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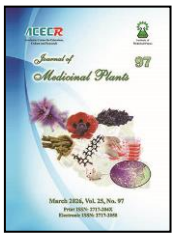

Research Article

Genetic diversity in six *Dracocephalum* L. species from Iran based on ISSR markers

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ABSTRACT

Background: *Dracocephalum* L., comprising 89 species worldwide, is the second-largest genus in the Nepetinae subtribe of the Mentheae tribe within the Lamiaceae family. In the flora of Iran, the genus is represented by 10 species. *Dracocephalum* species are known for their medicinal properties, including anticancer, antioxidant, and anti-inflammatory effects, as well as various other therapeutic uses. **Objective:** This study aimed to evaluate the effectiveness of Inter-Simple Sequence Repeat (ISSR) markers in differentiating *Dracocephalum* species and to assess the genetic diversity among various species within the genus. **Methods:** Thirteen populations from six *Dracocephalum* species were analyzed using eight ISSR primers. **Results:** A total of 128 bands were generated, of which 121 (94.3 %) were polymorphic. The highest polymorphism was observed with the IS7 and IS1 primers. The Polymorphism Information Content (PIC) values ranged from 0.286 (IS5) to 0.337 (IS1). Cluster analysis revealed distinct genetic groupings, with populations of *D. kotschyi* Boiss. (Isfahan), *D. lindbergii* Rech.f (Bojnourd), and *D. moldavica* L. (Urmia) forming separate clusters, indicating significant genetic differentiation. **Conclusion:** This study demonstrates the utility of ISSR markers in distinguishing *Dracocephalum* species and highlights the substantial genetic diversity within the genus. The high percentage of polymorphism underscores the rich genetic variability, which is essential for conservation efforts and potential medicinal applications. These findings provide valuable insights into the genetic structure of *Dracocephalum* species, laying the groundwork for future research in biotechnology and pharmaceutical development.

Abbreviations: ISSR, inter-simple sequence repeat; RAPD, random amplified polymorphic DNA; SAMPL, selectively amplified microsatellite polymorphic loci; ITS, internal transcribed spacer; PCR, polymerase chain reaction; PIC, polymorphic information content; MI, marker index; RP, resolving power

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1. Introduction

The genus *Dracocephalum* L., comprising 89 species worldwide, is the second-largest genus in the Nepetinae subtribe of the Mentheae tribe within the Lamiaceae family. It is commonly found in alpine and semi-arid regions [1, 2]. In the flora of Iran, the genus is represented by 10 species, most of which are perennial plants or woody bushes, with a few annual exceptions [3]. While the Flora of Iran records 10 species for the country, the genus is represented by a greater number of species across the broader Iranian plateau. *Dracocephalum* can be distinguished from other genera within the Nepetinae subtribe by several key morphological characteristics, including aristate-dentate bracts, swollen folds at the base of the sinuses between the calyx teeth, anther thecae diverging at 180° relative to each other, and a significantly broader median lobe on the posterior calyx lip [4]. Plants of this genus are aromatic medicinal species, rich in bioactive compounds such as phenols, flavonoids, alkaloids, and terpenes, which underpin their diverse therapeutic applications. The essential oils extracted from *Dracocephalum* species are typically composed of various monoterpenes (linalool, geraniol, camphor, and citronellal), sesquiterpenes (caryophyllene, and germacrene), and phenolic (eugenol) compounds [5]. The chemical composition can vary widely between species, and it is influenced by factors such as geographical origin, growth conditions, and extraction methods [6]. The key components such as linalool, geraniol, caryophyllene, and eugenol contribute to the oils' antimicrobial, anti-inflammatory, antioxidant, anxiolytic, anticancer, and immunomodulatory effects [5]. Further research, including clinical studies, is necessary to validate the therapeutic uses of *Dracocephalum* species and to explore their full pharmacological potential. However, current findings highlight the genus as a promising source of bioactive compounds for use in traditional and modern medicine. *Dracocephalum* species have been widely used in traditional medicine to treat various ailments, owing to their antioxidant, antihyperlipidemic, antimicrobial, anticancer, and antispasmodic properties, all attributed to these bioactive constituents [7].

