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Geometric morphometric approach to three species of tribe Phyllobiini (Curculionidae: Entiminae) from Turkey

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Abstract
Curculionidae is considered to be one of the species-rich families in Coleoptera. The taxonomic position of Phyllobini tribe in the family Curculionidae and the relationships, that is, phylogenetics of genera and the species pertaining to the genera in the tribe are unclear. However, no morphological study involving Phyllobiini has ever been conducted. We aimed to examine the diversity of pronotum and femur shape and interpret the taxonomic complexity of Phyllobiini using Geometric morphometrics. Three species of Phyllobiini (Curculionidae: Entiminae) used in this study are *Phyllobius glaucus*, *Parascythropus mirandus*, and *Oedecnemidius pictus*. Sexual size dimorphism was not significant in both femur and pronotum centroid size, on the other hand Manova results showed that there was sexual dimorphism in both femur and pronotum shape in Phyllobiini thus sexes were evaluated separately. Both canonical variate and principle component analysis were resulted in clear separation of femur and pronotum shape among species. Finally, based on the results obtained and the observed morphological traits, *O. pictus, P. mirandus* and *P. glaucus* can be considered a member of separate genus.

Keywords: Curculionidae; Phyllobiini; geometric morphometrics; shape variations

1. Introduction

Curculionidae is considered to be one of the species-rich families in Coleoptera. Recent articles suggesting that Phyllobini tribe, including our study samples, has been defined in the Entiminae subfamily of the family Curculionidae. The family Curculionidae has a great number of taxa and there is still no consensus on the taxonomic position of lots of categories in this family. Besides, both the taxonomic positions of Phyllobini tribe in the family Curculionidae and the relationships, that is, phylogenetics of genera and the species pertaining to the genera in the tribe are unclear [1]. The Phyllobiini Schoenherr, 1833 is a widespread beetle distributed in the Palearctic biogeographic region. Currently, it is recognized that 120 species belong to eight genera. It is known that thirty four species belong to eight genera occur in Anatolia [2, 3]. Members of weevil tribe Phyllobiini feed on a variety of plant species placed in the orders Urticales, Salicales, Betulales and Rosales [2]. Consequently, they can have a detrimental effect on agricultural crops and forest trees cause significant economic losses [2]. This group of curculionids has been investigated by Hoffmann [4]. Angelov [5]. Korotyaev and Egorov [6]. Dieckmann [7]. Pesarini [2]. Pişer [8]. As well as Yunakov & Korotyaev [9].

Geometric morphometrics [10, 11, 12]. is a very important procedure that helped scientists to test size and shape variation in the late 1980s and early 1990s. The main advantage of morphometrics data sets is containing two- or three-dimensional Cartesian coordinates of landmarks illustrates the form of morphological structure under study [13]. (Bookstein, 1996) [10 13 19]. Besides traditional techniques, geometric morphometrics has the ability to show shape changes like deviation of displacement vectors from the mean value or deformation grids in original sample space on each of the landmarks. Visualized shape variations can help to characterize populations within a species or sexes as biological features.

Beetles body (or a part of body head, pronotum, rostrum, femur and elytra) has been the subject of geometric morphometric analysis in the past [14, 15, 16]. Body morphometrics can help to characterize populations within species and sexes, as shown by the analysis of *Ceroglossus* (Carabidae) [14]. Body shape also studied using cryptic species to definitely determine *Nyctelia* (Coleoptera: Tenebrionidae) [15]. In literature there are also two remarkable review studies of Genus Oreoderus Burmeister (Coleoptera, Scarabidae) and Ablattaria Reitter (Coleoptera, Silphidae) [16]. Using diagnostic characters and geometric morphometrics however there is no study conducted on this species by using geometric morphometrics. Thus we aimed to interpret taxonomic complexity of Phyllobiini using Geometric morphometrics. The following questions were addressed: (1) is there morphological size and shape variation of pronotum and femur, in the species Phyllobiini; (2) is there any sexual dimorphism in Phyllobiini.

