Genetic and morphological diversity of three *Otostegia* species in Iran

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Abstract

Taxonomic and genetic diversity of the genus *Otostegia* (Lamiaceae), despite its medicinal importance, is not properly known. In the present study, therefore, 11 populations of three *Otostegia* species (*O. aucheri*, *O. michauxii*, and *O. persica*) collected from different localities across Iran. These localities are mostly situated in southern Iran, primarily in the Sistan and Baluchestan Province. Morphological divergence was assessed using 29 characters, complemented by multivariate clustering and ordination techniques for both morphological and molecular datasets. Furthermore, genetic diversity parameters were determined in all populations. Principal components analysis (PCA) was performed to identify plant height, leaf succulence and leaf margin as the primary diagnostic features driving inter-specific variation. Phenetic reconstruction via Neighbor-Joining (NJ) trees corroborated the morphological findings, effectively segregating the species into well-defined clusters. Notably, while most populations showed morphological and genetic affinities, the populations near the Sarbaz area emerged as outliers, exhibiting peak levels of polymorphism, Shannon diversity, and Nei's genetic index. While AMOVA confirmed significant molecular differentiation among species, Mantel test results indicated a lack of isolation-by-distance (IBD), as no significant correlation was observed between genetic divergence and geographical separation.

Keywords: Lamiaceae, ordination, population, ranking, Sistan and Baluchestan

تنوع ژنتیکی و ریخت‌شناسی سه گونه جنس گُلدِر در ایران

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خلاصه

تنوع طبقه‌بندی و ژنتیکی جنس گلدر (*Otostegia* Benth.) از نعنائیان، علیرغم اهمیت دارویی آن هنوز به خوبی شناخته نشده است. بنابراین، در مطالعه حاضر، ۱۱ جمعیت از سه گونه گیاه گلدر (*O. aucheri* Boiss.، *O. michauxii* Briq. و *O. persica* Boiss.) جمع‌آوری شده از مناطق مختلف ایران مورد بررسی قرار گرفت. این مناطق بیشتر در جنوب ایران به ویژه در استان سیستان و بلوچستان قرار دارند. واگرایی ریخت‌شناختی PCA از طریق ۲۹ صفت ارزیابی شد که با تکنیک‌های خوشه‌بندی و رسته‌بندی برای مجموعه داده‌های ریخت‌شناختی و مولکولی تکمیل شد. همچنین، پارامترهای تنوع ژنتیکی در همه جمعیت‌ها تعیین شد. ارتفاع گیاه، آبدار بودن برگ و حاشیه برگ به عنوان ویژگی‌های تشخیصی اصلی که باعث تنوع بین گونه‌ای می‌شوند نیز بررسی گردید. بازسازی فنتیکی از طریق درخت‌های NJ، یافته‌های ریخت‌شناختی را تأیید و گونه‌ها را به طور مؤثر در خوشه‌های کاملا مشخص تفکیک کرد. شایان ذکر است، در حالی که بیشتر جمعیت‌ها قرابت‌های ریخت‌شناختی و ژنتیکی نشان دادند، جمعیت‌های نزدیک منطقه سرباز سطوح بالایی از پلی‌مورفیسم، شاخص شانون و تنوع ژنتیکی را آشکار ساختند. این در حالی است که AMOVA تمایز مولکولی قابل‌توجهی را بین گونه‌ها تأیید کرد و نتایج آزمون مانتل نشان‌دهنده عدم جداسازی براساس فاصله بود، زیرا هیچ همبستگی معنی‌داری بین واگرایی ژنتیکی و جدایی جغرافیایی مشاهده نشد.

واژه‌های کلیدی: جمعیت، خوشه‌بندی، رسته‌بندی، سیستان و بلوچستان، نعنائیان

Introduction

Lamiaceae is one of the most prominent and distinctive families of flowering plants, comprising approximately 220 genera and almost 4,000 species worldwide (Naghibi *et al.* 2005, Benavides *et al*. 2010).

