



## *Dactylorhiza romana* (Sebast.) Soó subsp. *romana*: Sepal and bract morphometry

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Received : 04.11.2025  
Accepted : 13.12.2025  
Online : 26.01.2026

## *Dactylorhiza romana* (Sebast.) Soó subsp. *romana*: Sepal ve brakte morfometrisi

**Abstract:** This study aimed to compare the morphology of the sepal, and bract of dimorphic (pink and pale yellow-flowered) *Dactylorhiza romana* subsp. *romana* specimens used in salep production using classical and geometric morphometric methods. In classical morphometric analyses, criteria such as area, perimeter, aspect ratio, circularity, and solidity of flower parts collected from three different localities were examined; within-group differences were evaluated using analysis of variance (ANOVA) and discriminant analysis. In the geometric morphometric method, shape analyses were performed using homologous landmarks; procrustes analysis, principal component analysis (PCA), discriminant analysis, and regression analysis were performed. The findings showed that the Samsun specimens were distinctly different from the Çanakkale and Bolu groups, while Bolu exhibited transitional region characteristics. Furthermore, although the morphometric characteristics of these flower structures were effective in distinguishing between localities, no significant morphological variation was determined in dimorphic individuals. The differentiation between dimorphic specimens was thought to be due to genetic reasons.

**Key words:** Classical morphometry, geometric morphometry, *Orchidaceae*

**Özet:** Bu çalışma, salep üretiminde kullanılan dimorfik (pembe ve sarı çiçekli) *Dactylorhiza romana* subsp. *romana* örneklerinin sepal ve brakte morfolojisini klasik ve geometrik morfometrik yöntemlerle karşılaştırmayı amaçlamıştır. Klasik morfometrik analizlerde üç farklı lokaliteden toplanmış çiçek parçalarının alanı, çevresi, en-boy oranı, dairesellik ve sağlamlık gibi ölçütler incelenmiş; grup içi farklılıklar varyans analizi (ANOVA) ve diskriminant analizi ile değerlendirilmiştir. Geometrik morfometrik yöntemde homolog yer işaretleri aracılığıyla şekil analizleri yapılmış; procrustes analizi, temel bileşenler analizi (PCA), diskriminant analizi ve regresyon analizi gerçekleştirilmiştir. Elde edilen bulgular, Samsun örneklerinin Çanakkale ve Bolu gruplarından belirgin biçimde ayrıldığını, Bolu'nun ise geçiş bölgesi özelliği sergilediğini göstermiştir. Ayrıca bu çiçek yapılarının morfometrik özelliklerinin lokaliteler arasında ayırıcı etkiye sahip olmasına rağmen dimorfik bireylerde anlamlı morfolojik varyasyon belirlenmemiştir. Dimorfik örnekler arasındaki farklılaşmanın genetik nedenlerden kaynaklandığı düşünülmüştür.

**Anahtar Kelimeler:** Klasik morfometri, geometrik morfometri, *Orchidaceae*

**Citation:** Bakır A, Şenel G, Sungü Şeker Ş, Akbulut MK (2026). *Dactylorhiza romana* (Sebast.) Soó subsp. *romana*: Sepal and bract morphometry. *Anatolian Journal of Botany* 10: 15-22.

### 1. Introduction

The *Orchidaceae* family, with 736 genera (Chase et al., 2015) and approximately 28,000 species (Christenhusz and Byng, 2016) is one of the three largest plant families. Orchids have become popular models in studies on flower development, functional morphology, reproductive biology, and plant-pollinator interactions (Vignolini et al., 2012) due to the morphological diversity of their flowers, their traditional use, and their commercial importance. There is significant diversity in shape and colour among orchid species, and this complicates the identification of some taxa (Bradshaw et al., 2010). The arrangement of flowers, the number of parts, the size, colour, and proportions of flower parts such as bracts are important distinguishing features in orchid identification and diagnosis (Hürkul et al., 2020). In *Dactylorhiza* Neck. ex Nevski, taxonomic identification often relies on floral characters such as the shape and lobing of the labellum (lip), the presence, length, direction and shape of the spur, the form of the floral hood formed by sepals and petals, and

the pattern of markings (spots, lines, blotches) on the labellum (Akbulut et al., 2020).

*Dactylorhiza romana* (Sebast.) Soó subsp. *romana* is an orchid species belonging to the *Orchidaceae* family, adapted to the Mediterranean climate, exhibiting distinct habitat preferences and ecological adaptation abilities (Shipunov et al., 2004). This species has been recorded from Southern Europe and North Africa within the Mediterranean region, and its distribution extends eastwards to Iran and Turkmenistan (WFO, 2025). This species, which has high morphological diversity, is sensitive to genetic and environmental factors, and these factors cause variations in characteristics such as flower structure, colour distribution, and size. As populations of this species show diversity in different geographical regions, it stands out as an important element of biological diversity from a genetic and ecological perspective. The wide distribution of *D. romana* subsp. *romana* also contributes to the conservation of other orchid species and the balance of regional ecosystems. Studies on

*Dactylorhiza* and other orchid species are being conducted to understand the morphological, ecological, and genetic characteristics of these plants, and important results have been obtained. It has been noted that morphological characters such as bract length in *D. romana* subsp. *romana* subspecies can be used in taxonomic differentiation (Altundağ et al., 2012). In biological research, morphometry is a statistical method that analyses the shape variation of structures (Shipunov and Bateman, 2005; Slice, 2007; Shipunov and Efimov, 2015).