The quantity and quality of secondary metabolites in medicinal and aromatic plants (MAPs) are influenced by the interplay between genetic factors and environmental conditions [8]. Different ecological zones have given rise to distinct accessions of MAPs, resulting in the formation of unique ecotypes with specialized biological traits. When a plant population is introduced to a new environment, it undergoes physiological adaptations to ensure survival. Over time, evolutionary forces can lead to the development of distinct physiological, chemical, morphological, and genetic characteristics within the population [9].

To address the increasing demand for Medicinal and Aromatic Plants (MAPs) across various industries and to mitigate the risks of overharvesting and potential extinction of wild species, the adoption of sustainable cultivation practices is imperative. A crucial initial step in domestication programs involves the thorough evaluation of wild MAP accessions, with a focus on their morphological, genetic, and phytochemical characteristics [10]. One of the primary challenges associated with wild collection is the loss of genetic diversity, population extinction, and differentiation [11]. Accurate and detailed planning for the conservation and management of plant genetic

resources relies on effective analysis of genetic diversity and natural population differentiation [12]. DNA-based molecular markers provide the most precise and relevant information regarding the extent of genetic diversity [13]. Techniques such as RAPD, SAMPL, and ITS markers have been successfully employed to assess genetic variation and relationships among various *Satureja* species [14-16]. The inter-simple sequence repeat (ISSR) marker, a polymerase chain reaction (PCR)-based technique, utilizes microsatellite sequences primed by 2-4 arbitrary nucleotides anchored at either the 3′ or 5′ end [17]. ISSR markers combine the specificity of microsatellite markers with additional advantages, including cost-effectiveness, high polymorphism, and strong repeatability. Notably, ISSR markers do not require prior sequence information for primer synthesis, making them a versatile tool for genetic analysis [17]. By leveraging these molecular tools, researchers can better understand genetic diversity, support conservation efforts, and promote sustainable cultivation practices for MAPs.

Several studies have highlighted the importance of anatomical features and their implications in the systematics of the Lamiaceae family [18-24]. Within this family, the structural characteristics of roots, stems, leaves, and petioles have proven valuable for taxonomic classification, species delimitation, and subgeneric classification. For instance, morphological and anatomical traits have been used to investigate species relationships within the genus *Dracocephalum* in Iran [24]. However, despite these advances, research on the molecular characteristics of *Dracocephalum* remains limited. For example, Sheidai and Koohdar [25] reported the likelihood of two distinct varieties within *Dracocephalum thymiflorum* L. based on ISSR markers, morphological traits, and anatomical studies. Similarly, Koohdar et al. [26] provided evidence suggesting the existence of a hybrid between *D. kotschyi* Boiss. and *D. oligadenium* Bornm. & Gauba, supported by molecular, anatomical, and morphological data. These findings underscore the need for further molecular studies to better understand the relationships within this genus. To address this gap, the present study was conducted to elucidate the molecular relationships among 13 populations representing six species of the genus *Dracocephalum*. By integrating molecular data with existing morphological and anatomical insights, this research aims to contribute to a more comprehensive understanding of the taxonomic and evolutionary relationships within this genus.

2. Materials and methods

2.1. Plant materials and their origin

In this study, we investigated six populations of *D. kotschyi*, two populations each of *D. oligadenium* and *D. ghahremanii* Jamzad, and one population each of *D. lindbergii* Rech.f., *D. moldavica* L., and *D. subcapitatum* (Kuntze) Lipsky. These samples were collected from various well-documented growing regions across Iran for molecular analysis. DNA extraction and subsequent molecular analyses were conducted using silica-gel dried leaves. Furthermore, the plant specimens have been archived at the Herbarium of Medicinal Plants and Drug Research Institute at Shahid Beheshti University (MPH). The geographical details of the collected *Dracocephalum* populations are provided in Table 1.

Table 1. Geographical characteristics of the collected populations of *Dracocephalum* species

