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Seed Geometric Morphometrics of Neottioid Orchids

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Abstract

Cephalanthera, *Limodorum* and *Neottia*, which are known as primitive orchids, are rhizomatous orchids and commonly distributed in Turkey. This study aims to investigate orchid seed variation between some representatives of Neottieae, *Cephalanthera rubra*, *Limodorum abortivum*, *Neottia nidus-avis*, and *Neottia ovata*, naturally distributed in Turkey by using geometric morphometric analyses based on 2-dimensional landmarks. For this purpose, a total of 95 specimens were evaluated and photographed using scanning electron microscopy. Using software, 12 homologous landmarks were obtained to reflect the main aspects of the seed shape. Species were compared with various statistical methods by calculating the data obtained from shape and size variables with the Procrustes method. The difference in both size and shape were significant between the species. Shape differences were most prominent in the chalazal and micropylar regions of the seed as well as the whole seed width. Discriminant analysis and cross-validation scores were a highly powerful to distinguish the species with scores ranging from 60% to 88%. Regression analyses also revealed allometric effect of the size on seed shape with a similar trend across species. Based on current results, geometric morphometric analysis is encouraging in the research of structural variation within plant parts. The present study is also significant in terms of the widespread use of such studies in the field of botany, especially in the context of systematic or functional morphology.

Keywords: Epidenroid Orchid, Geometric Morphometric, Neottieae, Seed, Shape Variation

1. Introduction

Orchidaceae family have so many species distributed in a broad area with various vegetation types [1, 2] and has also a great species diversity in Turkey [3,4]. Tribe Neottieae (subfamily Epidendroideae) is often referred to as primitive orchids. Tribe Neottieae was firstly described by Lindley [5]. After that, different classifications have been proposed by many researchers [6-10]. Today Neottieae is more limited involved in only six genera recognized (*Aphyllorchis* Blume, *Cephalanthera* Rich., *Epipactis* Zinn, *Limodorum* Boehm., *Neottia* Guett., *Palmorchis* Barb. Rodr.) of which four are represented by various taxa in Turkey.

Orchid seeds are typically dispersed by the wind because they are extremely small, so-called dust seeds. The first detailed studies on orchid seeds were published as a review by Rasmussen [12] and Arditti and Ghani [13]. Although they are so small, have an undifferentiated embryo, and have not to contain endosperm, observations of orchid seed morphology using electron microscopy revealed detailed information on the seeds. Many studies such as descriptive morphology, and morphometry performed on the orchid seeds emphasized the distinguishing values of a few characters [14-18]. Especially, these studies revealed that the properties such as cell shape and number in the testa, and cell size in the medial or chalazal region, and periclinal wall pattern are systematically valuable.

On the other hand, in recent years there has been a growing attention in the usage of modern geometric morphometric (GMM) [19]. Beyond classical qualitative or quantitative definitions, GMM allows the researcher to quantify size and shape by analyzing relative landmark positions and point sets used for frames and surfaces [20]. Thus taxonomists and systematists tended to use geometric morphometry in their field. But, these analyses, which have mostly focused on insects, mammals, and fishes as not yet been greatly carried on plant [21]. Recently, GMM studies on the leaf shape comparison between different populations or taxon have got priority in the literature [19, 22].

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Unfortunately, such studies are very rare in Orchids. Firstly, Chemisquy et al. [23] studied the orchid seed shape applying the geometric morphometric approach and they stated that seed size, referred to centroid size, was a changeable features and illustrative at a phylogenetic level.

The current research aimed to analyse the seed morphology of a few representatives of Neottieae using scanning electron microscopy and geometric morphometrics. The aim was also to identify whether the geometric clue of seed shape or size differences is conserved among genera in the tribe and reflects the phylogenetic relationship of this group.

2. Materials and Methods

2.1. Seed acquisition

Mature seed samples of four different taxa classified in the genus *Cephalanthera*, *Neottia*, and *Limodorum* were gathered from various localities (Table 1). Species identification was completed using Flora of Turkey and Türkiye Bitkileri Listesi [3,4] and current plant names checked from the Plantlist database [24]. At least two different individuals from each locality were evaluated and different locality samples were examined together to eliminate intraspecific variation. The mature capsules were selected at random from different positions on the inflorescence of each individual and about five mature, undamaged capsules were dissected for each taxon and dried for about one month. The seeds of the collected specimens were put in the sterile tubes and stored in our laboratory collection with the specimen number (Table 1).