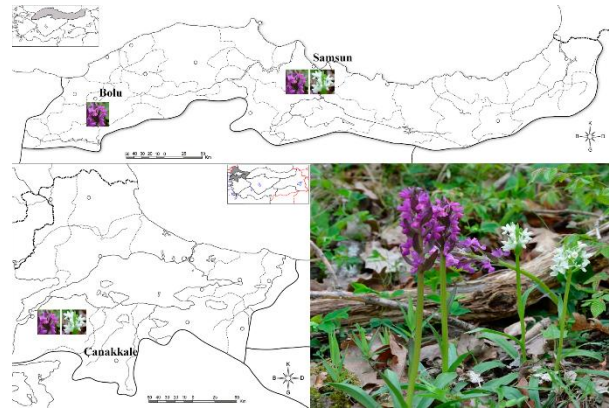
Traditional morphometry relies on measurements such as length, width, and angle, while geometric morphometrics (GMM) analyses shape information through landmarks and geometric coordinates (Rohlf and Marcus, 1993). GMM is a powerful tool for performing mathematical and geometric analyses of biological structures (Savriama, 2018). Although GMM studies in plants are limited compared to other organisms, interest in modern geometric morphometric research is increasing (Aytekin, 2017). This method has focused on insects and mammals. However, in recent years, it has also been applied to the flowers of various plant species.

Geometric morphometric studies have also emerged as a powerful tool in the analysis of morphological characteristics of orchids. The floral morphometric characteristics of *D. incarnata* (L.) Soó and *D. maculata* (L.) Soó species have drawn attention to the significant effect of environmental factors on flower morphology (Sumbembayev et al., 2021). The seeds of the *Cephalanthera rubra* (L.) Rich. and *Limodorum abortivum* (L.) Sw. species in Türkiye were examined using geometric morphometric methods; it was found that differences in size and shape were decisive in taxonomic differentiation (Süngü Şeker, 2022a). The effects of global change on plant morphology were evaluated, and the environmental adaptations of plant leaves and seeds were examined using geometric morphometry (Gao et al., 2023).

*Dactylorhiza romana* subsp. *romana* is an orchid species in which dimorphic individuals with two flower color forms, pink and pale yellow, grow in the same population and is widely used in salep production. This study aims to investigate the variation in morphological characteristics such as shape and size between dimorphic specimens by examining the dorsal sepal, lateral sepal, and bract morphology of *D. romana* subsp. *romana* specimens collected from the provinces of Bolu, Çanakkale, and Samsun (Türkiye) using classical and geometric morphometric methods. Is the morphological variation observed in flower colour also observed in shape or size? Could the morphological variation be due to differences in locality? Do both morphometric approaches yield consistent results? This study aims to comprehensively analyse the diversity in characteristics of dimorphic specimens.

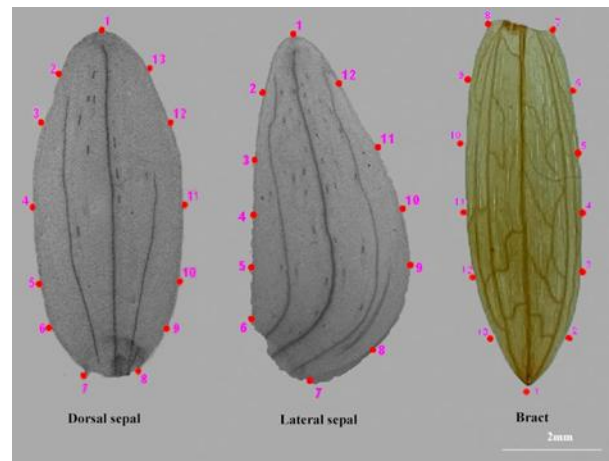
## 2. Materials and Method

This study was conducted on five groups representing both variants of *D. romana* subsp. *romana* from different localities: Çanakkale pink, Çanakkale pale yellow, Samsun pink, Samsun pale yellow, and Bolu pink. Considering the possible differences between localities, a total of five groups from three separate settlements were analysed (Fig. 1).



**Figure 1.** Collected samples localities and dimorphic individuals within the same population

In the study, the morphology of *D. romana* subsp. *romana* flower parts was examined using classical and geometric morphometric methods. For each group, thirty different images representing dorsal sepal, lateral sepal, and bract samples obtained from pink and pale yellow-flowered individuals from the provinces of Samsun, Çanakkale, and Bolu were analysed. This process was repeated for all groups, and size and shape variations were evaluated on a total of 450 flower part samples. The photographs and analysis of the samples were performed using a Leica DFC295 camera connected to a Leica S8APO stereomicroscope (Fig. 2).



