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Genetic diversity of *Satureja bachtiarica* Bunge species collected from north-west Iran

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Abstract: Sustained availability of genetic resources is essential for successful plant breeding. *Satureja bachtiarica* Bunge is an endemic species to Iran, widely dispersed throughout the country. The rocky mountainous terrain between Asalem and Khalkhal, situated in the Ardabil province, is one of them. The current research focused on the genetic classification of 11 different ecotypes of *S. bachtiarica* collected from northwest Iran using RAPD molecular markers. A significant genetic diversity was observed among the ecotypes, with 64 bands displaying substantial polymorphism. These polymorphic bands served as the foundation for genetic analyses conducted using NTYSYS-pc (2.02e) software. The genetic distance between the ecotypes was determined using the Dice similarity coefficient. Subsequently, a dendrogram was constructed based on the similarity matrix data, employing the unweighted pair-group method with arithmetic averages (UPGMA). Genetic clustering analysis of the molecular marker data from 11 studied ecotypes revealed the lowest genetic similarity among the Khoy and Ardabil ecotypes. In contrast, the Khalkhal and Meshgin Shahr ecotypes exhibited the highest similarity. After examining the dendrogram, it becomes clear that most clusters consist of ecotypes sharing entirely similar or relatively similar climatic conditions. This suggests that the molecular diversity outcomes align with the geographical diversity of the ecotypes.

Keywords: *Satureja bachtiarica*, RAPD molecular marker, classification, genetics distance.

Introduction

Rising global demand, fueled by population growth, necessitates increased food production. However, challenges such as genetic resource erosion and climate change loom large. Acknowledging the necessity to preserve botanical diversity signifies an increasing awareness that effective management of natural resources is crucial for sustained utilization (Arzani and Ashraf, 2016). The herbal species *Satureja bachtiarica* Bunge commonly referred to as Bakhtiari savory, exhibits a broad distribution across Iran, with notable prevalence in the western, central, and southwestern provinces. Bakhtiari savory (*S. bachtiarica*), a member of the *Lamiaceae* family, holds significance as a crucial Iranian herb. It is characterized by leaves folded lengthwise and arranged in a rectangular, linear form along the stem. The leaves, flowers, and calyx of *S. bachtiarica* contain secretory glands that hold essential oils. An Iranian accession of *S. bachtiarica*, has essential oil with phenolic compounds such as carvacrol (31.25%), thymol (23.50%), and o-cymene (13.87%) in the aerial parts during the flowering (Fathimoghaddam et al., 2020). Phenolic compounds, such as carvacrol and thymol, abundantly present in the *Lamiaceae* family, exert health-promoting effects on human beings (Soleimani et al., 2022).

Assessing genetic diversity is an effective means of exploiting germplasm resources for a breeding program. Genetic distance, whether determined by the frequency of different genotypes (genotypic distance) or the frequency of different alleles at specific gene locations (genetic distance), is directly linked to the phenomenon of heterosis (El Hafid et al., 2002). An investigation into the molecular analysis of selected *Thymus daenensis* clones revealed significant differences among them, empowering breeders with the ability to select and cultivate clones aligned with breeding objectives. The study also identified several ISSR markers associated with agro-morphological traits and phytochemicals (Heydari et al., 2019). In breeding programs, selecting parents with minimal similarity typically results in increased heterosis. Genetic distance, a multivariate statistical method calculated using various measurable traits, proves valuable in assessing gene or genotypic distance for genetic diversity evaluations.

Many studies have delved into evaluating genetic diversity and its application in breeding different plants (Zhang et al., 2010; Salehi et al., 2018; Baba Nitsa et al., 2023). The RAPD-PCR method, aside from its utility for estimating genetic distance and kinship relationships, can be effectively employed in the identification and classification of plant genotypes (Li et al., 2007). In a study, fifty-seven genotypes from eight populations of *S. bachtiarica* were evaluated using 15 ISSR markers and 11 RAPD markers. DNA profiling using RAPD primers resulted in the amplification of 84 loci, of which 81 were polymorphic, yielding an average of 7.36 polymorphic fragments per locus. Conversely, ISSR primers generated 136 bands, with 134 being polymorphic, and an average of 9.06 pieces per primer (98.52 percent) (Saidi et al., 2013). In a study, the diversity of morphological and phytochemical traits among Iranian and exotic populations of Marzeh (*Satureja hortensis*) grown in field conditions was examined. The research revealed that various Iranian and exotic populations of Marzeh exhibit substantial genetic diversity, presenting valuable potential for utilization in breeding programs. Specifically, cultivars originating from Khuzestan, Greece, and Uzbekistan were recommended based on their notable performance (Fathi et al., 2021). The current study aims to genetically classify various ecotypes of *S. bachtiarica* using RAPD molecular markers