2. Material and methods

2.1 Collection, Image-capturing and Landmark digitizing

In this study, species of Phyllobiini (Curculionidae: Entiminae) were used; *Phyllobius glaucus*, *Parascythropus mirandus*, *Oedecnemidius pictus*. Specimens collected from the central Anatolia and deposited at the Ahi Evran University Zoology Museum Entomology (AUZM-Ent.). Total of 60 specimens were used in this study. A single image was taken by a camera attached to Olympus SZX12 for each specimen, pronotum and femur separately. In order to digitize and save 8 landmarks for the pronotum and 10 landmarks for the femur, TPS-Dig2 program proposed by Rohlf [11,12,17] was used (Fig. 1). Further analyses of landmark configurations were done by using MophoJ v1.03a [18].

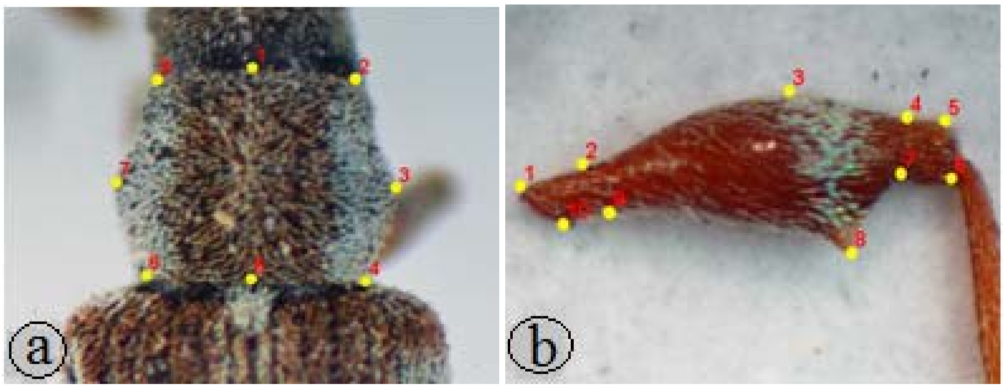


Fig 1: a- Pronotum; 1- middle point of anterior edge, 2- right point of anterior edge, 3- middle point of lateral edge (right), 4- right point of posterior edge, 5- middle point of posterior edge, 6- left point of posterior edge, 7- middle point of lateral edge (left), 8- left point of anterior edge. b- Femur; 1- basal point (dorsal), 2- beginning of extension (dorsal), back point (dorsal), 4- narrowed section (dorsal), 5- dorsal point of apex, 6- ventral point of apex, 7- cavity (ventral), 8- point of tooth, 9- beginning of extension (dorsal), 10- basal point (ventral).

2.2 Geometric morphometric analyses

Landmark-based morphometric methods were chosen as these are more effective in capturing information about the shape of an organism and lead to powerful statistical procedures for testing differences in shape. Moreover, these methods provide researchers with accurate tools for visualizing shape changes in a way that are both quantitatively correct and extremely suggestive [11] To compare overall pronotum and femur size among populations, the centroid size (the square root of the sum of the square distances between each landmark and the centroid) [19] was computed for each species and tested by analysis of variance (ANOVA) and visualized using a boxplot. Centroid size was also used to test for differences for sexual dimorphism through ANOVA.

Sexual dimorphism and the significance of shape differences among species were tested by means of two way (sex x species) analysis of variance for shape variables (MANOVA). We used two methods to describe the diversity of shapes: principle components analysis (PCA) and canonical variates analysis (CVA). PCA is a tool for simplifying description of variation among individuals, whereas CVA is used for simplifying descriptions of differences between groups [20]. CVA was also employed for testing shape differences between sexes and among species and for graphical illustrations of the MANOVA results. Permutation test applied for pairwise distances - 10000 iterations to obtain Mahalanobis distance and their statistical significance.

3. Results

3.1 Size Variation

For femur and pronotum, Shapiro-Wilk's test revealed a normal distribution of all species ($P>0.05$) and Levene's test showed that error variance of centroid size is equal across species (P =0,933, p=0,951 respectively).