**Figure 2.** Homologous landmark configuration selected for flower parts

### 2.1. Classical morphometric analysis methods

To determine intra-group differences morphometrically, quantitative characteristics such as area, perimeter, major and minor axes of the best-fit ellipse, aspect ratio, roundness, solidity, circularity, and logarithmic centre size were selected (Aoyagi et al., 2021; Süngü Şeker, 2022) (Table 1). Size and shape variations were calculated using the ImageJ/Fiji programme; multiple regression was applied to detect multiple linear relationships (multicollinearity). Variables with a Pearson correlation coefficient above 0.8 were excluded from the analyses (Das and Uddin, 2021). The analysis was continued with characteristics with a VIF (Variance Inflation Factor) value below 10 (Salmeron-Gomez et al., 2024). Subsequently, discriminant analyses were applied for a detailed examination of the differences between the groups. The study also determined which of

**Table 1.** Quantitative characteristics related to the examination of flower parts

| Morphological characteristics | Description                                                                                                                                         |
|-------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------|
| Area                          | The 2D area of each flower part                                                                                                                     |
| Perimeter                     | The length of the outer boundary of each flower part                                                                                                |
| Major axis                    | It is the fitting of the shape to an ellipse, thereby defining the line representing the longest distance passing through the center of the ellipse |
| Minor axis                    | The shortest diameter passing through the center of the fitted ellipse and extending perpendicular to the major axis                                |
| Aspect ratio                  | The ratio of the shape's width to its height: Major axis of fit ellipse / Minor axis of fit ellipse                                                 |
| Roundness                     | Calculating how round the shape is: $4 \times \text{Area} / \pi \times (\text{Major axis of fit ellipse})^2$                                        |
| Solidity                      | The ratio of the area of a shape to the area of the smallest convex shape that can enclose it: Shape area / Convex hull area                        |
| Circularity                   | Calculating how circular the shape is: $4\pi \times \text{Area} / (\text{Perimeter})^2$                                                             |
| Log centroid size             | The square root of the sum of the squares of the distances from the center points of all the landmarks on the shape, calculated logarithmically     |
| Log centroid size             | The square root of the sum of the squares of the distances from the center points of all the landmarks on the shape, calculated logarithmically     |

the examined characteristics were successful in distinguishing between groups. The analyses were performed using the SPSS 27 programme.

## 2.2. Geometric morphometric analysis methods

The tpsUtil 1.74, tpsDig2, and MorphoJ 1.08 programmes were used for geometric morphometric analyses. Homologous landmarks on flower parts were selected using tpsDig2 to examine shape differences between samples from different localities, and the distances of these landmarks from the centre of the shape were calculated. Shape and size components were separated using generalised Procrustes analysis (GPA). Size differences were evaluated using variance analysis. Principal component analysis (PCA) was used to visualise and summarise patterns of shape variation, whereas

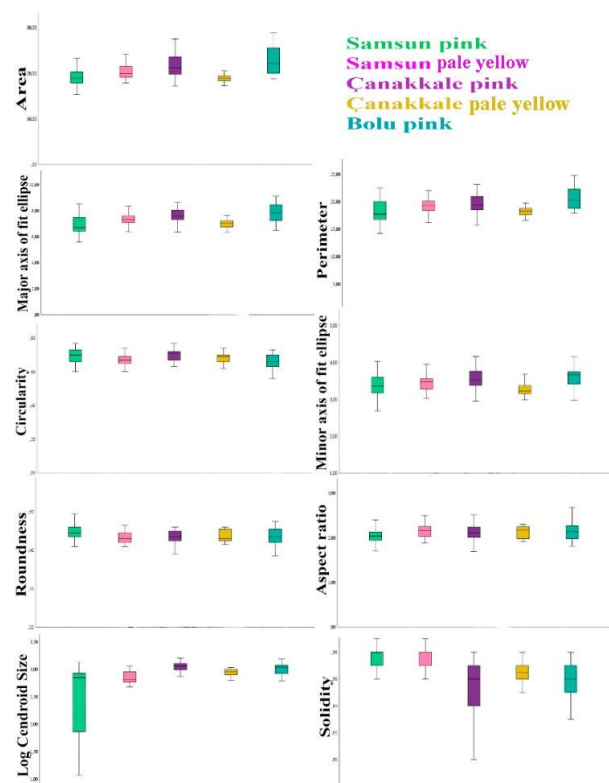
discriminant analysis was applied to test and quantify differences between groups according to shape differences. The relationship between shape and size variation was analysed using regression analysis, and all findings were statistically interpreted and validated using 10,000 permutation tests between different localities and dimorphic specimens.