Table 1. List of studied species, localities, voucher specimens

Taxon	Specimen number	Collection location	Collection date
Cephalanthera rubra (L.) Rich.	CepRubSss32	Turkey, Samsun Kurupelit, 209m.	July, 2017
Cephalanthera rubra	CepRubBss69	Turkey, Bolu, Abant, 839m.	August, 2015
Limodorum abortivum (L.) Sw.	LimAboSss64	Turkey, Samsun Kurupelit, 198m.	May, 2012
Neottia nidus-avis (L.) Rich.	NeoNidAviBss73	Turkey, Bolu, Abant, 1028m.	June, 2021
Neottia nidus-avis	NeoNidAviSmka29	Turkey, Samsun, Kurupelit, 209m.	May, 2014
Neottia ovata (L.) Bluff & Fingerh.	NeoOvaSmka39	Turkey, Samsun, Çarşamba, 18m.	June, 2015

2.2. Scanning electron microscopy

For electron microscopy, a small amount of sample was covered with 12.5–15 mm gold-palladium (scanning electron microscope (SEM) coating system, SC7620) on the stubs and was observed on the voltage of 5–15 kV in JEOL JMS-7001F SEM [25]. The seed shapes on about 30 photographs for each taxon were evaluated through SEM observation.

2.3. Geometric morphometric analysis

Thin-plate spline (TPS) series programs were used to prepare datasets using software tpsUtil version 1.74 [26,27]. All seed images were sequentially scaled and landmarked using tpsDig v. 2.16 [28]. The homologous landmarks set determined in this research (Figure 2) was chosen to explain the major aspects of the seed shape in taxa. These landmarks represent the clearest frame of the seed to aid with descriptions of shape changes: chalazal region (landmarks 1-3, 11-12), medial region (landmarks 4-5, 9-10), and micropyle (landmarks 6-8) on the lateral view of the seed (Figure 1).

The coordinates data sorted in txt file format were imported into the MorphoJ program [29] for analysis. To study shape, a Generalized Procrustes Analysis (GPA) was performed on the landmark configurations to extract shape data by removing information about size and orientation from each specimen [30, 31]. In this way, the coordinates representing the seed morphology were subdivided into shape and size variables. [32]. The Procrustes shape coordinates of each seed were superimposed to make a common profile [33].

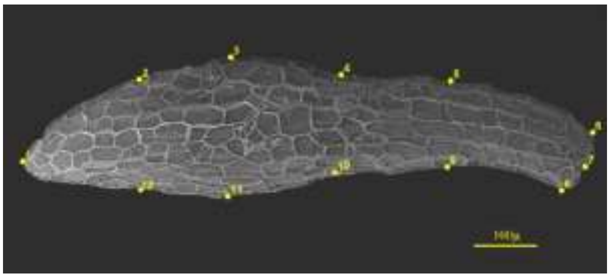

Figure 2. Landmark configuration on the seed

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2.4. Data analysis

For detecting differences in size among the species, the Kruskal-Wallis test was performed with centroid size. After being tested with Kruskal-Wallis, a Pairwise Dunn's test was conducted to determine which species made a significant difference. For shape analysis, principal component analysis (PCA) was carried out to Procrustes shape coordinates to determine the shape changes in the seed. After the dimension reduction of the data of the 12 landmarks, the combined variables that effected mainly to the variation in seed morphology were determined. Cross-validated discriminant function analysis was also subjected to statistically examine the success of seed shape variation for taxonomic assignment. Moreover, a multivariate regression analysis was used to estimate any allometric effect. The analyse was performed among species and within each species distinctly using a permutation test with 10,000 rounds. Output transformation grid and wireframe graph were obtained to analyze the direction of the shape compression or enlargement. All analyses were carried out by using the PAST version 4.03 [34] and MorphoJ version 1.07 packages [35].

3. Results and Discussion

3.1. Size variation

It was obtained chi-squared value = 61.46, df = 3, and p-value < 0.001 from Kruskal-Wallis test. The analysis result showed that the difference among seeds of the species is statistically significant. These differences can also be related to shape differences among the species due to allometric effects (see below). Pairwise Dunn's test showed that *C. rubra* gave a significant difference between *L. abortivum* ($p < 0.01$), and *N. nidus-avis* ($p = 0.01$). Other than that, *L. abortivum* gave significant difference between *N. nidus-avis* ($p < 0.01$), and *N. ovata* ($p < 0.01$). The difference between *N. nidus-avis* and *N. ovata* was a significant ($p<0.05$), but there were no significant differences between *C. rubra* and *N. ovata* ($p = 0.295$). Moreover, boxplot graphics showed that *L. abortivum* was the largest in centroid size, (Figure 2). Based on traditional morphometry, seed width and lengths were found to be the same in *C. rubra* and *N. ovata* and *L. abortivum* have the largest seed [36]. These result show that geometric morphometry yields results with the same trend as traditional morphometry. The present study was therefore compatible with the results of that previous study in terms of size.

