

Karyotype analysis in some accessions of Iranian borage (*Echium amoenum*)

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Abstract

The Iranian borage (*Echium amoenum*) is a biennial or perennial herb belonging to the family Boraginaceae. It is distributed mostly in the north of Iran and on the slopes of Alborz mountains. As a medicinal herb, Iranian borage is used for treatment of depression and some nervous diseases. Though many studies have focused on morphological and phytochemical diversity of various accessions of this plant, the number of studies focusing on cytotaxonomy of different accessions of this species is rather small. Thus, the present study focused on cytogenetic analysis of several accessions of Iranian borage. The terminal meristem of root tips was placed in a 0.002 mole 8-hydroxyquinolone for 4 hours, and then fixated in a fixator solution (3:1 acetic acid/alcohol) for 24 hours. The root tips were finally softened in 1 mole hydrochloric acid for 8 minutes in room temperature, dyed with 2% aceto-orcein stain for 10 minutes, and squashed on microscopic slide. The cells were photographed at the metaphase stage of mitosis, when the chromosomes were well separated. Long arm, short arm, long to short arm ratio and chromosome indices of metaphase cells were calculated. Figures summarized the idiogram based on the short arm length of eight pairs of chromosomes. According to our results, a basic chromosome counts of $x=8$ ($2n=2x=16$) was determined for all tested accessions. All analyzed plant cytotypes were diploid. The largest total chromosome length belonged to the accession from Jannat Roodbar region with 78.16 μm , while the shortest came from Esfahan with 51.10 μm . Analysis of metaphase karyotypes showed that centromeres were mainly located in the center (metacentric) or close to the center (submetacentric) of the chromosomes. The number of metacentric chromosomes was 10-12 out of 16 chromosomes. Accession of Roobdar had the largest centromeric index, i.e. 0.66, while accessions from Divrood, Qazvin and Mashhad had the lowest centromeric indices of 0.38-0.39. In addition, the long to short arm ratio varied between 1.51 and 1.66. Tested accessions showed lower diversity in terms of karyological characteristics and had symmetric karyotypes. However, given asymmetry indices in terms of chromosome types, overall chromosome shape, classification based on cluster and principal component analyses, and inclusion within three classes of Stebbins (A3, B2, B3), the studied accessions showed evolutionary differences with each other that could be a source of useful cytogenetic information for breeding purposes, and may be used to further understanding of evolution and accession genesis of Iranian borage species.

Keywords: Chromosome; Iranian borage; Karyotype; Metacentric; Submetacentric.

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Introduction

The main role of cytogenetic is to determine quantitative information on the evolutionary history of the plant, cytological evaluation, specification and species affinity of the plant. Some of this information is necessary to achieve the breeding goals and determine the appropriate strategy. Differences in the shape and size of the chromosomes can indicate a genetic difference

(Sheidai *et al.* 2002). Morphology of chromosomes is in fact one of the best criteria for the study of taxonomic relationships (Stebbins 1971). To accurately study the inter-species distances in a genus, counting the number of chromosomes, observing chromosomal similarities and preparing karyotype is necessary (Alishah and Omidi 2008).

Karyotype studies on accessions of a species or genus are very important, because various

accessions of each species have their special genomic adaptations with the environmental conditions in which they grow (Sheidai 1998). Karyotype may vary like other systematic traits, but in general, one karyotype may signify a species or even a genus.

A cytogenetic analysis is considered a primary step in eugenics and determining the number of chromosomes. Ploidy level is very critical for design of the proper gene transfer methods in breeding plans for transferring desirable genes of various species in a given species. Gene transfer is more successful between species with higher chromosomal similarities (McCoy and Echt 1992).

Iranian borage (*Echium amoenum*) is biennial or perennial herb belonging to family Boraginaceae (Soltani 2005). Its natural habitats range from 60 to 220 m above sea level (Rechinger 1967). Its major habitats are in north of Iran, e.g. Gorgan, Ramsar (Javaherdeh), Lashkan and Ashkevar (Grieve 2012). Iranian borage is a medicinal herb used to treatment of inflations, and as tranquilizer (Amin 2004) and anticonvulsant (Heidari *et al.* 2006).

There is little information on cytogenetic of *E. amoenum*. This experiment would provide additional information for identification of Iranian borage karyotype of seven accessions from various regions of Iran. The karyotype analysis provides numerical data obtained from the chromosome images.

