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Yakın İlişkili Orkide Cinsleri *Anacamptis*, *Neotinea* ve *Orchis*'in Karşılaştırmalı Damar Morfometrisi

Şenay SÜNGÜ ŞEKER [a,*]
[a] *Biyoloji Bölümü, Fen Fakültesi, Ondokuz Mayıs Üniversitesi, Samsun, TÜRKİYE*
** Sorumlu yazarın e-posta adresi: izham.kilinc@batman.edu.tr*
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Öz

Bu çalışmada farklı bir yaklaşımla yeni orkide sınıflandırmalarını test etmek, yaprak damar deseninin bu sınıflandırmaları ne derecede temsil ettiğini araştırmak ve diagnostik olan damarlanma özelliklerinin belirlenmesi amaçlanmıştır. Sistematik kategorisi değişen türler ile birlikte *Orchis* üyelerinden simpatrik yayılış gösteren 8 tür araştırmaya konu edilmiştir. Bitki örnekleri Karadeniz Bölgesi'ndeki çeşitli lokalitelerden toplanmıştır. Olgun yapraklara saydamlaştırma ve boyama işlemi uygulanarak stereo mikroskopta fotoğraflanmış, damar ve areol yapılarına ait 15 morfometrik özellik analiz edilmiştir. Yaprak damar özellikleri açısından türler arasındaki farklılığın anlamlı olup olmadığını test etmek amacıyla oneway ANOVA uygulanmış ve analiz sonucunda gruplandırmada etkili olanlar ayrım analizi ile belirlenmiştir. Analiz sonucunda yeni sınıflandırmayı destekleyen veriler elde edilmiştir. *Neotinea* üyelerini diğer taksonlardan ayıran özellikler arasında areollerin çevresi, daireselliği, en/boy oranı gibi areol özellikleri ön plana çıkarken *Anacamptis* üyeleri birim alandaki damar yoğunluğu, damar uzunluğu gibi özellikler açısından farklılaşmaktadır. Bu sonuçlar damarlanmanın topolojik ve morfometrik karakterlerinin orkidelerin sistematik ve filogenetik ilişkilerini yansıtabileceğini ve özellikle problemli gruplarda sınıflandırmaya ilişkin problemlerin çözümünde etkili olabileceğini işaret etmektedir.

Anahtar Kelimeler: *Anacamptis, Görüntü işleme, Neotinea, Orchis, Filogenetik sinyal, Yaprak damarlanma*

Comparative Vein Morphometry of Closely Related Orchid Genera *Anacamptis*, *Neotinea* and *Orchis*

Abstract

This research aimed to test new orchid classifications, investigate to what extent the leaf vein pattern represents this classification, and determine the diagnostic features. Along with the species whose systematic category changed, 8 taxa with sympatric distribution were the subject of the study. Samples were collected from various localities in the Black Sea Region. The vein structures were analysed by applying clearing and staining processes to leaves and photographed under a stereomicroscope, and 15 morphometric features were analysed. Oneway ANOVA was applied to test whether the difference between species in terms of leaf vein characteristics is significant, and the characteristics showing significant differences were used in discriminant analysis. It provided data supporting the new classification. Among the features that distinguish *Neotinea* members from other taxa, areole features such as perimeter, circularity, and aspect ratio of the areoles come to the fore, while *Anacamptis* members differ in terms of vein features such as vascular density and vascular length per unit area. These results indicate that the topological and morphometric characteristics of venation may reflect the systematic and phylogenetic relationships of orchids and may be effective in solving problems related to classification, especially in problematic groups.

Keywords: *Image processing, Leaf venation, Neotinea, Orchis, Phylogenetic signal*

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I. INTRODUCTION

The Orchidaceae family constitutes one of the largest Angiosperm groups in terms of biodiversity spreading over a wide geographical area [1], [2]. However, high biodiversity and variation in generative structures and hybridization abilities bring identification and classification problems to orchids. The genus *Orchis*, classified in the Orchidecaea family, is a large group distributed in Europe, North Africa, the Middle East and Central Asia [3]. *Orchis* genus, which includes more than 60 taxa according to the classical classification based on floral morphology, has been revised in recent years with different approaches such as molecular phylogenetic, karyology or reproductive biology. [4]-[6]. Phylogenetic studies, especially using marker genes, have shown that the genus is polyphyletic and some species form monophyletic groups with closely related genera (*Anacamptis*, *Neotinea*). For this reason, some taxa have been included in these genera that were previously classified as monotypic. [4], [7]-[9]. There are also different views on the classification of *Orchis* members. For example, Tyteca and Klein [5] rejected the new phylogenetic classification and divided the genus into 4 sections (*Odondorchis*, *Herorchis*, *Androrchis* and *Orchis*). The group, including the same researcher, later accepted the new phylogenetic classification and added *Odondorchis* to the *Neotinea* genus; they accepted that *Herorchis* should be included in *Anacamptis*, but insisted on the distinction between *Orchis* and *Androrchis* [10]. In addition, other studies using additional molecular markers and clustering algorithms have also revealed data supporting this distinction [11].

