International Journal of Horticultural Science and Technology (2027) Vol. 14, No. 1, pp. 71-88 doi.org/10.22059/10.22059/ijhst………………….

International Journal of Horticultural Science and Technology

Journal homepage: https://ijhst.ut.ac.ir

Integrated Analysis of Genetic Diversity in Iranian Cyclamen Populations: Morphological Variation and Phylogenetic Insights from ITS and trnL Molecular Markers

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ARTICLE INFO

Article history:

Received: 5 April 2025,
Received in revised form: 22 November 2025,
Accepted: 22 November 2025,

Article type:

Research paper

Keywords:

Chloroplast region,
Conservation genetics,
Genetic diversity,
Internal transcribed spacer region,
Population differentiation,
Species identification

ABSTRACT

Cyclamen, known for its ornamental appeal and medicinal applications, is one of the most significant potted plants worldwide. However, all wild species within this genus face various levels of endangerment, making their conservation crucial. Identifying different wild species of cyclamen is essential for enhancing conservation efforts and optimizing breeding programs. Previous studies on cyclamen populations native to Iran have utilized morphological, phytochemical, and cytogenetic markers, all confirming notable variations among these populations. Molecular markers in particular have provided more accurate insights into species identification and taxonomic relationships. In this study, we used morphological characteristics alongside the *ITS* molecular marker to precisely identify the species within Iranian cyclamen populations. We further analyzed chloroplast *trn*L (UAA) intron sequence variation to investigate distinctions among *Cyclamen coum* subspecies. Comparative analysis of *ITS1* and *ITS4* ribosomal DNA sequences, along with phylogenetic tree construction, categorized wild Iranian cyclamen as *C. coum*, aligning with morphological findings. The *trn*L sequence data also provided a new perspective on subspecies differentiation in cyclamen and showed a close affinity with *Cyclamen elegans*. Morphological and molecular analyses revealed clear regional differentiation, with the 'Pasand' population showing distinct structural and genetic characteristics. This suggests that 'Pasand' may represent an ecologically specialized or partially isolated group within *C. coum*, emphasizing its importance for conservation and future breeding programs. However, due to the complexities of taxonomic classification within this genus, further studies are needed to clarify the species and subspecies of cyclamen in Iran.

Introduction

Cyclamen species are spread across regions from the Mediterranean through parts of Europe, reaching into areas of Syria, Lebanon, Turkey, and even northern Iran in Asia, and parts of Somalia in Africa (Jalali et al., 2012; Thulin and Warfa, 1989). Global warming and climate change, human activities like road building, dam construction, tourism, agricultural expansion, urban development, harvesting of Cyclamen hypocotyls for medicinal purposes or export as ornamental plants, and livestock grazing have significantly damaged the natural habitats of various plant species, including Cyclamen, especially over the past 50 years. These pressures stressed plant populations, highlighting the need to conserve this germplasm to avoid extinction and ensure future evolution (TasKin et al., 2012; Yavari Kondori et al., 2024). All wild species of Cyclamen are categorized in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). This

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classification means that while these species are not currently endangered, they could become so if unsustainable harvesting and habitat destruction continue. Specifically, *C. libanoticum* is listed as vulnerable on the International Union for Conservation of Nature (IUCN) Red List, while *C. somalense* is recognized as a species at risk of extinction that urgently requires conservation efforts (Species, 2024). Consequently, conservation studies should be carried out on this genus, as it represents a significant genetic resource for ornamental plants (Secretariat, 2023). Therefore, assessing the level and distribution of genetic diversity in rare and endemic species populations is crucial for maintaining genetic diversity within a species as part of established conservation strategies (TasKin et al., 2012). To enhance the germplasm bank of the genus *Cyclamen*, particularly for the commercially significant species *C. persicum*, it is essential to access the germplasm of other Cyclamen species that can be utilized in future breeding efforts (Prange et al., 2010). For example, *C. coum* originates from mountainous regions and demonstrates excellent cold resistance, making it a valuable genetic resource for Cyclamen breeders. Wild Cyclamen exhibit traits such as frost tolerance, cold hardiness, floral scent, varied flower colors, distinct leaf shapes, autumn and winter flowering, and pest and disease resistance—traits often lacking in commercial varieties. As a result, these wild species are leveraged to improve these characteristics in commercial Cyclamen (Aalaei et al., 2007). Globally, *Cyclamen* is among the most commercially significant ornamental potted plants, cultivated for its diverse floral forms and prolonged blooming period. According to international horticultural trade reports, cultivated *Cyclamen persicum* and its hybrids represent a multi-million-euro industry in Europe and Asia, with breeding programs focusing on color diversity, fragrance, and stress tolerance. Beyond its economic value, *Cyclamen* also serves as a key genetic resource for studies on plant adaptation and evolution due to its wide distribution across contrasting climatic zones. However, many wild *Cyclamen* species are now threatened by habitat loss and over-collection. Understanding their genetic and morphological diversity is therefore crucial both for in-situ conservation and for incorporating resilient traits from wild populations into breeding programs aimed at improving cultivated varieties. For example, Yavari-Kondori et al. (2022) conducted a study in 2022 on the morphological traits of Cyclamen populations in Iran and found that the Cyclamen in the Kordkuy region outperformed many other wild Cyclamen species in terms of flower count, leaf count, and leaf area. Therefore, they could interest breeders (Yavari Kondori et al., 2022). Evaluating diversity is essential for enhancing the effectiveness of breeding programs. Consequently, research on morphological, molecular, phytochemical, and cytological characteristics can contribute to assessing of genetic diversity (Yavari Kondori et al., 2024). In this regard, analyses of molecular markers can validate traditional classifications based on morphological and physiological traits, clarify genetic relationships both within and between species, establish connections among populations, and investigate the presence of genetic drift or migration between them (Alhajjar et al., 2011; Doležalová et al., 2003). Moreover, the climatic variations of the Quaternary period and the subsequent alterations in habitats and vegetation may have led to increased species diversity, as proposed for a group of steppe species within the diverse genus *Stipa* (Bagheri et al., 2017). The diversity of some *Cyclamen* species has been studied through morphological research as well as using cytological, biochemical and molecular markers (Curuk et al., 2016). For instance, research has shown that morphological similarities between native Iranian *Cyclamen* and other *Cyclamen* species worldwide may indicate the presence of a broader range of *Cyclamen* species and subspecies in Iran (Yavari Kondori et al., 2022). To validate this hypothesis, molecular marker analysis can evaluate the reliability of traditional classifications based on morphological and physiological traits, identify genetic distances within and across species, clarify relationships among populations, and detect genetic drift between them (Doležalová et al., 2003). Integrating morphological, biochemical, and molecular data can lead to more accurate classification outcomes for the *Cyclamen* genus (Curuk et al., 2016). Iran is home to populations of *Cyclamen*, yet despite visible diversity, little research—particularly at the molecular level—has been conducted on this germplasm. This study aims to examine previously unstudied *Cyclamen* populations through both morphological and molecular markers. Operating under the hypothesis that Iran hosts more than one *Cyclamen* species and that part of the diversity observed in Iranian *Cyclamen* is genetically based, this research aimed to determine whether significant morphological and molecular diversity exists among Iran's *Cyclamen* populations and, if so, to explore the relationships among them and other cyclamen species.

Despite the growing number of studies on *Cyclamen* morphology and tissue culture, limited research has combined morphological and molecular approaches to evaluate the diversity and taxonomic relationships of wild *Cyclamen* populations in Iran. Most previous investigations have focused on *C. persicum* or relied solely on morphological or cytological markers, leaving uncertainties about the genetic structure and subspecies differentiation within *C. coum* populations. Furthermore, the extent to which ecological variation across northern Iran contributes to morphological divergence remains poorly understood.

Therefore, the present study aimed to (1) assess the morphological diversity among *Cyclamen coum* populations from distinct ecological regions of northern Iran, (2) determine their genetic relationships using ITS and trnL molecular markers, and (3) integrate these datasets to clarify potential subspecies differentiation and provide insights for breeding and conservation strategies.

Materials and Methods

Sampling and morphological examination

After two years of preliminary study, all plant samples were collected from the natural habitats in three northern provinces of Iran: Gilan, Mazandaran, and Golestan. The coordinates of the sampling locations were recorded using GPS, and a total of 44 samples, including 10 from each region and four off-type samples, were collected (Table 1). All samples were collected during a single period in December 2024, when all Cyclamen populations were in the active flowering stage to ensure uniform phenological conditions. Morphological data for all plants were recorded within the same week of sampling (Fig. 1), thereby minimizing temporal variation in growth or morphology among regions. This synchronization ensured that observed morphological differences reflect inherent population diversity rather than seasonal or developmental discrepancies. Leaves were stored at -80 °C in a freezer after being frozen with liquid nitrogen until DNA extraction. All morphological traits were measured on-site within the plant's natural habitat. Twentythree traits in total were recorded, comprising 13 quantitative and 10 qualitative characteristics. The traits included leaf number (LN), lamina length (LL), lamina width (LW), lamina length-to-width ratio (LL/LW), leaf margin (LM), leaf tip shape (LTS), leaf shape (LS), flower number (FN), petal number (PN), petal length (PL), petal width (PW), pistil length (PL), stamen length (ST), basal corolla ring color (BCRC), petal color (PC), darker petal margin (DPM), petal tip shape (PTS), pedicel coiling (PEC), basal spot with a white or pale pink eye (BSC), hypocotyl diameter (HD), hypocotyl thickness (HT), rooting zone on the hypocotyl (RZ), surface of the hypocotyl (SH). The surface of the hypocotyl refers to the external texture of the tuber, ranging from smooth to rough, and is indicative of adaptation to environmental conditions such as soil texture and humidity.