Materials and Methods

Plant Materials

The seeds of landraces of Iranian borage were collected from different regions of Iran, including

Jannat Roodbar, Javaherdeh, Divrood, Qazvin (Alamut), Mashhad, Shahrood and

Esfahan, from late spring to summer. The seeds were treated with 10% (v/v) sodium hypochlorite (Whitex) + few drops of Tween solution for 15 minutes at room temperature; then were washed three times with distilled water, 5 minutes for each time. The water-absorbed seeds were placed in Petri dishes fitted with a wet filter paper and then transferred to germinator with constant temperature of 25 °C. When the length of the radicle reached to about 1.5 cm, it was cut and pre-treated to increase the number of metaphase cells.

Pre-treatment

For pre-treatment, the samples were treated with a solution of 2 mM 8-hydroxyquinoline. Due to increased mitotic divisions in the terminal meristem tissues of the roots, the root tips (3-5 cm in length) were taken between 8:00 and 11:30 a.m. to prevent terminal meristem from damage. To hold the cell division process in the metaphase stage and improve the metaphase index, the taken radicles were first washed in distilled water and 0.002 mole 8-hydroxyquinoline and then placed inside refrigerator at 4 °C for 4 hours.

Fixation and storage

Once the pre-treatment phase was completed, the radicles were washed with distilled water for 5-10 minutes and then treated in a fixator solution for 24 hours. The fixator solution consisted of glacial acetic acid and 96% alcohol ethanol with the ratio of 3:1. When the radicles were fixated, they stored inside the same solution at room temperature for several days. The bottles containing the fixated

materials were closed to prevent alcohol evaporation. Once out of the fixating solution, the radicles were first washed in distilled water for 10 minutes; then dried with filter paper, and immediately placed inside bottles containing 70% alcohol. The samples remained at 2-5 °C inside refrigerator until they were used.

Hydrolysis and staining

Fixated roots were placed in hydrochloric acid (HCl 1 N) for 7-10 minutes at 20-25 °C. They were then taken out of the hydrolysis solution and washed again with distilled water. Acetocarmine 2% was used for staining. About 1-2 ml of dying solution was added to the root samples, washed and dried after hydrolysis, and stored in refrigerator for 24-48 hours (Mousavizadeh *et al.* 2016).

Microscopic Analysis

One drop of 45% glacial acetic acid was placed on a clean film. Root caps were separated by scalpel, and 1-2 mm of root was placed on the film as sample. The tissues were then crushed with a needle and another film was placed on the sample. The slide was softly beaten with the end of a pen to separate the cells and make them dispersed under the slide. The sample evaluated by microscope for primary analysis aimed at finding metaphase cells. An optical microscope was used at low magnification (objective lens 10, eyepiece lens 10) to find locations containing suitable cells, then 100x magnification (objective lens 100 with immersion oil) was used to photograph the cells.

Data Analysis

For karyotype analysis, suitable metaphase photos

were selected and saved with bmp file extension. Required measurements were performed with Micromasure software (Reeves 2001). The information produced by this procedure was saved in Excel. For each population the following karyotypic features were recorded for analysis: length of longest arm (L), length of shortest arm (S), mean chromosome length (CL), total chromosome length (TCL), ratio of L to S (L/S), centromere index (CI) which indicates the ratio of S to TCL. Type of chromosome was determined according to Levan *et al.* (1964). Populations were classified by the Stebbins bidirectional table. The percentage of total karyotype shape (TF%) was calculated as follows:

$$\text{Total Percent of Shape (Huziwara *et al.* 1962)} = \\ (\text{total length of short arms/TCL}) \times 100$$

Principal component analysis was performed to draw two-dimensional diagrams. Furthermore, cluster analysis based on cytological data was carried out using the Ward method and Euclidean distances. Means were compared by Duncan's Multiple Range Test at 5% probability level. Statistical analyses were performed by SAS v.9.1 and SPSS v.21. Diagrams were drawn by Excel.

Results and Discussion

Analysis of variance of studied traits showed significant differences among accessions in terms of length of short arm (S), length of long arm (L), chromosome length (CL) and centromere index (CI). This was despite the fact that the arm ratio and polymorphism percent among accessions were not statistically significant (Table 1).