Many studies conducted on orchid seed morphometry have demonstrated that orchid seeds vary enormously in size from 150 mm to 6000 mm [37]. This diversity may be related to seed distribution as well as reflecting the phylogenetic relationship. Chemisquy et al., (2009) analyzed the seed morphology of some taxa of the tribe Chloraeae performing tools of geometric morphometrics and they emphasized that seed size, namely centroid size, was a variable feature and illuminating at a phylogenetic level [23]. On the other hand, Nakanishi (2022) found that the dispersibility values calculated by proportioning the seed length by embryo length indicate the anemochorous potential and the seed size and dispersibility of terrestrial orchids are between epiphytes and saprophytes. [38]. These results indicate that, at least in the species subject to the present study, seed size may be related to seed distribution and indirectly reproductive success rather than reflecting the phylogenetic relationship between the genera. For this reason, in these species with different seed sizes, it may be interesting to evaluate the seed distribution about size in future studies.

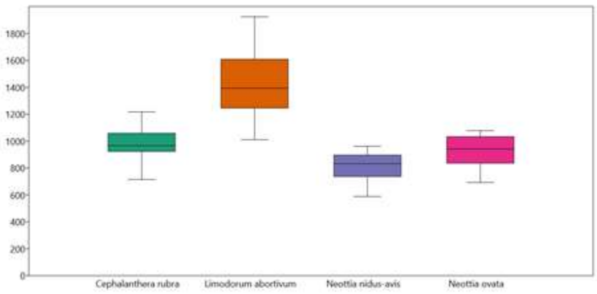

Figure 2. Boxplot graphics show the variation of centroid size for seed between the four species.

3.2. Shape variation

PCA was carried out on the Procrustes shape coordinates of seed morphology of the species and revealed that the first six principal components used for the shape analyses explained 83.7% of overall shape variation of the seed among the species. Figure 3 displays the scatter plot graphic of the PC1 and PC2 scores accounted for 56.4% of the total seed shape variables (PC1 accounted for 34.8%, and PC2 accounted for 21.6%). Based on the graphic, specimens of *C. rubra* and *L. abortivum* created different groups, whereas those of the other species were scattered between these two species. Along with the positive extremes of both PC1 and PC2 axes, the transformation grid and wireframe graphs were visualized. Based on this, the seed was curved at the medial region and, the chalazal pole was narrowed towards the positive value of the PC1 axis. There was also clear variation along the PC2 axis separating the specimens of *C. rubra* and *L. abortivum*. The chalazal region tip was gradually shortened and the medial region increasingly became narrower in the positive PC2 direction.

Discriminant analysis (Figure 4) revealed that the seed shape of *C. rubra* displayed a small overlap with that of *L. abortivum* and *N. ovata* and a large overlap with that of *N. nidus-avis*. Likewise, the seed shape of *L. abortivum* showed relatively greater overlap with *Neottia* sp. In these pairs of taxa, *L. abortivum* and *N. ovata* were more accurately classified based on seed