Table 1. Sampling locations of cyclamen populations collected from northern Iran.

Region	Province	Coordinates (GPS)	Number of Samples	Plant Species
Lavij	Mazandaran	36°35'N, 52°03'E	10	*C. coum*
Hamsafa	Golestan	36°46'N, 54°28'E	10	*C. coum*
Visadar	Gilan	37°13'N, 49°09'E	10	*C. coum*
Pasand	Golestan	36°45'N, 54°35'E	10	*C. coum*
Unique 1–4	Mixed sites	—	4	*C. coum*

Fig. 1. Representative images of *Cyclamen coum* collected from natural habitats in northern Iran. (A) Flowering individual growing under forest canopy in a humid Hyrcanian habitat; (B) Field measurement and data recording of morphological traits during sampling; (C) Whole plant specimen including leaves, flowers, and tuber after collection; (D) Dissected plant parts showing tuber, petiole, floral organs, and leaf morphology used for trait analysis.

The selected regions represent ecologically diverse sites within the Hyrcanian forest belt of northern Iran, extending along the southern coast of the Caspian Sea. These areas differ in altitude, microclimate, and vegetation structure, allowing a broad representation of Cyclamen habitats. Lavij (Mazandaran Province) is a humid montane forest located at approximately 1,400-1,600 m above sea level, dominated by *Fagus orientalis*, *Carpinus betulus*, and *Alnus subcordata*, with rich humus soil and annual precipitation exceeding 1,200 mm. Hamsafa (Golestan Province) lies at the eastern margin of the Hyrcanian range (around 800-1,000 m), characterized by a transitional climate with warmer summers, mixed deciduous vegetation, and loam-clay soil with moderate moisture. Visadar (Gilan Province) represents a low-elevation site (200-400 m) within the humid temperate zone, featuring dense broadleaf forest canopies and persistently high air humidity throughout the year. Pasand (Golestan Province) is situated in a semi-humid zone (500-700 m) with lighter forest cover and a higher proportion of herbaceous ground flora, where partial habitat fragmentation due to agricultural activities is evident. These ecological variations likely contribute to the observed morphological and genetic diversity among Cyclamen populations by imposing different selective pressures related to temperature, soil composition, and canopy light availability.

DNA extraction and molecular analysis

The DNA extraction followed a modified Doyle and Doyle protocol (Doyle, 1991), using a CTAB buffer prepared with 1.21 g of 1M Tris Base, 8.18 g of 5M NaCl, 0.75 g of 0.5M EDTA, 2 g of CTAB, and 4 g of PVP in a 100 mL solution (Yang, 2017). The collected samples were ground using mortar and pestle. The samples were incubated at 65 °C for approximately 60 min with CTAB buffer and 20 µL β-mercaptoethanol, and extracted with chloroform-isoamylalcohol (24:1). Following the addition of cold isopropanol, the overnighted precipitated DNA was washed with 70% ethanol, then diluted with distilled water for storage. The quality of the extracted DNA was evaluated through agarose gel electrophoresis and Nanodrop analysis. The DNA extracted from all samples within each region was pooled at an equal concentration before amplification. The pooling of DNA samples from each region was performed to obtain an averaged genetic profile representative of the regional population rather than of individual plants. This approach reduces random intra-population variation and facilitates the detection of broader inter-population differences, which aligns with the study's objective of assessing genetic divergence among regions. Although pooling may limit the resolution of individual-level polymorphisms, it is a widely accepted strategy in preliminary phylogeographic studies where the focus is on population-level relationships and cost-effective sequencing(Futschik and Schlötterer, 2010; Hawliczek et al., 2020). The pooled DNA from each region, along with the DNA extracted from the unique samples, were amplified using PCR. Double-stranded DNA amplification was performed in a 20 µL reaction volume containing 10 µL of Ampliqon Taq DNA Polymerase 2x Master Mix (composed of Tris-HCl pH 8.5, (NH4)2SO4, 4 mM MgCl2, 0.2% Tween® 20, 0.4 mM of each dNTP, Ampliqon Taq DNA polymerase, an inert red dye, and stabilizers), 1 µL of each primer, 1 µL of DNA template, and 7 µL of distilled water. The primers used for amplifying the ITS and trnL regions are listed in Table 1. The PCR program started with an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 47 °C for 30 s, and extension at 72 °C for 1 min, performed on a PeQlab thermocycler. To remove excess primers and deoxynucleotide triphosphates after amplication, PCR products were purified on QIAQuick PCR columns (Qiagen), according to the manufacturer's instructions.

Sequencing and phylogenetic analysis

Sequencing was performed, both strands, using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) in a 20 ll volume containing 20 ng of purified DNA and 3.2 pmol of primer, according to the manufacturer's guidelines. Sequencing reactions underwent 25 cycles of 30 s at 96 °C, 30 s at 50 °C, and 4 min at 60 °C on a Perkin Elmer thermocycler. Following this step, excess dye terminators were removed by a spin-column purication. Sequencing reactions were subject to electrophoresis for 6 h on an ABI ABI 3500XL genetic analyzer in a 5% Long Ranger gel (FMC).

The *ITS* and *trn*L region sequences obtained were observed using Chromas 2.6.6 software. Both DNA strands of each sample were sequenced and aligned. Ambiguities were resolved by aligning the forward and reverse sequences of the same sample. A matrix was constructed by aligning the sequences using Clustal X 2.1 (Thompson et al., 1994). Finally, a phylogenetic tree for the sequences analyzed in this study, along with sequences downloaded from the NCBI database (accession numbers: AF163998_AF164019 and AJ236987.1_AJ236989.1), was constructed using RAxML v8.2 software. Phylogenetic trees were constructed via the maximum likelihood method with bootstrap analysis (1000 replicates) to assess clade support.

Statistical analysis

Morphological data were analyzed separately using Python software (version 3.13).

Results

Morphological diversity

The statistical analysis of morphological traits based on variance analysis among different groups indicates that the grouping variable has a significant effect on certain traits, while its impact on others is not substantial. Specifically, traits such as lamina width, lamina length-to-width ratio, hypocotyl diameter, petal length, petal width, pistil length, leaf margin, leaf tip shape, leaf shape, and petal tip shape showed significant differences. However, traits such as flower number and leaf number did not exhibit significant variation.

Descriptive statistics highlighted differences among locations. The first three principal components explained 55.54% of the total variance. Traits like lamina length, petal width, pistil length, leaf tip shape and petal tip shape had the highest loadings on PC1, differentiating samples by location (Fig. 2). Moreover, the overlap of clusters indicates that plants from different regions share similar morphological characteristics. Regions such as Lavij, which appear as tightly clustered groups, suggest a higher degree of morphological similarity among the samples within that region. Additionally, Pasand, which forms a distinct cluster on the right, reflects the unique morphological traits of Pasand plants, particularly in significant characteristics. More concentrated populations are likely more suitable for breeding programs aimed at achieving pure lines in a shorter period, while more dispersed populations may be better suited for breeding programs focused on enhancing diversity through crossbreeding. The contribution of individual morphological traits to the first three principal components is illustrated in Figure 3.

