22	0.21	0.20	0.22	0.22	0.16	0.20	0.16
CI	0.39	0.40	0.42	0.46	0.49	0.40	0.41	0.44	0.42	0.41	0.47	0.48	0.35	0.43	0.37
AR	1.58	1.47	1.40	1.18	1.02	1.52	1.44	1.28	1.39	1.45	1.14	1.07	1.86	1.31	1.67
Levan	m	m	m	m	m	m	m	m	m	m	m	m	sm	m	m

Fig. 3 Schematic representation of haploid chromosome complements and quantitative chromosome parameters of six Zataria multiflora ecotypes. The parameters include: Chromosome number (Chr), Total chromosome length (CL), Long arm (LA), Short arm (SA), Centromeric Index (CI), Arm ratio (AR), and Levan's classification of chromosomes based on centromere position (Levan).

The indices of interchromosomal size heterogeneity, including the interchromosomal asymmetry index (A_2), difference of relative length (DRL), and coefficient of variation of chromosome length (CV_{CL}), were significantly higher in the Arsanjan (A_2 = 0.21, DRL = 3.80, CV_{CL} = 17.46%) and Tabas (A_2 = 0.15, DRL = 3.88, CV_{CL} = 14.61%) ecotypes, indicating greater variation in chromosome size within their complements. In contrast, the Sarvestan ecotype exhibited the lowest values for these indices (A_2 = 0.11, DRL = 2.18, and CV_{CL} = 11.18%), indicating the greatest uniformity in chromosome size among the studied ecotypes.

Significant variation in karyotype asymmetry indices was observed among the ecotypes, indicating differences in chromosome structure and karyotype evolution. The Darab ecotype exhibited the most asymmetrical karyotype, characterized by the highest values of the intrachromosomal asymmetry index (A_1 = 0.39) and the Arano's index (AsK = 62.39%), together with the lowest total form percentage (TF = 37.61%). Conversely, the Sarvestan and Kavar ecotypes displayed the most symmetric karyotypes, exhibiting the lowest A_1 (0.14 and 0.18) and AsK (53.66% and 55.00%), as well as the highest TF (46.34% and 45.00%). In addition, the Darab ecotype possessed the highest mean arm ratio (r = 1.67), consistent with its greater karyotype asymmetry, whereas the Sarvestan ecotype showed the lowest mean arm ratio (r = 1.16), indicating a highly symmetrical karyotype (Table 3).

The degree of karyotype asymmetry (A) further revealed the karyotypic differentiation among the ecotypes, with the highest value observed in Darab (0.25) and the lowest in Sarvestan (0.07). The asymmetric index (AI), which integrates variation in chromosome length and centromere position, was also highest in Darab (66.30) and lowest in Sarvestan (39.40), providing a comprehensive measure of karyotype asymmetry that was consistent with the results of the other asymmetry indices. Similarly, the symmetry index (S%) varied significantly among

the ecotypes, ranging from 56.14% to 72.14%. The Sarvestan and Kavar ecotypes exhibited the highest S% values (72.14% and 71.71%, respectively) (Table 3), further confirming their highly symmetrical karyotypes. Notably, although all quantitative asymmetry indices showed statistically significant differences among the ecotypes, the Stebbins karyotype classification (SC) [31] assigned all six ecotypes to the 1A symmetry class (Table 3), which represents the most symmetrical karyotype class. This finding suggests that, while significant quantitative variation in chromosome morphology and karyotype asymmetry have occurred during ecotypic differentiation, the overall structural karyotype organization has remained highly conserved.

Table 2 Analysis of variance (ANOVA) for the chromosomal parameters and karyotype asymmetry indices measured in the six ecotypes of *Zataria multiflora* Boiss.

