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COMPARISON OF DIFFERENT MORPHOMETRIC METHODS ON THREE TAXA OF THE GENUS CERATOCEPHALA (RANUNCULACEAE) IN IRAN

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The genus *Ceratocephala* with about 14 species in the world, has two species in Iran, including *C. falcata* and *C. testiculata* which are widely distributed in different parts of the country. *Ceratocephala falcata* has variations in fruit shapes. Some botanists have considered several subspecies and varieties for this species. In this research, traditional morphometric and geometric morphometric methods have been used to determine the taxonomic position of these taxa. Traditional morphometric studies based on nineteen quantitative characters (including leaf and fruit traits) of 78 populations were performed using SPSS software with the Ward method. Geometric morphometry based on fruit traits was performed in 22 specimens using tps Dig 2.12 and morpho J software. Traditional morphometry was not successful in distinguishing varieties, but different varieties including *C. falcata* var. *falcata* and var. *incurva* were identified using geometric morphometric methods.

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Keywords: Iran; flora; Ranunculaceae; morphometry; *geometric morphometry*

مقایسه روش‌های مورفومتری مختلف بر روی سه آرایه از جنس Ceratocephala، (Ranunculaceae) در ایران

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جنس Ceratocephala با حدود ۱۴ گونه در جهان، دارای دو گونه C. falcata و C. testiculata در ایران است که در نقاط مختلف کشور پراکنش گسترده‌ای دارد. C. falcata دارای اشکال مختلف میوه است. برخی گیاه‌شناسان چندین زیرگونه و واریته برای این گونه در نظر گرفته‌اند. در این تحقیق از روش‌های مورفومتری هندسی و سنتی برای تعیین موقعیت طبقه‌بندی این آرایه‌ها استفاده شده است. مطالعات مورفومتری سنتی بر اساس ۱۹ صفت کمی شامل صفات برگ و میوه در ۷۸ جمعیت با استفاده از نرم افزار SPSS با روش وارد انجام شد. مورفومتری هندسی بر اساس صفات میوه در ۲۲ نمونه با استفاده از نرم افزار tps Dig 2.12 و morphoJ انجام گرفت. مورفومتری سنتی در تمایز واریته‌های شناخته شده موفق نبود، اما واریته‌های var. falcata و var. incurva با استفاده از روش‌های مورفومتری هندسی مجزا شدند.

INTRODUCTION

The genus *Ceratocephala* Moench. belongs to the family Ranunculaceae. It is closely related to the genus *Ranunculus* L. Phylogenetic analyses based on nuclear and plastid markers have been studied by Emadzadeh & al. (2010) and provided new information about relationships and character evolution within the tribe *Ranunculeae*. Their results showed that in the maximum parsimony, tree topology based on the combined data set, *Ceratocephala* and *Myosurus* L. produced a clade that was sister to the *Ranunculus* clade with high bootstrap support and based on Neighbor Net

analysis, *Ceratocephala*, and *Myosurus* were not only separated from the core *Ranunculus* clade, but they were also highly diverged from each other.

The special morphological characteristics of *Ceratocephala* (achenes have inflated empty chambers on both side, and an elongated beak) support its segregation from *Ranunculus*. Also, the karyotype of *Ceratocephala* with the basic chromosome number x=7 is different from *Ranunculus* species often with x=8 basic chromosome number (Baltisberger & Hörandl 2016).

Cerathocephala has 4-12 species in the world which are distributed from the South and center of Europe to North West Africa, South West Asia, USA, and Canada (Tuma & Weedon 1989). Also, one species of *Ceratocephala* is reported from New Zealand (Garnock-Joens 1984). *Cerathocephala* has two representatives in Iran: *Ceratocephala falcata* and *C. testiculata* (Crantz) Besser. Their flowering occurs in the spring (Tutin, 1964; Iranshahr & al. 1992). Both species are annual with similar leaves, flowers, and achenes, but they differ in the size of their flowers, achene, and achene beak (Fig. 1). *Ceratocephala testiculata* has achenes with a narrow straight beak, but *C. falcata* has achenes with a broad falcate beak (Tutin 1964). The distribution of these species in Europe is disputed. Pollen grains, fruit, indumentum and anatomical traits of the stem and leaf of Iranian species have been studied by Keshavarzi & al. (2017).