Otostegia Benth. (Lamiaceae) includes about 33 species in the Mediterranean region and adjoining Asia Minor (Khan *et al.* 2009). In Iran, three species of this genus are so far recorded, namely, *O. aucheri* Boiss., *O. michauxii* Briq., and *O. persica* Boiss.. *Otostegia persica* and *O. aucheri* are endemic to Iran and Pakistan (Ayatollahi *et al.* 2009), with their distribution being concentrated mainly in Sistan and Baluchestan Province (southeast of Iran). This region is considered as one of the tropical zone of Iran. Most localities where these species occur are characterized by a warm-dry climate and saline soil. This province has three distinct climates: viz., hot-humid, hot-dry, and temperate. These climatic conditions have caused a high diversity of plants in the region to the extent that unique plant species can be found there. Some species of this region are rare, unique and sometimes endemic with valuable medicinal properties (Ebrahimzadeh 2006).

The local names for *O. persica* is "Gol-e-kharu" and "Golder" (Asgarpanah & Mohammadi 2013). Many medicinal properties of *Otostegia*, such as antibacterial, antioxidant, anti-plasmodial, anti-diabetic, and anti-glycation activities, have been reported in clinical studies (Ayatollahi *et al.* 2007, 2009, 2010, Sharififar *et al.* 2007, Nateghpour *et al.* 2008, Ebrahimpoor *et al.* 2011). It has been traditionally used to treat various ailments such as toothache and arthritis (Sadeghi *et al.* 2014). The genus exhibits strong antioxidant properties due to presence of flavonoids (Sharififar *et al.* 2003). While various bioactive compounds, such as flavonoids and tannins have been identified, no alkaloids and saponins have been reported from *O. persica* (Asadipour *et al.* 2004).

It is widely recognized that, native species vary morphologically and genetically due to adaptation to local environmental conditions. This result is revealed in several ecotypes among populations. These morphological and genetic variations play a crucial role the survival and adaptability of plants under fluctuating environmental conditions such as light, water, temperature, and rainfall level (Thompson & Wilson 1992). This diversity is driven by mutations, gene translocations, and gene flow between populations, thereby contributing to the resilience of the species.

Morphological diversity refers to variations in plant characteristics. In *Otostegia*, morphological characters can vary significantly among different populations. These variations may be formed by environmental factors, genetic backgrounds, and evolutionary pressures, providing insight into the adaptive strategies employed by these plants.

There is a significant lack of information regarding the genetic and morphological characterization of *Otostegia*, which limits our understanding to the environmental adaptation processes resulting more complications of its taxonomic delimitation. Previous studies in Iran have predominantly focused on morphological, edaphic, phytochemical, and cytogenetic traits (chromosome counts) of this genus. However, these traditional approaches lack the phylogenetic resolution required to clarify the complex evolutionary lineages and genetic diversity within the genus, particularly among its populations in the Middle East and Iran. For instance, investigations of *O. persica* populations in Fars Province (Iran) revealed substantial morphological variability, clustering genotypes into distinct groups based on multiple phenotypic characters. Similarly, research in the Baluchistan region has highlighted significant inter-population differences in morphological and phytochemical profiles, suggesting a complex interplay between environmental and genetic factors (Nematinejad *et al.* 2022).

Most existing research has been conducted at the broader family level in the Lamiaceae, within which *Otostegia* represents only one of hundreds of the investigated genera. Therefore, there is a critical need for molecular-based studies to bridge this gap leading to provide a robust phenetic understanding of the genus. This study represents the first

comprehensive attempt to characterize the genetic and morphological diversity of *Otostegia* collected from diverse localities in Iran.

In the present study, therefore, three species of the *Otostegia*, which are mostly native to Sistan and Baluchestan Province, were investigated and discussed here.