Materials and Methods

Plant materials

Eleven ecotypes of *S. bachtiarica* were used in this study (Table 1). The seeds were cultivated in the plastic pots. At the five-leaf stage, fresh and young leaf tissues were collected and kept at 4°C during transportation to the laboratory. The plant materials were subsequently washed with distilled water to eliminate external contamination and allowed to dry at room temperature before being used for DNA extraction. To check the genetic diversity, DNA extraction was performed using the STE method (Azad and Nematadeh, 2013). The reagents and chemicals used for DNA extraction were Merck products.

Table 1. Eleven *Satureja bachtiarica* accessions used along with their collection area attributes.

No.	Accessions	Province	Code	N Latitude	E Longitude
1	Malekan	East Azerbaijan	M	37.148670	46.094492
2	Marand	East Azerbaijan	R	38.444456	45.749005
3	Ahar	East Azerbaijan	A	38.474632	47.084016
4	Sarab	East Azerbaijan	S	37.943017	47.513255
5	Ardabil	Ardabil	B	38.225497	48.142412
6	Meshgin Shahr	Ardabil	N	38.391922	47.647310
7	Khalkhal	Ardabil	G	37.625130	48.515529
8	Astara	Ardabil	L	38.454065	48.855583
9	Arshag	Ardabil	I	38.729863	48.030296
10	Khoy	West Azerbaijan	X	38.535244	44.968960
11	Mako	West Azerbaijan	K	39.283039	44.444270

The quality and quantity of the extracted genomic DNA were evaluated using two methods, spectrophotometry and electrophoresis on a 0.8% agarose gel. In the electrophoresis of DNA on a 0.8% agarose gel, four microliters of extracted DNA were mixed with two microliters of loading buffer and discharged into the wells of the agarose gel under TBE buffer conditions. The agarose gel was electrophoresed with a constant voltage of 85 for one hour. After staining with ethidium bromide, the DNA was observed under UV light and photographed in the Gel-doc device of the American UVP company. Following the interpretation of the gel, qualitatively and quantitatively suitable samples were selected as template DNA for PCR.

RAPD analysis

The RAPD marker used in this study was based on the method of Monika et al. (2006), which was optimized with some modifications to the PCR reaction. The 14 RAPD primers used in this project were obtained from Sinaclon company. The primers had 5-7 OD at 260 nm wavelength.

Table 2. List of RAPD primers and their sequences.

No.	Primer	Number of nucleotides	Sequences
1	OPD-03	10	5'-GTCGCCGTCA-3'
2	OPD-05	10	5'-TGAGCGGACA-3'
3	OPB-05	10	5'-CAGGCCCTTC-3'
4	OPB-13	10	5'-TTCCCCCGCT-3'
5	OPB-14	10	5'-TCCGCTCTGG-3'
6	OPB-20	10	5'-GGACCCTTAC-3'
7	OPA-01	10	5'-CAGGCCCTTC-3'
8	OPA-10	10	5'-CTGCTGGGAC-3'
9	OPA-04	10	5'-AATCGGGCTG-3'
10	OPH-20	10	5'-GGGAGACATC-3'
11	OPH-04	10	5'-GGAAGTCGCC-3'
12	OPH-15	10	5'-AATGGCGCAG-3'
13	OPH-05	10	5'-AGTCGTCCCC-3'
14	OPH-01	10	5'-GGTCGGAGAA-3'

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All primers were prepared as lyophilized. The names of the primers along with their ten nucleotide sequences are given in Table 2.

PCR condition and data analysis

All the chemicals mentioned in this section were obtained from Sinaclon company, whose specifications are given in Supplementary Table 1.

To enhance the reproducibility of RAPD markers, all necessary precautionary factors related to PCR reaction conditions were taken into consideration. These factors include DNA quality and quantity, the prevention of biological contamination from *Taq* DNA polymerase, primer and magnesium chloride concentrations, pipetting accuracy, and PCR conditions to perform PCR reactions. The equipment used in the autoclave was sterilized at a temperature of 121 °C and a pressure of 1.5 bar for 15 minutes. The main reaction mixture was prepared as outlined in Supplementary Table 1.

The PCR apparatus used in this project was a T100 model (T100 Thermal Cycler, Bio-Rad) with gradient capabilities. The set program was the same for all primers, except for the annealing temperature, which was determined by the melting temperature of the primers (Supplementary Table 2). The results obtained by molecular markers the GC method were used to estimate the binding temperature of primers to single-stranded DNA. Many companies provide these specifications along with primers to researchers. In this project, the PCR program was adjusted according to the method of Monika et al. (2006).