Source of Variation	df	Mean squares							
		LA	SA	DRL	HCL	A	A_1	A_2	TF
Ecotype	5	4.524 **	1.050 **	2.424 **	8.779 **	0.015 **	0.033 **	0.002 **	37.842 **
Error	18	0.082	0.058	0.042	0.359	0.000	0.000	0.000	7.423
C.V.%		5.22	5.95	6.96	6.30	6.16	6.04	5.46	6.39
Source of Variation	df	Mean squares							
		AsK	S	X_{CI}	X_{CA}	CV_{CL}	CV_{CI}	AI	r
Ecotype	5	37.842 *	221.825 **	0.004 **	146.281**	21.569 **	13.795 **	516.188 **	0.126 **
Error	18	11.206	12.206	0.001	0.658	0.693	0.145	8.315	0.005
C.V.%		5.84	5.38	6.40	5.47	6.15	5.23	5.36	5.02

Long Arm (LA), Short Arm (SA), Difference of relative length (DRL), Total chromosome length of the haploid complement (HCL), Degree of karyotype asymmetry (A), Intrachromosomal asymmetry index (A_1), Interchromosomal asymmetry index (A_2), Total form percentage (TF%), Arano index of karyotype asymmetry (AsK), Symmetry index (S%), Mean centromeric index (X_{CI}), Mean centromeric asymmetry (X_{CA}), Coefficient of variation of chromosome length (CV_{CL}), Coefficient of variation of centromeric index (CV_{CI}), Asymmetric index (AI), mean arm ratio (r).

Table 3 Mean values of chromosomal parameters and karyotype asymmetry indices for the six ecotypes of *Zataria multiflora* Boiss.

Ecotype	LA (µm)	SA (µm)	DRL	HCL (µm)	A	A_1	A_2	TF (%)	
Darab	6.81 ± 0.39 a	4.11 ± 0.33 a	2.34 ± 0.19 c	10.92 ± 0.77 a	0.25 ± 0.01 a	0.39 ± 0.03 a	0.12 ± 0.01 c	37.61 ± 1.01 b	
Arsanjan	5.69 ± 0.46 b	4.38 ± 0.33 a	3.80 ± 0.33 a	10.07 ± 0.18 ab	0.13 ± 0.01 c	0.23 ± 0.01 c	0.17 ± 0.01 a	43.49 ± 3.62 ab	
Kavar	4.29 ± 0.17 c	3.51 ± 0.07 b	2.29 ± 0.10 c	7.80 ± 0.44 d	0.10 ± 0.00 d	0.18 ± 0.01 d	0.12 ± 0.01 c	45.00 ± 1.39 a	
Sarvestan	4.93 ± 0.21 c	4.26 ± 0.25 a	2.18 ± 0.12 c	9.19 ± 0.68 bc	0.07 ± 0.00 e	0.14 ± 0.00 e	0.11 ± 0.01 c	46.34 ± 3.40 a	
Tabas	6.60 ± 0.20 a	4.64 ± 0.18 a	3.88 ± 0.26 a	11.24 ± 0.73 a	0.18 ± 0.01 b	0.30 ± 0.00 b	0.15 ± 0.01 b	41.26 ± 2.98 ab	
Chupanan	4.55 ± 0.13 c	3.33 ± 0.17 b	3.18 ± 0.10 b	7.88 ± 0.58 cd	0.15 ± 0.01 c	0.26 ± 0.02 c	0.14 ± 0.01 b	42.21 ± 2.85 ab	
Ecotype	AsK (%)	S (%)	X_{CI}	X_{CA}	CV_{CL} (%)	CV_{CI} (%)	AI	r	SC
Darab	62.39 ± 3.99 a	70.18 ± 4.89 ab	0.38 ± 0.02 b	24.70 ± 0.76 a	11.97 ± 0.76 c	7.93 ± 0.33 b	66.30 ± 3.23 a	1.67 ± 0.08 a	1A
Arsanjan	56.51 ± 2.45 ab	56.14 ± 2.05 c	0.43 ± 0.03 ab	13.41 ± 0.37 d	17.46 ± 0.63 a	8.84 ± 0.24 a	50.66 ± 2.30 bc	1.33 ± 0.05 bc	1A
Kavar	55.00 ± 3.00 ab	71.71 ± 3.28 a	0.45 ± 0.02 a	10.31 ± 0.77 e	12.05 ± 0.55 c	6.43 ± 0.38 c	53.38 ± 3.86 b	1.24 ± 0.05 cd	1A
Sarvestan	53.66 ± 3.19 b	72.14 ± 0.63 a	0.46 ± 0.03 a	7.43 ± 0.49 f	11.18 ± 1.10 c	4.41 ± 0.25 d	39.40 ± 1.82 d	1.16 ± 0.03 d	1A
Tabas	58.74 ± 4.76 ab	56.43 ± 3.89 c	0.41 ± 0.04 ab	17.61 ± 0.92 b	14.61 ± 0.74 b	6.60 ± 0.19 c	45.16 ± 3.52 cd	1.44 ± 0.08 b	1A
Chupanan	57.79 ± 1.85 ab	63.37 ± 4.35 bc	0.42 ± 0.02 ab	15.45 ± 1.24 c	14.00 ± 1.06 b	9.50 ± 0.67 a	67.83 ± 1.88 a	1.39 ± 0.10 bc	1A