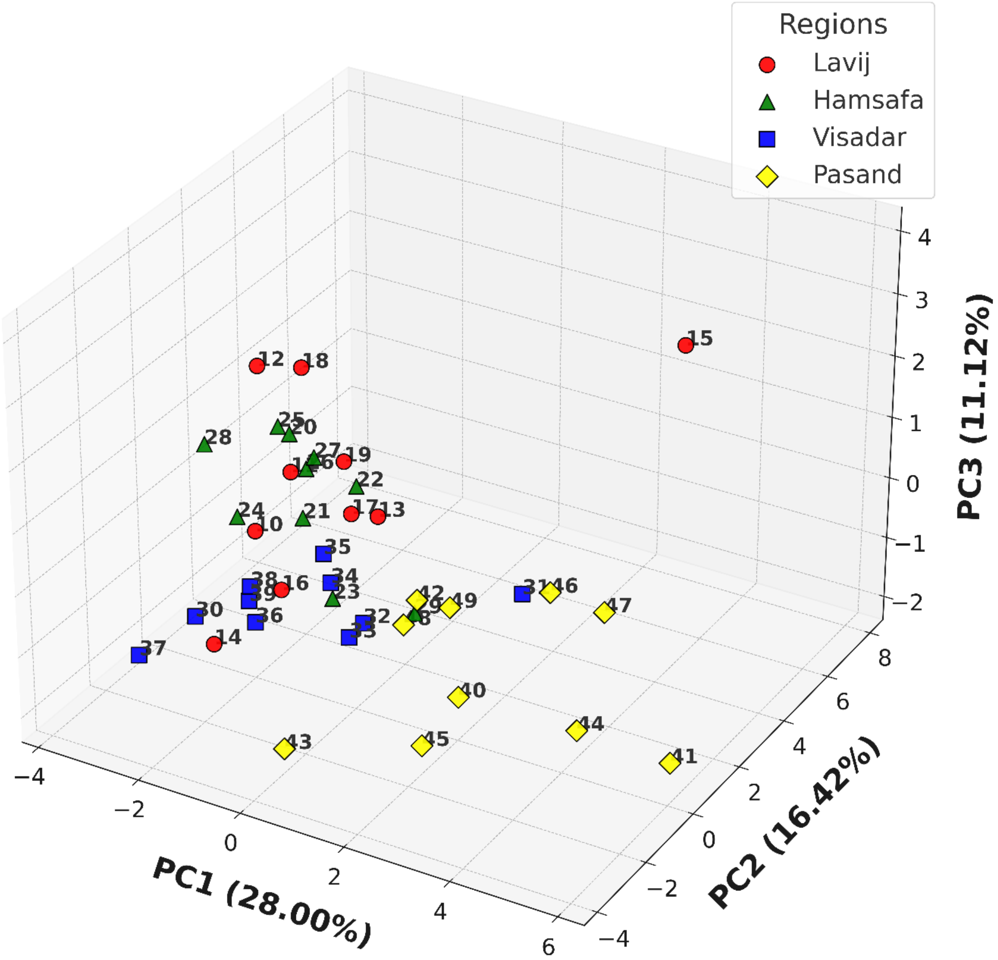


Fig. 2. 3D principal component analysis (PCA) of morphological traits in cyclamen populations from four regions (Lavij, Hamsafa, Visadar, and Pasand). The first three components (PC1–PC3) explain 55.5% of total variance, showing clear morphological separation among regional populations.

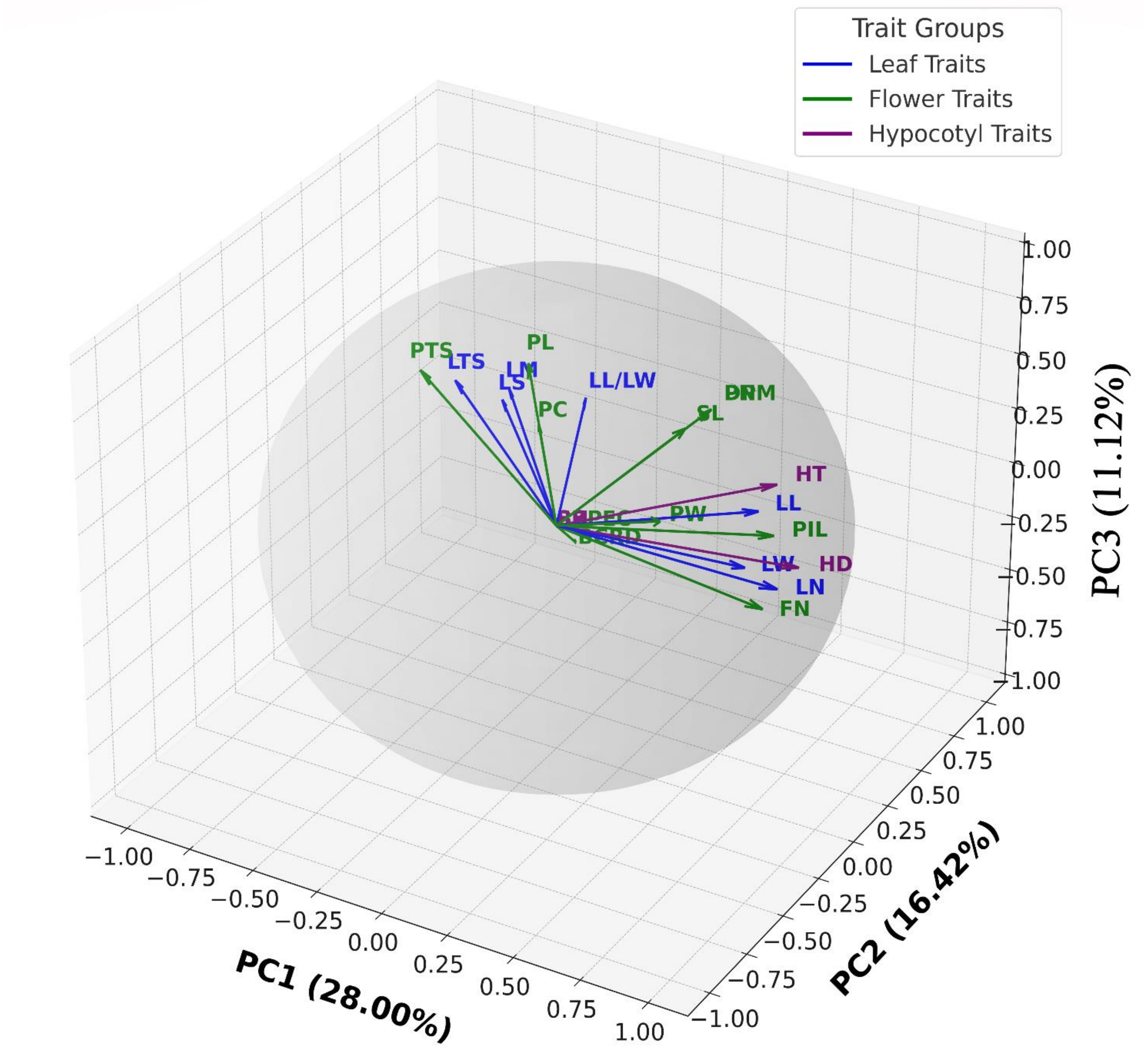
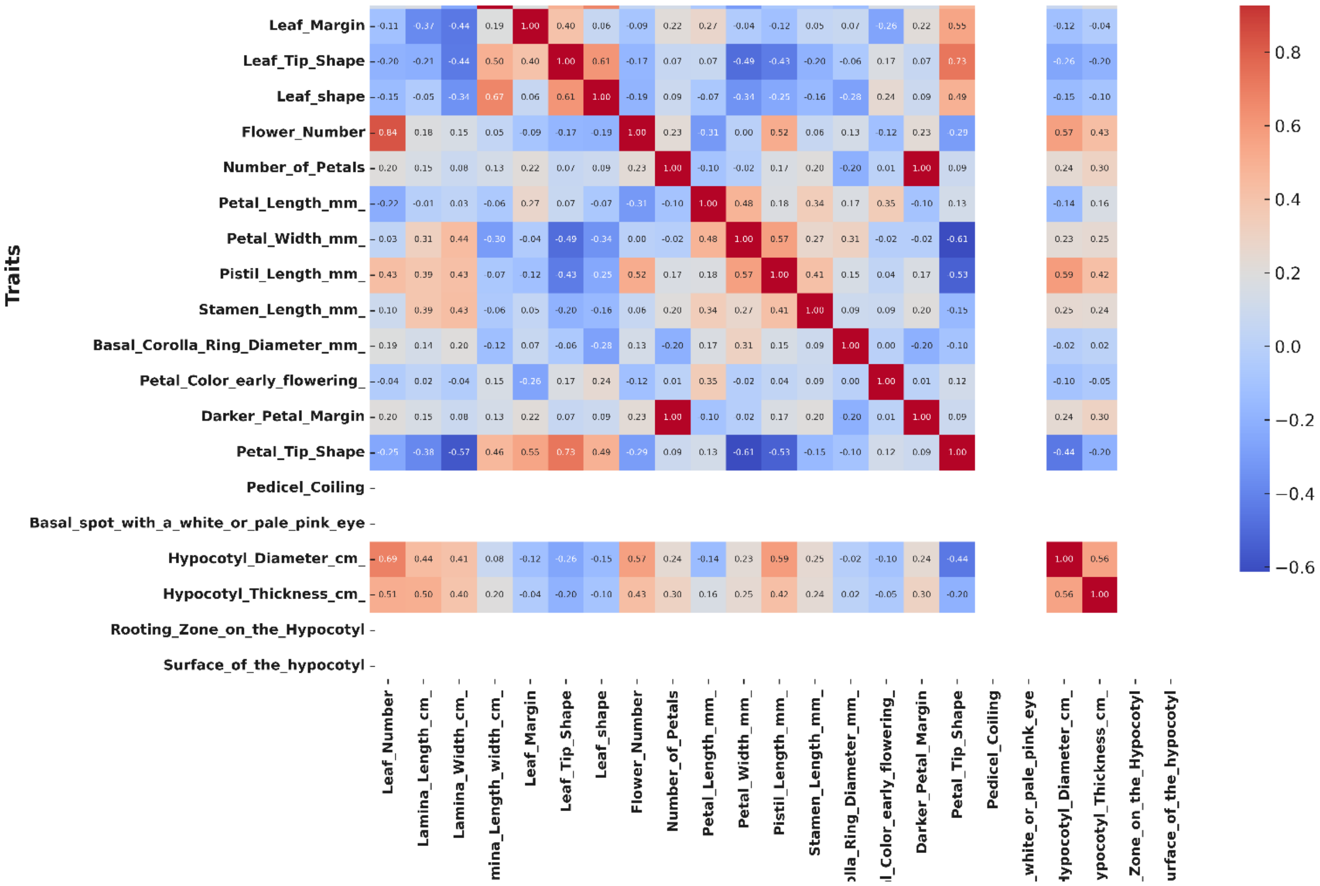


Fig. 3. 3D PCA biplot showing the contribution of morphological traits to the first three principal components (PC1–PC3). Arrows represent variable loadings grouped by trait type: leaf (blue), flower (green), and hypocotyl (purple), illustrating their relative influence on population differentiation.

