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Pericarp anatomy and surface micromorphology of some orchids in the Black Sea Region

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Karadeniz bölgesinde yayılış gösteren bazı orkidelerin perikarp anatomisi ve yüzey mikromorfolojisi

Abstract: In this study, we have investigated the anatomy and ultrastructure of the pericarp to determine important characters of the fruits belonging to some Turkish orchidoid species, and to determine which features are related to ecological or habitat preferences. For the purpose, the samples belonging to 19 orchid taxon were collected in the Black Sea Region. SEM and light microscopy photographs were taken with the standard techniques. Variations among taxa were evaluated using various statistical methods such as correlation and discrimination analysis. Among the investigated characteristics, fruit surface ornamentation is related to habitat preferences of the species while morphometric properties of epidermal cells and structural features such as the type of crystal inclusions are important characters at the genus level.

Key words: Anatomy, discrimination analysis, micromorphology, *Orchidaceae, Orchidoidae*

Özet: Bu çalışmada, bazı orkide türlerine ait meyvelerin önemli özelliklerini belirlemek ve hangi özelliklerin ekolojik veya habitat tercihleriyle ilişkili olduğunu tespit etmek için perikarp anatomisi ve mikromorfolojisi incelenmiştir. Bu amaçla, Karadeniz Bölgesi'nde 19 orkide taksonuna ait örnekler toplanmıştır. Standart tekniklerle SEM ve ışık mikroskobu fotoğrafları çekilmiştir. Taksonlar arasında çeşitlilik arz eden özellikler korelasyon ve ayrım analizleri gibi çeşitli istatistiksel yöntemler kullanılarak değerlendirilmiştir. İncelenen özellikler arasında meyve yüzey desenlenmesi türlerin habitat tercihleri ile ilişkili iken epidermal hücrelere ait morfometrik özellikler ile kristal inklüzyonların çeşidi gibi yapısal özellikler cins seviyesinde önemli ayırt edici karakterlerdir.

Anahtar Kelimeler: Anatomi, ayrım analizi, mikromorfoloji, *Orchidaceae, Orchidoidae*

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1. Introduction

Orchidaceae is one of the largest families in terms of biodiversity and is flowering plants with wide area of distribution excluding extreme habitats (Dressler, 1993). With more than 800 genera and nearly 27.000 species, new members are added to the orchid family every year (Chase et al., 2015; The Plant List, 2018). In Turkey, it is possible to encounter over 200 taxa from sea level to over 2000 m elevation (Davis and Davis, 1982; Güner et al., 2012). Additionally, the economic value of these plants is increased through widespread use in food, cosmetic and pharmaceutical industries (Sezik, 1984).

Structural studies of orchids have focused on flower parts generally due to the morphological variety and unrivaled pollination strategies of flowers (Stpiczyńska, 2003; Cozzolino and Widmer, 2005; Schiestl, 2005; Bell et al., 2009; Vereecken, 2009; Anton et al., 2012; Nunes et al., 2015). Furthermore, studies about the internal and surface structure of seeds rather than fruit, symbiotic-asymbiotic germination trials or orchid-fungus relationships are seen to be more notable (Gamarra et al., 2007; Chemisquy et al., 2009; Chen et al., 2012; Gamarra et al., 2012; Tesitelová et al., 2012; Bektaş et al., 2013). Though seeds are very small, they show high rates of variety in testa structure. The physical properties of seeds and the functional outcomes of these properties were revealed in detail by Arditti and Ghani (2000). Also, some studies have revealed the developmental features of seed coats (Molvray and Chase, 1999; Lee et al., 2005, 2007). Many micromorphological studies have stated that seed shape, surface patterning, anticlinal and periclinal wall structures or shape of testa cells are characteristic at different systematic categories and are reliable character to contribute to solving systematic problems in the family (Gamarra et al., 2012; Barthlott et al., 2014; Akbulut and Şenel, 2016; Şeker and Şenel, 2017; Gamarra et al., 2018).

The most accepted opinion related to developmental features of orchid fruit is that they comprise three carpels and the midvein of the carpel is located immediately below the stigma lobes. Orchids have marginal placentation (Brown, 1831; Payer, 1857; Van Tieghem, 1871). Additionally, according to the split-carpel model, the fruit is thought to occur by joining of other flower parts like sepal and petals. In this model, the sepals divide the carpels into two equal pieces and each petal joins to two separate carpel sections to form the fruit (Rasmussen and Johansen, 2006). In spite of detailed investigation of the internal structure of the carpel in these studies, the pericarpic surface morphology or morphometric features have been ignored compared to the seed.