Cerathocephala testiculata has several synonyms: *C. reflexa* Steven, *C. orthoceras* DC., *Ranunculus testiculatus* Crantz. (Tutin & al. 1964). Its original distribution was reported in South-Eastern Europe and Asia Minor. There are many variations in the length of the stem, flower size, beak size, and shape in various populations of *C. falcata*. Bossier (1861) introduced four varieties based on the variation of these characters. Plants without stems have been named var. *excapus* Boiss., (recorded from Syria and Palestine) those with small achene, short and broad beaks named var. *minus* Boiss., (recorded from Afghanistan and Palestine), those with achene terminating in a falcate beak from the basal part named var. *falcata* and those terminating in a beak that is falcate at the top named var. *incurva* (Steven) Chrtek & Chrtková (recorded from Gilan province of Iran, Crimean Peninsula, and Armenia). After Boissier, no research has been carried out to distinguish these varieties. These are considered as intraspecific variations. Two species of *Ceratocephala* are reported from Iran (Iranshahr & al. 1992), one of them comprises two varieties.

Distinguishing different varieties, undoubtedly, is essential in taxonomy and can be used for further applications such as conservation projects. A new plant variety cannot be protected unless it has been taxonomically described and is distinguishable from other varieties.

Fruit and seed characteristics provide useful phylogenetic information and are therefore often used to distinguish taxa at different taxonomic levels. Several studies on fruits and seeds have proven the taxonomic value of these organs in many members of the Ranunculaceae family (Chaudhary & Trifonova 1988; Maciejewska-Rutkowska & Antkowska 2013; Jung & Heo 2017; Pakravan & al. 2021).

In 1990s morphometric analysis was developed so that shape variation studies became possible (Adamd & al. 2004). Geometric morphometrics is a powerful method to visualize morphological changes and differences (Webster & Sheets 2010). Using both traditional morphometrics and geometric morphometrics could give us new explanation of biological designs. Geometric morphometrics provide analytical and graphical analysis for phenotypic differences so the interpretation would be easier.

Since different species of *Ceratocephala* are used as medicinal plants, it is necessary to identify the varieties in order to determine the taxa with more useful chemical compounds (Khalmatov 1964; Zaurov & al. 2013).

This study aims to discriminate three taxa: *Ceratocephala falcate* var. *falcata*, *C. falcata* var. *incurva*, and *C. testiculata* utilizing geometric morphometric analysis based on fruit characteristics and traditional morphology methods based on morphological characters.

MATERIALS AND METHODS

Morphological analysis

The study was conducted in two parts. Part one was a geometric morphometric study of achene in *C. falcata* var. *incurva*, var. *falcata* and *C. testiculata*. Many specimens of *Ceratocephala* from different parts of Iran such as: Alborz, Ardabil, Azarbayejan, Esfahan, Fars, Golestanan, Hamedan, Hormozgan, Kermanshah, Khorassan, Kurdestan, Lorestan, Markazi, Mazandaran, Qazvin, Qom, Tehran, Semnan, Sistan & Baloochstan, Yazd and Zanjan, provinces were analyzed (Table 1). In the second part 19 morphological variables were measured and then analyzed in order to find out the relationship between these three taxa.

Number of specimens studied from each population varied from 3 to 7. Wherever possible, the same individuals were used to obtain both traditional and geometric morphometric data. Thus 256 specimens were studied in total and 61 fruit were studied for geometric morphometric analysis.