Additionally, a heatmap with hierarchical clustering was created to group traits and samples based on their similarities (Fig. 4). The dendrogram on the left illustrates how the samples are clustered together, while the dendrogram at the top shows the relationships among the traits. In the clustering analysis of morphological traits, certain traits, such as “Lamina Length,” “Lamina Width,” and “Lamina Length-to-Width Ratio,” were grouped together, indicating a correlation among these traits and likely reflecting similar morphological dimensions, such as size. Similarly, petal-related traits, including “Petal Length,” “Petal Width,” and “Petal Shape,” formed a distinct cluster, potentially suggesting a shared functional or evolutionary origin. Samples that appear closer to each other in the dendrogram generally exhibit similar trait profiles, which may be associated with shared environmental, genetic, or regional factors.

Based on the correlation matrix of the analyzed traits, a positive correlation (0.83) was observed between leaf number and flower number, indicating that plants with more leaves tend to produce more flowers. Additionally, a positive correlation (0.69) between lamina length and hypocotyl diameter is likely related to nutrient transport. Moreover, a positive correlation (0.57) between hypocotyl diameter and petal width, as well as a negative correlation (-0.57) between flower number and petal apex shape, were also detected (Fig. 5).

Fig. 4. Hierarchical clustering heatmap of *Cyclamen coum* samples based on standardized morphological traits. Rows represent individual plants, and columns correspond to measured leaf, flower, and hypocotyl traits. The color gradient indicates standardized trait values (Z-scores), revealing morphological similarity patterns and grouping of samples with shared characteristics.



	Leaf_Number	Lamina_Length_cm_	Lamina_Width_cm_	mina_Length_width_cm_	Leaf_Margin	Leaf_Tip_Shape	Leaf_shape	Flower_Number	Number_of_Petals	Petal_Length_mm_	Petal_Width_mm_	Pistil_Length_mm_	Stamen_Length_mm_	olla_Ring_Diameter_mm_	l_Color_early_flowering_	Darker_Petal_Margin	Petal_Tip_Shape	Pedicel_Coiling	_white_or_pale_pink_eye	lypocotyl_Diameter_cm_	ypocotyl_Thickness_cm_	Zone_on_the_Hypocotyl	urface_of_the_hypocotyl
Leaf_Margin	-0.11	-0.37	-0.44	0.19	1.00	0.40	0.06	-0.09	0.22	0.27	-0.04	-0.12	0.05	0.07	-0.26	0.22	0.55			-0.12	-0.04		
Leaf_Tip_Shape	-0.20	-0.21	-0.44	0.50	0.40	1.00	0.61	-0.17	0.07	0.07	-0.49	-0.43	-0.20	-0.06	0.17	0.07	0.73			-0.26	-0.20		
Leaf_shape	-0.15	-0.05	-0.34	0.67	0.06	0.61	1.00	-0.19	0.09	-0.07	-0.34	-0.25	-0.16	-0.28	0.24	0.09	0.49			-0.15	-0.10		
Flower_Number	0.84	0.18	0.15	0.05	-0.09	-0.17	-0.19	1.00	0.23	-0.31	0.00	0.52	0.06	0.13	-0.12	0.23	-0.29			0.57	0.43		
Number_of_Petals	0.20	0.15	0.08	0.13	0.22	0.07	0.09	0.23	1.00	-0.10	-0.02	0.17	0.20	-0.20	0.01	1.00	0.09			0.24	0.30		
Petal_Length_mm_	-0.22	-0.01	0.03	-0.06	0.27	0.07	-0.07	-0.31	-0.10	1.00	0.48	0.18	0.34	0.17	0.35	-0.10	0.13			-0.14	0.16		
Petal_Width_mm_	0.03	0.31	0.44	-0.30	-0.04	-0.49	-0.34	0.00	-0.02	0.48	1.00	0.57	0.27	0.31	-0.02	-0.02	-0.61			0.23	0.25		
Pistil_Length_mm_	0.43	0.39	0.43	-0.07	-0.12	-0.43	-0.25	0.52	0.17	0.18	0.57	1.00	0.41	0.15	0.04	0.17	-0.53			0.59	0.42		
Stamen_Length_mm_	0.10	0.39	0.43	-0.06	0.05	-0.20	-0.16	0.06	0.20	0.34	0.27	0.41	1.00	0.09	0.09	0.20	-0.15			0.25	0.24		
Basal_Corolla_Ring_Diameter_mm_	0.19	0.14	0.20	-0.12	0.07	-0.06	-0.28	0.13	-0.20	0.17	0.31	0.15	0.09	1.00	0.00	-0.20	-0.10			-0.02	0.02		
Petal_Color_early_flowering_	-0.04	0.02	-0.04	0.15	-0.26	0.17	0.24	-0.12	0.01	0.35	-0.02	0.04	0.09	0.00	1.00	0.01	0.12			-0.10	-0.05		
Darker_Petal_Margin	0.20	0.15	0.08	0.13	0.22	0.07	0.09	0.23	1.00	-0.10	-0.02	0.17	0.20	-0.20	0.01	1.00	0.09			0.24	0.30		
Petal_Tip_Shape	-0.25	-0.38	-0.57	0.46	0.55	0.73	0.49	-0.29	0.09	0.13	-0.61	-0.53	-0.15	-0.10	0.12	0.09	1.00			-0.44	-0.20		
Pedicel_Coiling																							
Basal_spot_with_a_white_or_pale_pink_eye																							
Hypocotyl_Diameter_cm_	0.69	0.44	0.41	0.08	-0.12	-0.26	-0.15	0.57	0.24	-0.14	0.23	0.59	0.25	-0.02	-0.10	0.24	-0.44			1.00	0.56		
Hypocotyl_Thickness_cm_	0.51	0.50	0.40	0.20	-0.04	-0.20	-0.10	0.43	0.30	0.16	0.25	0.42	0.24	0.02	-0.05	0.30	-0.20			0.56	1.00		
Rooting_Zone_on_the_Hypocotyl																							
Surface_of_the_hypocotyl																							

Fig. 5. Correlation matrix of quantitative morphological traits in *Cyclamen coum.* Color scale represents Pearson correlation coefficients (r), with red and blue indicating positive and negative relationships, respectively. White cells correspond to non-numerical (qualitative) traits for which correlation values could not be calculated.

Based on the results of the Tukey HSD analysis, significant and distinct differences were observed in the morphological traits among the regions, particularly between Pasand and the others. Specifically, the lamina width in Pasand was significantly greater than in Hamsafa (by approximately 1.28 cm) and Lavij (by approximately 1.46 cm). These differences were statistically significant at the 5% level for Hamsafa and the 1% level for Lavij, highlighting the unique characteristics of Pasand in this regard. Additionally, Visadar exhibited a significantly lower lamina width compared to Pasand (with a difference of approximately -1.52 cm), which was also significant at the 1% level. These findings underscore clear differences in leaf traits among the regions.

For the lamina length-to-width ratio, Pasand differed significantly from Visadar (by approximately 0.10), which was statistically significant at the 5% level. This provides further evidence of morphological differentiation between these regions. Finally, the hypocotyl diameter in Pasand was significantly greater than in Hamsafa (with a difference of approximately 1.33 cm), a difference that was statistically significant at the 5% level. These comparisons demonstrate that Pasand exhibits distinct morphological traits that differentiate it from other regions. The notable differences in lamina width, lamina length-to-width ratio, and hypocotyl diameter, which are significantly higher in this region, provide strong evidence for the biological and morphological uniqueness of Pasand.

A comparative analysis of all 44 examined samples revealed significant differences in several morphological traits between unique and regional samples, which may indicate ecological adaptations, phenotypic or genetic diversity, or evolutionary divergence. One such trait is the number of leaves, which was significantly lower in the unique samples compared to the regional samples. Because all plants were examined simultaneously during the active flowering period, these differences cannot be attributed to developmental stage or sampling time. This difference may reflect the ecological adaptation of the unique samples to specific environments or optimization for specialized conditions. This trait, likely influenced by genetic or phenotypic diversity, could be indicative of subspecies or unique variants within the population.