Due to excessive number of species, high rate of variation in flowers or hybridization capacity, revealing characters rapidly and reliably for solutions to systematic problems carries great importance for orchids (Arditti, 1977;

Dressler, 1993). Many problems have not been solved due to identification based on flower morphology. With the more common use of molecular markers in recent years, systematic categories of many species have changed (Bateman et al., 2003).

However, the main disadvantages of these methods are high cost and requirement for additional approaches to differentiate closely-related taxa. In this study, the aim was to determine distinguishing features for the pericarp of closely-related orchid species, identification of whether these features are useful for classification and research of the relationships with habitat and ecological preferences of taxa.

2. Materials and Method

Samples were collected from different localities in the Black Sea Region from 2012-2016 (Table 1). Species identification was completed using Flora of Turkey and Türkiye Bitkileri Listesi (Davis and Davis, 1982; Güner et al., 2012). Fruits were fixated in formalin-acetic acid-alcohol (FAA) and then stored in 70% ethyl alcohol.

Table 1. List of *Orchidaceae* taxa and localities.

Taxa	**Locality**	**Voucher**
Anacamptis laxiflora (Lam.) R.M.Bateman, Pridgeon & M.W.Chase	Ondokuzmayıs, Samsun	Ss,36
Anacamptis laxiflora	Terme, Samsun	Ss,37
Anacamptis papilionacea (L.) R.M.Bateman, Pridgeon & M.W.Chase	Avdan, Canik, Samsun	Omuhb,7226
Anacamptis papilionacea	Kurupelit, Samsun	Omuhb,7827
Anacamptis pyramidalis (L.) Rich.	Kurupelit, Samsun	Omuhb,4141
Anacamptis pyramidalis	Avdan, Canik, Samsun	Omuhb,4142
Coeloglossum viride (L.) Hartm.	Köprübaşı, Trabzon	Omuhb,8253
Dactylorhiza euxina (Nevski) H.Baumann & Künkele	Köprübaşı, Trabzon	Mka,15
Dactylorhiza euxina	Kavron, Rize	Mka,18
Dactylorhiza romana (Seb.) Soó	Kurupelit, Samsun	Mka,11
Dactylorhiza romana	Abant, Bolu	Ss,10
Dactylorhiza saccifera (Brongn.) Soó	Ayder, Rize	Mka,19
Dactylorhiza saccifera	Köprübaşı, Trabzon	Mka,20
Gymnadenia conopsea (L.) R.Br.	Köprübaşı, Trabzon	Mka,27
Himantoglossum caprinum (M. Bieb.) Spreng.	Bayabat, Sinop	Mka,28
Himantoglossum caprinum	Kurupelit, Samsun	Omuhb,7739
Neotinea tridentata (Scop.) R.M.Bateman, Pridgeon & M.W.Chase	Maraşlı, Çaykara, Trabzon	Mka,42
Neotinea tridentata	Kurupelit, Samsun	Ss,26
Neotinea tridentata	Fatsa, Ordu	Ss,25
Ophrys apifera Huds.	Kurupelit, Samsun	Ss,39
Ophrys apifera	Avdan, Canik, Samsun	Omuhb,7716
Ophrys oestrifera M. Bieb.	Kurupelit, Samsun	Omuhb,7717
Ophrys oestrifera	Kavak, Samsun	Omuhb,7727
Orchis mascula L.	Kurupelit, Samsun	Omuhb,7712
Orchis mascula	Abant, Bolu	Ss,16
Orchis mascula	Çambaşı, Ordu	Omuhb,7829
Orchis pallens L.	Abant, Bolu	Ss,17
Orchis pallens	Köprübaşı, Trabzon	Mka,22
Orchis purpurea Huds.	Kurupelit, Samsun	Ss,14
Orchis purpurea	Fatsa, Ordu	Ss,21
Orchis purpurea	Abant, Bolu	Ss,22
Orchis purpurea	Kavak, Samsun	Ss,24
Platanthera chlorantha (Cruster) Rchb.	Kurupelit, Samsun	Omuhb,4123
Platanthera chlorantha	Kavak, Samsun	Mka,33
Serapias orientalis Greuter	Kurupelit, Samsun	Ss,35
Spiranthes spiralis (L.) Chevall	Kurupelit, Samsun	Mka,38
Spiranthes spiralis	Köprübaşı, Trabzon	Mka,49
Steveniella satyrioides (Spreng.) Schltr.	Kurupelit, Samsun	Omuhb,3041
Steveniella satyrioides	Ünye, Ordu	Mka,50
Steveniella satyrioides	Bafra, Samsun	Mka,40