## 3. Results

### 3.1. Classical morphometric findings

In dorsal sepal analyses, the Samsun groups generally showed distinct differences from the others. Samsun pink specimens generally exhibited the highest variance, while Çanakkale pale yellow specimens showed the lowest variance (Fig. 3). In discriminant analysis, both the first and second canonical functions were found to be significantly discriminating between groups (correlation correlations for DF 1 and DF 2 was 0,584 and 0,392, respectively,  $p < 0.001$ ) and explained 95.3% of the variation. Cross-validation results showed that the overall model accuracy was 48.7%. The highest classification success was observed in the Çanakkale pale yellow group at 90%, while the lowest success was found in the Bolu pink group at 26.7%.

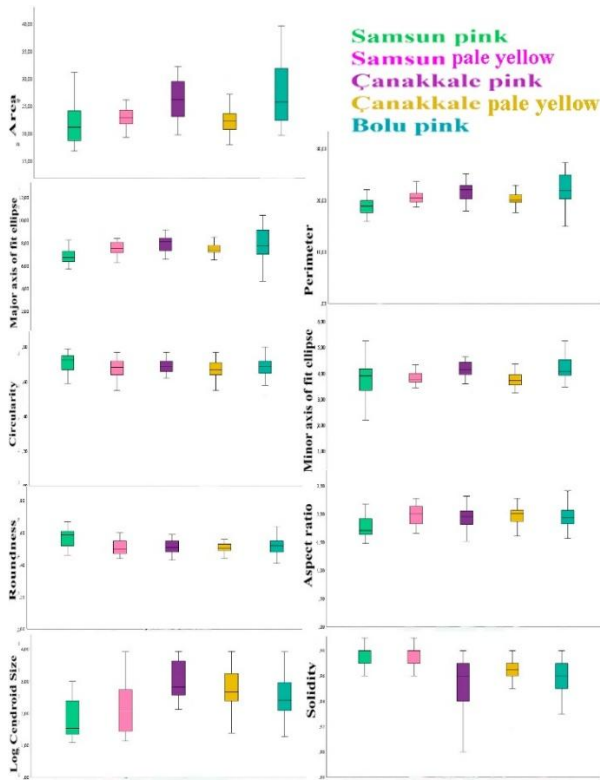
No significant variance differences were generally observed between groups in lateral sepal samples (Fig. 4). Discriminant analysis revealed that both the first and



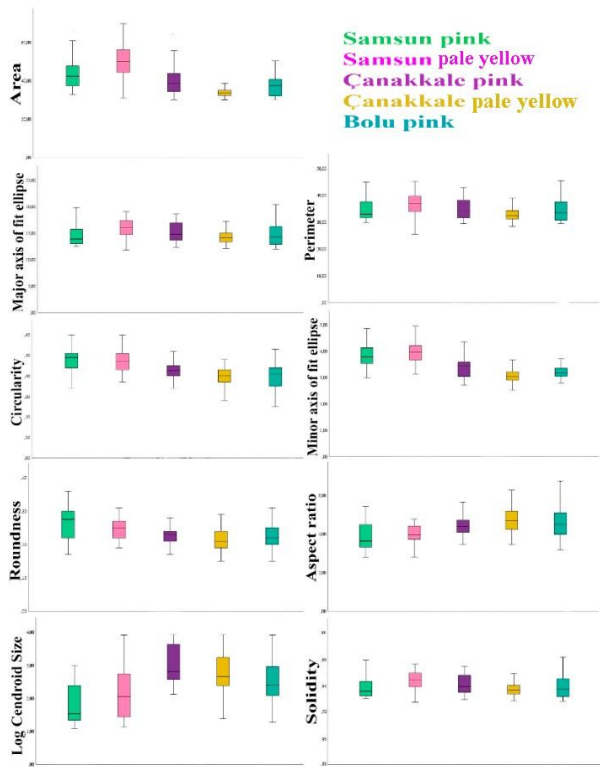
**Figure 3.** Box plot for dorsal sepal morphometric characteristics of dimorphic samples, showing the range from the first to the third quartile of the distribution. The horizontal line inside the rectangle represents the median, while the whiskers represent the lower and upper 25th percentiles, typically indicating the minimum and maximum values

second canonical functions were effective in distinguishing between groups (correlation correlations for DF 1 and DF 2 was 0,652 and 0,439, respectively,  $p < 0.001$ ), and this analysis explained 88.4% of the variation. The overall accuracy rate in cross-validation classification was 54.7%; the highest success rate was 73.3% in the Samsun pink group, while the lowest success rate was 26.7% in the Çanakkale pale yellow group.

Among the bract samples, Samsun pale yellow samples generally showed the highest variance, while Çanakkale pale yellow samples exhibited the lowest variance (Fig. 5).



**Figure 4.** Box plot for lateral sepal morphometric characteristics of dimorphic samples, showing the range from the first to the third quartile of the distribution. The horizontal line inside the rectangle represents the median, while the whiskers represent the lower and upper 25th percentiles, typically indicating the minimum and maximum values.