Similarly, for flower number, unique samples produced significantly fewer flowers than the regional samples. This reduction in flower production may reflect a resource allocation trade-off, where plants in harsher or resource-limited environments invest more in maintenance and stress tolerance rather than maximizing reproductive output at a given time. Such patterns are commonly observed in populations exposed to drought, nutrient deficiency, or limited light availability, where reproductive effort is adjusted to ensure long-term persistence. Along the same lines, the hypocotyl diameter was also significantly smaller in the unique samples, which may be interpreted as an adaptation to environmental stresses, such as drought or nutrient scarcity. These differences could also result from distinct growth strategies and physiological adjustments in the unique samples.The three-dimensional PCA plot (Fig. 6) illustrates the distribution of all 44 samples, including regional and unique individuals, based on their morphological traits. The plot shows that unique samples are partly separated from regional clusters, suggesting potential morphological divergence. Based on the results of the PCA analysis for all samples, this separation of unique samples may indicate the presence of a distinct subspecies or a phenotypic type specifically shaped by environmental conditions. These clustering patterns could reflect the ecological adaptations of the unique samples or may serve as evidence of evolutionary divergence within these groups. Based on the results of the PCA analysis for all samples, the separation of unique samples may indicate the presence of a distinct subspecies or a phenotypic type specifically shaped by environmental conditions. These clustering patterns could reflect the ecological adaptations of the unique samples or may serve as evidence of evolutionary divergence within these groups.

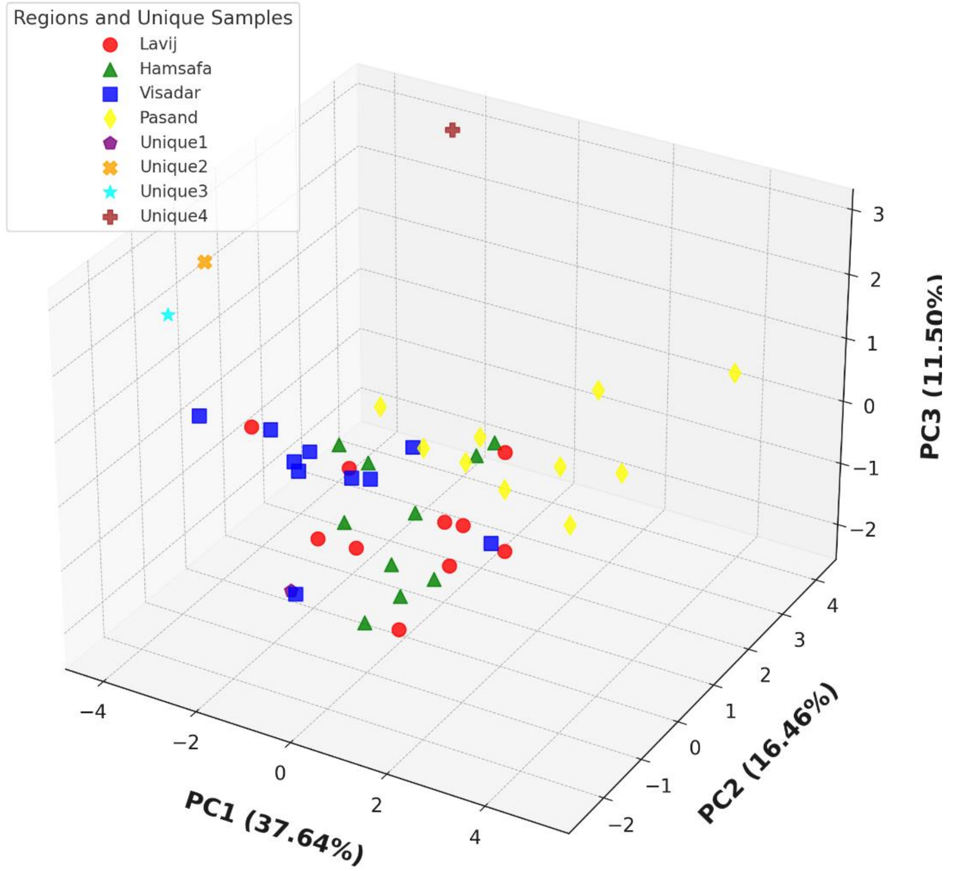


Fig. 6. 3D PCA of morphological traits in *Cyclamen coum* populations from four regions (Lavij, Hamsafa, Visadar, and Pasand) and four unique samples (Unique 1–4). The first three principal components (PC1–PC3) explain 65.6% of total variance. The spatial distribution highlights morphological differentiation, with Pasand and unique samples showing partial separation from other regional populations.

Figure 7 displays the variation of key quantitative morphological traits—including petal width and length, pistil length, lamina dimensions, and hypocotyl diameter—across four regions and unique samples. The boxplots reveal clear regional differentiation, with Pasand showing generally higher values in hypocotyl diameter and lamina width, while Visadar and Hamsafa display lower mean values for petal traits. Unique samples exhibit distinct trait ranges compared to regional populations, emphasizing their morphological divergence.

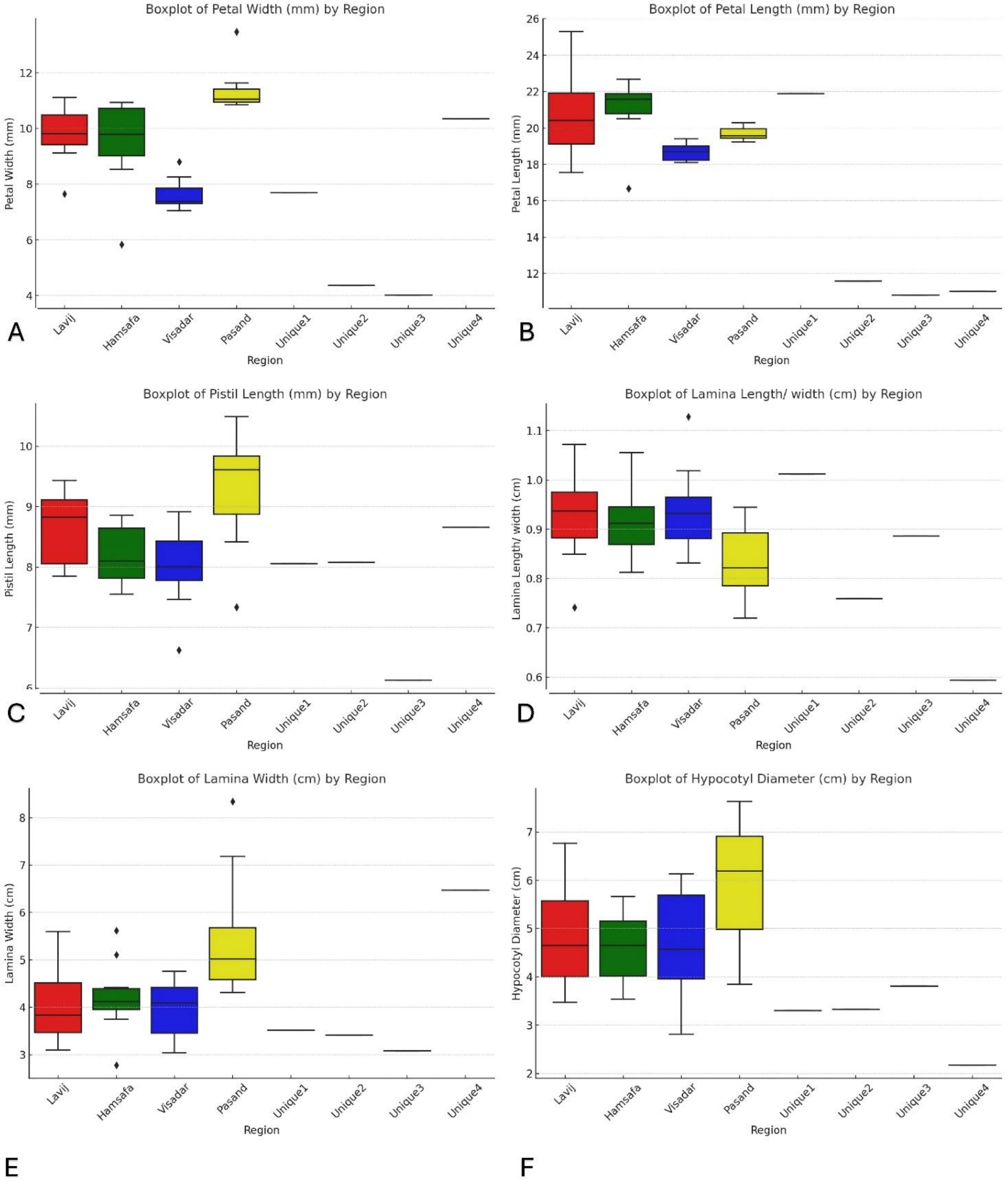


Fig. 7. Regional variation in selected morphological traits of *Cyclamen coum*. (A) Petal width; (B) petal length; (C) pistil length; (D) lamina length-to-width ratio; (E) lamina width; and (F) hypocotyl diameter across four regions (Lavij, Hamsafa, Visadar, and Pasand) and four unique samples. Boxes represent interquartile ranges with median lines; dots indicate outliers.