To determine the anatomical and morphometrical characters, cross-section and surface sections were taken from fruit samples from at least three different individuals in each taxon. Anatomical features were investigated using a light microscope (Zeiss AxioLab A1 microscope and the Zeiss Axiocam 105 imaging system) and morphometric features were assessed with at least 30 measurements for each character. Furthermore, pericarp surface micromorphology was identified with scanning electron microscope studies. Samples were passed though alcohol series (30%, 50%, 70% and 95%) to remove excess water from tissues and dried with a critical point dryer. After coating with 15 mm gold-palladium (SEM coating system, SC7620), surface investigations were

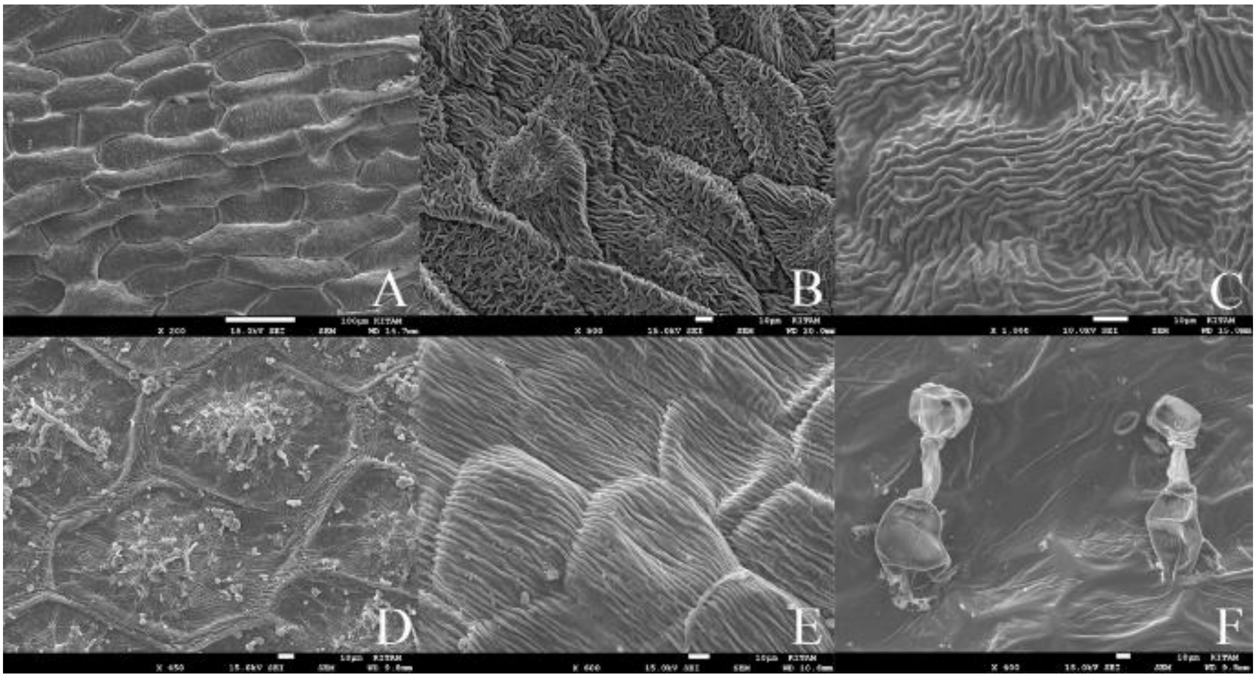

Figure 1. SEM image of the fruit surface: (A) *H. caprinum,* (B) *S. orientalis*, (C) *O. pallens,* (D) *N. tridentata,* (E) *D. euxina,* (F) *S. spiralis.*

completed with a JEOL JMS-7001F brand scanning electron microscope (SEM, 5-15 kV voltage). For organization of photographs the Photoshop CS6 program was used.