No.	Population	Species	Locality/Collector
1	Damavand	*D. kotschyi* Boiss.	Tehran: Damavand, 3200 m, Gholipour 1218 (MPH)
2	Rineh	*D. kotschyi* Boiss.	Mazandaran: Haraz road, Rineh, Nova, 2200 m, Sonboli & Amini Rad 462 (MPH)
3	Dena	*D. kotschyi* Boiss.	Kohgiluyeh & Boyerahmad: Yasouj-Sisakht, Dena, 3000-3200 m, Sonboli, Gholipour & Kanani 1163 (MPH)
4	Isfahan	*D. kotschyi* Boiss.	Isfahan: Khansar - Boean, Miandasht, Hossein Abad Mountain, 2700-2900 m, Termeh & Karavar 22789 (IRAN)
5	Polour	*D. kotschyi* Boiss.	Mazandaran: Haraz road, Polour to Lasem, 2700 m, Sonboli & Yousefzadi 1217 (MPH)
6	Elika	*D. kotschyi* Boiss.	Mazandaran: Elika, 2500-3200 m, Termeh, Daneshpazhooh & Zargani 22805 (IRAN)
7	Mazandaran 1	*D. oligadaenium* Bornm. & Gauba	Mazandaran: Siah Bisheh, Chalus road, 2480 m, Sonboli, Gholipour & Mirjalili 1703 (MPH)
8	Mazandaran 2	*D. oligadaenium* Bornm. & Gauba	Mazandaran: Chalus road, Pol-e Zanguleh, 2300 m, Gauba 22810 (IRAN)Sonboli, Hadian & Moridi 1627 (MPH)
9	Shahrud	*D. ghahremanii* Jamzad	Shahroud: Mojan, Sangban, Shahkuh Mountain, 2400 m, Termeh, Mousavi & Habibi 56256 (IRAN)
10	Semnan	*D. ghahremanii* Jamzad	Semnan: Shahmirzad, Chashm, Nizva, 3200 m, Sonboli & Gholipour 1219 (MPH)
11	Bojnourd	*D. Lindbergii* Rech. f.	Khorasan: Bojnourd, Rein, Aladagh, 1700 m, Sonboli, Kanani & Gholipour 1737 (MPH)
12	Mashhad	*D. subcapitatum* (Kuntze) Lipsky	Mashhad: Kalat, 1860 m, Sonboli & Gholipour 917 (MPH)
13	Urmia	*D. moldavica* L.	Urmia: Ashnabad village, 1710 m, Sonboli & Mojarad 1626 (MPH)

2.2. DNA extraction and ISSR analysis

DNA extraction was carried out using the Bioflux kit following the manufacturer's protocol. The concentration of the extracted DNA was measured using a NanoDrop spectrophotometer (Labtech International, Ringmer, UK), and its quality was assessed via agarose gel electrophoresis. The DNA samples were diluted to a concentration of 10 ng/µl using TE buffer. ISSR amplification was performed according to the method described by Heydari et al. [7]. The amplified products were analyzed by electrophoresis on a 1.5 % agarose gel using 1 × TBE buffer (Tris – Acetate), with a DNA ladder ranging from 100 to 3000 kb for size comparison.

To evaluate polymorphism among the studied populations, 12 primers were initially screened. Ultimately, 8 primers demonstrating high polymorphism were selected for ISSR molecular analysis. Each PCR reaction mixture, with a final volume of 10 µl, consisted of 1.4 µl of DNA template (10 ng/µl), 0.6 µl of ISSR primer, 3 µl of sterile distilled water, and 5 µl of 2X PCR master mix. The PCR amplification program was as follows: initial denaturation at 94 °C for 4 minutes, followed by 30 cycles of 30 seconds at 94 °C, 40 seconds of annealing at 47–55 °C, and 60 seconds of extension at 72 °C, with a final extension step at 72 °C for 10 minutes. The amplified DNA fragments were then separated by electrophoresis on a 1.5 % agarose gel and visualized using a DNA green viewer.

2.3. Data analysis

The amplified bands were scored as either 0 (absence) or 1 (presence). For each marker, the total number of amplified bands, the number of polymorphic bands, and the percentage of

polymorphism were calculated. Key parameters, including the effective multiplex ratio (EMR), polymorphic information content (PIC), marker index (MI), and resolving power (RP), were determined based on established methods from previous literature [7]. A dendrogram was constructed using the UPGMA method in NTSYS software. The PCO plot was also drawn using R software.

3. Results

3.1. Molecular evaluation

To assess polymorphism among the studied populations, 12 ISSR primers were initially tested. Based on the pretest results, 8 primers exhibiting high polymorphism were selected for ISSR molecular analysis (Fig. 1).