Our research showed that all studied accessions of Iranian borage were diploid and had 16 chromosomes (Figure 1). Works of Babakhanzadeh Sajirani (2016) also has indicated that all studied accessions were diploid with 16 chromosomes. There was no difference among accessions in terms of chromosome count and diploid level, but chromosome size was different among various accessions. Such changes in chromosome size among various accessions seemed normal. It may thus be deduced that the evolution process had no similar effect on chromosome morphology in various geographical regions. In terms of short arm length, there were differences among accessions. The longest length of short arm was in accessions of Jannat Roodbar (1.95 μm) and Javaherdeh (1.86 μm), and minimum short arm length was in accession of Esfahan (1.20 μm). In terms of long arm length, maximum long arm length was observed in Jannat Roodbar accession and the minimum length in Mashhad and Esfahan accessions. The mean long arm lengths of Javaherdeh, Divrood, Qazvin and Shahrood accessions were in the medium range. Variations in length of short arm was 1.20 to 1.95 μm and for the long arm was from 1.98 to 2.93 μm . It seems that structural variation was higher in length of long arm rather than the short arm. Comparison of mean chromosome length (CL) showed that the accessions of Jannat Roodbar and Esfahan had maximum and minimum CLs, respectively. Thus, Esfahan accession had smaller chromosomes and Jannat Roodbar and Javaherdeh accessions had larger chromosomes. Mean centromeric index (CI) was maximum for

Jannat Roodbar (0.66), Esfahan (0.61) and Javaherdeh (0.59), and minimum for Qazvin, Divrood and Mashhad (ranging from 0.38 to 0.39). Therefore, accession of Jannat Roodbar showed good similarities to those of Javaherdeh and Esfahan in terms of CI (Table 2). Hosseinpour Azad *et al.* (2011) studied eight accessions of Iranian borage in the northern and northwestern regions of Iran, and their works showed maximum similarity between Qazvin and Lorestan and minimum similarity between Jannat Roodbar and Gorgan accessions. Mirjani *et al.* (2004) speculated that the difference in chromosome size among various accessions of a given species may be associated with the quantitative changes in DNA. In view of our results, it might be concluded that there is a rather fine genetic diversity among accessions of Iranian borage.

Karyotype symmetry parameters

Results of estimated parameters of karyotype symmetry are provided in Table 3. In terms of karyotype formula, accessions were metacentric and subcentric. Thus, accessions of Jannat Roodbar, Divrood, Qazvin, Mashhad and Shahrood were the most symmetric. Works of Babakhanzadeh Sajirani (2016) showed that chromosomes of Iranian borage are of metacentric and subcentric type, and in the studied accessions, chromosomes were of the same type. The number of metacentric chromosomes were higher than subcentric ones. Metacentric chromosome count was highest in accessions of Jannat Roodbar, Divrood, Qazvin, Mashhad and Shahrood in comparison to Esfahan and Javaherdeh accessions.

Table 1. Analysis of variance of chromosome characteristics of Iranian borage accessions.

Source of variation	Degrees of freedom	Short arm length	Long arm length	Chromosome length	Arm ratio	Centromere index	TF
Accession	6	0.20*	0.30**	0.99**	0.009 ^{ns}	0.043*	3.8 ^{ns}
Error	14	0.049	0.037	0.142	0.026	0.01	6.21
CV (%)	-	13.97	7.48	9.24	10.25	20.57	6.40

ns, * and **: non-significant and significant at 5 and 1% probability levels, respectively; TF: percentage of total karyotype shape.

Table 2. Mean comparison of measured chromosome characteristics of Iranian borage accessions.

Accessions	Short Arm Length (μm)	Long Arm Length (μm)	Chromosome Length (μm)	Arm Ratio	Centromere Index	TF (%)
Jannat Roodbar	1.95a	2.93a	4.88a	1.54a	0.66a	39.67a
Javaherdeh	1.86a	2.68ab	4.55ab	1.51a	0.59a	40.93a
Divrood	1.66ab	2.65ab	4.31ab	1.60a	0.38b	38.48a
Qazvin	1.58abc	2.45bc	4.04bc	1.54a	0.39b	39.30a
Mashhad	1.37bc	2.18cd	3.55cd	1.62a	0.38b	38.55a
Shahrood	1.53abc	2.51bc	4.05bc	1.63a	0.47ab	37.89a
Esfahan	1.20c	1.98d	3.19d	1.66a	0.61a	37.64a

Means with similar letters in each column are not significantly different at 5% probability level, using Duncan's Multiple Range Test; TF: percentage of total karyotype shape.