Correlation analyses were performed to assess the correlation between structural features and ecological or habitat preferences and ordination tests (CDA: canonical discrimination analysis) were used to identify the effect of characters on taxa differentiation. Tests were completed with the aid of SPSS (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.) program.

3. Results

The fruit structure of 19 studied orchid taxa were investigated in terms of a variety of features like epidermal cell size, cell shape, surface ornamentation, presence of hairs or papillary structures, and inclusion of crystal or starch grains. On the valves of *S. spiralis* species, secretory hairs of a variety of sizes were identified. However, hairs were not encountered in the other taxa.

Additionally, the epidermal cell margins were unclear in *S. spiralis* and there was no surface ornamentation. In other taxa, ornamentation was longitudinal, transversal or in both directions, with striate or undulate. However, SEM photographs of the fruit surface of *N. tridentata* show reticulate pattern and accumulations similar to secretions on the surface. Similar accumulations were present in *S. satyrioides* (Figure 1, Table 3).

According to correlation analysis results applied with the aim of identifying the correlation between fruit structural character and ecological needs or habitat preferences of taxa, there was a positive correlation between fruit surface ornamentation and habitat preferences (Table 2).

Table 2. Correlation between structural features and ecological or habitat preferences.

Correlations				
			Elevation	Habitat
Spearman's rho	Epidermal cell shape	Correlation Coefficient	.126	.011
		Sig.	.607	.966
	Ornamentation	Correlation Coefficient	.296	.654**
		Sig.	.218	.002
	Ornamentation direction	Correlation Coefficient	.094	.364
		Sig.	.701	.126
	Surface secretion	Correlation Coefficient	-.393	.067
		Sig.	.096	.786
	Hair	Correlation Coefficient	-.270	.046
		Sig.	.264	.852
	Papillary structure	Correlation Coefficient	-.270	-.391
		Sig.	.264	.098
	Crystal	Correlation Coefficient	-.071	.374
		Sig.	.779	.126
	Starch	Correlation Coefficient	-.303	.183
		Sig.	.222	.468
	Placentha	Correlation Coefficient	-.024	-.248
		Sig.	.924	.322
	Elevation	Correlation Coefficient	1.000	.192
		Sig.		.432
	Habitat	Correlation Coefficient	.192	1.000
		Sig.	.432	
Pearson	Epidermal cell length	Pearson Correlation	-.354	.300
		Sig.	.150	.227
	Epidermal cell width	Pearson Correlation	-.107	-.349
		Sig.	.672	.156
	Epidermal cell heigth	Pearson Correlation	-.099	-.159
		Sig.	.696	.528
	*. Correlation is significant at the 0.05 level (2-tailed).			
	**. Correlation is significant at the 0.01 level (2-tailed).			

Table 3. Qualitative characteristics and ecological preferences of taxa.

Taxa/ characters	Epidermal cell shape	Ornamentation	Ornamentation direction	Surface secretion	Hair	Papillary structure	Crystal	Starch	Elevation (m)	Habitat
A. laxiflora	Isodiametric to elongated	Striate	Transversal	-	-	+	Raphide	+	0-500	Wet meadows
A. papilionacea	Isodiametric to elongated	Striate	Vertical	-	-	-	Raphide	+	1500-2000	Meadows, Open areas, Edges of forests
A. pyramidalis	Elongated	Striate	Double direction	-	-	-	Raphide	+++	500-1000	Meadows, Open areas, Edges of forests
C. viride	Isodiametric to elongated	Undulate	Transversal	-	-	-	Prismatic	+	1000-1500	Meadows
D. euxina	Isodiametric	Striate	Transversal	-	-	-	Raphide	+	1500-2000	Wet meadows
D. romana	Isodiametric	Striate	Vertical	-	-	-	Raphide	+++	500-1000	Meadows, Open areas, Edges of forests
D. saccifera	Isodiametric to elongated	Undulate	Double direction	-	-	-	Raphide	+	1500-2000	Meadows, Open areas, Edges of forests
G. conopsea	Isodiametric to elongated	Undulate	Vertical	-	-	-	Raphide	+	1500-2001	Meadows
H. caprinum	Elongated	Undulate	Double direction	-	-	-	Raphide	+++	1500-2002	Meadows, Open areas, Edges of forests
N. tridentata	Isodiametric	Reticulate	Double direction	+	-	-	Raphide	+++	0-500	Meadows
O. apifera	Isodiametric to elongated	Striate	Vertical	-	-	-	Raphide	+++	0-500	Forest
O. oestrifera	Isodiametric to elongated	Striate	Vertical	-	-	-	Raphide	+	0-500	Forest
O. mascula	Isodiametric to elongated	Striate	Transversal	-	-	-	Absent	+++	500-1000	Meadows, Open areas, Edges of forests
O. pallens	Isodiametric	Undulate	Double direction	-	-	-	Raphide	+	1000-1500	Meadows, Open areas, Edges of forests
O. purpurea	Elongated	Undulate	Vertical	-	-	-	Raphide	+	500-1000	Meadows, Open areas, Edges of forests
P. clorantha	Isodiametric to elongated	Striate	Vertical	-	-	-	Raphide	+++	500-1000	Forest
S. orientalis	Isodiametric to elongated	Undulate	Double direction	-	-	-	Prismatic	+++	0-500	Meadows
S. satyrioides	Isodiametric to elongated	Striate	Vertical	+	-	-	Raphide	+	0-500	Forest
Spiranthes spiralis	Isodiametric	Absent	Transversal	-	Secretory hair	-			0-500	Meadows, Open areas, Edges of forests