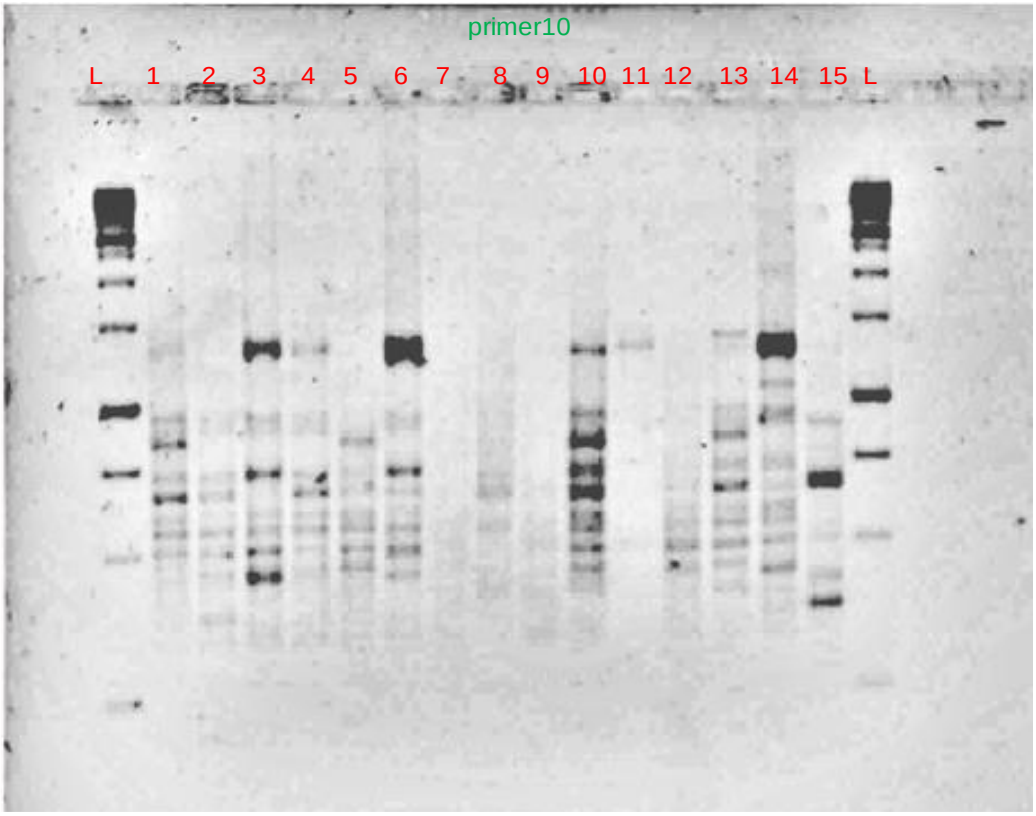

Fig. 1. Gel electrophoresis banding pattern of the studied *Dracocephalum* species using the IS10 primer.

These primers generated a total of 128 bands, of which 121 were polymorphic, yielding an average polymorphism percentage of 94.3 % across all primers. The highest polymorphism was observed for the IS7 and IS1 primers, both showing 100 % polymorphism. The IS21 primer produced the highest number of polymorphic bands [20], while the IS9 primer produced the lowest [11]. The lowest polymorphism percentage (84.6 %) was recorded for the IS9 primer. On average, each ISSR primer produced 15.12 polymorphic bands (Table 2). The polymorphic information content (PIC) ranged from 0.286 (IS5) to 0.337 (IS1). The highest marker index (5.09) was recorded for IS1, while the lowest value was observed for IS5. The resolving power of the primers varied between 4.62 and 9.85.

Table 2. Specificity of the studied primers and the polymorphism value in the studied *Dracocephalum* species

Primer	Motif	Annealing temperature	Total number of amplified bands	Number of polymorphic bands	Polymorphic percentage	PIC	MI	RP
IS5	(AC)9G	55	16	15	93.7	0.286	4.32	4.62
IS6	(AC)9C	55	16	15	93.7	0.335	5.06	8.31
IS7	(ACG)5G	51	17	17	100	0.294	4.44	7.69
IS9	(TCG)5G	51	13	11	84.6	0.321	4.85	7.46
IS10	(TCG)5C	51	15	14	93.3	0.330	4.99	9.85
IS11	(AC)8G	49	18	17	94.4	0.322	4.87	4.85
IS21	(AG)8RC	53	21	20	95.2	0.296	4.47	4.92
IS1	(CA)9G	55	12	12	100	0.337	5.09	7.69
Total			128	121				
Mean			16	15.12 ^{EMR}		0.315	4.76	6.92