Monocot plants have main veins arranged parallel to the midrib and show a pattern of transverse secondary veins that anastomoses to form short interconnections between the main veins at the distal and basal ends of the leaf blade [12]. Veining pattern is affected by phylogenetic or genetic structure together with the ecological conditions in which the plant is found. In addition, the vascularization pattern is associated with many physiological processes such as resistance to injury, lignin-induced mechanical support, photosynthesis and transpiration led by carbohydrate and water carrying capacity. [13]-[23]. Therefore, in recent years, hypotheses have emerged that leaf veining features may offer useful characters for species identification. In order to test these hypotheses, morphometric and topological analyses of the veining pattern are performed on leaf photographs, especially in dicot groups, with the help of various programs. However, there is a need for such studies in monocot plants, especially in systematically problematic groups such as orchids. The aim of this study was to test new classifications using a varied approach, to determine the degree to which leaf vein pattern represents this classification, and to determine the diagnostic features of leaf veining.

II. MATERIALS AND METHODS

Plant samples were collected from various localities in the Black Sea Region and were stocked in 70% ethanol for long-term preservation. (Table 1). A total of 8 species (*Anacamptis coriophora*, *A. laxiflora*, *A. papilionacea*, *Neotinea tridentata*, *Orchis pallens*, *O. provincialis*, *O. purpurea*, *O. simia*) whose systematic category changes and sympatric distribution with *Orchis* members were examined and thus ecological factors were eliminated. The vein structures were analysed by applying clearing and staining processes to mature leaves in two different locality samples for each species.

The clearing method was applied with reference to Vasco et al., [24]. Accordingly, leaf samples were incubated in 5% NaOH solution at 40-54 °C for 12-24 hours depending on leaf size and thickness. The samples were washed with distilled water and treated with 4.5-5.5% sodium hypochlorite for 20 seconds to 10 minutes. After the leaf samples were passed through graded ethanol series (50%, 70%, 95%) for 30 minutes, the veins were stained with 1% safranin solution for 3 hours by applying standard protocol.

Table 1. *List of studied species, localities, voucher specimens, and leaf size.*

Taxon	Grup	Specimen number	Locality Altitude	Habitat	Leaf width *(cm)*	Leaf length *(cm)*
Anacamptis coriophora (L.) R.M.Bateman, Pridgeon & M.W.Chase	*Anacamptis*	AnaCorBss75	Bolu, Abant, Ömerler, 1171m.	Edges of coniferous forests, meadows	1,5	8,0
Anacamptis coriophora	*Anacamptis*	AnaCorSss13	Samsun, Bafra, 300m.	Meadows	1,7	9,0
Anacamptis laxiflora (Lam.) R.M.Bateman, Pridgeon & M.W.Chase	*Anacamptis*	AnaLaxBss74	Bolu, Abant, Ömerler, 1171m.	Edges of coniferous forests, meadows	2,0	20,0
Anacamptis laxiflora	*Anacamptis*	AnaLaxS ss61	Samsun, Çakırlar, 9m.	Wet meadows	1,6	17,0
Anacamptis papilionacea (L.) R.M.Bateman, Pridgeon & M.W.Chase	*Anacamptis*	AnaPapSss65	Samsun, Kurupelit, 147m.	*Quercus* forests edges, meadows	1,5	9,0
Anacamptis papilionacea	*Anacamptis*	AnaPapSss19	Samsun, Avdan, 600m.	Meadows	1,1	10,6
Neotinea tridentata (Scop.) R.M.Bateman, Pridgeon & M.W.Chase	*Neotinea*	NeoTriSss56	Samsun, Kurupelit, 218m.	*Quercus* forests edges, meadows	2,0	9,0
Neotinea tridentata	*Neotinea*	NeoTriOss25	Ordu, Fatsa, 140m.	*Corylus* plantations, meadows	1,8	8,0
Orchis pallens L.	*Androrchis*	OrcPalBss17	Bolu, Abant Gölü, 1345m.	Coniferous and deciduous forests	3,8	11,4
Orchis pallens	*Androrchis*	OrcPalTmka22	Trabzon, Köprübaşı, 2300m.	Meadows	4,0	10,8
Orchis provincialis Balb. ex Lam. & DC.	*Androrchis*	OrcProSss57	Samsun, Kurupelit, 166m.	*Quercus* forests edges, meadows	1,5	8,0
Orchis purpurea Huds.	*Orchis*	OrcPurSss60	Samsun, Kurupelit, 216m.	*Quercus* forests	4,0	18,0
Orchis purpurea	*Orchis*	OrcPurBss22	Bolu, Abant yolu, 986m.	*Quercus* forests	7,6	22,8
Orchis simia Lam.	*Orchis*	OrcSimSss27	Samsun Kavak, 330m.	Forests edges, meadows	2,8	6,7
Orchis simia	*Orchis*	OrcSimSss23	Samsun, Ondokuz Mayıs, 880m.	Meadows	3,1	7,0

Five different regions on the leaves were photographed using a Leica S8APO stereo microscope with a Leica DFC295 digital camera attachment. After a series of cropping and cleaning process, the photos were converted from RGB form to grayscale. By using automatic or locally adaptive thresholding approaches, binary images were obtained with segmentation defining vessel regions as white and areoles as black. Metric calculations were performed on these binary images, and 15 morphometric features of vessel and areole structures (vascular density, vascular length density, vessel diameter, vessel length, areole area, areole perimeter, width of areole bounding rectangle, height of areole bounding rectangle, major axis of fitted ellipse to areole, minor axis of fitted ellipse to areole, circularity, areole ferret, areole minferet, AR (aspect ratio) were calculated with the help of ImageJ/Fiji program (Figure 1) [25]