Materials and Methods

- Plant collections

A total of 11 populations of three *Otostegia* species (viz., *O. persica*, *O. aucheri*, and *O. michauxii*) were sampled from various localities in Iran (Table 1). Sampling was conducted during two seasons (autumn and spring) from 2019 to 2021, with five individuals collected from each population. Fresh samples were transported to the laboratory and cleaned under a dissecting microscope (Olympus, Model SZH). For subsequent analyses, healthy specimens were selected for morphological studies and samples were preserved in silica gel for molecular analysis.

Table 1. Populations of the studied *Otostegia* species in Sistan and Baluchestan Province (Iran) along with its related data

Taxon	No. of population	Sampling location	Geographical coordinates	
Otostegia aucheri	1	Karevandar	60˚ 45′ E	27˚52′ N
	2	Zahedan to Mirjaveh Road	61˚ 16′ E	29˚19′ N
	3	Mirjaveh	61˚ 17′ E	29˚09′ N
	4	15 km south of Zahedan	60˚ 47′ E	29˚21′ N
O. persica	5	Sarbaz Bridge	61˚ 21′ E	26˚58′ N
	6	Enzā Village	61˚ 23′ E	26˚59′ N
	7	Itak to Sarbaz Road	61˚ 24′ E	27˚01′ N
	8	Kalat to Suran Road	61˚ 28′ E	27˚03′ N
	9	Karevandar to Khash Road	60˚ 46′ E	28˚25′ N
O. michauxii	10	South of Kazerun	51˚ 41′ E	29˚35′ N
	11	Dasht-e Arzhan	51˚ 38′ E	29˚39′ N

- Morphometry

In total, 29 morphological characters were evaluated for the morphometric analysis (Table 2).

Table 2. Morphological characters and their codings

No.	Character/Code
1	Habit: 1. Pavement areas, 2. Hilly areas
2	Plant height: 1. > 80 cm, 2. ≤ 80 cm
3	Leaf shape: 1. Narrow elliptical, 2. Ovate to circular, 3. Obovate-cuneate
4	Number of leaves per node: 1. ≤ 4, 2. > 4
5	Leaf lobes: 1. Lobed, 2. Entire
6	Leaf margin: 1. Entire/Non-dentate, 2. Dentate, 3. Dentate with spines

Table 2 (contd)

7	Petioles: 1. Short, 2. Absent
8	Leaf succulence: 1. Succulent, 2. Non-succulent
9	Leaf shape: 1. Length ≈ 2–3 × width, 2. Length ≈ width
10	Leaf trichomes: 1. Short, 2. Absent
11	Leaf apex: 1. Acute, 2. Barbate
12	Leaf veins: 1. Present, 2. Absent
13	Number of spines per node: 1. > 4, 2. ≤ 4
14	Size of spines: 1. ≥ 2 mm, 2. < 2 mm
15	Number of flowers per inflorescences: 1. > 10, 2. ≤ 10
16	Bract size: 1. ≥ 10 mm, 2. < 10 mm
17	Calyx trichomes: 1. Absent, 2. Short, 3. Long
18	Upper lip of calyx shape: 1. Egg-shaped, 2. Bayonet-shaped
19	Upper lip of calyx apex: 1. Acute, 2. Barbate
20	Number of lobes on lower lip of calyx: 1. > 3, 2. 3
21	Lobes of lower lip of calyx: 1. Acute apex, 2. Barbate apex
22	Calyx perianth size: 1. Corolla ≈ Calyx, 2. Corolla > Calyx
23	Corolla lobe size: 1. Upper lip of flower cup > 10 mm, 2. Upper lip of flower cup ≤ 10 mm
24	Corolla color: 1. White, 2. White to yellow
25	Corolla spines: 1. Present, 2. Absent
26	Fruit length (mm)
27	Corolla length (mm)
28	Calyx length (mm)
29	Internode length (mm)