PIC: main values of polymorphic information content; MI: Marker Index; RP: resolving power; EMR: effective multiplex ratio

3.2. Cluster analysis

The dendrogram generated using the UPGMA method, based on cluster analysis of the studied *Dracocephalum* populations, revealed four major clusters (Fig. 2). Cluster I was divided into two subclusters: the first subcluster included ten populations, comprising Damavand, Polour, Rineh, and Dena from *D. kotschyi*; Mazandaran 1 and Mazandaran 2 from *D. oligadaenium*; Semnan (*D. ghahremanii*); and Mashhad (*D. subcapitatum*). The second subcluster consisted of Shahrud (*D. ghahremanii*) and Elika (*D. kotschyi*). Clusters II, III, and IV were each represented by a single population: Isfahan (*D. kotschyi*), Bojnourd (*D. Lindbergii*), and Urmia (*D. moldavica*), respectively. The highest similarity coefficient was observed between the Damavand and Polour populations. Notably, *D. moldavica* was distinctly separated from the other species, as expected. Similarly, *D. Lindbergii* was genetically distinct from the *D. kotschyi* populations. The genetic distance between *D. moldavica* and species such as *D. kotschyi* and *D. subcapitatum* was confirmed, while *D. subcapitatum* showed closer genetic affinity to *D. kotschyi*. The Principal Coordinate Analysis (PCO) plot confirmed the results obtained by cluster analysis, where each of the populations of Urmia (*D. moldavica*), Bojnourd (*D. Lindbergii*), and Isfahan (*D. kotschyi*) were distinct from the other populations (Fig. 3).

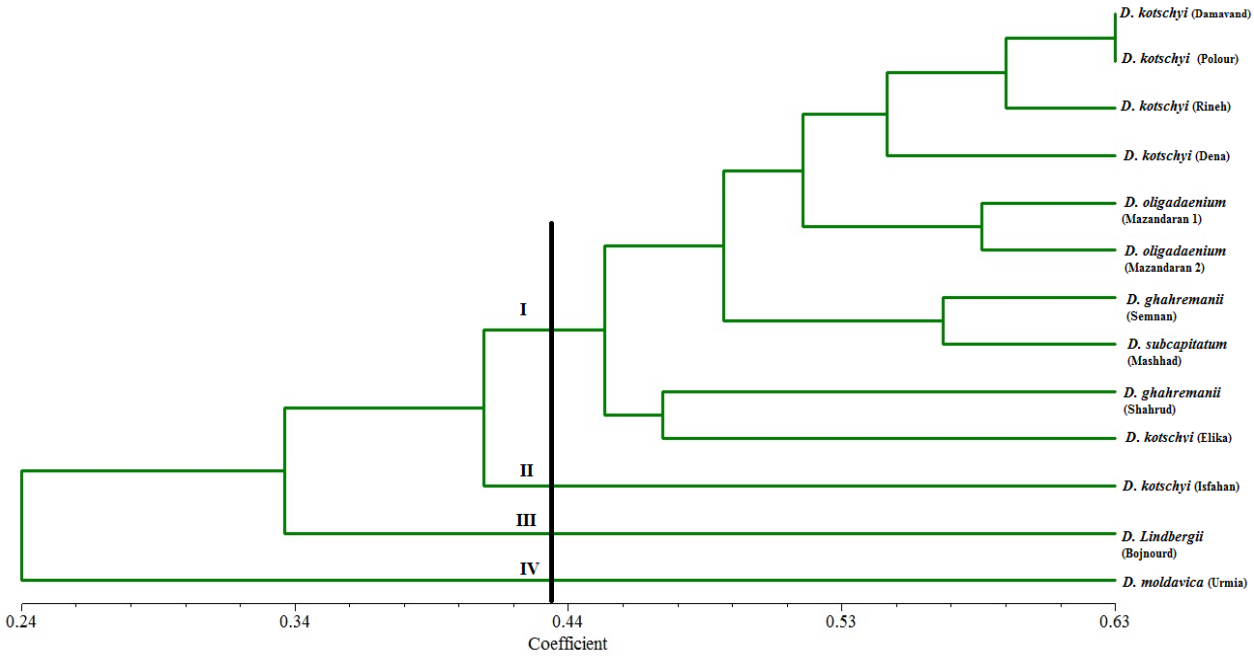

Fig. 2. Dendrogram obtained by Cluster analysis of the studied populations of *Dracocephalum* in Iran