From an evolutionary perspective, these inter-population morphological differences suggest the action of local selection pressures and potentially restricted gene flow among geographically isolated populations. The distinct clustering of Pasand in the PCA and its morphological divergence support the possibility of incipient ecological differentiation, which could be an early step toward subspecies formation. Such phenotypic differentiation in geographically fragmented populations is consistent with the patterns observed in other Cyclamen taxa distributed across the Mediterranean and Near Eastern regions, where environmental heterogeneity and historical isolation have promoted both morphological plasticity and genetic diversification (Curuk et al., 2016; TasKin et al., 2012).The integration of morphological and molecular findings provides valuable information for both conservation and breeding initiatives. The clear regional differentiation—particularly the distinctiveness of the Pasand population—indicates potential genetic isolation, making these populations important candidates for in situ and ex situ conservation. Preserving these genetically and morphologically distinct groups could help maintain adaptive traits essential for the long-term survival of Cyclamen in changing environments. Moreover, the identification of populations such as Lavij and Visadar, which exhibit high morphological uniformity and apparent gene flow, provides a useful genetic pool for breeding programs aimed at improving cold tolerance, floral characteristics, or ornamental quality in cultivated Cyclamen. By identifying ecologically resilient and genetically unique populations, this study contributes to a more targeted and sustainable approach to Cyclamen germplasm conservation and utilization.

In addition to quantitative traits, qualitative morphological characters were analyzed using Chi-square tests to determine their association with sampling regions (Table 2). Significant regional variation was observed for leaf margin, leaf tip shape, and petal tip shape ($P < 0.01$), while other traits such as petal color and surface of the hypocotyl showed no significant differences. These results confirm that both quantitative and categorical traits contribute to morphological differentiation among Cyclamen populations.

Table 2. Results of Chi-square analysis of qualitative morphological traits of *Cyclamen coum* populations from four regions (Lavij, Hamsafa, Visadar, and Pasand). Significant Chi-square values ($P < 0.05$) indicate differences in trait distribution among regions.

Trait	χ^2	*df*	*P-value*	*Significance*
Leaf margin	25.88	9	0.0021	**
Leaf tip shape	21.18	3	9.68E−05	***
Leaf shape	20.27	6	0.002	**
Petal color (early flowering)	7.54	9	0.27	ns
Darker petal margin	3.08	3	0.38	ns
Petal tip shape	40.00	6	1.07E−08	***
Pedicel coiling	0.00	9	1.00	ns
Central corolla with white/pale pink eye	0.00	3	1.00	ns
Rooting zone on hypocotyl	0.00	3	1.00	ns
Surface of hypocotyl	0.00	3	1.00	ns

Molecular insights

The phylogenetic tree was constructed using ITS sequences from the collected samples and supplemented with sequences of other cyclamen species from the NCBI database. It provides robust evidence for clustering our samples within the species and subspecies of *C. elegans* and *C. coum*. This cluster, which includes eight samples, has a bootstrap value of 89, indicating a close genetic relationship among them. Notably, the cluster is closely associated with *C. elegans* and *C. coum*, with *C. elegans* identified as the sister taxon, supported by a very high bootstrap value of 98. These findings suggest that the studied samples may represent a subgroup or population of *C. elegans*, sharing similarities with *C. coum* while potentially exhibiting subtle distinguishing traits.

The ITS-based phylogenetic dendrogram (Fig. 8) illustrates the relationships among Cyclamen species and the regional populations sampled in this study. Samples collected from the four Iranian regions—Lavij (Mazandaran Province), Hamsafa (Golestan Province), Visadar (Gilan Province), and Pasand (Golestan Province)—together with Unique 1–4 plants, form a cohesive red clade positioned within the *Cyclamen coum*–*C. elegans* complex. This grouping indicates a close genetic affinity among wild Iranian cyclamen populations and confirms their placement within the *C. coum* lineage. Within this red clade, subtle sub-branching reflects geographic structure: Pasand and Hamsafa populations cluster near the *C. elegans* node, while Lavij, Visadar, and Unique 4 group more tightly together, suggesting more recent shared ancestry or gene flow among western populations.

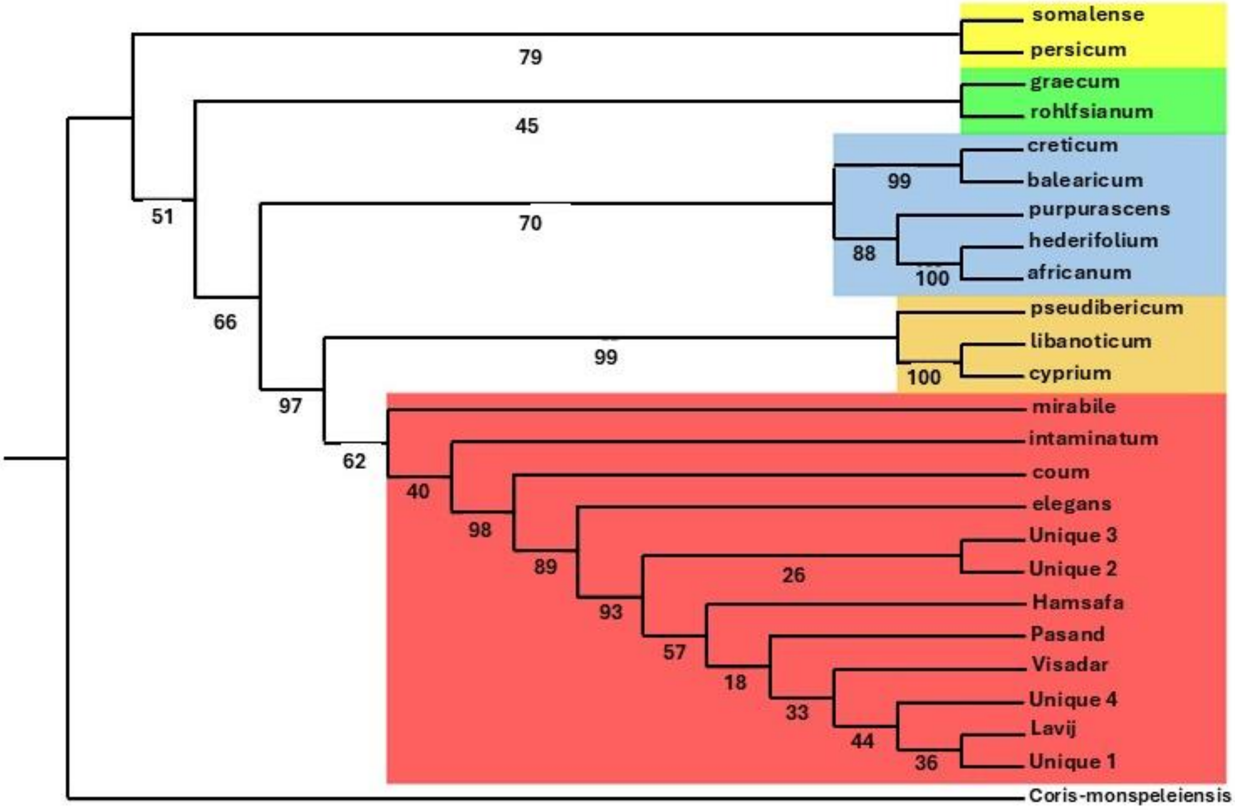


Fig. 8. ITS-based phylogenetic tree of Cyclamen species showing the relationships of Iranian populations (red clade) with reference taxa. Samples from Lavij, Hamsafa, Visadar, Pasand, and four unique individuals cluster within the *C. coum*–*C. elegans* complex, supported by high bootstrap values.

Higher-level clustering patterns correspond to known species relationships. For instance, the *C. graecum*–*C. persicum*–*C. somalense* (yellow–green clade) and *C. creticum*–*C. purpurascens*–*C. hederifolium* (blue clade) groups are consistent with previously published ITS-based phylogenies. The moderate bootstrap support (40–98) among Iranian accessions suggests partial lineage sorting or recent divergence, while their monophyly supports recognition as an intraspecific variant or potential subspecies within *C. coum*.