Long Arm (LA), Short Arm (SA), Difference of relative length (DRL), Total chromosome length of the haploid complement (HCL), Degree of karyotype asymmetry (A), Intrachromosomal asymmetry index (A_1), Interchromosomal asymmetry index (A_2), Total form percentage (TF%), Arano index of karyotype asymmetry (AsK), Symmetry index (S%), Mean centromeric index (X_{CI}), Mean centromeric asymmetry (X_{CA}), Coefficient of variation of chromosome length (CV_{CL}), Coefficient of variation of centromeric index (CV_{CI}), Asymmetric index (AI), Arm ratio (r), Stebbins karyotype classification (SC). The values are means ± SE. In each column, common letters indicate no significant difference between means (Tukey's HSD test; $P \leq 0.05$).

Principal Component Analysis (PCA) was performed using the chromosomal parameters and asymmetry indices to evaluate the structure of karyotypic diversity and to elucidate relationships among the six ecotypes. The analysis revealed that the first two principal components (PCs) together explained 81.1% of the total variance, with the first component (PC1) accounting for 56.8% and the second component (PC2) explaining 24.3% (Fig. 4).

The biplot visualized the contribution of each variable to the first two principal components revealing distinct biological interpretations for each PC (Fig. 4). PC1 was positively and strongly associated with the indices of intrachromosomal asymmetry, including the intrachromosomal asymmetry index (A_1), the Arano index (AsK%), arm ratio (r), and the degree of karyotype asymmetry (A). Conversely, PC1 showed a strong negative association with the total form percentage (TF%), and the mean centromeric index (X_{CI}). Therefore, PC1 primarily represents a gradient of intrachromosomal asymmetry, in which positive scores correspond to more asymmetric karyotypes.

PC2 showed high positive loadings from the variables reflecting interchromosomal variation, including the difference of relative length (DRL), the interchromosomal asymmetry index (A_2), and the short arm (SA). Thus, PC2 delineates a gradient of heterogeneity in chromosome size within the complement, with higher scores indicating greater disparity in chromosome lengths.

The biplot clearly separated the ecotypes highlighting their distinct karyotypic profiles (Fig. 4). The Darab ecotype was positioned at the extreme positive end of PC1, confirming its status as the most asymmetric karyotype characterized in this study. In contrast, the Sarvestan and Kavar ecotypes were located at the negative end of PC1, visually supporting their highly symmetric karyotypes. The Arsanjan and Tabas

ecotypes were distinctly separated along the positive PC2 axis with high DRL and A_2 values indicating greater heterogeneity in chromosome size. The Chupanan ecotype was placed in intermediate positions showing a combination of moderate asymmetry and chromosome size variation.