After the completion of the PCR, a 1.5% agarose gel was used to verify the PCR products. We utilized agarose from Sigma-Aldrich in our experiment. Ten microliters of the mixture of PCR product and loading buffer were dispensed into each of the wells and subsequently loaded onto the gel. We used the weight indicator marker 3 (Lambda DNA / *EcoR* I+*Hind* III from Thermo Fisher Scientific Inc.) to determine the size of the bands in the gel. Electrophoresis conditions were set at 80 V for 1.5 hours. After the electrophoresis and staining of the gel with ethidium bromide, the gel was washed with distilled water, and the DNA was observed and photographed under the light (UV) using UVP gel documentation systems exemplified in Figure 1. The amplification products were assessed for the presence (1) or absence (0) of bands, and a binary matrix for RAPD markers was constructed. The software NTSYS-pc 2.02e was used for genetic analysis. A numerical value for the similarity coefficients was determined, ranging between zero and one, where zero indicates the absence of common bands (genetic dissimilarity) and one indicates identical band patterns (complete genetic similarity). The similarity matrix was entered into the SAHN clustering for dendrogram construction using the UPGMA algorithm.

Results

Fourteen PAPD primers were used in this study to assess the diversity of 11 NW Iranian *S. bachtiarica* ecotypes. Figure 1 represents the electrophoretic profile of agarose gel PCR products, of 11 ecotypes of *S. bachtiarica* using OPD-03 RAPD primer, illustrating the genetic variability among the genotypes analyzed. The Dice similarity coefficient approach was used to calculate the genetic distance between the ecotypes. Among the ecotypes, a notable genetic diversity was identified, marked by 64 bands displaying substantial polymorphism. Genetic clustering analysis of the molecular marker data from 11 studied ecotypes revealed the highest genetic similarity between the Khalkhal and Meshgin ecotypes, whereas Shahr Khoy and Ardabil ecotypes appeared more distantly related.

The dendrogram obtained from cluster analysis using the average distance algorithm (UPGMA) revealed five groups at the level of similarity (0.45) (Figure 2). The first group included the Mako ecotype (K) and the second group was formed separately by the Khoi ecotype (X). The third group had two subgroups of Marand (R) and Malekan (M) ecotypes. The fourth group included five different ecotypes and had three subgroups. The sub-group of the Arshq ecotype (I) was completely separate from the sub-sub-subgroups of the Ahar ecotype (A) and the Sarab ecotype (S). The sub-group Sub-sub-sub-types of Khalkhal (G) and Meshgin Shahr (N) ecotypes were placed. Astara (L) and Ardabil (B) ecotypes were classified in the fifth group (Figure 2). Upon reviewing the resulting dendrogram, it is evident that most groups contain ecotypes with either completely similar or relatively similar climatic conditions.