To further investigate the relationships between the studied samples and other subspecies of *C. coum* and related Cyclamen species, molecular comparisons were repeated using *trnL* sequences (Fig. 9). The resulting phylogenetic tree, depicted in the figure, reveals notable findings. The red clade demonstrates substantial intraspecific variation within *C. coum*, likely driven by geographic isolation or adaptation to distinct ecological niches and close relationship with *C. elegans*. This diversity suggests that *C. coum* is undergoing ongoing evolution, with certain populations potentially differentiating into subspecies. Within the blue clade, *C. coum subsp. caucasicum* diverges markedly from *C. coum subsp. elegans* (red clade), indicating adaptations to unique ecological conditions or ranges. Additionally, *C. coum subsp. coum*, located near *C. coum subsp. caucasicum*, forms the core of the blue clade. Its placement implies that it may represent the ancestral or widely distributed form of the *C. coum* species.

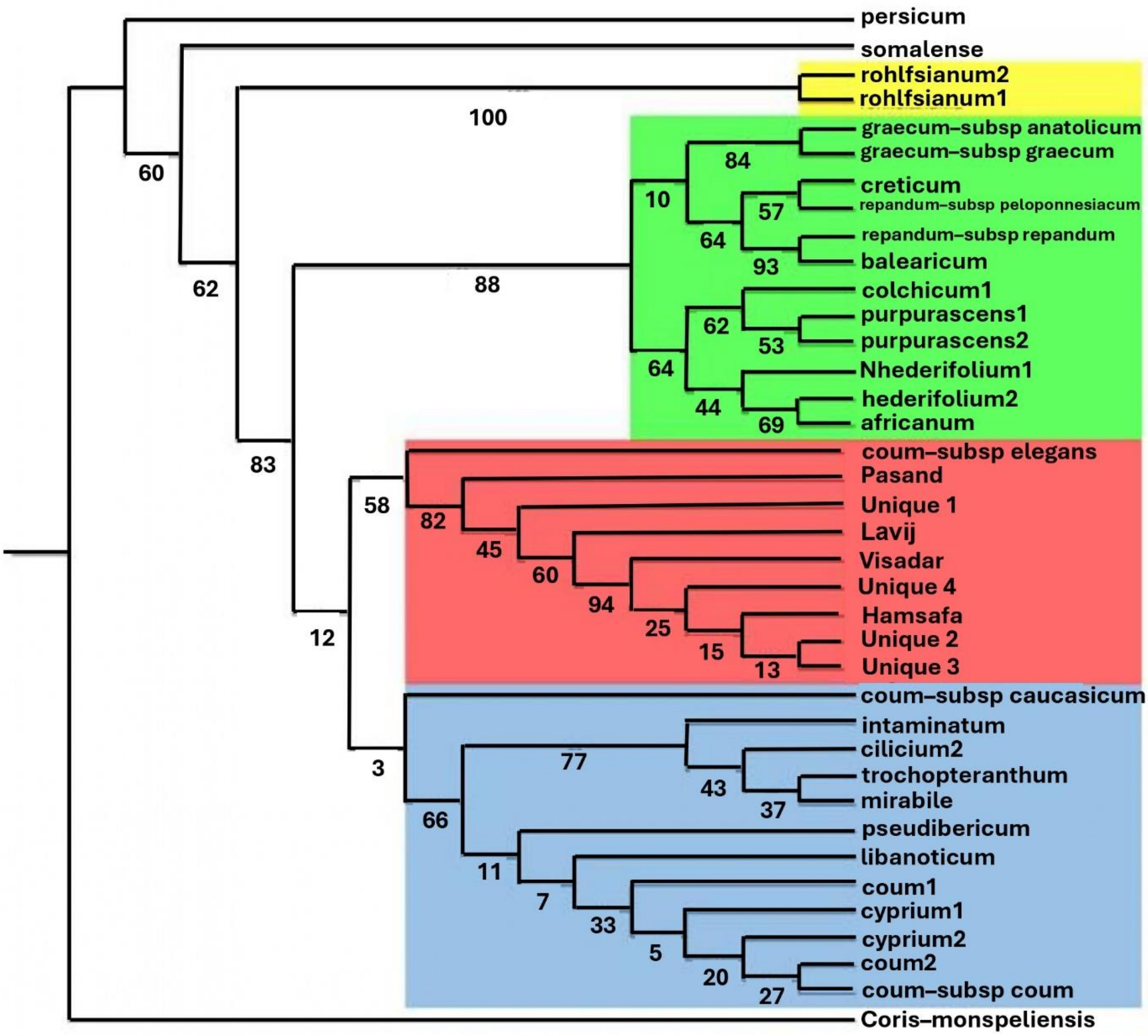


Fig. 9. trnL-based phylogenetic tree of cyclamen species and subspecies. The red clade represents Iranian *C. coum* populations analyzed in this study, showing intraspecific variation and a close relationship with *C. elegans*. The blue clade includes *C. coum* subsp. caucasicum and *C. coum* subsp. coum, indicating divergence linked to geographic and ecological factors.

The separation of Pasand within the red clade indicates notable genetic divergence from other regions, such as Lavij, Visadar, and Hamsafa, as well as the Unique 1–4 samples. Although bootstrap support in this region is modest (45–60), it suggests a mix of shared ancestry and sufficient divergence to position Pasand as a distinct group. This separation is likely driven by geographic or ecological isolation, limiting gene flow and promoting local adaptation. Pasand may represent an isolated population shaped by genetic drift, possibly originating from a small founder group or experiencing a population bottleneck. In contrast, the tighter clustering of Lavij, Visadar, and Hamsafa suggests stronger genetic connectivity or recent divergence. Pasand's distinctiveness highlights its unique evolutionary trajectory, potentially influenced by historical events and environmental factors. Further genetic and ecological studies could provide deeper insights into the processes driving this divergence.

Discussion

According to heatmap with hierarchical clustering, the correlated traits, such as “Lamina Length” and “Lamina Width,” may indicate a shared growth mechanism or selective pressure acting on these traits (Bradshaw et al., 1991). In contrast, the clustering of petal-related traits reflects their common evolutionary pathway or functional linkage (Bai, 2017).

Overall, PCA analyses demonstrate that the unique samples possess distinctive morphological traits, potentially arising from adaptation to specific environments, varying selective pressures, or genetic differences (Bradshaw et al., 1991). These findings provide evidence of intra-species or subspecies diversity within the studied Cyclamen populations.

rDNA sequences are highly conserved among different species due to their critical role in protein synthesis by ribosomes. As a result, these sequences are frequently used in phylogenetic studies to compare genetic relationships between species. Ribosomal RNA sequences also have the potential to aid in species identification, particularly in cases where lineages have been separated for extended periods with minimal morphological changes, such as in protozoan parasites and fungal pathogens (Keith Hamby et al., 1988).

The ITS1 and ITS2 regions have been extensively utilized in phylogenetic reconstructions. For example, studies by (Baldwin, 1993), (Suh et al., 1993), (Baldwin et al., 1995), and (Eldenäs et al., 1998) have demonstrated their variability to be suitable for species-level research. Additionally, chloroplast DNA (cpDNA) is recognized as one of the most powerful tools in phylogenetic and evolutionary studies. The non-coding region known as the trnL (UAA) intron has been widely employed to infer phylogenetic relationships at family and genus levels (Gielly and Taberlet, 1996; Jork and Kadereit, 1995; Susanto et al., 2020), within genera (Böhle et al., 1996; Kita et al., 1995; van Ham et al., 1994), and even within species (FERRIS et al., 1993; Garcia-Suarez et al., 1997; Liston and Kadereit, 1995; McCauley et al., 1996; Susanto et al., 2020). The high mutation rates often observed in this region (Gielly and Taberlet, 1994) make it a particularly valuable tool for clarifying phylogeographic relationships.

The integration of these molecular markers data highlights the rich genetic diversity. As shown in Figure 10, the red clade demonstrates significant internal diversity, highlighting extensive evolutionary diversification within *C. coum* and related taxa. Bootstrap values vary, with higher values (91–98) supporting major groupings and lower values (20–53) in subclusters, indicating incomplete lineage sorting or recent divergence. *C. intaminatum*, *C. mirabile*, and *C. coum* form a basal subgroup, likely representing ancestral lineages or early offshoots of the *C. coum* complex. Meanwhile, *C. elegans* shows evolutionary distinction despite its proximity to the core *C. coum* populations.