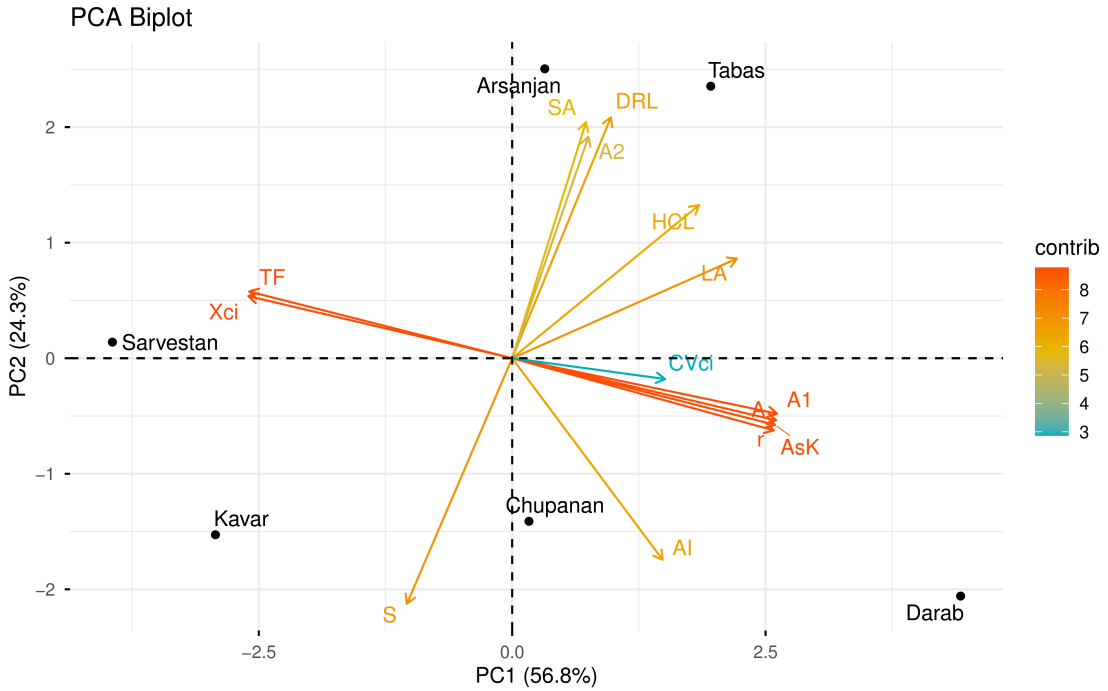


Fig. 4 Principal component analysis (PCA) biplot of six *Zataria multiflora* ecotypes based on chromosomal parameters and karyotype asymmetry indices. Vectors represent karyotype indices including: long arm (LA), short arm (SA), mean arm ratio (r), difference of relative length (DRL), total form percentage (TF%), total chromosome length of the haploid complement (HCL), symmetry index (S%), Arano index of karyotype asymmetry (AsK%), intrachromosomal asymmetry index (A_1), interchromosomal asymmetry index (A_2), mean centromeric index (X_{CI}), degree of karyotype asymmetry (A), coefficient of variation of centromeric index (CV_{CI}), and asymmetry index (AI).

Cluster analysis using Ward's method supported the patterns revealed by PCA, grouping the *Z. multiflora* ecotypes based on overall karyotypic similarity (Fig. 5). The dendrogram showed a primary division of the ecotypes into two major clusters. Cluster I comprised the two most symmetric ecotypes, Kavar and Sarvestan, indicating a highly similar and conserved chromosomal structure. Cluster II contained the remaining four ecotypes, which displayed greater internal karyotypic variation. Within this cluster, Arsanjan and Tabas formed a sub-cluster, suggesting close karyotypic affinity. Chupanan joined this sub-group at a higher distance, followed by Darab, with the most asymmetric karyotype, which fell into the most distinct position within this cluster. This clustering pattern suggests that intrachromosomal asymmetry is a major driver of karyotypic differentiation among *Z. multiflora* ecotypes.