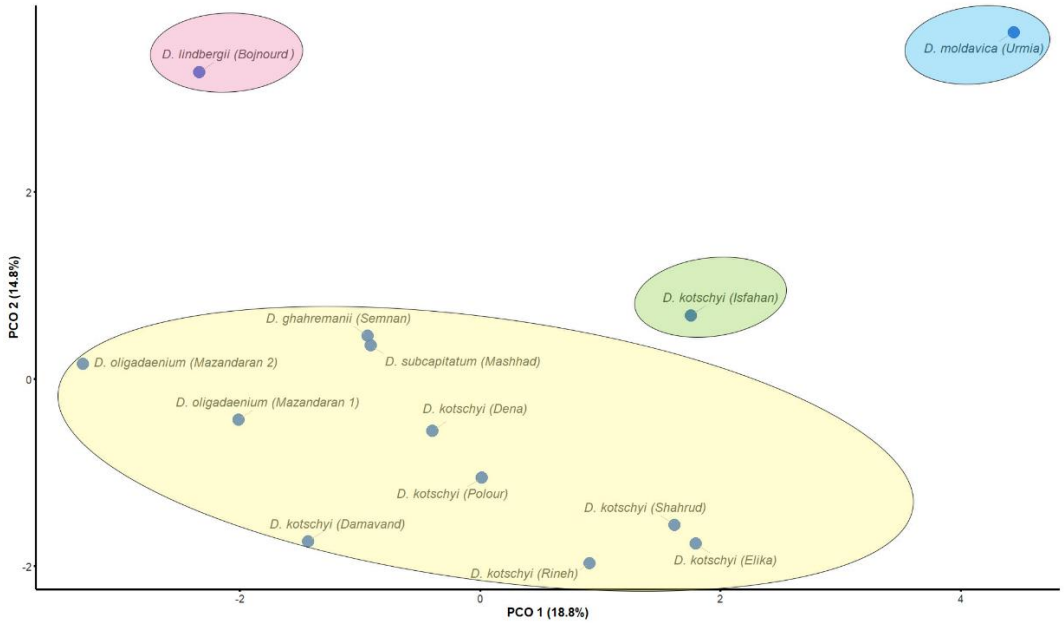

Fig. 3. Principal Coordinate Analysis (PCO) for the studied populations of *Dracocephalum* in Iran

4. Discussion

This study investigated the genetic diversity of several Iranian species within the *Dracocephalum* genus using ISSR markers. The ISSR marker system proved highly effective, generating a large number of polymorphic bands (121 bands), which underscores its utility for assessing genetic diversity in *Dracocephalum* populations. ISSR markers have been widely recognized as a reliable tool

for studying genetic diversity in various medicinal plants, including *Papaver bracteatum* L. [27], *Magnolia officinalis* Rehder & E. Wilson [28], *Lepidium sativum* L. [29], *Satureja rechingeri* Jamzad [30], *Glycyrrhiza glabra* L. [31], and *Artemisia dracunculus* L. [32]. Furthermore, Borghei et al. [33] highlighted the potential of ISSR markers in breeding programs to improve key traits of *D. moldavica*. Genetic differentiation within and between populations is influenced by factors such as biological traits, life cycle, population size, gene flow, mutation rate, genetic drift, and mating system. For a more comprehensive analysis of plant genetic diversity, next-generation sequencing (NGS) techniques are increasingly valuable [12]. These techniques are particularly suitable for crops like *Dracocephalum* species, especially given the declining costs of sequencing and the absence of a reference genome.

The Lamiaceae family, the sixth-largest family of economically important flowering plants, comprises seven subfamilies as identified by Harley et al. [34]. This classification is based on recent molecular data and morphological features. However, understanding the relationships among species within Lamiaceae genera remains challenging due to inconsistencies in phylogenetic trees derived from different gene sets. These complexities are further compounded by extensive inter-specific hybridization and polyploidy events during their evolution [2]. Cluster analysis of molecular data from studied populations revealed clear distinctions among species of the *Dracocephalum* genus. While Rechinger [3] reported eight species of this genus in Iran, the findings of this study suggest the presence of more than eight species distributed across the region.