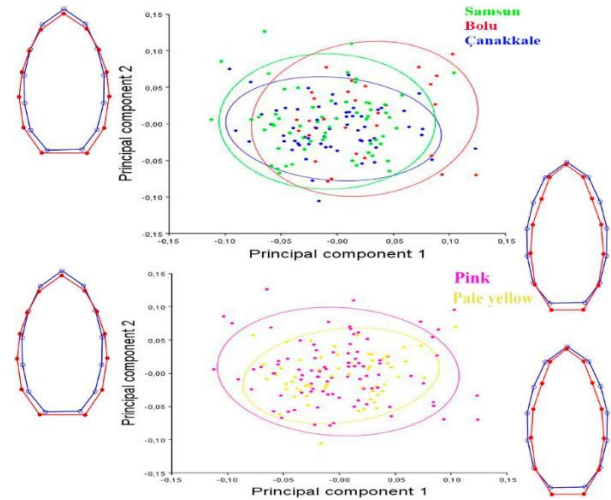
**Figure 5.** Box plot for the bract morphometric characteristics of dimorphic specimens, showing the range from the first to the third quartile of the distribution. The horizontal line inside the rectangle represents the median, while the whiskers represent the lower and upper 25th percentiles, generally indicating the minimum and maximum values.

The first and second canonical functions were found to be effective in distinguishing the groups (correlation correlations for DF 1 and DF 2 was 0,780 and 0,387, respectively,  $p < 0.001$ ) and explained 97.4% of the variation. According to cross-validation results, the overall accuracy of the model was determined to be 58%; the highest success rate was 83.3% in the Çanakkale pale yellow group, while the lowest success rate was 30% in the Bolu pink group.

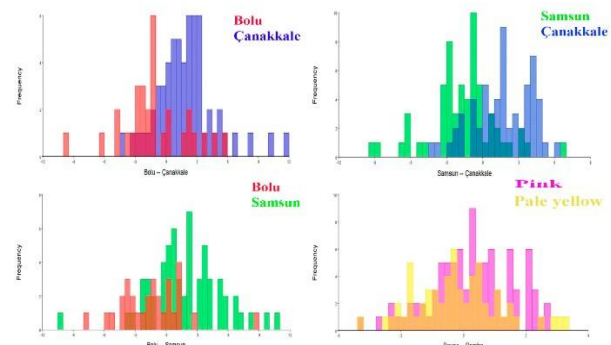
Morphometric characteristics such as logarithmic centre size, circularity, fullness, and aspect ratio were found to be the most effective characters in determining morphological differences between settlements.

### 3.2. Geometric morphometric findings

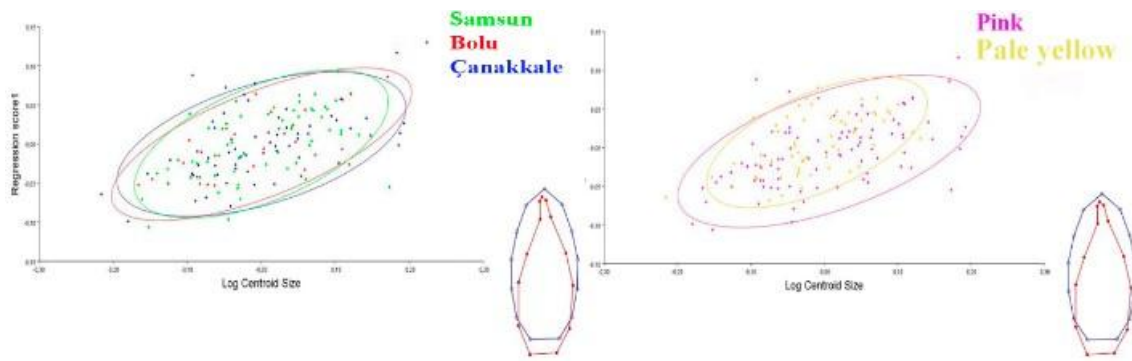
In the Procrustes shape coordinate-based dorsal sepal PCA analysis, the first ten components explained 94.9% of the shape variation. PC1 (32.1%) and PC2 (24.8%) explained 56.9% of the total shape variation. It was observed that dorsal sepals narrowed and elongated along the PC1 axis, while its base widened along the PC2 axis (Fig. 6). Discriminant analysis revealed that the Çanakkale samples formed a distinct group, while the Samsun and Bolu samples overlapped with the Çanakkale group (Fig. 7). Regression analysis determined that as the size of the dorsal



**Figure 6.** Principal Component Analysis (PCA) scatter plot of dimorphic specimens for the dorsal sepal, visualising the positive ends of the PC axes with shape transformation graphs. The right and left sides of the graphs represent the change in these axes.