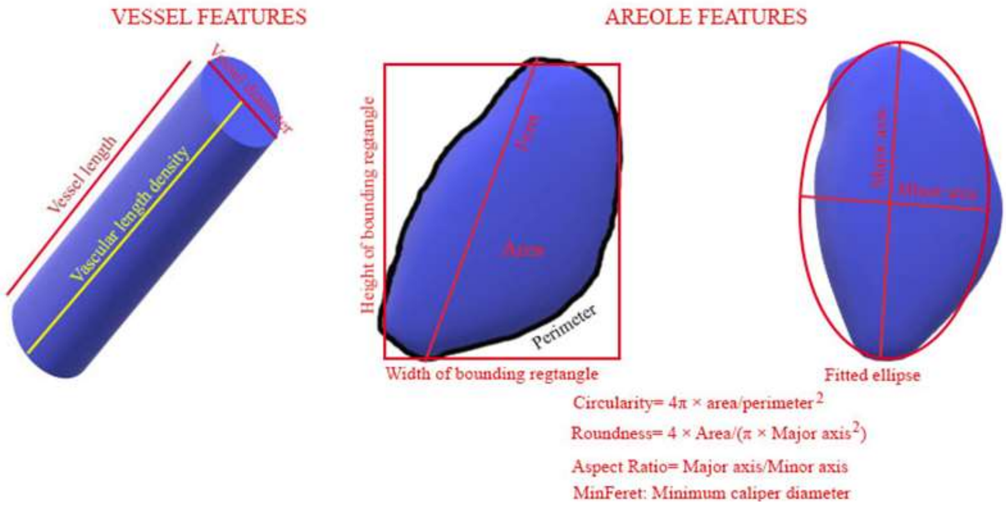

Figure 1. *Definition of quantitative vessel and areole features.*

ANOVA was applied to test whether the difference between species in terms of leaf vein characteristics is significant, and by using the characteristics that showed significant difference as a result of the analysis, Linear discriminant analysis were performed. In addition, the correlation between leaf sizes, the elevations of the taxa, and the morphometric vein features were evaluated with the Pearson correlation test. All statistical analyses were performed with the help of SPSS software (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.).

III. RESULTS

Leaf vein characteristics of some taxa belonging to the closely related genera *Anacamptis*, *Neotinea* and *Orchis* were investigated. It was seen that secondary vein density increases towards the leaf tip in all leaves. While free-ended veins, which are frequently seen in dicots, were mostly not observed in orchids. The shape of secondary vessels and areoles in *O. purprea* and *O. simia* were also more irregular than in other species (Figure 2).

As a result of ANOVA, it was seen that leaf vein and areole metric properties differ significantly among the orchid species that are the subject of the study. All the quantitative character mean values were significantly different in at least one taxon (Table 2). Box plots of 15 veins and areole characters are shown in Figure 3. Accordingly, values such as vascular density, vascular length density, and vessel diameter were higher in *Anacamptis* members. In *Neotinea tridentata*, vessel length, areole perimeter and area, height of areole bounding rectangle, major axis of fitted ellipse to areole, and areole minFeret were measured at the highest value. On the other hand, while major axis of fitted ellipse to areole, ferret and aspect ratio were the lowest in the areoles of *Orchis* taxa; the circularity and roundness of the areoles were of high value. According to these results, all metric features measured were included in linear discriminant analysis.