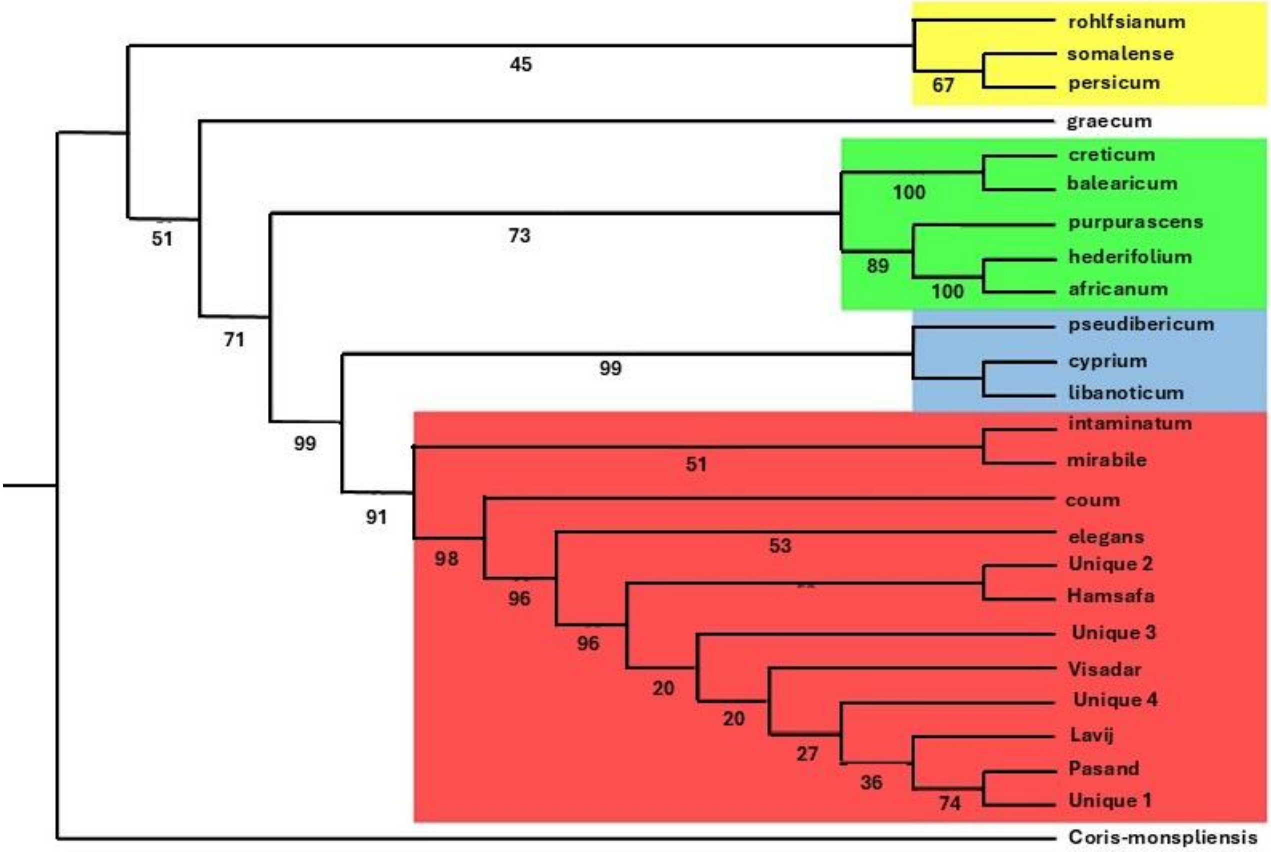


Fig. 10. Combined ITS + trnL phylogenetic tree of Cyclamen species showing the relationships among Iranian populations (red clade) and reference taxa. The Iranian samples cluster within the *C. coum*–*C. elegans* complex, with the Pasand population forming a distinct, well-supported sub-branch, indicating partial genetic differentiation within *C. coum.*

The geographically defined populations (Unique 1–4, Pasand, Lavij, Visadar, Hamsafa) form a closely related cluster with moderate to high bootstrap support, suggesting recent diversification. Among these, Pasand exhibits strong divergence (bootstrap 74), likely due to geographic or ecological isolation driving local adaptation. Lavij and Visadar populations cluster tightly with Unique 4, indicating frequent gene flow or recent shared ancestry, while Hamsafa occupies an intermediate position between Unique 2 and the Lavij-Visadar cluster, reflecting moderate genetic distinction (bootstrap 53).

Unique populations (1–4) show scattered placement within the red clade, reflecting notable genetic diversity. For instance, Unique 1 clusters with Pasand, while Unique 2 aligns with Hamsafa and the core *C. coum* group. In contrast, Unique 3 and 4 exhibit high genetic similarity with Lavij and Visadar. The patterns observed suggest genetic isolation in populations like Pasand and Hamsafa, potentially driven by physical barriers or limited gene flow. In contrast, the tight clustering of Lavij, Visadar, and Unique 3/4 points to recent divergence influenced by ecological factors. Divergent populations, such as Pasand, may reflect unique local adaptations, while lower bootstrap values in some branches suggest ongoing gene flow or incomplete lineage sorting within the red clade.

The patterns of morphological and molecular divergence observed among Cyclamen populations align with classical principles of evolutionary ecology and conservation genetics. Populations such as Pasand, which exhibit both morphological distinctiveness and genetic divergence, may represent incipiently isolated gene pools—an early stage of evolutionary differentiation. According to evolutionary theory, geographic isolation combined with ecological heterogeneity promotes divergent selection, leading to adaptive differentiation and possibly subspeciation over time (Nosil, 2012). In the Hyrcanian region, steep environmental gradients in temperature, humidity, and soil composition likely act as micro-refugia, maintaining local adaptations and preventing complete homogenization of gene flow across populations.

From a conservation standpoint, the existence of genetically and morphologically distinct Cyclamen populations underscores the need for regionally tailored conservation strategies. Preserving both widespread and isolated populations can safeguard not only current genetic diversity but also the evolutionary processes that generate it. Conservation programs should therefore consider maintaining in situ populations across different ecological zones, complemented by ex situ germplasm collections to capture unique alleles and phenotypic variants. Integrating molecular data such as ITS and trnL diversity with morphological and ecological information can enhance the identification of evolutionarily significant units (ESUs), which are essential for the long-term sustainability of Cyclamen biodiversity in Iran and beyond. While this study provides valuable insights into the morphological and genetic diversity of Iranian cyclamen populations, certain limitations should be acknowledged. The molecular analysis was based primarily on two loci—ITS and trnL—which, although widely used for phylogenetic reconstruction, may not fully capture fine-scale genetic differentiation or recent divergence events. Both markers represent relatively conserved genomic regions; thus, subtle population-level variations or hybridization signals could remain undetected. Future studies could benefit from incorporating high-resolution molecular markers such as chloroplast intergenic spacers, microsatellites (SSRs), or next-generation sequencing (NGS) approaches like RAD-seq or genome skimming. These techniques would provide a more detailed understanding of gene flow, population structure, and adaptive variation. Additionally, expanding the sampling across a broader geographic range and including environmental variables (e.g., soil pH, light intensity, precipitation gradients) could further elucidate the ecological drivers of morphological and genetic diversity in cyclamen.

Conclusion

This study highlights significant morphological and genetic diversity among Cyclamen populations, with distinct patterns emerging across regions. Morphological analyses revealed clear differentiation in traits such as lamina width, petal shape, and hypocotyl diameter, particularly in Pasand, which displayed unique characteristics compared to other regions. Principal Component Analysis and clustering identified strong correlations among traits, suggesting shared evolutionary pathways or functional linkages. The distinct trait profiles of Pasand and the unique samples indicate possible ecological adaptation or genetic drift due to geographic isolation or selective pressures.

Molecular data corroborated these findings, with phylogenetic analyses showing the studied samples clustered within *Cyclamen elegans* and *C. coum*, reflecting close genetic relationships. However, populations like Pasand exhibited significant divergence, potentially due to geographic barriers, limited gene flow, or local adaptation. Unique samples showed genetic variation, with scattered placement within the red clade, suggesting potential subspecies differentiation or ecological specialization.

Overall, this study underscores the importance of integrating morphological and molecular approaches to uncover the evolutionary trajectories of cyclamen populations. The observed diversity and divergence highlight the role of geographic isolation and environmental factors in shaping the evolutionary pathways of these populations. Further studies incorporating additional genetic markers and ecological data could provide deeper insights into the mechanisms driving this diversity. The findings of this study have direct implications for both cyclamen breeding and conservation strategies. The identification of distinct morphological and genetic patterns among regional populations—particularly the differentiation of Pasand and other isolated groups—provides valuable information for developing targeted breeding programs. Populations with desirable adaptive traits, such as cold tolerance, unique leaf or flower morphology, and stress resilience, can serve as genetic resources for the improvement of cultivated cyclamen varieties.

From a conservation perspective, the observed regional differentiation highlights the need to protect not only widespread populations but also ecologically and genetically unique ones that may harbor locally adapted traits crucial for long-term species survival. Integrating molecular and morphological data, as demonstrated in this study, can help define conservation units and guide ex situ collections or reintroduction efforts in threatened habitats. Therefore, this research not only clarifies the taxonomic and evolutionary relationships within cyclamen populations in Iran but also contributes to their sustainable use and preservation in the face of environmental change.

Acknowledgments

The authors would like to thank all individuals who provided technical and administrative support during sample collection and laboratory procedures.

Author contributions

Conceptualization, EA; methodology, EA; software, EA; validation, EA, RN, and MSA; formal analysis, EA; investigation, EA; resources, RN and MSA; data curation, EA; writing—original draft preparation, EA; writing—review and editing, EA, RN, and MSA; visualization, EA; supervision, EA, RN, and MSA; project administration, RN and MSA. All authors have read and agreed to the published version of the manuscript.

Funding

This research received no external funding.

Conflict of Interest

The authors indicate no conflict of interest in this work.

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