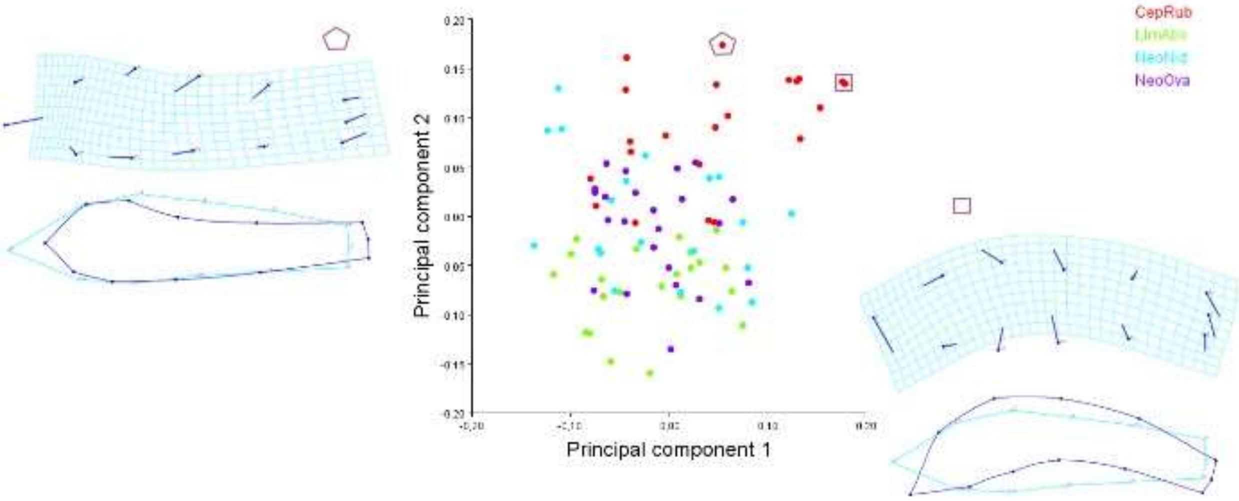

Figure 3. PCA scatter plot graphics (on the middle) show the shape variations in the studied species; shapes on the right and left sides visualize the positive extremes of PC axes using transformation grids and wireframe graphs and square/polygon symbols are used to show the positions of visualized shapes in the PCA scatterplot.

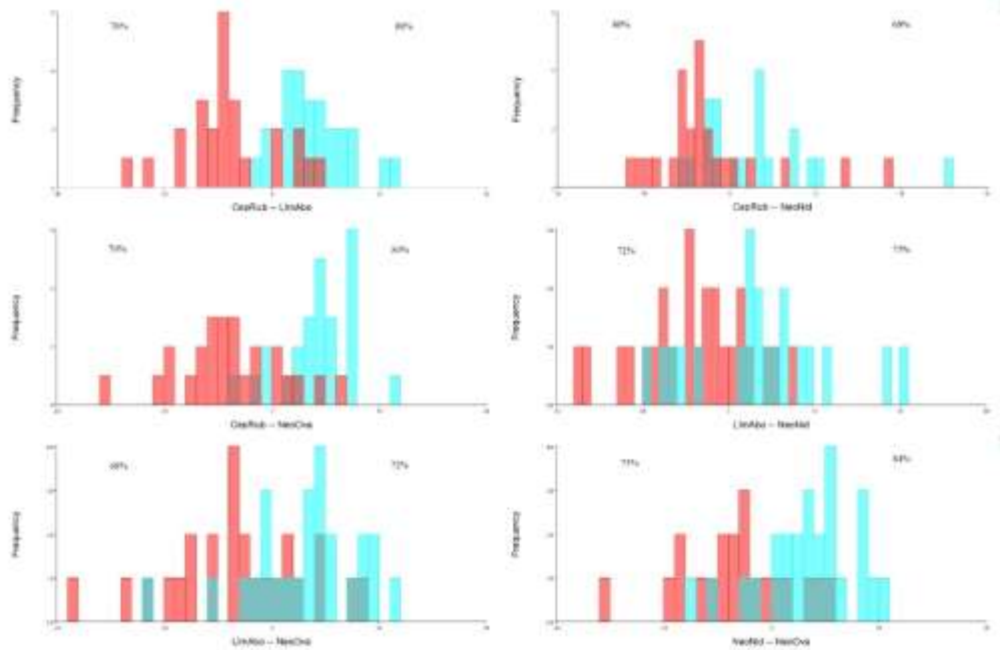

Figure 4. The cross-validation results of discriminant analysis using the seed shapes of *C. rubra, L. abortivum, N. nidus-avis,* and *N. ovata*. The red and blue color represent specimens of species with abbreviations at the bottom of each graph, respectively.

shape than the others (Figure 4). Of 95 admixed individuals, ca. 60–88% were correctly classified as true species using seed shape. Qualitative micromorphological characters of seeds were analyzed in many studies. These characters appear very useful at the supraspecific level in the subtribe Orchidinae [39,40]. However, in these studies, the seed shape was evaluated as a whole and defined qualitatively as clavate, fusiform, filiform, etc. On the other hand, a geometric morphometric study allows us to examine the shape changes in the chalazal or micropylar regions separately and to prevent the errors arising from the researcher's observation by digitizing the shape data.

In their geometric morphometric study on the tribe Chloraeeae, Chemisquy et al. (2009) showed that seed shape resulted in a continuum among the taxa studied, and in only a few cases could genera or groups of species be discriminated on the basis [23]. But the present study was not compatible with the results of that Chemisquy et al. (2009) in terms of shape. The result revealed that Procrustes shape were different among the studied species in present research. In addition, Magrini et al. (2010) 's findings with her outline study on *Limodorum* support the current study [41]. They aimed to verify the taxonomic value of *Limodorum* seeds, particularly of the shape of two close species (*L. abortivum* and *L. trabutianum*) growing sympatrically. The outline analysis confirmed a low intraspecific variability of seed shape, but show a very high interspecific variability, thus geometric morphometry allowed us to distinguish between these two species even during the fruiting phase, simply using seed shape as a diagnostic character. In addition, the last study [42] explained the relationship between closely related parent species and their hybrids belonging to the *Orchis* Tourn. ex L. genus with a geometric morphometric approach with labellum morphology. This situation is inspiring for the evaluation of species with systematic problems, such as orchids, whose systematic category often changes, using geometric morphometric approaches.