- ISSR Analysis

Genomic DNA extraction was carried out using the CTAB-activated charcoal protocol on dried leaves (Krizman *et al.* 2006). Seven ISSR primers: UBC807, UBC810, UBC811, (GA) 9A, (GA) 9C, (GA) 9T, and (GT) 7CA were employed. Polymerase chain reaction (PCR) was performed in a 23 µL reaction volume containing the following reaction mixture: 18.25 µL H_2o, 2.5 mM Tris-HCl buffer at pH 8, 0.35 mM MgCl2, 0.5 mM of each dNTP (Bioron, Germany), 1 µM of a single primer, 20 ng of genomic DNA, and 0.4 µL of Taq DNA polymerase (Bioron, Germany). Amplification reactions were performed in a Techne thermal cycler (Germany), under the following cycling conditions: an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of pre-amplification (denaturation at 94 °C for 30 s, annealing at 52 °C for 45 s, and extension 2 min at 72 °C), and final extension at 72 °C for 10 min. PCR products were visualized

via 2% agarose gel electrophoresis followed by ethidium bromide staining. Fragment sizes were estimated using a 1kb molecular weight ladder (Fermentas, Germany).

- Data analyses

Morphometry: The morphological characters were coded as binary and multistate characters. Clustering methods, namely, unweighted paired groups with Arithmetic Mean (UPGMA), Neighbor-Joining (NJ), and Ward methods were applied. In addition, principal components analysis (PCA) was performed to identify the most variable morphological characters. In the PCA analysis, plots of the first and second components were used to visualize the species grouping. Euclidean distance and Gower distance were used for clustering (Podani 2000). Data were analyzed using PAST Ver. 2.17 (Hamer *et al.* 2012).

ISSR Analysis: Presence and absence of ISSR bands were coded as binary characters 1 and 0, respectively. Genetic diversity parameters including gene diversity (He), Shannon index (I), the number of effective alleles (Ne), and polymorphic percentage (P%) were determined for each population (Weising 2005, Freeland *et al.* 2011). These analyses were performed using GenAlex 6.4 (Peakall & Smouse 2006).

The clustering (UPGMA and NJ) and ordination (PCA) methods were employed to evaluate the relationships between populations (Podani 2000) using NTSYS Ver. 2.02 (1998) and DARwin Ver. 5 (2008). Analysis of molecular variance (AMOVA) was performed to determine the molecular differentiation between populations. Consensus tree was constructed to combine both morphological and molecular trees (Podani 2000). The Mantel test with 999 random permutations was employed to evaluate the correlation between the geographical, morphological, and molecular distances using GenAlex Ver. 6.4.

Results

- Morphometry

All clustering methods produced highly consistent results. Therefore, only the UPGMA analysis is presented here. The clustering results are illustrated in figure 1. The studied species were assigned to separate clusters. Populations of *O. aucheri*, *O. michauxii*, and *O. persica* comprised distinct subclusters. The populations of *O. aucheri* and *O. michauxii* showed greater morphological affinity to each other than *O. persica*. In general, populations of *O. persica* located in the Sarbaz area (including Sarbaz Bridge, Anza Village, the Itak-Sarbaz Road, and the Kalat-Suran Road) are well-separated from the populations along the Karavandar-Khash Road.