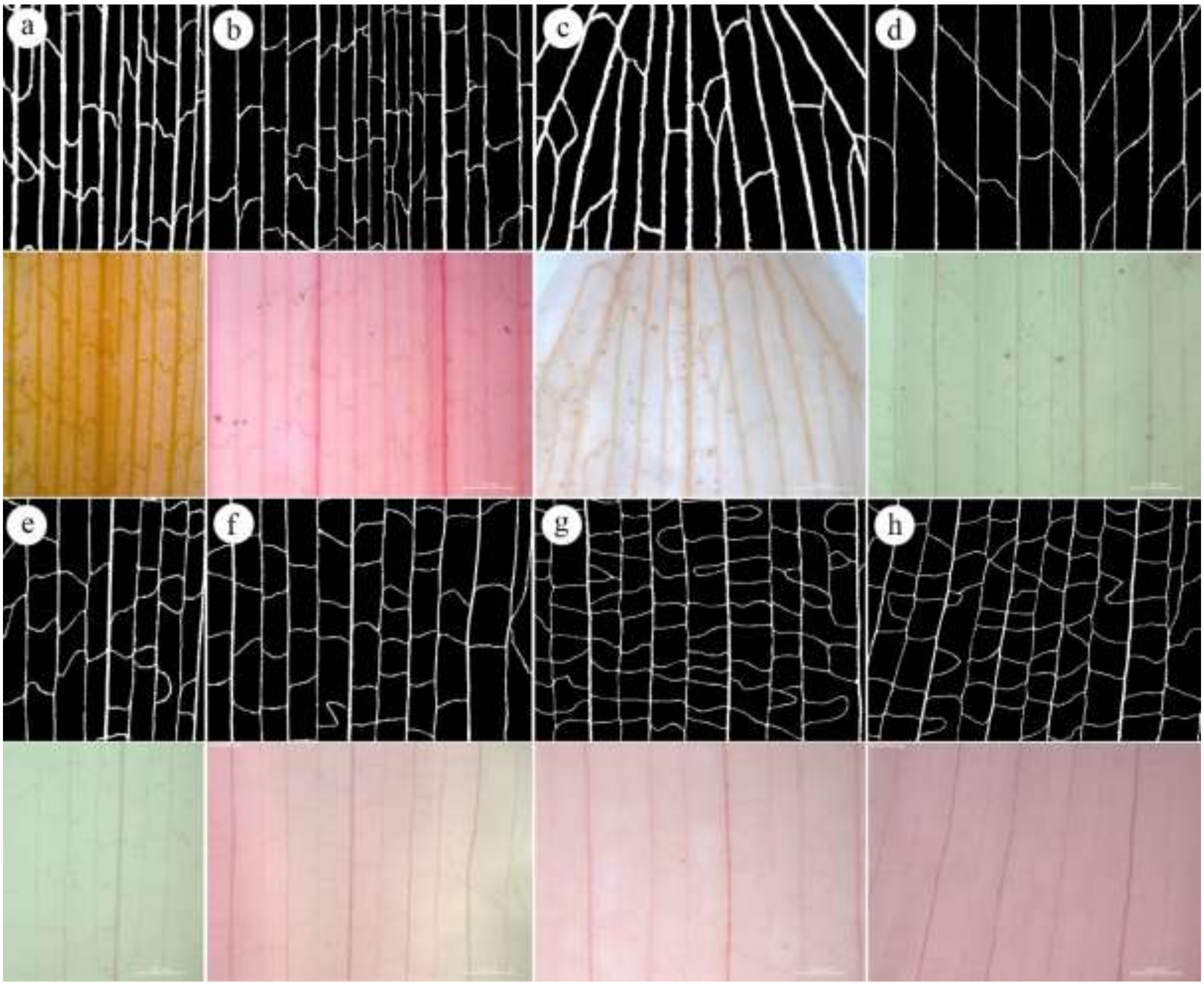

Figure2. *Binary and RGB images showing the vein and areole pattern. a:Anacamptis coriophora, b: A. laxiflora, c: A. papilionacea, d: Neotinea tridentata, e: Orchis pallens, f: O. provincialis, g: O. purpurea, h: O. simia.*

Table 2. *Oneway ANOVA outputs of vascular features.*

Vascular features	**F (7, 232)**	**Sig.**
Vascular density	151,523	,000
Vascular length density	59,464	,000
Vessel diameter	21,771	,000
Vessel length	13,221	,000
Areole area	34,622	,000
Areole perimeter	42,786	,000
Width of areole bounding rectangle	169,586	,000
Height of areole bounding rectangle	83,953	,000
Major axis of fitted ellipse to areole	54,174	,000
Minor axis of fitted ellipse to areole	24,347	,000
Circularity	89,039	,000
Areole feret	53,479	,000
Areole minFeret	23,285	,000
AR (Aspect ratio)	45,791	,000
Roundness	92,959	,000

The distribution of taxa on the first two discriminant components calculated as a result of canonical discriminant analysis is given in Figure 4. As a result of the analysis, it was seen that primary and secondary components explain 93.9% variation, cumulatively at the rate of 67.5% and 26.4%, respectively (Table 3). Although there were partial overlaps between the groups on the graph, the success rate of identifying groups of leaf vein and areole features was 90% (Table 4). According to Table 5, while the characters with the highest load on the canonical discriminant function, which explain the variation at a higher rate, were circularity, roundness, major axis of fitted ellipse to areole, aspect ratio, areole ferret, areole perimeter, while the most loaded features to the second discriminant function were the vessel characters such as vascular density, vascular length density, vessel length, as well as areole features such as areole area, height of areole bounding rectangle and minor axis of fitted ellipse to areole.