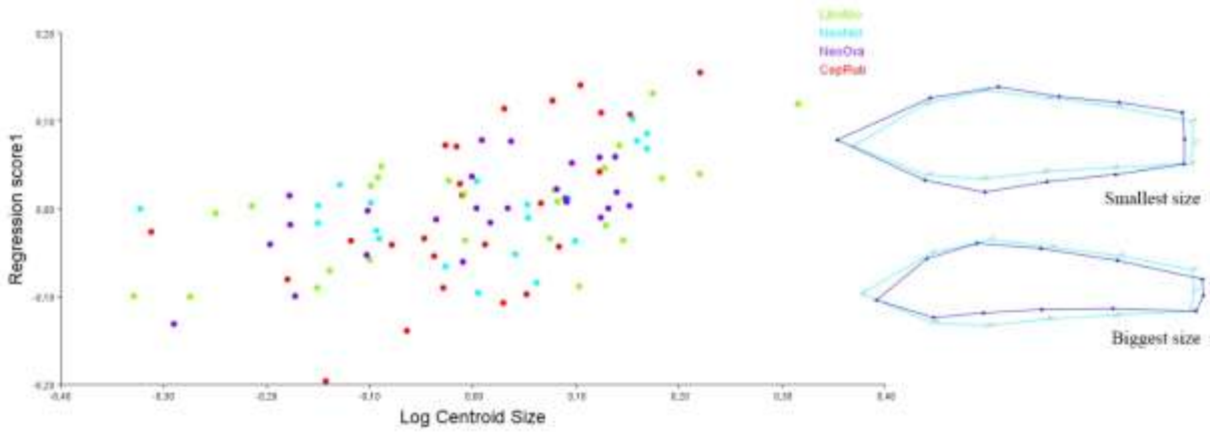

Figure 5. The Scatterplot of regression scores vs centroid size; shapes at the opposite extremes of the range of allometric variations are visualized using a seed wireframe.

3.3. Allometric effect

Before proceeding with full regression analysis, the significance of allometry within groups was evaluated by splitting species into separate samples and, then applying multivariate regressions of the shape onto size one group if at least one of the groups is statistically significant. Regressions of the shape onto size one species were marginally significant (P<0.01 – P = 0.01, 12.7 – 20.7 % of variance explained) for all species. For the seed, multivariate regression of the Procrustes coordinates on log centroid size for the four species showed a highly significant result ($p < 0.001$), with allometry explaining 28.5% of total shape variation. This test as well as the large overlap between species in the scatterplot (Figure 5) suggested that the effect of size on shape was similar in the four species: bigger seeds tend to be shorter in the chalazal region, but be longer at the micropyle and all the seed tend to be narrower. On the contrary, as can be also seen from the wireframe graphs, at the negative extreme of the centroid size, the seed became shorter at the micropyle but became longer at the chalazal region and all the seeds tend to be wider (Figure 5).

Because orchid seeds are very small, they are called dust seeds and are dispersed by the wind. The anemochorous potential of the seed is estimated based on the ratio of seed length to embryo length [43]. As the seed size increases, the shrinkage of the seed means that the spherical embryo also gets smaller. The ratio of seed size to embryo length increases, thus it is logical that this allometric effect may be a positive adaptation to the reproductive biology of the species by improving the buoyancy of the seed for wind dispersal.

4. Conclusion

Geometric morphometric analysis is a powerful procedure that determines and display the shape differences effectively. Orchid seeds are commonly used in taxonomic analyses in many other groups at diverse taxonomic levels. However, orchidologists avoid using the method for this purpose in their studies as much as zoologists have done. Using free software and a dataset from SEM observation of orchid seeds, a detailed but simple analysis computing size and shape variables using Procrustes methods was performed. The present result is impressive that Procrustes-based methods for the analysis of landmarks were extremely effective in determining the differences in shape and size and in revealing very small-scale variations. For this reason, botanists need to illuminate functional morphology as well as taxonomy.

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Author's Contributions

Şenay Süngü Şeker: Collected the seed, performed the experiment and geometric morphometric analysis, interpreted the results, wrote the manuscript.

Ethics

There are no ethical issues after the publication of this manuscript.

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