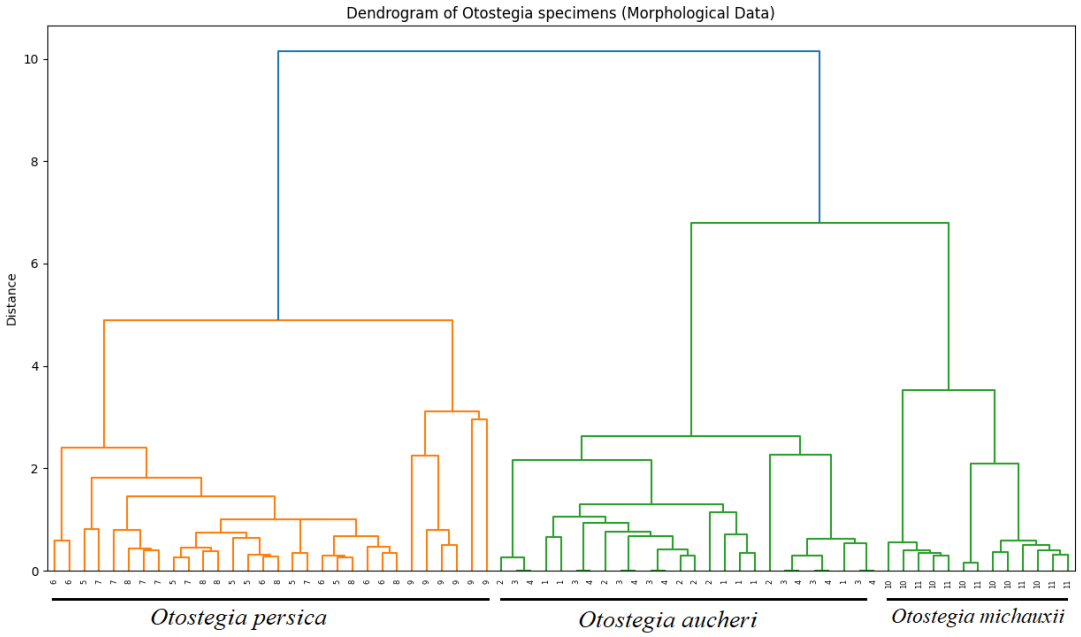

Fig. 1. UPGMA morpho-analysis of *Otostegia* species.

Both PCA and PCoA methods produced similar results, therefore, only the PCA results are presented here (Fig. 2). The PCA biplot and loadings identified the most significant diagnostic morphological characters for differentiating populations. The PCA revealed that, the first two principal components accounted for 98.65% of the total variance. Based on these results, these factors were selected for subsequent analysis. Plant height (accounting for approximately 64.04% of the total variance) had the highest correlation with the first principal component. The characters contributing to the second factor accounting for 34.61% of the total variance were leaf succulence and leaf margin. These characters exhibited high discriminatory power for species identification. Consequently, these morphological characters are highly effective for species differentiation.

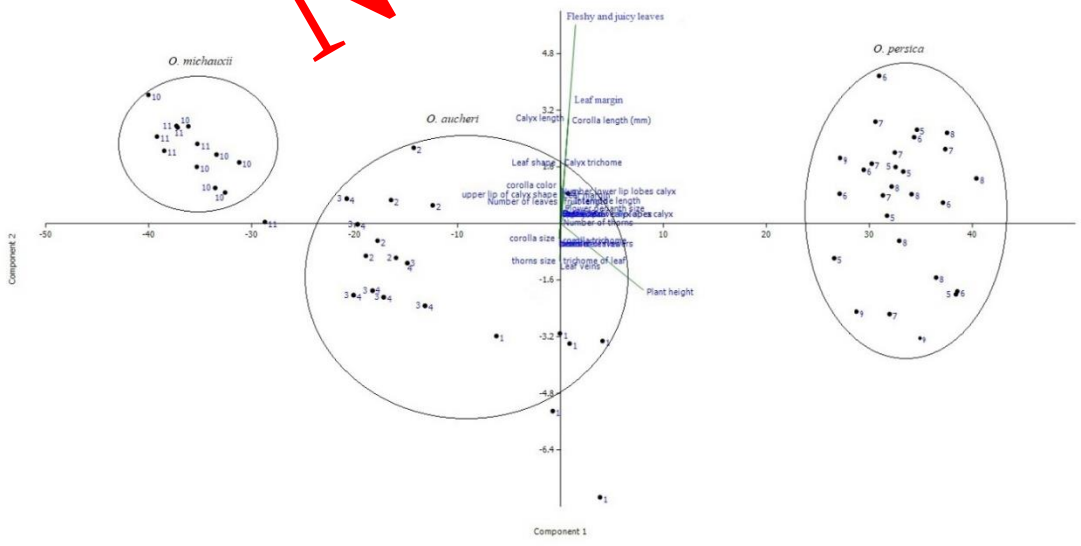

Fig. 2. PCA plot of *Otostegia* species.