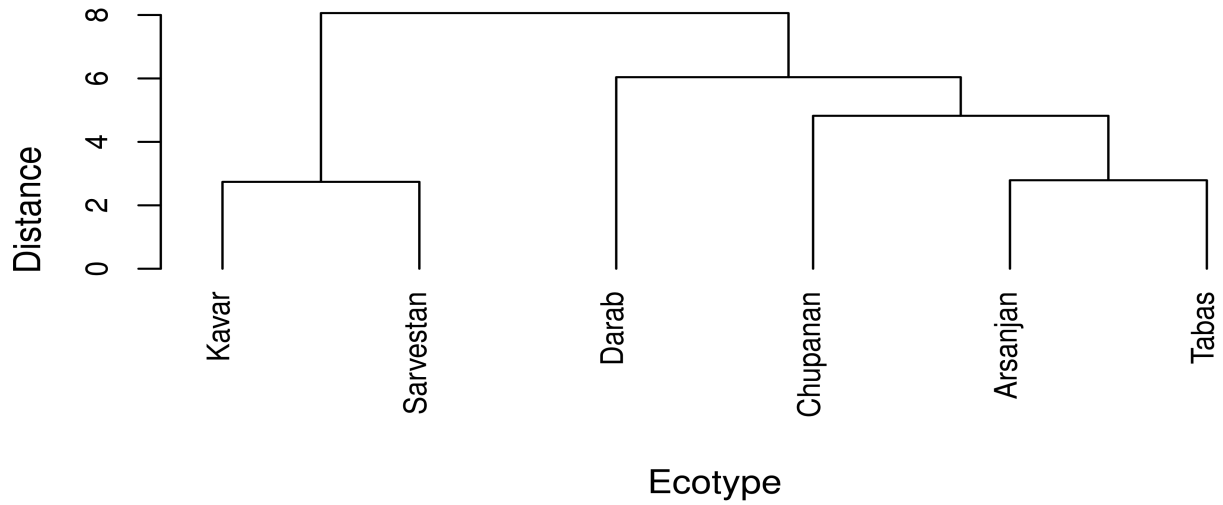


Fig. 5 Dendrogram showing the relationships among six ecotypes of *Zataria multiflora* Boiss. based on chromosomal parameters and karyotype asymmetry indices using Ward's clustering method and Euclidean distance.

DISCUSSION

The present study provides the first comprehensive karyotype identification and characterization of the medicinally significant plant species *Zataria multiflora* Boiss., establishing a fundamental cytogenetic framework for this monotypic genus. The discovery of a consistent diploid chromosome number of 2n = 2x = 30 across all six Iranian ecotypes is in agreement with reports for several species within the Lamiaceae family, particularly in the closely related genus *Thymus* [15, 16]. For instance, *Thymus vulgaris*, a species with notable phytochemical similarity to *Z. multiflora*, commonly possesses 2n = 30 chromosomes, although tetraploid and diploid karyotypes have also been reported in other *Thymus* species such as *T. kotschyanus* and *T. praecox* [24, 25]. The uniform chromosome number observed among the studied ecotypes suggests that polyploidy has not contributed substantially to the cytogenetic differentiation detected in the present material, whereas polyploidy and dysploidy are known as common evolutionary drivers in some other Lamiaceae taxa [14]. The predominance of metacentric and submetacentric chromosomes in Z. *multiflora* further parallels the conserved chromosomal morphology observed in *Thymus*, suggesting broadly similar chromosomal organization between the two genera [15].

Despite the conserved diploid number among the *Z. multiflora* ecotypes, our study detected substantial karyotypic variation at the intraspecific level. This inter-ecotypic variation was quantitatively captured by various asymmetry indices, including A_1, AsK%, and TF%. Indeed, the ecotypes displayed a clear spectrum of chromosomal asymmetry, ranging from the highly symmetrical and exclusively metacentric karyotype of Kavar to the more asymmetric karyotype of Darab, which contained a higher proportion of submetacentric chromosomes. Differences in karyotype asymmetry have long been used to characterize patterns of chromosomal differentiation among plant taxa [12, 31]. The observed variation in asymmetry indices, together with significant differences in centromeric asymmetry (X_{CA}) and the coefficient of variation of the centromeric index (CV_{CI}), indicates that chromosome morphology and centromere position vary among the *Z. multiflora* ecotypes despite the conservation of chromosome number. Similar patterns of karyotypic variation without changes in chromosome number have been reported in other medicinal plant species and are generally associated with chromosome morphological differentiation rather than changes in ploidy level [11]. Furthermore, the occurrence of both relatively symmetrical and asymmetrical karyotypes among the *Z. multiflora* ecotypes indicates measurable cytogenetic differentiation within the species. Although environmental variables were not directly analyzed in the present study, the ecotypes were collected from geographically diverse regions of Iran, including semi-arid regions of southern Iran and arid desert regions of central-eastern Iran that differ in climatic and edaphic characteristics. Therefore, the observed cytogenetic variation may be associated with the environmental heterogeneity across the natural distribution range of the ecotypes. However, the extent to which environmental factors contribute to the observed karyotypic variation remains unclear. Further studies integrating cytogenetic, ecological, and molecular data would be valuable for elucidating the mechanisms underlying karyotype variation in *Z. multiflora*.