The TF of chromosomes was calculated to be 37-40% (Table 2), which indicated the accessions were similar in terms of evolutionary level. The TF of chromosomes is used for comparison and analysis of karyotype symmetry, karyotype relations between species and genera and as an index for karyotype classification (Pazco 2006). This factor has a positive relationship with karyotype symmetry, and is calculated from the ratio of total length of short arm to the total length of all chromosomes. The closer the TF to 50, the larger the number of metacentric chromosomes in relation to other chromosome modes. If the value approaches zero, the number of acrocentric and telocentric chromosomes is larger and the karyotype is of asymmetric type. In this experiment, the large geographical distances between the accessions of Iranian borage was not considered as a restricting factor on the gene flow among accessions and distinctions of accessions. Rather, the relationships among people from

various provinces of Iran caused a gene flow among accessions of Iranian borage. Furthermore, the highest total chromosome length belonged to Jannat Roodbar accession (78.16 μm), and the lowest belonged to Esfahan accession with 51.10 μm (Table 3). The topic of karyotype symmetry is one of the major factors in studying species evolution rooted in chromosome studies. To perceive the importance of karyotype asymmetry, the relationship of chromosome asymmetry with other traits such as morphological characteristics needed to be studied at several environmental conditions.

According to the bidirectional table of Stebbins (1971), the studied Iranian borage accessions were classified into three classes of 3A, 3B and 2B (Table 4). Stebbins bidirectional table is regarded as one of the most effective tools for classification of karyotypes, for it turns asymmetric from left to right and from top to bottom (Stebbins 1971).

Table 3. Number of somatic and base chromosomes, ploidy level and karyotype formulae for Iranian borage.

Accessions	2n	X	Formula	Total chromosome length (μm)
Jannat Roodbar	16	8	12m+4sm	78.16
Javaherdeh	16	8	10m+6sm	72.83
Divrood	16	8	12m+4sm	68.99
Qazvin	16	8	12m+4sm	64.75
Mashhad	16	8	12m+4sm	56.89
Shahrood	16	8	12m+4sm	64.82
Esfahan	16	8	10m+6sm	51.10

m = metacentric, sm = submetacentric.