**Figure 7.** Cross-validation results of the discriminant analysis performed using dorsal sepal shapes of dimorphic specimens collected from different locations. The colours in the graphs represent specimens corresponding to provinces and dimorphic specimens, as shown on the right side of the figure



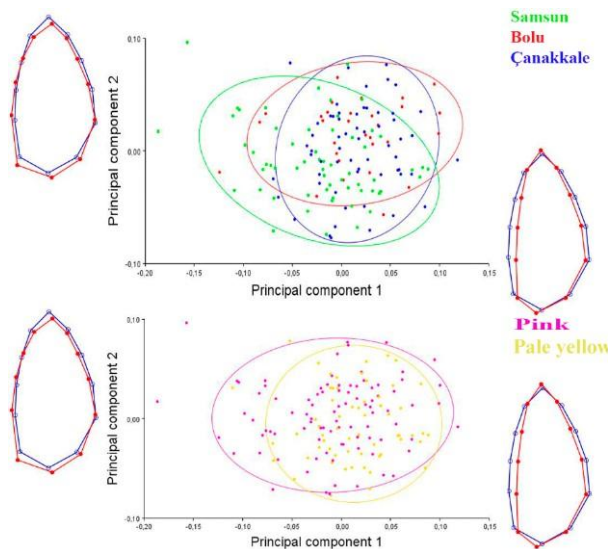
**Figure 8.** Distribution graph of regression scores of dorsal sepal samples belonging to dimorphic specimens collected from different locations against the logarithmic centre dimension

sepal increases, the distal part of the sepal narrows and elongates slightly; this trend was observed similarly in both settlements and dimorphic specimens (Fig. 8).

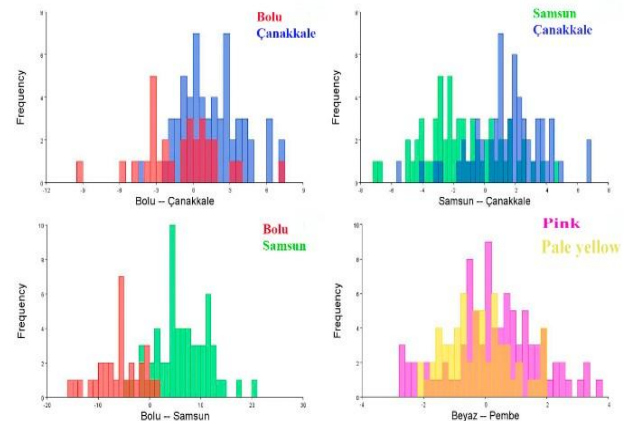
In the PCA analysis of lateral sepal samples based on Procrustes shape coordinates, the first ten components explained 94.3% of the shape variation. PC1 (34.9%) and PC2 (17%) explain 51.9% of the total shape variation. Slight changes in shape were observed along both axes (Fig. 9). Discriminant analysis showed that the Çanakkale

and Samsun samples formed separate groups, while the Bolu samples exhibited a wide distribution covering both groups. No clear distinction was found between the Çanakkale and Bolu samples, and a large degree of overlap was observed (Fig. 10). In the regression analysis examining the relationship between size and shape, it was found that as the lateral sepal size increased, the shape narrowed and the distal part elongated (Fig. 11).

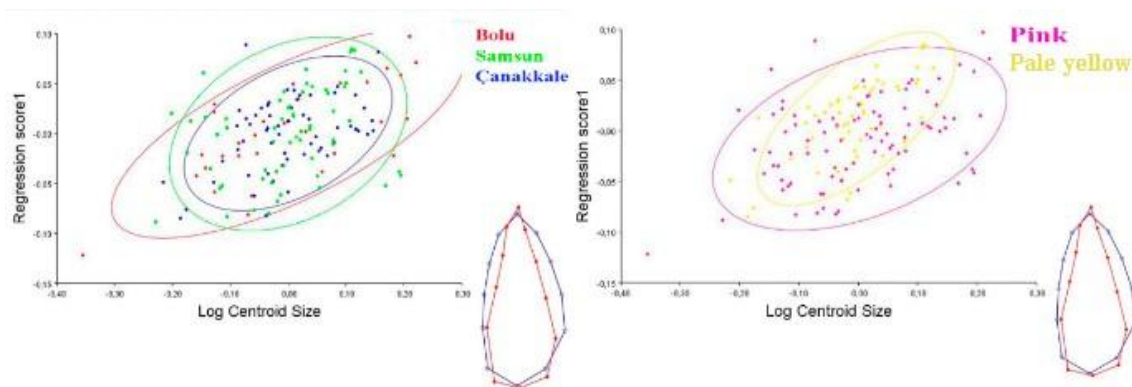
In the PCA analysis of bract samples based on Procrustes shape variations, the first ten components were found to explain 94.1% of the total shape variation. PC1 (43.3%) and PC2 (21.6%) explain 64.9% of the total variation. It was