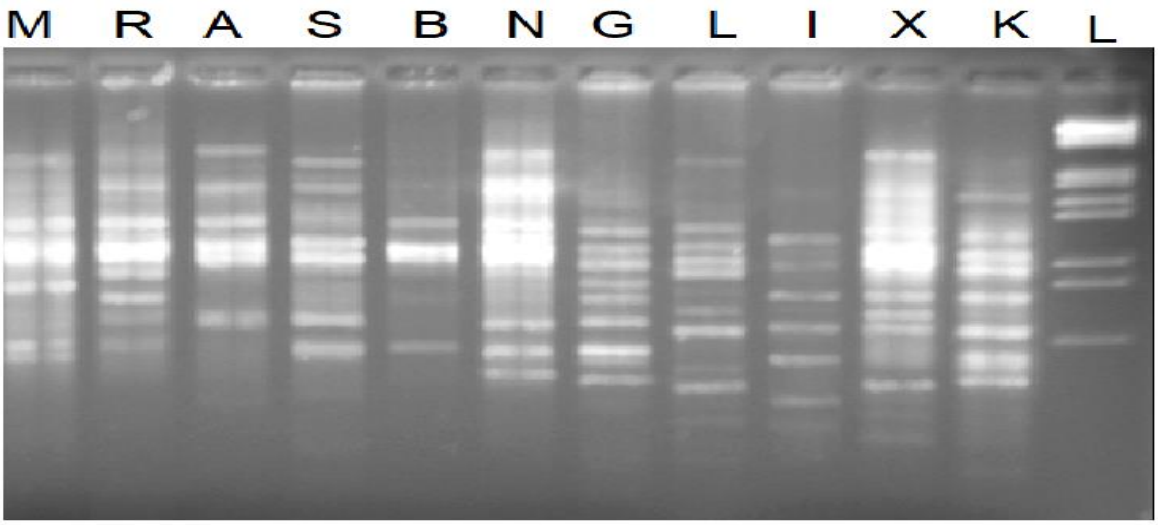

Figure 1. Polymorphic bands within the gel profile generated by the OPD-03 marker in the studied ecotypes. The codes correspond to the initials of the ecotypes as follow: M: Malekan, R: Marand, A: Ahar, S: Sarab, B: Ardabil, N: Meshgin Shahr, G: Khalkhal, L: Astara, I: Arshag, X: Khoy, K: Mako, L: Ladder.

1

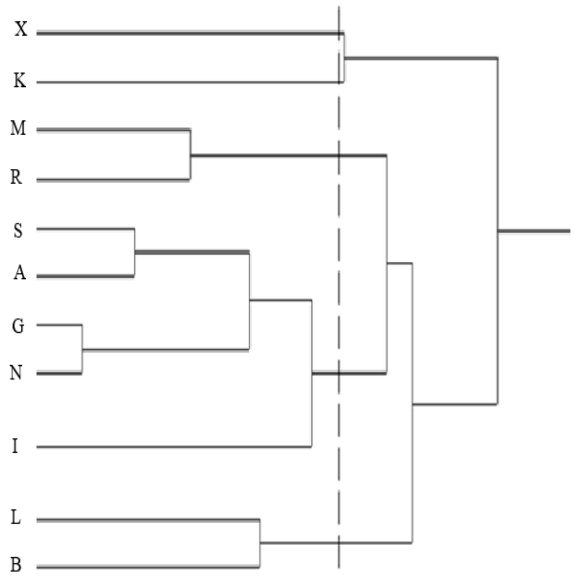

Figure 2. Dendrogram illustrating the clustering of data obtained from RAPD molecular markers using the Dice similarity matrix and UPGMA algorithm**.** The codes correspond to the initials of the ecotypes as follows: M: Malekan, R: Marand, A: Ahar, S: Sarab, B: Ardabil, N: Meshgin Shahr, G: Khalkhal, L: Astara, I: Arshag, X: Khoy, K: Mako, L: Ladder.

This indicates that the molecular diversity results of the ecotypes are in harmony with their geographical diversity. However, it is essential to conduct additional studies, such as Bister's research, which is based on specific markers, to enhance the classification of savory plant ecotypes further and complete the overall research. In addition, the EIGEN program was employed to conduct principal component analysis, and a resulting three-dimensional plot was generated as shown in Figure 3. This multivariate method, a grouping method, was chosen to complete the cluster analysis information. The decomposition results into main components were consistent with the results of cluster analysis to a large extent, although there were differences in this field in some clusters.

Discussion

The effectiveness of RAPD molecular markers in genetically classifying a wide range of plants has been demonstrated, aligning well with the data obtained from the present study. Numerous studies have concentrated on exploring the genetic diversity of various plant species, including those classified as savory plants. In a comprehensive study, Kameli et al. (2013) used the ISSR molecular markers to assess the genetic diversity of multiple ecotypes of savory plants.

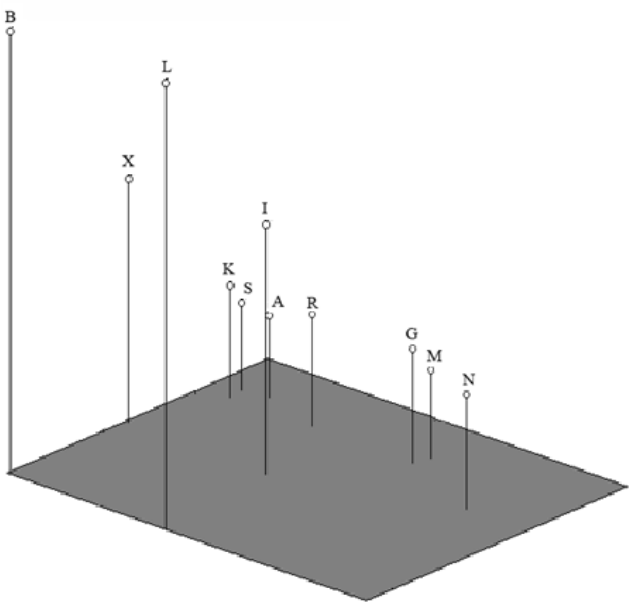

Figure 3. Three-dimensional plot resulting from principal component analysis. (The codes refer to: M: Malekan, R: Marand, A: Ahar, S: Sarab, B: Ardabil, N: Meshgin Shahr, G: Khalkhal, L: Astara, I: Arshag, X: Khoy, K: Mako, L: Ladder)