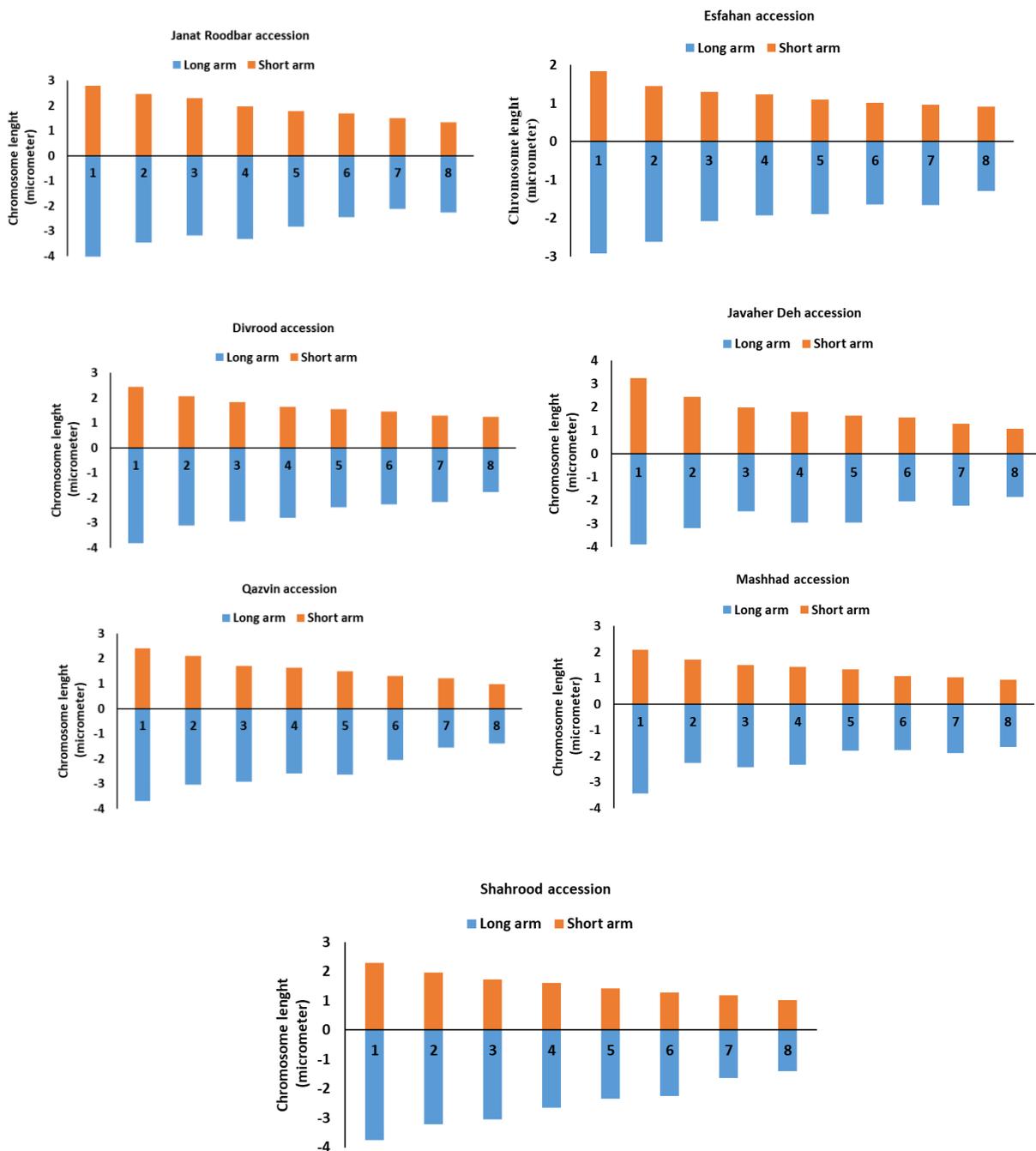


Figure 1. Idiogram of haploid chromosomes of Iranian borage accessions at metaphase stage.

Table 4. Karyotype symmetry (Stebbins bidirectional classification) in accessions of Iranian borage.

Ratio of chromosomes with L/S above 2		Longest/shortest chromosome		
1	0.51-0.99	0.01-0.5	0.00	
4A	3A, Jannat Roodbar	2A	1A	2>
4B	3B, Qazvin	2B, Esfahan, Javaherdeh, Divrood, Mashhad, Shahrood	1B	2-4
4C	3C	2C	1C	4<

Multivariate analysis

Based on cluster analysis (Figure 2) the accessions were divided into three main clusters at the Euclidean distance of about 11. The first cluster contained accessions of Qazvin and Shahrood with mean chromosome length, total chromosome length and Stebbins classification serving as main factors in their grouping. The second cluster included Javaherdeh, Divrood and Jannat Roodbar accessions which were grouped due to ratio of short to long arm and long to short arm. The third group included Mashhad and Esfahan accessions; their common traits being TF, mean chromosome length and length of the short arm.

The 2D diagrams were drawn based on the first and second principal components. Results of 2D diagram corresponds results of cluster analysis to a large extent (Figure 3). Samples of Esfahan, Mashhad, and Shahrood were placed in group A, while samples from Jannat Roodbar, Javaherdeh, Divrood and Qazvin were placed in group B. Movement from positive to negative side of PCA1 (from group B to group A) results in gradual increase of ratio of short to long arm, decrease of chromosome length, decrease of length of short and long arms.

The study of accessions demonstrated that they were different mainly in terms of chromosome length. Chromosome length of Jannat Roodbar accession was maximum (4.85 μm), but Esfahan

accession had a minimum chromosome length of 3.12 μm . Results showed that geographical conditions affected chromosome length. In reality, plant breeding requires more general or specific cytological methods and information for classification of populations. This information is related to microscopic structures, number and diversity of chromosomes and chromosome behavior. Chromosomal differences reflect the genetic differences, but morphological, physiological and chemical differences reflect the differences about the gene products, which are subject to variation due to environmental effects. Accessions of a given species have their own specific genome adapted to the environment in which they grow, which may result in the appearance of new varieties or even new species in the habitats. Thus, chromosomal studies may be helpful in revealing the evolutionary processes of plant species.