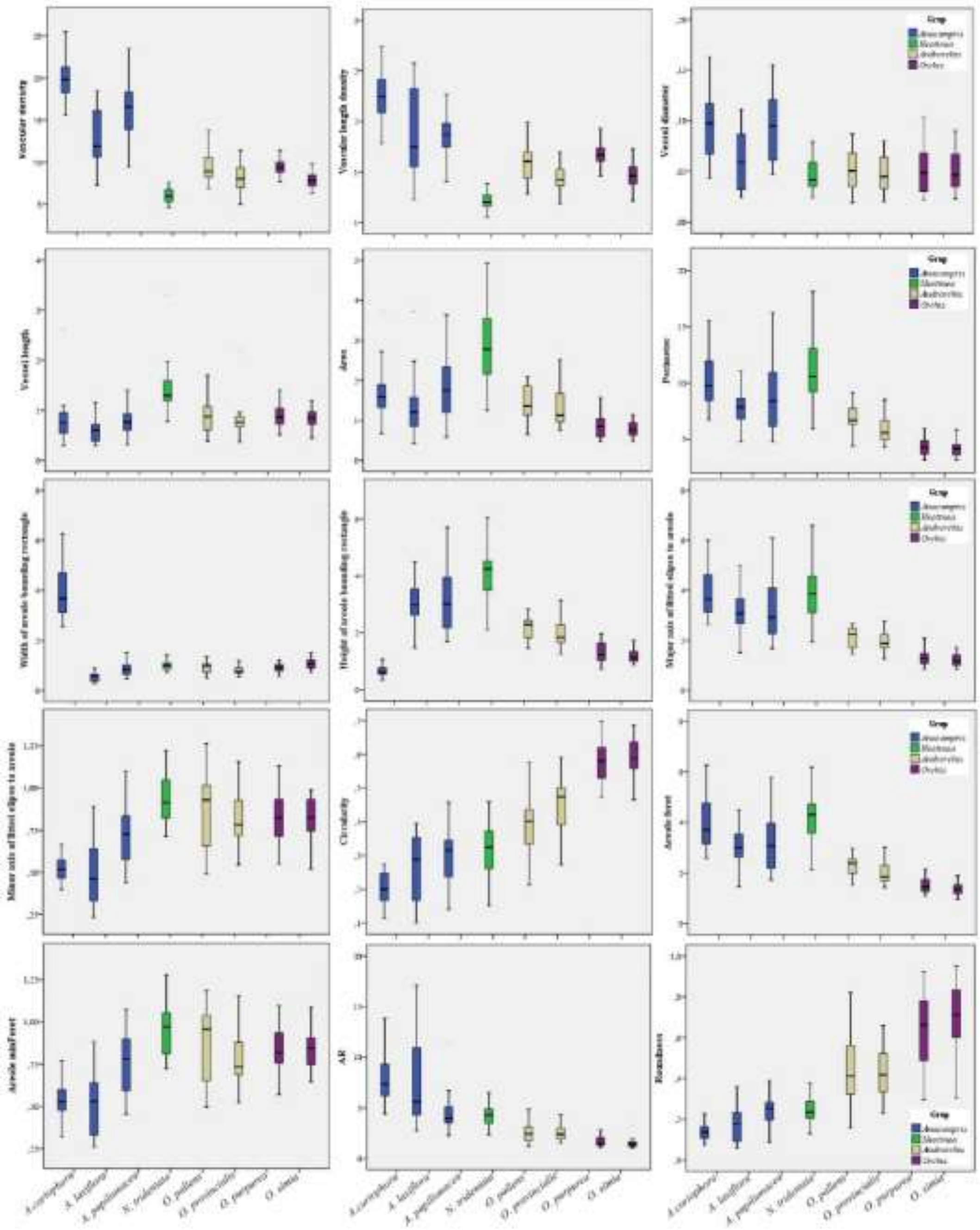

Figure 3. *Boxplots for each quantitative vessel and areole feature among species. The rectangles span from the first quartile to the third quartile of the distribution. The full horizontal lines in rectangles represent median and whiskers show the lower and the upper 25% of the distribution.*

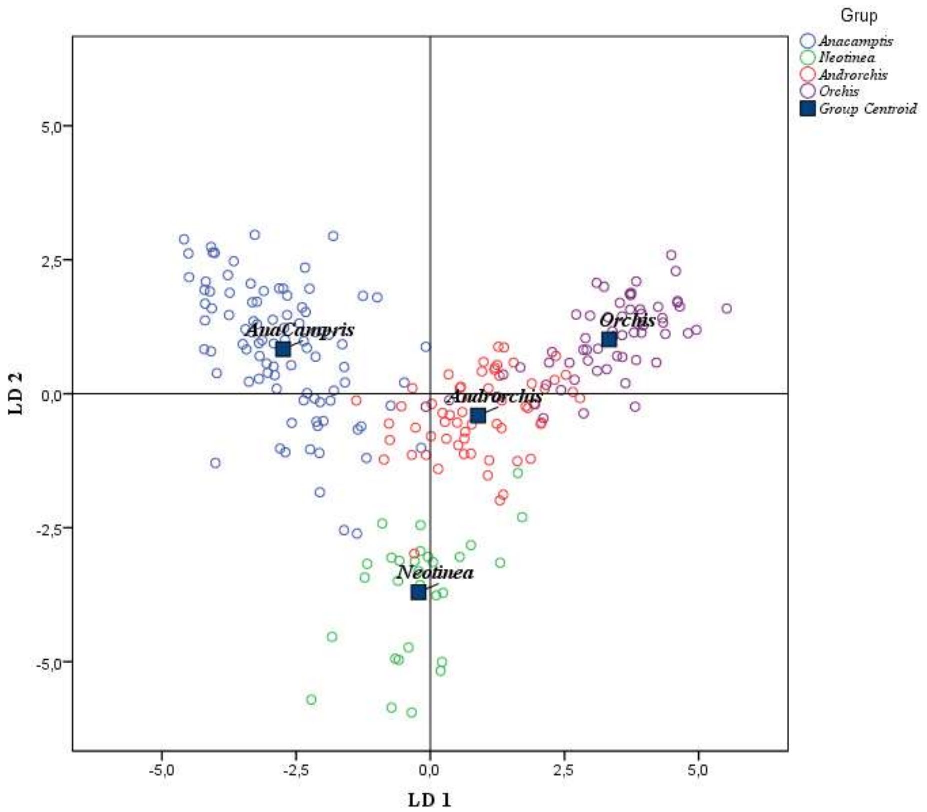

Figure 4. *Linear discriminant analysis (LDA) plots show individuals' distribution for the first two discriminant functions (LD1 and LD2).*