Epidermal cells are isodiametric or elongated, and are pentagonal or hexagonal. In the cross-sections of *A. laxiflora*, elongated papillary structures is notable between the epidermal cells. Also, starch grains or crystals are clear with density on the mesocarp varying from species to species. While fruits are commonly observed to have raphide bundles, prismatic crystals found in species like *S. orientalis* and *C. viride*. Vascular bundles are collateral. In all taxa, the placenta is characterized by a single row cells with large nuclei and dense cytoplasm (Figure 2, Table 2).

Circular graphs were used with the aim of comparing the mean epidermal cell sizes between taxa (Figure 3). Accordingly, among the three features, the largest epidermal cells were found in *O. mascula*.

However, the cell length for *D. euxina*, cell width for *C. viride* and cell height for *G. conopsea* had smallest values measured.

Canonical discrimination analysis was used with the aim of determining the degree of accuracy of groupings according to fruit structural features and which feature was more effective in this grouping. Figure 4 shows the distribution of taxa based on the first two components. Accordingly, at genus level, taxa were accurately grouped with 94.4% success rate.

The most effective characters in canonical discrimination analysis were the morphometric features of epidermal cells like length, width and height. Additionally, the presence and variety of crystal in the tissue or habitat preferences of taxa came to the fore during taxon grouping (Table 4).