- ISSR analysis

A total of 153 polymorphic and reproducible bands (comprising 150 polymorphic and three monomorphic bands) were generated by seven ISSR primers. Among the studied populations, the *O. persica* population from Sarbaz Bridge

exhibited the highest number of ISSR bands (35), whereas the *O. aucheri* population from Mirjaveh showed the lowest (8). Each primer yielded between five and 35 bands, with an average of 21 bands per primer across a size range from 100 bp (UBC807) to 2000 bp (GA) 9T. The ISSR primer, generated the highest number of polymorphic bands (35), while ISSR primers (GA) 9A produced only 5 (minimum observation). Population-specific ISSR bands were also identified; for instance, the *O. persica* population at Sarbaz Bridge exhibited the highest number of specific bands (5). Overall, populations located in the Sarbaz area (including Sarbaz Bridge, Anza Village, the Itak-Sarbaz Road, and the Kalat-Suran Road) possessed a higher frequency of both total and specific bands. The genetic diversity parameters of the species are shown in table 3.

The mean percentage of polymorphic loci (P) was notably high. The heterozygosity values obtained, revealed substantial genetic variability. Consequently, the highest genetic diversity was observed in the Sarbaz Bridge population.

The populations of *O. persica* along the Itek-Sarbaz Road, and Sarbaz Bridge had the most value of percentage polymorphism (55.17%). These populations also had the highest values of the Shannon index (I) and Nei's genetic diversity (He), at 0.272 and 0.175, respectively. However, the populations of *O. michauxii* south of Kazerun (Fars Province, Iran) exhibited the lowest values for percentage polymorphism (20.25), the Shannon index (0.112), and He (0.077).

Analysis of molecular variance (AMOVA) revealed significant molecular differences among the studied species (PhiPT = 0.35, P = 0.01). Furthermore, the results indicated that, 33% of the total genetic variability was attributable to the variation among populations, while 67% was due to the variation within these populations.

Table 3. Genetic diversity parameters in the studied populations

No. of population	Ne	I	He	P%
1	1.242	0.202	0.137	35.56
2	1.157	0.142	0.094	26.67
3	1.157	0.142	0.094	26.67
4	1.151	0.138	0.091	25.17
5	1.351	0.272	0.175	55.17
6	1.322	0.251	0.165	48.28
7	1.351	0.272	0.175	55.17
8	1.311	0.250	0.170	47.28
9	1.220	0.242	0.155	45.25
10	1.142	0.112	0.077	20.25
11	1.147	0.135	0.085	24.23

Ne: No. of effective alleles; I: Shannon index; He: Gene diversity; P%: Percentage of polymorphism

Plant grouping based on both UPGMA and NJ methods yielded highly consistent results. Therefore, only the NJ tree, based on Nei's genetic distance, is explained here (Fig. 3). Consisting with the morphological clustering, two major clusters were identified in the molecular tree. Populations of *O. aucheri* and *O. michauxii* were placed in the first major cluster with their respective populations forming separate subclusters. Various populations of *O. persica* formed the second major cluster. In this cluster, populations located in the Sarbaz area formed a distinct subcluster that was distant from other populations.

In the present study, both morphological and molecular analyses revealed that, *O. persica* populations located in the Sarbaz area were clearly differentiated from other populations, exhibiting higher levels of both morphological and genetic diversity.