Table 3. *Summary of linear discriminant analysis.*

Function	Eigenvalue	% of Variance	Cumulative %	Canonical Correlation
1	5,902[a]	67,5	67,5	,925
2	2,309[a]	26,4	93,9	,835
3	,534[a]	6,1	100,0	,590

a. First three canonical discriminant functions were used in the analysis.

Table 4. *Predicted memberships of each individual to the groups.*

Grup			Predicted Group Membership			
			Anacamptis	*Neotinea*	*Androrchis*	*Orchis*
Original	**Count**	*Anacamptis*	80	3	7	0
		Neotinea	0	28	2	0
		Androrchis	1	1	56	2
		Orchis	0	0	8	52
	%	*Anacamptis*	88,9	3,3	7,8	0,0
		Neotinea	0,0	93,3	6,7	0,0
		Androrchis	1,7	1,7	93,3	3,3
		Orchis	0,0	0,0	13,3	86,7

a. **90.0%** of original grouped cases correctly classified.

Table 5. *Coefficients (correlation) between each quantitative vascular feature and three linear discriminant functions.*

	Function		
	1	**2**	**3**
Roundness	,637*	,244	,038
Circularity	,608*	,175	-,013
Major axis of fitted ellipse to areole	-,434*	-,325	,338
Aspect ratio	-,400*	,044	,253
Areole feret	-,398*	-,384	,361
Areole perimeter	-,361*	-,349	,251
Vessel diameter	-,227*	,200	,153
Vascular density	-,478	,564*	,191
Areole area	-,201	-,532*	,239
Vascular length density	-,288	,515*	,101
Height of areole bounding rectangle	-,164	-,486*	,134
Vessel length	,048	-,373*	,214
Minor axis of fitted ellipse to areole	,229	-,306*	-,130
Areole minferet	,212	-,293*	-,004
Width of areole bounding rectangle	-,141	,104	,183*

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

Pearson correlation was performed to test the relationship between the morphometric features of the vascularization and the leaf size of the species and the elevations at which they spread. As a result of the analysis, no correlation was found between the areole and vein features and size or elevation. In addition, there was no similarity between different groups growing in the same area in terms of any quantitative feature (Table 6).

Table 6. *Correlation matrix for leaf vascular descriptive features and elevation preferences of the species.*

	Vascular density	Vascular length density	Vessel diameter	Vessel length	Area	Perimeter	Width	Height	Major	Minor	Circularity	Feret	MinFeret	AR	Round	Leaf width	Leaf length	Elevation
Vascular density	1	,958**	,965**	-,511	,009	,456	,683	-,303	,515	-,812*	-,667	,448	-,757*	,728*	-,602	-,363	,018	,074
Vascular length density	,958**	1	,856**	-,645	-,204	,274	,658	-,452	,354	-,868**	-,549	,280	-,826*	,685	-,475	-,163	,214	,203
Vessel diameter	,965**	,856**	1	-,342	,174	,551	,657	-,179	,580	-,668	-,676	,529	-,595	,666	-,613	-,440	-,141	-,006
Vessel length	-,511	-,645	-,342	1	,759*	,398	-,062	,505	,290	,693	,001	,391	,735*	-,222	-,022	,030	-,322	-,173
Area	,009	-,204	,174	,759*	1	,874**	,134	,709*	,808*	,188	-,618	,859**	,238	,370	-,646	-,486	-,346	-,257
Perimeter	,456	,274	,551	,398	,874**	1	,450	,487	,988**	-,292	-,907**	,996**	-,236	,751*	-,904**	-,635	-,262	-,171
Width	,683	,658	,657	-,062	,134	,450	1	-,554	,471	-,491	-,462	,463	-,445	,525	-,382	-,219	-,307	,077
Height	-,303	-,452	-,179	,505	,709*	,487	-,554	1	,451	,261	-,353	,475	,273	,153	-,428	-,363	,021	-,269
Major	,515	,354	,580	,290	,808*	,988**	,471	,451	1	-,422	-,942**	,994**	-,369	,839**	-,942**	-,665	-,191	-,205
Minor	-,812*	-,868**	-,668	,693	,188	-,292	-,491	,261	-,422	1	,611	-,329	,990**	-,834*	,580	,384	-,269	,038
Circularity	-,667	-,549	-,676	,001	-,618	-,907**	-,462	-,353	-,942**	,611	1	-,907**	,579	-,923**	,989**	,666	,082	,024
Feret	,448	,280	,529	,391	,859**	,996**	,463	,475	,994**	-,329	-,907**	1	-,272	,781*	-,906**	-,626	-,222	-,200
MinFeret	-,757*	-,826*	-,595	,735*	,238	-,236	-,445	,273	-,369	,990**	,579	-,272	1	-,803*	,559	,398	-,262	,025
AR	,728*	,685	,666	-,222	,370	,751*	,525	,153	,839**	-,834*	-,923**	,781*	-,803*	1	-,904**	-,594	,090	-,087
Round	-,602	-,475	-,613	-,022	-,646	-,904**	-,382	-,428	-,942**	,580	,989**	-,906**	,559	-,904**	1	,704	,064	,126
Leaf width	-,363	-,163	-,440	,030	-,486	-,635	-,219	-,363	-,665	,384	,666	-,626	,398	-,594	,704	1	,567	,442
Leaf length	,018	,214	-,141	-,322	-,346	-,262	-,307	,021	-,191	-,269	,082	-,222	-,262	,090	,064	,567	1	,117
Elevation	,074	,203	-,006	-,173	-,257	-,171	,077	-,269	-,205	,038	,024	-,200	,025	-,087	,126	,442	,117	1