A two-way ANOVA of mean centroid sizes showed a significant femura size variation among species ($F_{species}$=62.30, P =0.000). A significant sexual size dimorphism was absent among species (F_{sex}=0.25, P =0.618904) and the interaction between these two effects was negligible ($F_{sex*species}$=2.75, P =0.073165). The results of Tukey HSD as a post hoc test on centroid sizes are summarized in Table 1a, as pair-wise differences. Oedecnemidius pictus was significantly different and smaller than all other species (P <0.001), (Table 1a**)** (Fig. 2a). For femura size, Oedecnemidius pictus males, on average, larger than females whereas Oedecnemidius mirandus and Phyllobius glaucus females were larger than males (Fig. 2a) The result of homogeneous subsets of centroid sizes extracted from Tukey HSD identified two groups with significantly different centroid size: Oedecnemidius pictus (first group) has the smallest size, and Oedecnemidius mirandus and Phyllobius glaucus (second group) have the biggest.

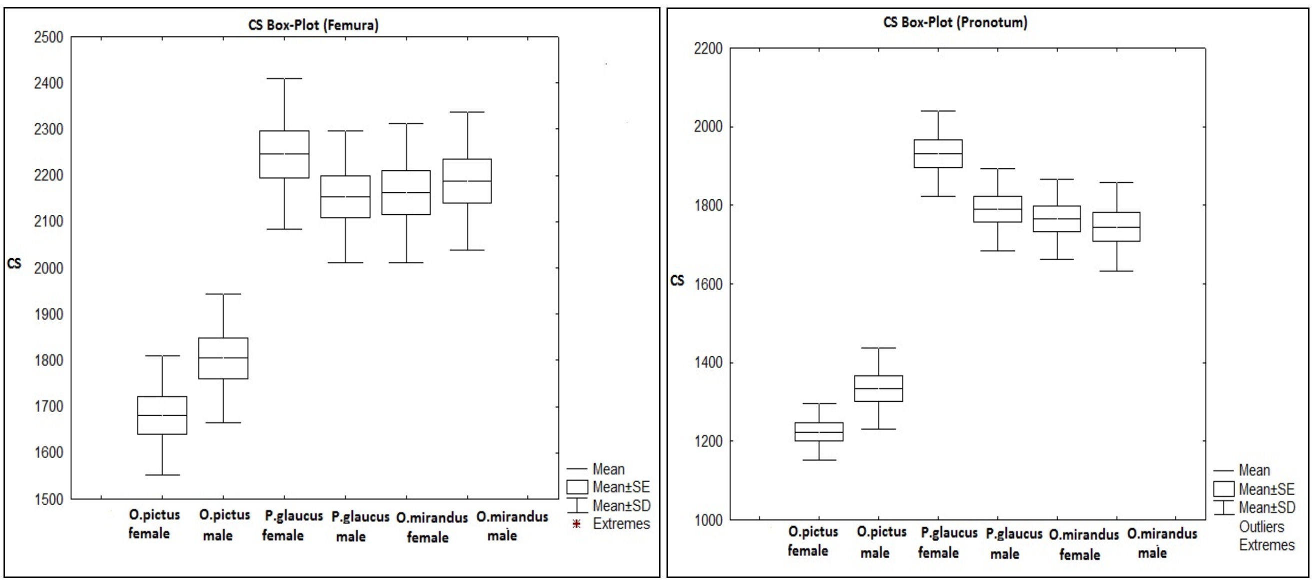
A two-way ANOVA of mean centroid sizes showed a significant pronotum size variation among species ($F_{species}$=187.43, P=0.000). A significant sexual size dimorphism was absent among species (F_{sex}=0.42, P=0.517674) and there was a significant interaction between sex and species of pronotum size ($F_{sex*species}$=7.80, P=0.001059). The results of Tukey HSD as a post hoc test on centroid sizes are summarized in Table 1b, as pair-wise differences. Both male and female Oedecnemidius pictus were significantly different and smaller than all other species (p<0.001). Phyllobius glaucus of females were significantly different and bigger than all other species (Table 1b), (Fig. 2b). For pronotum size, Phyllobius glaucus females, on average, larger than males whereas Oedecnemidius pictus and Oedecnemidius mirandus males were larger than females (Fig. 2b). The result of homogeneous subsets of centroid sizes extracted from Tukey HSD identified three groups with significantly different centroid size. Oedecnemidius pictus (first group) has the smallest size, Oedecnemidius mirandus (second group) has medium size and Phyllobius glaucus (third group) has the biggest pronotum centroid size.