Conclusions

The present study was carried out to study the cytological features including chromosome number and karyotypic characteristics of seven accessions of Iranian borage. Basic chromosome number was determined as $x = 8$ ($2n = 2x = 16$). All studied accessions were diploid. The highest total chromosome length belonged to the accession from Jannat Roodbar region with 78.16 μm , while

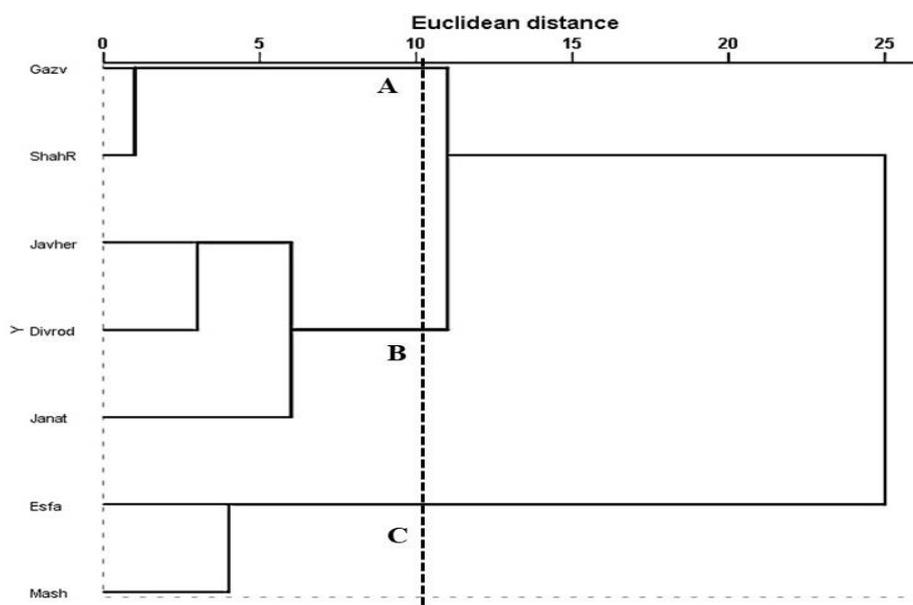


Figure 2. Dendrogram of Iranian borage accessions grouped based on cytological traits using Ward method and Euclidean distance.

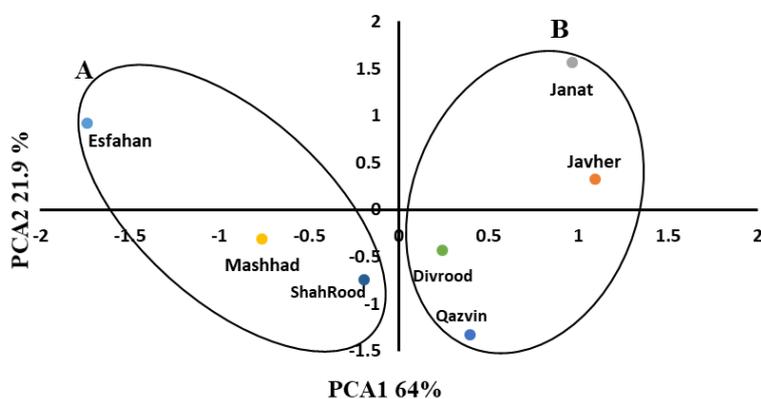


Figure 3. Scores of the first two principal components (PCA) for seven accessions of Iranian borage.

the shortest came from Esfahan with $51.10 \mu\text{m}$. The long arm of chromosome ranged from 1.98 to 2.93 and the short arm measured between 1.20 to 1.95 micrometer. Metaphase karyotype analysis revealed that centromeres were mainly located at the middle (metacentric) or near the middle (submetacentric) of chromosomes. The karyotype

formulae were obtained as $12\text{ m} + 4\text{ sm}$ for Jannat Roodbar, Shahrood, Divrood, Qazvin and Mashhad, and $10\text{ m} + 6\text{ sm}$ for Javaherdeh and Esfahan accessions. Accession of Jannat Roodbar had the largest centromeric index, i.e. 0.66. Accessions from Divrood, Qazvin and Mashhad had the minimum centromeric indices of 0.38-0.39.

In addition, the long to short arm ratio varied between 1.51 and 1.66. The studied accessions were evolutionary located in one level according to asymmetrical karyotype, value of TF and separating to 3B and 2B classes (except Jannat Roodbar in 3A class) based on Stebbins categories.

The karyological data may provide useful information to better understand the taxonomy, evolution and speciation in the genus *Echium* and to identify candidate species for breeding programs.

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مطالعه کاربوتیبی چند اکوتیپ گل گاوزبان ایرانی (*Echium amoenum* Fisches & Mey.)