**Figure 9.** Principal Component Analysis (PCA) scatter plot of lateral sepals for dimorphic specimens, visualising the positive ends of the PC axes with shape transformation graphs. The right and left sides of the graphs represent the change in these axes

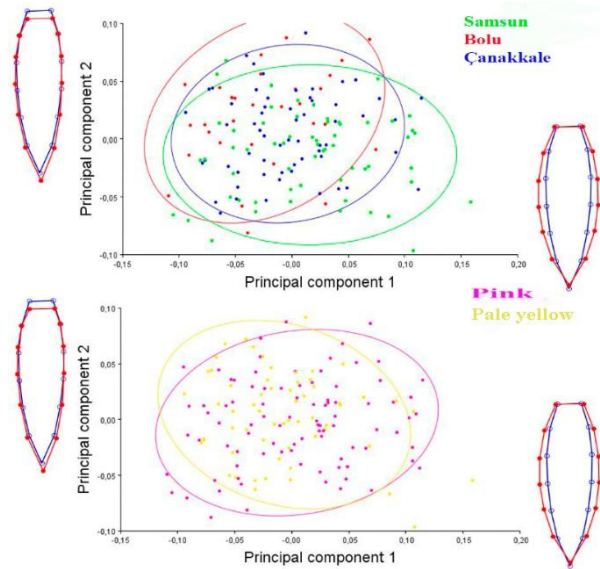


**Figure 10.** Cross-validation results of the discriminant analysis performed using lateral sepal shapes of dimorphic specimens collected from different locations. The colours in the graphs represent the specimens corresponding to the provinces and dimorphic specimens, as shown on the right side of the figure



**Figure 11.** Distribution graph of regression scores of lateral sepal samples from dimorphic specimens collected from different locations against the logarithmic centred dimension

observed that the lengths of the bracts widened without changing along the PC1 axis; however, they shortened along the PC2 axis (Fig. 12). Discriminant analysis showed that the Samsun samples formed a distinct group; the Bolu and Çanakkale samples were scattered within this group (Fig. 13). Regression analysis revealed that as bract size increases, their shape narrows and lengthens slightly; this trend was observed similarly in both the settlement and dimorphic groups (Fig. 14).

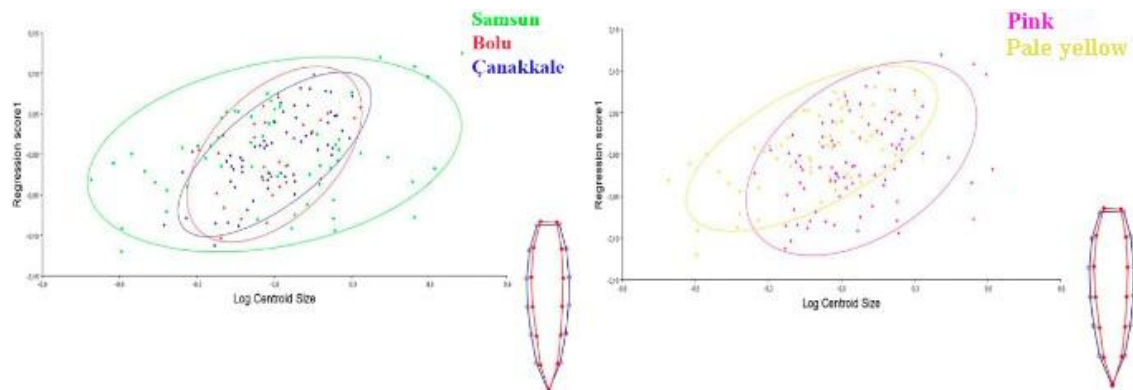


**Figure 12.** Principal Component Analysis (PCA) scatter plot for dimorphic bract samples, visualising the positive ends of the PC axes with shape transformation graphs. The right and left sides of the graphs represent the change in these axes

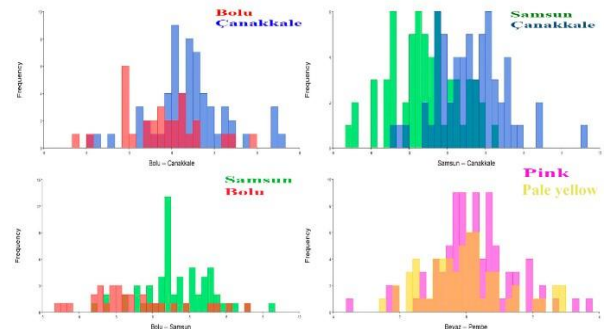
#### 4. Discussions

*Dactylorhiza romana* subsp. *romana* is a dimorphic species in which pink and pale yellow flowers are distributed within the same population. In this study, the morphology of *D. romana* subsp. *romana* flower parts was examined using classical and geometric morphometric methods to identify structural diversity between dimorphic specimens and different localities.