Additionally, Namayandeh et al. (2017) employed RAPD molecular marker primers to estimate the polymorphic information content (PIC) in diverse savory plant ecotypes, yielding an average of 0.34. In a study that accurately identified different ecotypes of savory plant species using the barcode method, genetic materials were extracted and used as templates for amplification with matK, rbcl, and ITS genetic primers. Among these, only the matK primer demonstrated satisfactory amplification. The amplified product was then sequenced to serve as the basis for genetic analysis, and the study's findings indicated that the *matK* gene can be utilized as part of the barcode system for identifying savory species (Peiri and Fazeli, 2022). In another research, the genetic diversity of Iranian stands of *S. hortensis* L. was studied based on horticultural traits and RAPD markers. The results showed that both environmental and genetic factors are influential in the observed changes. Also, their results show that the RAPD approach together with horticultural analysis seems to be more suitable for fingerprinting and evaluating genetic relationships among *S. hortensis* populations with high accuracy (Hadian et al., 2008).

Conclusion

The method employed for isolating high-quality DNA from the *S. bachtiarica* plant was suitable, even in the presence of inhibitory polyphenolic compounds in the cellular contents of the plant. This inference is supported by the generation of distinct and high-resolution band patterns observed during the electrophoresis separation of polymerase chain reaction products. Moreover, the efficiency of RAPD molecular marker-based primers in the genetic classification of the studied ecotypes was evident. Most Bakhtiari savory plant ecotypes in comparable climatic and geographical conditions, were grouped into dendrogram patterns with close genetic distances. Nonetheless, additional investigations employing specific and semi-specific markers are imperative for a more refined classification of ecotypes.

Supplementary Materials:

The Supplementary Material for this article can be found online at: https://www.jpmb-gabit.ir/article_710709.html.

Supplementary Table 1. Specifications of the PCR reaction mixture for a final volume of 18 microliters.

Supplementary Table 2. PCR primer adjusted program for RAPD.

Author contributions

Not applicable.

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Conflict of interest statement

The authors declare no conflict of interest.

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مقاله پژوهشی

تنوع ژنتیکی گیاه مرزه بختیاری (*Satureja bachtiarica* Bunge) جمع‌آوری شده از شمال غرب ایران

نورالدین حسین‌پورآزاد *

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چکیده: در دسترس بودن پایدار منابع ژنتیکی برای اصلاح گیاهان ضروری است. گیاه مرزه بختیاری *Satureja bachtiarica* یکی از گونه‌های بومی ایران با پراکنش گسترده در کشور بوده و در مناطق کوهستانی صخره‌ای بین اسالم و خلخال در استان اردبیل نیز پراکنده است. پژوهش حاضر بر طبقه‌بندی ژنتیکی ۱۱ اکوتیپ مختلف *S. bachtiarica* جمع‌آوری شده از شمال غرب ایران با استفاده از نشانگرهای مولکولی RAPD متمرکز بود. در این بررسی تعداد ۶۴ باند الکتروفورزی تنوع ژنتیکی قابل توجهی در بین اکوتیپ‌ها نشان دادند که به عنوان مبنای آنالیز ژنتیکی با نرم افزار NTYSYS-pc (2.02 e) مورد استفاده قرار گرفتند. فاصله ژنتیکی بین اکوتیپ‌ها با استفاده از ضریب تشابه دایس تعیین شده و سپس دندروگرام بر اساس داده‌های ماتریس شباهت، با استفاده از الگوریتم (UPGMA) ساخته شد. تجزیه و تحلیل خوشه‌بندی ژنتیکی داده‌های نشانگر مولکولی از ۱۱ اکوتیپ مورد مطالعه کمترین شباهت ژنتیکی را در بین اکوتیپ‌های خوی و اردبیل نشان داد. در مقابل، اکوتیپ‌های خلخال و مشگین‌شهر بیشترین شباهت را داشتند. پس از بررسی دندروگرام، مشخص شد که بیشتر خوشه‌ها در برگیرنده اکوتیپ‌هایی هستند که در شرایط آب و هوایی کاملاً مشابه یا نسبتاً مشابهی رویش دارند. بر این اساس، نتایج از هماهنگی بین تنوع مولکولی با تنوع جغرافیایی اکوتیپ‌ها حکایت داشته است.

کلمات کلیدی: مرزه بختیاری، نشانگر مولکولی RAPD، دسته‌بندی ژنتیکی، فواصل ژنتیکی.

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