Table 1a: Results of Tukey HSD (post-hoc) test on femura centroid size, significant values bolded * P <0.01

	***O. pictus* female**	***O. pictus* male**	***P. glaucus* female**	***P. glaucus* male**	***O. mirandus* female**	***O. mirandus* male**
O. pictus female						
O. pictus male	0.4179					
P. glaucus female	0.0001*	0.0001*				
P. glaucus male	0.0001*	0.0002*	0.7168			
O. mirandus female	0.0001*	0.0001*	0.7911	1.0000		
O. mirandus male	0.0001*	0.0001*	0.9460	0.9952	0.9988	

Table 1b: Results of Tukey HSD (post-hoc) test on pronotum centroid size, significant values bolded * *P* <0.01

	***O. pictus* female**	***O. pictus* male**	***P. glaucus* female**	***P. glaucus* male**	***O. mirandus* female**	***O. mirandus* male**
O. pictus female						
O. pictus male	0.1601					
P. glaucus female	0.0001*	0.0001*				
P. glaucus male	0.0001*	0.0001*	0.0311*			
O. mirandus female	0.0001*	0.0001*	0.0068*	0.9942		
O. mirandus male	0.0001*	0.0001*	0.0019*	0.9950	0.9981	



(a) (b)

Figs 2a, b: Box-plot showing the average of centroid size of femur (a) and pronotum (b) each species. The inner line represents the median. Box margins are at 25th and 75th percentiles bars extend to 5th and 95th percentiles, circles represent outliers.

Shape Variation

Univariate normality of shape variables was tested by Shapiro-Wilk's test which revealed a normal distribution of all species (*P*>0.05) of femur and pronotum and MANOVA design was balanced so that there was an equal number of observations in each cell, the robustness of the MANOVA tests was guaranteed for homogeneity of covariance matrix. Levene's test showed that error variance of shape variables was equal across species.

The factorial MANOVA of femur and pronotum shape found significant differences between species and sexes, and the interaction between these two effects was significant (Table. 2a and 2b). In a comparison of Mahalanobis distances between the sexes of all species were sexually dimorphic for femur and pronotum shape (Table 3a, b), except *Oedecnemidius pictus* for pronotum shape.

Table 2a: MANOVA of sex x species for the femur shape

Effect	γWilks	F	df	p
species	0.009117	23.09076	32	0.000000
sex	0.494294	2.49377	16	0.010112
species*sex	0.289343	2.09396	32	0.004329

Table 2b: MANOVA of sex x species for the pronotum shape

Effect	γWilks	F	df	p
species	0.038397	14.70346	24	0.000000
sex	0.472967	3.99296	12	0.000373
species*sex	0.379151	2.23611	24	0.003642

Table 3a: Hotelling's pair-wise comparisons (p values) and Mahalanobis distances for all species on upper and lower diagonal respectively 1 * <0.05; 1 ** <0.01(femur)

	O. pictus female	*O. pictus* male	*P. glaucus* female	*P. glaucus* male	*P. mirandus* female	*P. mirandus* male
O. pictus female		0.00030**	<.0001**	<.0001**	<.0001**	<.0001**
O. pictus male	4.26650		<.0001**	<.0001**	<.0001**	<.0001**
P. glaucus female	8.97660	9.45640		0.01290*	<.0001**	<.0001**
P. glaucus male	7.39160	7.86980	2.40000		<.0001**	<.0001**
P. mirandus female	7.45400	8.91800	6.59660	5.88450		0.006**
P. mirandus male	6.87910	8.57080	6.25680	5.35950	2.6553	

Table 3b: Hotelling's pair-wise comparisons (p values) and Mahalanobis distances for all species on upper and lower diagonal respectively 1 * <0.05; 1 ** <0.01(pronotum).