The pattern of conserved chromosome number accompanied by variation in karyotype morphology observed in *Z. multiflora* is consistent with findings reported for several other members of the Lamiaceae. Cytogenetic studies in *Salvia* have demonstrated that chromosome evolution within the genus involves both chromosome number changes and variation in karyotype structure among taxa [17]. Similarly, analyses of *Origanum* [18], *Nepeta* [19], and *Satureja* [20] have revealed differences in chromosome morphology, asymmetry indices, and karyotype formulas among species and populations despite relatively conserved chromosome complements. The range of karyotype asymmetry observed among the *Z. multiflora* ecotypes therefore falls within the broader pattern of cytogenetic diversity documented across the Lamiaceae and suggests that variation in chromosome morphology may represent an important component of chromosomal differentiation within the family.

Principal Component Analysis and cluster analysis further demonstrated the existence of karyotypic diversification among the *Z. multiflora* ecotypes. The PCA biplot revealed that intrachromosomal asymmetry affecting centromere position has played a more prominent role in shaping the karyotype diversity of these six *Z. multiflora* ecotypes than interchromosomal heterogeneity affecting overall chromosome sizes. This pattern is consistent with previous cytogenetic studies emphasizing the evolutionary significance of intrachromosomal asymmetry in plants [20, 39]. The cluster analysis revealed clear cytogenetic differentiation among the ecotypes, separating the highly symmetrical ecotypes (Kavar and Sarvestan) from the ecotypes characterized by greater karyotype asymmetry and chromosome-size heterogeneity (Darab, Arsanjan, Tabas, and Chupanan). These groupings are consistent with the variation detected by the asymmetry indices and PCA and indicate the existence of measurable karyotypic diversity within the species.

The conserved diploid chromosome number and relatively symmetrical karyotypes among the *Z. multiflora* ecotypes support the cytological uniformity of this species, whereas differences in chromosome morphology and asymmetry indices provide additional characters that may be useful for distinguishing geographically separated populations and improving the cytogenetic resolution of taxonomic studies. From a breeding perspective, characterization of chromosome number and karyotype structure represents an essential baseline for future investigations of genetic diversity and the utilization of divergent germplasm. Similarly, documenting cytogenetic variation among natural ecotypes contributes to the characterization and conservation of the species' genetic resources. However, direct relationships between karyotypic variation, adaptive significance, and breeding performance require further investigation integrating cytogenetic, molecular, ecological, and breeding approaches.

Although the present study establishes a valuable cytogenetic baseline for *Z. multiflora*, analysis of a larger number of metaphase plates would permit a more comprehensive assessment of within-ecotype cytogenetic variability. Indeed, broader population sampling and the application of molecular cytogenetic techniques, such as fluorescence in situ hybridization (FISH), would provide additional insights into chromosome organization and evolutionary relationships within the species.

CONCLUSION

The six Iranian ecotypes of *Zataria multiflora* exhibited a conserved chromosome number (2n = 30), indicating cytological stability at the species level. Based on the Stebbins karyotype classification, all six ecotypes exhibited symmetrical karyotypes. However, significant variation in chromosome morphometric parameters and asymmetry indices revealed the presence of measurable cytogenetic differentiation among these

geographically diverse ecotypes. Multivariate analyses further highlighted clear cytogenetic differentiation among the studied ecotypes. Principal component analysis indicated that intrachromosomal asymmetry contributed more strongly to karyotypic differentiation than variation in chromosome size. Consistent with these results, cluster analysis resolved two major groups, separating the more symmetrical ecotypes from those exhibiting relatively greater karyotype asymmetry. Collectively, these findings contribute to a better understanding of chromosome organization and karyotype variation in this medicinally important species and provide a useful cytogenetic baseline for future studies of *Z. multiflora*.

Consent for Publication
Not Applicable.

Data Availability
Not Applicable.

Conflict of Interests
The authors have not declared any conflict of interests.

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Authors' contributions
M. I.: Investigation; Formal analysis; Visualization; Writing- original draft preparation. S. S.: Investigation. H. R.: Conceptualization; Supervision; Validation; Writing- review and scientific editing. V. R., M. E., and A. A.: Writing- review and scientific editing.

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