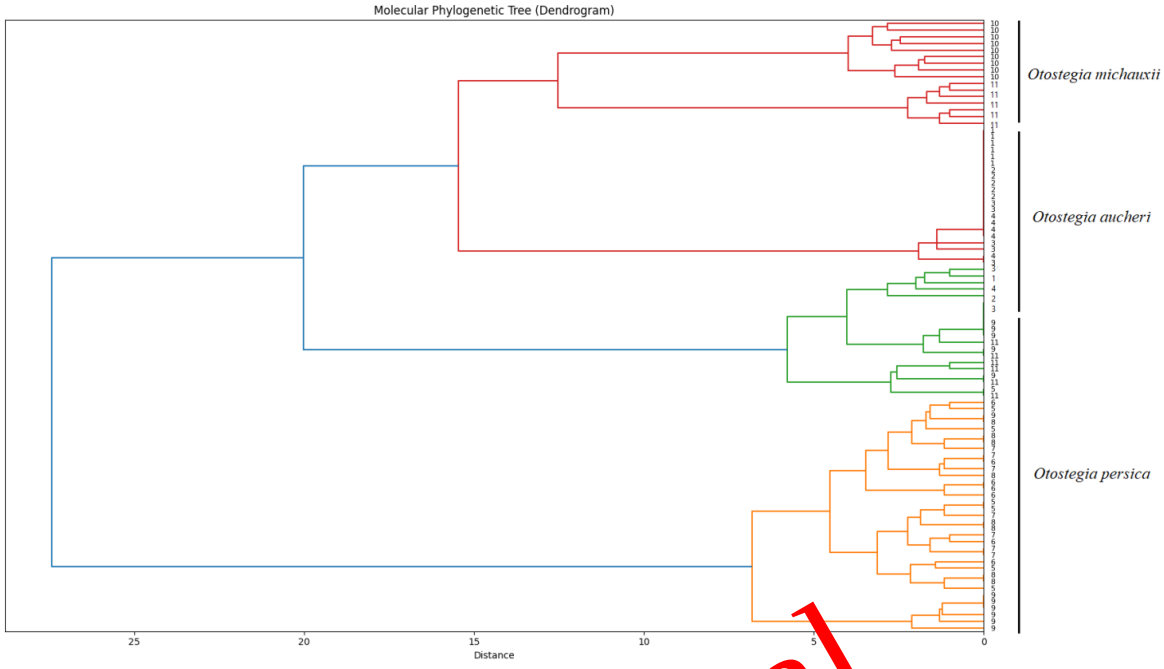

Fig. 3. NJ molecular analysis of *Otostegia* species

The Mantel test revealed no relationship between the genetic and geographical distance among the populations ($P > 0.05$). These results suggest that, gene flow within *Otostegia* populations is not restricted by geographic distance, potentially due to efficient pollen or seed dispersal mechanisms.

Discussion

The present study provides an integrated perspective on the diversity within the genus *Otostegia*, highlighting the potential links between morphological variation and molecular genetic structure. Results derived by present study, demonstrate a notable congruence between morphometric clustering (UPGMA) and molecular groupings (NJ). While previous studies (e.g., Nematinejad *et al*. 2022) have relied primarily on morphological characters to differentiate *Otostegia* populations, the results here suggest that, these phenotypic distinctions may be supported by underlying genetic divergence. The separation of *O. aucheri*, *O. michauxii*, and *O. persica* in both analyses indicated that, the observed morphological characters are likely more than mere environmental plasticity, and potentially reflecting stable taxonomic differences.

Data derived from the present study establish that, vegetative and reproductive morphological traits serve as stable taxonomic markers. For instance, the height of the shrub in *O. persica* is above 80 cm, while in *O. aucheri* and *O. michauxii*, it is below 50 cm. The leaf margin of *O. persica* is dentate and lobed, while in *O. aucheri* and *O. michauxii*, lobes and teeth are absent. The leaves of *O. persica* are broad and have thorns at the tips of the teeth. The leaf shape is ovate to obovate or orbicular. There are more than four leaves per node along the stem. The petiolate leaves are not fleshy or juicy. The length of the leaf blade is 2–3 times of its width. Short trichomes can be seen in the leaf blade. The venation is dense and can be seen on both leaf surfaces. There are 10 or more flowers per inflorescence cycle. The upper lip of the