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۲- مرکز تحقیقات کشاورزی شاهرود، شاهرود.

چکیده

گل گاوزبان ایرانی گیاهی دو یا چند ساله است و به خانواده Boraginaceae تعلق دارد. پراکندگی آن بیشتر در شمال ایران و دامنه زاگرس است. گل گاوزبان به عنوان یک گیاه دارویی در درمان بیماری‌های افسرده‌گی و اعصاب مورد استفاده قرار می‌گیرد. تحقیقات بسیاری روی تنوع مورفولوژیکی و فیتوشیمیایی گونه‌های مختلف جنس گل گاوزبان صورت گرفته است ولی مطالعه‌های سیتوتاکسونومی کمی روی گونه‌های مختلف این جنس انجام شده است. بنابراین، در این تحقیق به بررسی سیتوژنتیکی اکوتیپ‌های مختلف گل گاوزبان ایرانی پرداخته شده است. برای انجام آزمایش، مریستم‌های انتهایی نوک ریشه ابتدا با $0.02 \times$ مول ۸-هیدروکسی کوئینولین به مدت چهار ساعت قرار گرفتند و سپس در مدت زمان ۲۴ ساعت در محلول فیکساتور (۱:۳ استیک اسید/الکل) در دمای ۴ درجه سانتی‌گراد تثبیت شدند. در نهایت نوک ریشه با اسید هیدروکلریک یک مول در دمای اتاق به مدت ۸ دقیقه نرم شده و به مدت ۱۰ دقیقه با استفاده از رنگ اورسینین ۲ درصد رنگ‌آمیزی گردید و در ادامه روی لام اسکواش شد. سلول‌ها در مرحله متافاز میتوز، که دارای کروموزوم‌های خوب جدا شده بودند، عکس‌برداری شدند. بازوی بلند، بازوی کوتاه، نسبت بازوی بلند به بازوی کوتاه و همچنین شاخص‌های کروموزوم از سلول‌های متافازی محاسبه شدند. بر اساس نتایج به دست آمده، تعداد کروموزوم پایه ۸ ($2n=2x=16; x=8$) بین اکوتیپ‌ها مورد تایید قرار گرفت. تمام توده‌های مورد مطالعه، دیپلوئید بودند. بزرگترین طول کل کروموزوم متعلق به اکوتیپ جنت-رودبار یعنی $78/16$ میکرومتر بود و کوچکترین طول کل کروموزوم در توده اصفهان به میزان $51/10$ میکرومتر ثبت گردید. آنالیز کاربوتیب متافاز نشان داد که سانترومرها به‌طور عمده در وسط (متاسنتریک) یا نزدیک وسط (ساب‌متاسنتریک) کروموزوم قرار دارند. تعداد کروموزوم‌های متاسنتریک بین ۱۰ و ۱۲ از کل ۱۶ جفت کروموزوم متغیر بود. اکوتیپ جنت-رودبار با داشتن نسبت 0.66 از بزرگ‌ترین نسبت شاخص سانترومری برخوردار بود. از طرف دیگر اکوتیپ‌های دیورود، قزوین و مشهد با داشتن نسبت $0.39 - 0.38$ حداقل شاخص سانترومری را داشتند. همچنین نسبت بازوی بلند به کوتاه بین $1/51$ تا $1/66$ متغیر بود. اکوتیپ‌های مورد آزمایش به لحاظ خصوصیات کاربوتیبی تنوع کمتری داشته و از کاربوتیب متقارنی برخوردار بودند. اما، با توجه شاخص‌های عدم تقارن از نظر تیپ کروموزوم، درصد شکل کلی کروموزوم، طبقه بندی بر اساس تجزیه‌های خوشه‌ای و مولفه‌های اصلی و قرارگیری در سه کلاس A۳، B۳ و B۲ طبق کلاس‌بندی استبینز، اکوتیپ‌های مورد بررسی از نظر تکاملی تفاوت‌هایی با یکدیگر داشتند. این تفاوت‌ها با فراهم کردن اطلاعات سیتوژنتیکی، می‌تواند در برنامه‌های اصلاحی برای درک بهتر طبقه‌بندی، تکامل و شکل‌گیری اکوتیپ‌ها مورد استفاده قرار گیرد.

واژه‌های کلیدی: ساب‌متاسنتریک؛ کاربوتیب؛ کروموزوم؛ گل گاوزبان ایرانی؛ متاسنتریک.