The effectiveness of logarithmic centre size and area data in distinguishing groups based on the dorsal sepal base was found to be consistent with previous studies demonstrating the effectiveness of dorsal sepal structure in distinguishing *Habenaria parviflora* taxa (Cruz-Lustre et al., 2020). Another study indicated that dorsal sepal morphometric



**Figure 14.** Distribution graph of regression scores of bract samples belonging to dimorphic specimens collected from different locations against the logarithmic centred dimension.



**Figure 13.** Cross-validation results of discriminant analysis performed using bract shapes of dimorphic specimens collected from different locations. The colours in the graphs represent specimens corresponding to provinces and dimorphic specimens, as shown on the right side of the figure.

characteristics are important in the taxonomic differentiation of *Dolabrifolia* (Pfitzer) Szlach. & Romowicz species, but that classification accuracy is limited; our research results support this finding (Simo-Droissart et al., 2016). Geometric morphometric analyses of dorsal sepal shapes revealed that the Çanakkale specimens showed limited differentiation from the Samsun and Bolu specimens, and a weaker differentiation was observed between the Samsun and Bolu groups. It has been stated that dorsal sepal shapes play a role in interspecific differentiation in *Delphinieae* species, but intraspecific variation is limited (Chen et al., 2018). Similarly, in *Cattleya walkeriana* Gardner dorsal sepal variation is limited and shows less variability compared to other flower structures (Aguiar et al., 2020). These findings are consistent with the low morphological differences observed between different local specimens in this study. The weak distinction between dimorphisms in our study is also consistent with these findings. Logarithmic centre size and fullness characteristics were found to be effective in distinguishing groups based on lateral sepals. *Pomatocalpa maculosum* (Lindl.) J.J.Sm. specimens from different geographical regions were successfully distinguished based on lateral sepal morphology, and differences in environment, length, and width were identified. However, our research results showed that while the Samsun groups differed significantly from the other groups, the Çanakkale and Bolu groups were found to be similar (Wattana, 2006). Similarly, it was observed that lateral sepal morphology alone did not provide sufficient discriminatory power in the *Diuris punctata* Sm. species complex, which was consistent

with our research results (Smith et al., 2008), despite the wide variation in shape and size among all specimens of the genus *Diuris* Sm.

Geometric morphometric analyses revealed a limited distinction between Çanakkale and Samsun specimens in lateral sepal shapes, while Bolu specimens behaved as an intermediate transition group. A study concluded that lateral sepal variation is related to genetic and environmental factors, which explains the wide variation observed in the specimens in our study (Roy, 2022). In bract specimens, logarithmic centre size and circularity were found to be effective in distinguishing groups. It has been reported that *Platanthera hyperborea* Lindl. populations can be distinguished based on bract morphology, but no significant differentiation was observed in some populations (Bateman et al., 2015). Similarly, in our study, the Samsun groups could be distinguished from other groups, but sufficient differentiation could not be achieved between the Çanakkale and Bolu groups. Geometric morphometric analyses also show that the bract shapes of the Samsun specimens differ from those of other specimens. Altundağ et al. (2012) suggested that bract shapes could be an important criterion in subspecies differentiation in *D. romana*. However, the fact that only the Samsun specimens were clearly differentiated from the other groups contradicts the existing findings in the literature. It has been noted that bract shapes may be influenced by environmental factors, which could complicate morphological differentiation (Gardner et al., 2016). This may explain the wide variation observed in the Bolu specimens in our study.

The findings obtained from all the flower parts studied have made significant contributions to the literature on understanding the morphological diversity of the *D. romana subsp. romana* species. The analyses revealed that the specimens collected from Samsun province were morphologically distinct from those collected from Çanakkale and Bolu Provinces; conversely, the specimens

from Çanakkale and Bolu exhibited more similar morphological characteristics. This indicates that the Samsun specimens possess a more unique morphological structure, while the Bolu specimens exhibit a transitional morphology. The morphometric analyses revealed that structural criteria such as roundness, logarithmic centre size, and area were significantly influential in the differentiation between locations. The variation in these criteria has been decisive in evaluating the differentiation observed in flower structures depending on geographical locations. However, no significant morphological distinction was found between dimorphic specimens evaluated based on flower colour. The fact that colour variations are not discriminatory at the morphological level suggests that dimorphism has a limited effect on morphometric characters. The results indicate that the observed morphological differences may originate not only from environmental conditions but also from genetic factors. In particular, the large Bolu sample, which includes specimens from Samsun and Çanakkale, supports the possibility that this region is a genetic transition zone.

In general, while it is understood that flower structures are effective in classification at the location level, further molecular-based genetic analyses are needed at the dimorphic specimen level. Such studies will be instructive for future research in terms of revealing the genetic basis of morphological variations.

#### Conflict of Interest

Authors have declared no conflict of interest.

#### Authors' Contributions

Aydanur Bakır: Investigation; Visualization; Resources; Writing – original draft. Gülcan Şenel: Writing – review and editing. Şenay Süngü Şeker: Plant material supply; Writing – review and editing. Mustafa Kemal Akbulut: Plant material supply and editing.

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