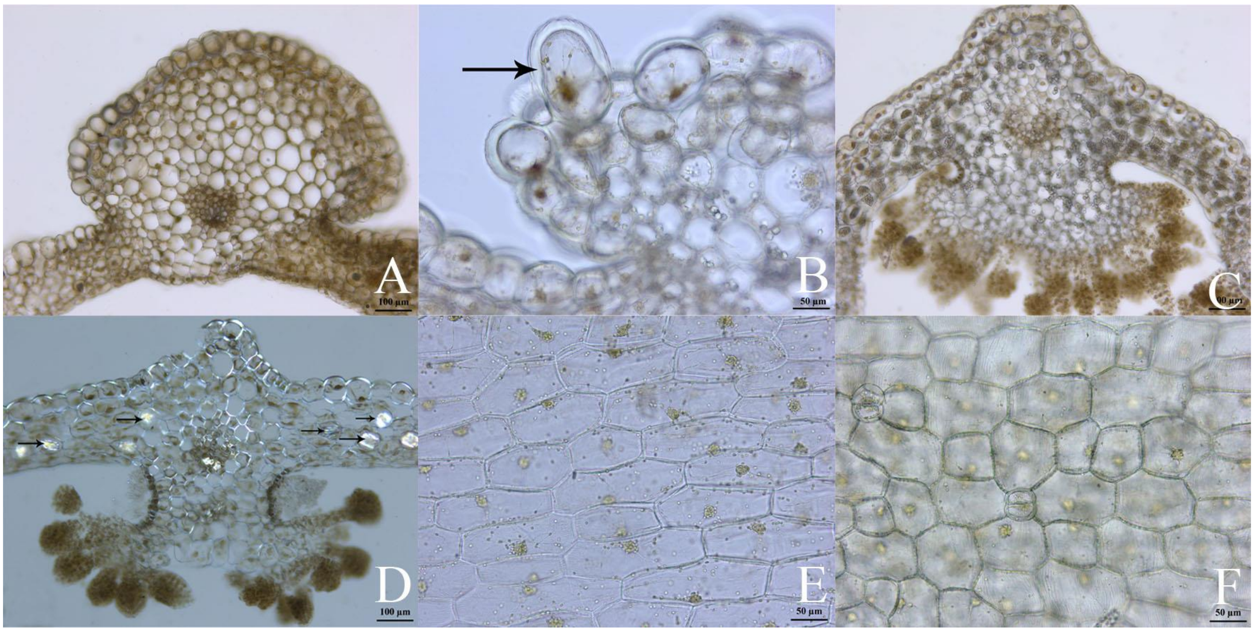

Figure 2. Light microscope image of pericarp in transverse and surface sections: (A) *O. purpurea,* (B) *A. laxiflora*, (C) *P. chlorantha,* (D) *D. saccifera,* (E) *A. papilionacea,* (F) *N. tridentata.*

4. Discussions

In the literature, hair with a variety of sizes and structures were reported on the vegetative and generative organs of orchids. Mainly researched for floral structures, these secretory hairs are emphasized to have functions in attracting pollinators acting as osmophore or nectarium due to secretions like mucilage, lipophilic compounds, pectic acid or phenolic compounds (Stpiczyńska and Davies, 2009; Nunes et al., 2014, 2015). In the literature, endocarpic hairs are mentioned in epiphytic species and it was emphasized that this type of trichome was only found on epiphytes (Beer, 1857; Horowitz, 1901-1902). As seen in our anatomical investigations, the lack of these types of structure in terrestrial species supports this view.