Based on the study of Sonboli et al. [35] who studied 35 quantitative and qualitative morphological traits of *Dracocephalum* populations in Iran, it was found that the populations were divided into three distinct groups. The first group included the species *D. moldavica,* while the second cluster included species *D. lindbergii* and populations of species *D. oligadenium*. The third group included *D. kotschyi*, *D. ghahremanii* and *D. subcapitatum*. The grouping presented in the study of Sonboli et al. [35] for species of the genus *Dracocephalum* was consistent with the results obtained from the present study. The taxonomic status of *D. oligadenium* remains a subject of discussion. Although it is treated as a synonym of *D. kotschyi* in some global databases (e.g., POWO), it was previously described as a distinct species from Iran by Esfandiari [36]. Our ISSR data, which show *D. oligadenium* populations clustering closely with, but not entirely intermixed with, *D. kotschyi* populations, provide a molecular perspective that aligns more closely with Esfandiari's report than with a full synonymization. The genetic proximity suggests a very recent divergence or ongoing gene flow, but the presence of a distinct sub-structure indicates that *D. oligadenium* may not be completely synonymous with *D. kotschyi*. The placement of *D. oligadenium* within the same major cluster as *D. kotschyi*, yet as a distinct sub-cluster, is highly informative. This pattern is consistent with several evolutionary scenarios: (1) *D. oligadenium* is a recently diverged lineage from *D. kotschyi* that has not yet accumulated significant genetic distance but is morphologically distinct; (2) it represents a subspecies or variety within a broader *D. kotschyi* complex; or (3) there is historical or contemporary introgressive hybridization

between the two taxa, as previously suggested by Koohdar et al. [26], which would lead to shared genetic markers while maintaining a degree of distinctness. Our ISSR data, while highly informative for diversity studies, cannot definitively distinguish between these scenarios. However, they clearly indicate that the relationship is too complex to be dismissed as simple synonymy and warrants recognition and further study. To conclusively determine the taxonomic rank of *D. oligadenium*, further investigation using more conserved DNA regions, such as the Internal Transcribed Spacer (ITS) or chloroplast markers, is recommended.

Studying genetic diversity in medicinal plant genera provides a roadmap for optimizing phytochemical production through breeding. By linking genetic markers to metabolite profiles, scientists can engineer plants with enhanced medicinal properties, improve stress resilience, and ensure sustainable utilization. Advances in genomics, coupled with conservation strategies, are critical for unlocking the full potential of medicinal plants in pharmaceutical and nutraceutical industries [37, 38]. Given the effectiveness of ISSR markers in distinguishing *Dracocephalum* species and assessing genetic variation, a comprehensive study of these species along with an exploration of the correlation between ISSR markers and morphological and phytochemical traits could significantly enhance breeding efforts.

5. Conclusion

This study highlighted the effectiveness of ISSR markers in assessing the genetic diversity of Iranian *Dracocephalum* species, as evidenced by the generation of 121 polymorphic bands. The results demonstrate the utility of ISSR markers not only for differentiating species within the *Dracocephalum* genus but also for broader applications in studying genetic diversity across various medicinal plants. The clear separation of species, such as *D. lindbergii* and *D. moldavica* from *D. kotschyi*, underscores the potential of molecular tools in resolving taxonomic complexities and understanding population structures. Furthermore, the findings suggest that the diversity of *Dracocephalum* species in Iran may be greater than previously reported, warranting further investigation. The challenges in understanding species relationships within the Lamiaceae family, particularly due to inter-specific hybridization, polyploidy, and incongruent phylogenetic data, emphasize the need for advanced techniques like next-generation sequencing (NGS). These methods, coupled with ISSR markers, could provide deeper insights into genetic variation, phylogenetic relationships, and trait correlations, ultimately supporting breeding programs aimed at improving desirable traits in *Dracocephalum* species. Overall, this study contributes to the growing body of knowledge on the genetic diversity of medicinal plants and underscores the importance of integrating molecular, morphological, and phytochemical approaches for effective conservation and breeding strategies.

Author contributions

A. S: Supervision, Methodology, Validation, Review and Editing. S. H: Conceptualization, Investigation, Plant material collection, Statistical analysis, Extraction. S. F: Supervision, Validation, Review and editing. H. E: Validation, Formal analysis, Writing and Editing. All authors approved the final manuscript.

Conflicts of interest

The authors declare that they have no competing interests.

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