calyx is rectangular to bayonet-shaped. The number of upper and lower calyx lip lobes is 3 or 3–7, respectively. The corolla (which is taller than the calyx), is white to yellow. Some appendages, such as hairs, rise from the inner wall of the corolla. In *O. aucheri*, leaves are narrowly lanceolate to narrow elliptical. The number of fleshy and juicy leaves is less than four per stem node. The leaves are entire (without teeth or lobes) with a narrowed petiole-like base. The length of leaf blade is not greater than its width. The leaf blade is without trichomes, and the venation is indistinct. The number of flowers per inflorescence cycle is below 10. The upper lip of the calyx is ovate with pointed apex. The number of lower calyx lip lobes is up to three. The calyx has approximately the same size as the corolla. The corolla is white, and its inner surface is devoid of trichomes. *Otostegia michauxii* has a height of about 30–40 cm. The leaves are fleshy, veined, petiolate, and obovate-cuneate with entire margins. Short-appressed hairs cover the leaves. The number of flowers per inflorescence cycle is about 2–4. The calyx covers the corolla tube. The lower lip of the calyx is deeply trilobate.

A particularly interesting finding is the elevated genetic diversity observed in the *O. persica* populations located near the Sarbaz area. These populations exhibited the highest levels of polymorphism (55.17%), Shannon index (0.272), and Nei's genetic diversity (0.175). The presence of private ISSR bands in these populations suggests that, the Sarbaz area might function as a potential evolutionary refugium or a center of diversification for *Otostegia* in Iran-a possibility consistent with the role of diverse microhabitats-in maintaining unique genetic lineages in arid regions.

It is hypothesized here that, the elevated polymorphism in Sarbaz area may be associated with the environmental heterogeneity and complex microclimatic variations characteristic of the Baluchestan topography. It is possible that, such an allelic landscape, potentially influenced by recombination and outcrossing, facilitates the maintenance of high genetic diversity. These dynamics could potentially underlie the observed morphological variations, suggesting that, the highly diverse *O. persica* populations in this region may represent candidate ecotypes adapted to localized ecological pressures.

Interestingly, despite this localized diversity, the Mantel test revealed no significant correlation between genetic and geographic distances, indicating an absence of a clear Isolation-by-Distance (IBD) pattern. This lack of spatial structure suggests that, gene flow may not be strictly limited by geographic distance. Such connectivity could be potentially facilitated by long-distance dispersal mechanisms, such as wind-mediated seed movement or the activity of specialized insect pollinators, which might counteract the effects of habitat fragmentation.

This complex evolutionary pattern-characterized by high localized diversity alongside an absence of IBD-may reflect the unique interplay of climatic and topographical factors in Sistan and Baluchestan. The region's environmental stressors could act through a dual mechanism: 1. While fragmented landscapes and extreme conditions may drive localized adaptation (potentially leading to the formation of distinct populations in Sarbaz area), regional wind patterns or pollinator behaviors might simultaneously maintain a degree of gene flow that prevents strict isolation. Despite these insights, certain limitations must also be acknowledged. First, the reliance on ISSR markers, while useful for assessing genome-wide polymorphism, provides a snapshot of dominant DNA sequences and lacks the higher resolution offered by single-nucleotide polymorphism (SNP) markers or whole-genome sequencing. Consequently, the finer-scale genetic architecture of these species remains fully elucidated; and 2. While sampling here provides a broad overview, it may not capture the complete extent of intra-specific variation across the entire Irano-Turanian range. Future studies employing high-resolution genomic tools (such as RAD-seq or GBS) and more extensive sampling would be instrumental in further refining our understanding of the evolutionary dynamics of *Otostegia* in arid environments.

In conclusion, the research here confirms that, morphological and molecular markers are highly congruent in *Otostegia*, providing a robust basis for its taxonomic classification. The high genetic diversity found in the Sarbaz populations highlights the urgent need for targeted conservation efforts in this region to preserve unique evolutionary

lineages. Future studies should incorporate a broader range of molecular markers and additional ecological data to further refine the conservation strategies for these medicinal species.

Acknowledgments

This paper is derived from MSc thesis of the 1st. author jointly supervised by the 2nd. and 3rd. authors submitted to the University of Sistan and Baluchestan (Zahedan, Iran). The authors thank the Faculty of the Biology Department (University of Sistan and Baluchestan) for providing facilities and support during the present research.

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