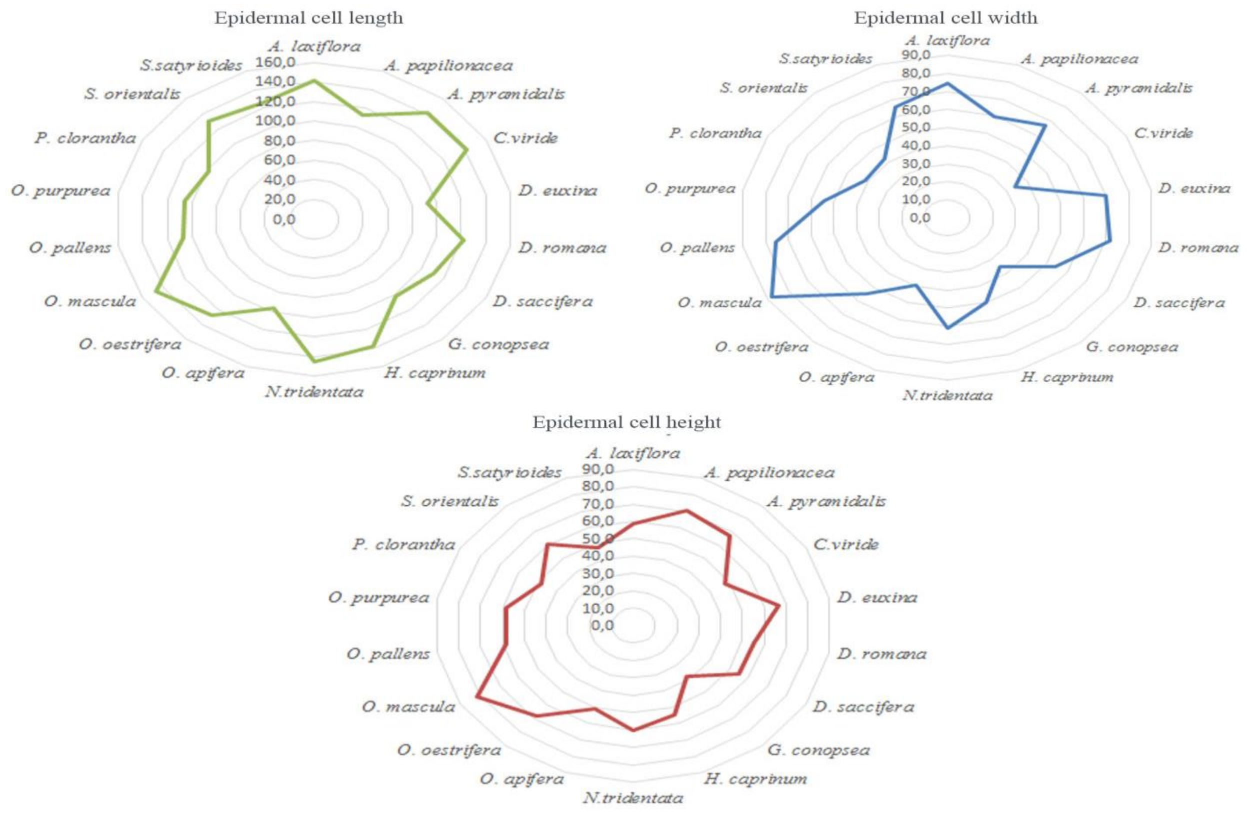

Figure 3. Circular plots of quantitative epidermal features.

Correlation analysis results mean that in habitats with higher humidity rates, the cuticle layer or secondary wall thickness reduces on the fruit surface and indicates that the surface ornamentation may be related to ecological adaptation rather than being a differentiating character for species.

As emphasized in previous research, though orchid fruit display homogeneous structure in terms of basic structural features, there is variety in terms of features like the shape, size and content of pericarp cells (Mayer et al., 2011). Though studies about orchid fruit have noted different features for identification of taxa, micromorphological and morphometric features have been ignored. For example, Prillieux (1857) identified 7 different fruit according to dehiscence type and stated that this variety may be useful to characterize species according to some criteria like apically fused vs. free, recurving valves, and valve number. Horowitz (1901–1902) in a study of many orchids classified taxa according to number and distribution of vascular bundles.

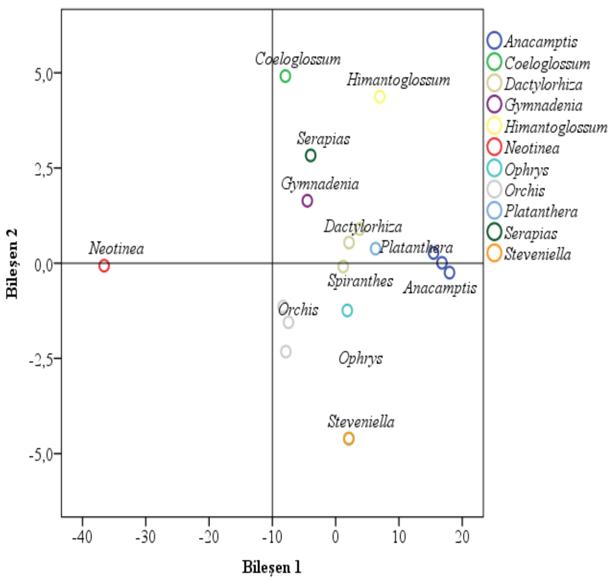

Figure 4. Distribution of genera on canonical discrimination components.

In morphometrical investigation, the cell sizes for *D. euxina*, *C. viride* and *G. conopsea* had smallest values. A study using molecular markers like ITS proved the close phylogenetic relationships between these three taxa (Shipunov et al., 2004). Moreover, Bateman et al. (1997) included the *C. viride* (*D. viridis*) species in the *Dactylorhiza* species and this new classification has been adopted by many researchers. In our study, this common morphometric feature in the pericarp epidermal cells of the three taxa support this view.

In this study, though structural character has low differentiation power for *Ophrys* taxa, discrimination analysis was found that fruit morphometric features have high rates of success for differentiation of other taxa. According to this finding, the pericarp features of orchidoid species may be effective for classification of genus and upper categories.

Table 4. Characters associated with discrimination components.

Characters	Function	
	1	2
Epidermal cell length	.024	-.752*
Epidermal cell widt	.271	-.587*
Epidermal cell heigth	.199	-.568*
Epidermal cell shape	.031	.051
Papillae	.021	.000
Starch	-.009	.056
Habitat	-.479	-.147
Ornamentation direction	-.011	.024
Ornamentation	-.085	.243
Crystal	-.005	.301
Elevation	.018	.229
Pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions Variables ordered by absolute size of correlation within function.		
*. Largest absolute correlation between each variable and any discriminant function		

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