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

IV. DISCUSSION AND CONCLUSION

In this study, the leaf veining pattern of orchid taxa, some of which showed sympatric distribution in the same vegetation period and whose systematic category changed according to new classification approaches, was examined in detail. As a result of the research, it was clearly seen that the metric properties of vein and areole on the leaves may reflect phylogenetic relationships, especially in the related genera like *Orchis*, *Anacamptis* and *Neotinea*. In most phylogenetic studies based on nuclear or chloroplast DNA, *Orchis* members formed two separate clades on well supported in phylogenetic trees [3]-[4], [7]-[9], [11] Thus, genus *Orchis* has been accepted as two different section named as *Orchis* with anthropomorphic labellum and the *Androrchis* where the perianth parts do not form a hood. In addition, it was stated that the seed morphology showed variation between these sections [26]. Vascularization data also support division between sections. The most obvious difference between these two subgroups was due to the circularity, roundness and aspect ratio of the areoles. Although *O. provincialis* and *O. pallens* from the *Androrchis* section have quite different demands in terms of elevation and habitat, the same veining pattern reflects their phylogenetic relationships. The same situation was observed in *O. purpurea* and *O. simia*, members of the *Orchis* section.

On the other hand, *O. provincialis* spreads together with *A. paplinionacea* and *N. tridentata* in the same flowering period. However, when the vascularization characteristics were examined, there was a clear distinction between these three taxa. The features that have a high load on the secondary component in the discriminant analysis and distinguish *N. tridentata* from other taxa were low vascular density and vascular length density, and high values such as vessel length, areole area, height of areole bounding rectangle and minor axis of fitted ellipse to areole. Among *Anacamptis* members, *A. coriophora* and *A. laxiflora* Bolu specimens distributed together at the wet or moist meadow grassland habitat. *A. papilionacea* grows in open areas of the forests or open meadows. In the discriminant analysis, the characters with higher loadings on the primary function and especially distinguish *Anacamptis* members from other taxa areole features such as circularity, roundness, and major axis of fitted ellipse areole ellipse, aspect ratio, feret and perimeter. In other words, *Anacamptis* members differ from other groups thanks to the longer and narrower areole structures which they share.

As a result, the secondary vascularization pattern of orchids seems to be influenced by the phylogenetic structure of taxa rather than ecological or ecophysiological processes. As a result of the analysis, data supporting the new classification were obtained.

This research has indicated that the topological and morphometric characteristics of vascularization may reflect the systematic and phylogenetic relationships of orchids and may be effective in solving problems related to classification, especially in problematic groups such as orchids with high hybridization ability. In addition, evaluating measurable morphological features and digitizing the morphological descriptions will prevent the subjective data based on the researcher's observation and the errors arising from the qualitative descriptions.

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V. REFERENCES

[1] P. Delforge, *Orchids of Europe, North Africa and the Middle East*, London, England: A&C Black, 2006, pp. 623.

[2] *The Plant List* (2018). [Online]. Available from http://www.theplantlist.org/.

[3] R. L. Dressler, *Phylogeny and Classification of the Orchid Family*, Cambridge, UK: Cambridge University Press, 1993, pp. 314.

[4] R. M. Bateman, P. M. Hollingsworth, J. Preston, L. Yi-Bo, A. M. Pridgeon, and M. W. Chase, "Molecular phylogenetics and evolution of Orchidinae and selected Habenariinae (Orchidaceae)," *Botanical journal of the Linnean Society*, vol. 142, pp. 1-40, 2003.

[5] D. Tyteca and E. Klein, "Genes, morphology and biology-The systematics of Orchidinae revisited," *Journal of European Orchids*, vol. 40, no. 3, pp. 501-544, 2008.

[6] G. Scopece, S. Cozzolino, and R. M. Bateman, "Just what is a genus? Comparing levels of postzygotic isolation to test alternative taxonomic hypotheses in Orchidaceae subtribe Orchidinae," *Taxon*, vol. 59, pp. 1754–1764, 2010.

[7] S. Aceto, P. Caputo, S. Cozzolino, L. Gaudio, and A. Moretti, "Phylogeny and evolution of *Orchis* and allied genera based on ITS DNA variation: morphological gaps and molecular continuity," *Molecular Phylogenetics and Evolution*, vol. 13, no. 1, pp. 67-76, 1999.