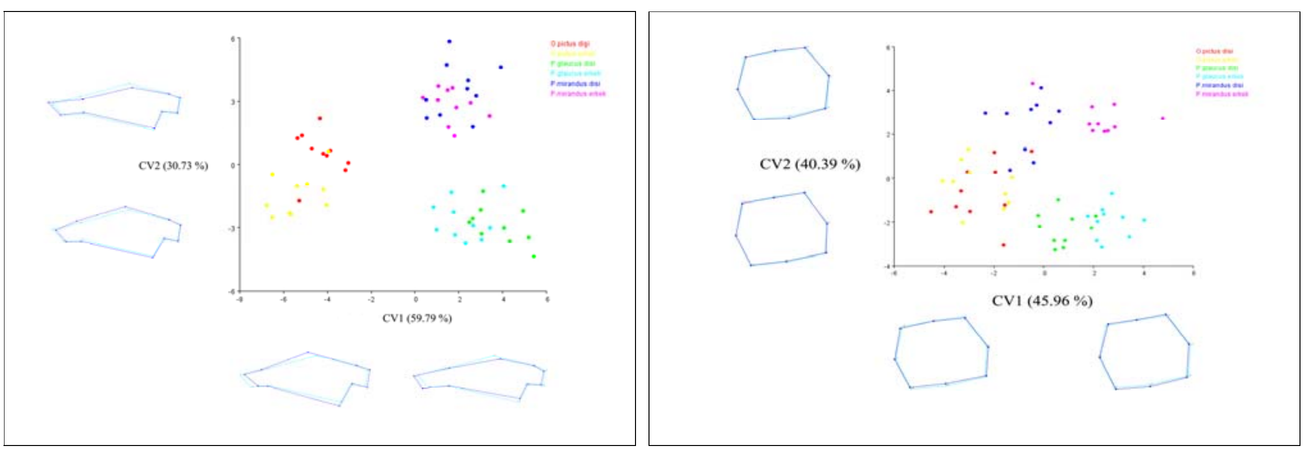
	O. pictus female	*O. pictus* male	*P. glaucus* female	*P. glaucus* male	*P. mirandus* female	*P. mirandus* male
O. pictus female		0.5886	<.0001**	<.0001**	0.0002**	<.0001**
O. pictus male	1.5877		<.0001**	<.0001**	<.0001**	<.0001**
P. glaucus female	4.3039	4.4409		0.0001**	<.0001**	<.0001**
P. glaucus male	5.5312	5.5933	3.1776		<.0001**	<.0001**
P. mirandus female	4.2649	3.7639	5.1577	5.8105		0.006**
P. mirandus male	5.8917	5.9008	5.5946	4.9784	3.6694	

Because of sexual dimorphism in shape was significant, following analysis were performed with subgroups (including both species and sexes). For femura, PCA of all subgroups explained 76.22% of shape variation within samples by the two first PC axes extracted from the variance-covariance matrix (PC1 explains 65.47% and PC2 explains 10.75%). At least five axes were required to cover more than 90% of the femur shape variation. PC1 scores showed that *O. pictus* was characterized by positive values whereas *P. mirandus* was characterized by negative values. By contrast *P. glaucus* showed intermediate position.

PCA analysis of all specimens of pronotum explained 55.28% of shape variation within samples by the two first PCA axes extracted from the variance-covariance matrix (PC1 explains 43.72% and PC2, 11.56%). At least eight axes were required to cover more than 90% of the shape variation.

For femur and pronotum, differences between subgroups were well illustrated by CVA plot (Figs. 3a, b). For femur, first two CV axes explaining the 90.521% of total shape variation (CV1 explains 59.793% and CV2, 30.728%). Three major clusters were obtained: *O. pictus*, *P. glaucus* and *P. mirandus* were clearly separated from each other. A completely good discrimination of *O. pictus* from *P. glaucus* and *P. mirandus* is provided by the first Canonical axis. CV1 displays that *O. pictus* was in negative side of CV1 whereas *P. glaucus* and *P. mirandus* were in positive side of CV1. Moreover CV1 may help to distinguish the sexes of *P. mirandus*. *O. pictus*, had relatively low values on CV1, which was clearly separated from others with stout short femur shape (revealed with landmarks 3, 8, 1, 10). CV2, clearly separated *P. mirandus* and *P. glaucus*, which contributed to distinguish female and male of *O. pictus*. *O. pictus* males were in negative side of CV2 whereas *O. pictus* females were in positive side of CV2. Females of *O. pictus* had higher values than males on the second canonical axis which showed that femurs of females getting narrow elongated femura shape (revealed with landmarks 2, 3, 8, 1, 10). On the other hand, *P. glaucus* was clearly separated from *P. mirandus* with stout short femur shape (revealed with landmarks 2, 3, 8, 1, 10).