[8] R. Bateman, A. Pridgeon, and M. Chase, "Phylogenetics of subtribe Orchidinae (Orchidoideae, Orchidaceae) based on nuclear ITS sequences. 2. Infrageneric relationships and reclassification to achieve monophyly of *Orchis* sensu stricto," *Lindleyana*, vol. 12, pp. 113-141, 1997.

[9] A. Pridgeon, R. Bateman, A. Cox, J. Hapeman, and M. Chase, "Phylogenetics of subtribe Orchidinae (Orchidoideae, Orchidaceae) based on nuclear ITS sequences: 1. Intergeneric relationships and polyphyly of *Orchis* sensu lato," *Lindleyana*, vol. 12, pp. 89-109, 1997.

[10] D. Tyteca, M. Ceinos, J. L. Gathoye, R. Brys, and H. Jacquemyn, "On the morphological, biological and genetic heterogeneity of the genus *Orchis* (Orchidaceae, Orchidinae)," *Phytotaxa*, vol. 75, no. 1, pp. 19-32, 2012.

[11] L. A. Inda, M. Pimentel, and M. W. Chase, "Phylogenetics of tribe Orchideae (Orchidaceae: Orchidoideae) based on combined DNA matrices: inferences regarding timing of diversification and evolution of pollination syndromes," *Annals of Botany*, vol. 110, no. 1, pp. 71-90, 2012.

[12] P. A. Conklin, J. Strable, S. Li, and M. J. Scanlon, "On the mechanisms of development in monocot and eudicot leaves," *New Phytologist*, vol. 221, no. 2, pp. 706-724, 2019.

[13] K. J. Niklas, "A mechanical perspective on foliage leaf form and function," *New Phytologist*, vol. 143, no. 1, pp. 19-31, 1999.

[14] T. J. Givnish, J. C. Pires, S. W. Graham, M. A. McPherson, L. M. Prince, T. B. Patterson, ... and K. J. Sytsma, "Repeated evolution of net venation and fleshy fruits among monocots in shaded habitats confirms a priori predictions: evidence from an ndhF phylogeny," *Proc. of the Royal Society B: Biological Sciences*, vol. 272, no. 1571, pp. 1481-1490, 2005.

[15] T. J. Brodribb, T. S. Feild, and G. J. Jordan, "Leaf maximum photosynthetic rate and venation are linked by hydraulics," *Plant Physiology*, vol. 144, no. 4, pp. 1890-1898, 2007.

[16] E. Katifori, G. J. Szöllősi, and M. O. Magnasco, "Damage and fluctuations induce loops in optimal transport networks," *Physical Review Letters*, vol. 104, no. 4, pp. 048704, 2010.

[17] T. J. Brodribb, D. Bienaimé, and P. Marmottant, "Revealing catastrophic failure of leaf networks under stress," *Proc. of the National Academy of Sciences*, vol. 113, no. 17, pp. 4865-4869, 2016.

[18] B. Blonder, N. Salinas, L. P. Bentley, A. Shenkin, P. O. Chambi Porroa, Y. Valdez Tejeira, ... and Y. Malhi, "Structural and defensive roles of angiosperm leaf venation network reticulation across an Andes-Amazon elevation gradient," *Journal of Ecology*, vol. 106, no. 4, pp. 1683-1699, 2018.

[19] B. Blonder, C. Violle, L. P. Bentley, and B. J. Enquist, "Venation networks and the origin of the leaf economics spectrum," *Ecology Letters*, vol. 14, no. 2, pp.91-100, 2011.

[20] A. Ohtsuka, L. Sack, and H. Taneda, "Bundle sheath lignification mediates the linkage of leaf hydraulics and venation," *Plant, Cell and Environment*, vol. 41, no. 2, pp. 342-353, 2018.

[21] G. P. John, C. Scoffoni, T. N. Buckley, R. Villar, H. Poorter, and L. Sack, "The anatomical and compositional basis of leaf mass per area," *Ecology Letters*, vol. 20, no. 4, pp. 412-425, 2017.

[22] A. A. Agrawal and K. Konno, "Latex: a model for understanding mechanisms, ecology, and evolution of plant defense against herbivory," *Annual Review of Ecology, Evolution, and Systematics*, vol. 40, pp. 311-331, 2009.

[23] J. F. Vincent, "The mechanical design of grass," *Journal of Materials Science*, vol. 17, no.3, pp. 856-860, 1982.

[24] A. Vasco, M. Thadeo, M. Conover, and D. C. Daly, "Preparation of samples for leaf architecture studies, a method for mounting cleared leaves," *Applications in Plant Sciences*, vol. 2, no. 9, pp. 1400038, 2014.

[25] J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, … and A. Cardona, "Fiji: an open-source platform for biological-image analysis," *Nature Methods*, vol. 9, no.7, pp. 676–682, 2012.

[26] R. Gamarra, E. Ortúñez, P. G. Cela, and V. Guadaño, "*Anacamptis* versus *Orchis* (Orchidaceae): seed micromorphology and its taxonomic significance," *Plant Systematics and Evolution*, vol. 298, no. 3, pp. 597-607, 2012.