For pronotum, first two CV axes explaining the 86.35% of total shape variation (CV1 explains 45.96% and CV2, 40.39%). Three major clusters were obtained: *O. pictus*, *P. glaucus* and *P. mirandus* were clearly separated from each other. The first Canonical axis provided a good discrimination of sexes in *P. glaucus* and *P. mirandus* but sexes of *O. pictus* partially overlapped each other. On the other hand, CV2 clearly separated *P. mirandus* and *P. glaucus.*



(a) (b)

Figs 3a, b: Canonical variate analyses (CVA) of femur (a) and pronotum (b) configurations. Colors represent different both sex and species: *P.mirandus* female (blue), *P.mirandus* male (pink) *O.pictus* female (red), *O.pictus* male (yellow) *P.glaucus* female (green) *P.glaucus* male (turquoise). Shape differences along the CV1 and CV2. The dark lines show the extreme shape change in positive and negative direction of the PC shown above. The gray lines are the mean shape of femur and pronotum respectively. The scale for each figure is; femur Cv1 (-6,.8), CV2 (-4, 6) pronotum PC1 (+0.08, -0.06).

4. Discussions

Geometric Morphometrics was applied to this genus for the first time. This study revealed that Geometric Morphometrics is a promising tool to figure out sexual dimorphism and taxonomic complexity of Phyllobiini. Numerous placements of some species in this tribe have been made based mostly on morphological traits. For instance, Hoffmann [4] subdivided the species of *Phyllobius* into seven subgenera. Subsequently, Dieckmann [7] separated *Phyllobius* into eight subgenera. Pesarini [2] and Lodos *et al*. [3] elevated all Dieckmann's subgenera to genera. At the species level, while Dieckmann [7] and Lodos *et al*. [3] placed *Phyllobius pictus* in *Phyllobius*, Pesarini [2] placed *Ph. pictus* in a different genus, as *Oedecnemidius pictus*. Also, while Pesarini [2] removed *Parascythropus mirandus* from *Phyllobius*, Lodos *et al*. [3] proposed that *Par. mirandus* belongs to the genus *Phyllobius*. Because these studies proposed different placements, we decided to combine morphological characters and Geometric Morphometrics to resolve the relationships.

Femur and pronotum are morphologically important characters and generally use in diagnositic keys. According to results, femur seems to be more decisive than pronotum as a taxonomic character. Because, femur using in copulation and generally improve in Phyllobiini members.

For both femur and pronotum centroid size, females are larger than males except *O. pictus* using Box-Plot (Figs. 2a, b) but sexual size dimorphism is significant only in *Phyllobius glaucus* pronotum in ANOVA results (Table 1a, 1b). The shape space of femur and pronotum showed clearly separation of both sexes and species. Although differences among species and between sexes are significant for femur and pronotum shape variables, sexual dimorphism is negligible for pronotum of *Oedecnemidius pictus* (Table 2a, b).

The shape of external traits (femur and pronotum) were clearly different among species in CVA. Moreover sexual dimorphism also may be present in femur and pronotum shape of Phyllobiini but it was difficult to detect because of small sample size. Considering to CVA, *O. pictus* showed that different direction of shape variation: with short stout femur shape. This suggests *P. mirandus* and *P. glaucus* seems to be more related than *O. pictus*. Erbey *et al.* [1] researched to the species of phyllobiini tribe according to morphological characters and 18S rRNA sequence analysis. They expressed *O. pictus* different from *P. glaucus* and *P. mirandus*.

Finally based on the results obtained and the observed morphological traits, *O. pictus, P. mirandus* and *P. glaucus* can be considered as member of separate genus. By applying larger sample size, and different ecological and genetic data sets into the shape analysis, we can reveal the evolutionary processes of Phyllobiini.

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