Voucher specimens for each species in the analyses were deposited at the Alzahra University Herbarium (ALUH), herbarium of Research Institute of Forests and Rangelands of Iran (TARI), and Kharazmi University herbarium (T), (Table 1) (acronyms according to Thiers, 2019).

Geometric morphometric analysis

Images of achene (dorsal side) were captured using a digital Dino capture. Photographs were taken from 22 specimens. Since ripen achenes were needed for this study, some populations were left out. The digitization was performed using tpsDig2.12 software (Rohlf 2008). Best quality achenes and their images were selected for further analysis which consisted of 20 images for three taxa from 22 specimens.

Images were used to record 10 semi landmarks on each achene due to the shape of the achene (Fig. 2). Semi landmarks were chosen to putatively represent homologous points (Jensen 1990; Jensen & al. 1993), and to embed diagnostic features of each species following various authors (Jensen 1990; Bruschi & al. 2000; Borazan & Babac 2003; Gonzalez-Rodriguez & al. 2004). Semilandmark coordinates were used in Morpho J software (Klingenberg 2011). Semi landmark configurations were aligned using partial generalized Procrustes analysis (GPA) (Rohlf & Slice 1990; Dryden & Mardia 1998). Matrix of covariance was used as datasets for subsequent multivariate statistical analyses such as principle component analysis and canonical variate analysis. Canonical variate analysis (CVA) is a statistical method employed for three or more groups (Klingenberg 2011). CVA was performed with permutation tests using Mahalanob is distances (10,000 permutations per test). Result of CVA is presented in a scatter plot for significant canonical variate scores.

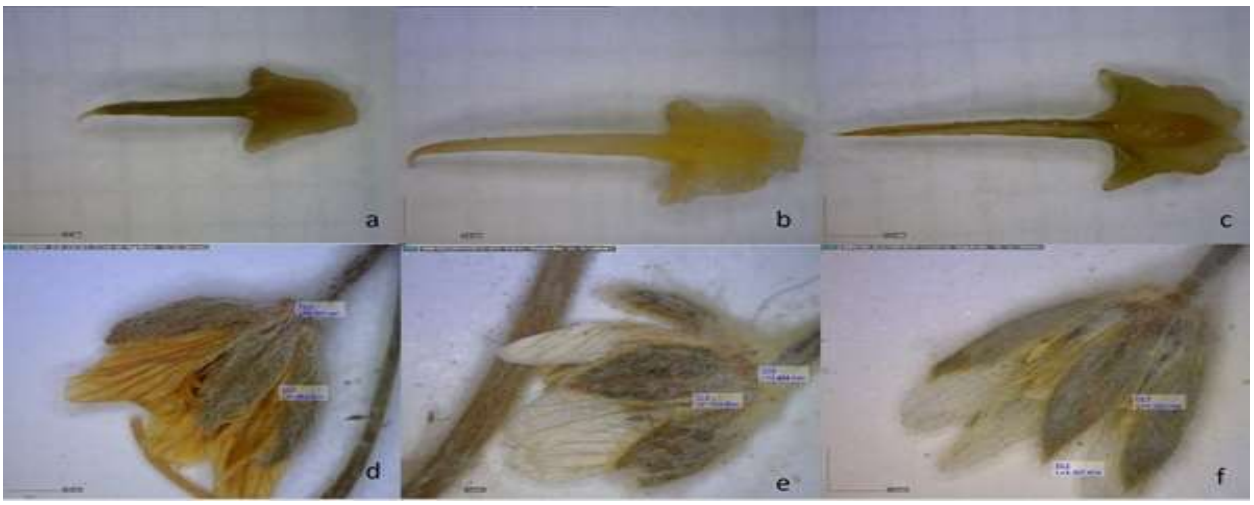

Fig. 1. Fruits and flowers of *Ceratocephala* species and varieties. a & d, *C. falcata* var. *incurva; b & e, C. falcata* var. *falcata;* c & f, *C. testiculata.*

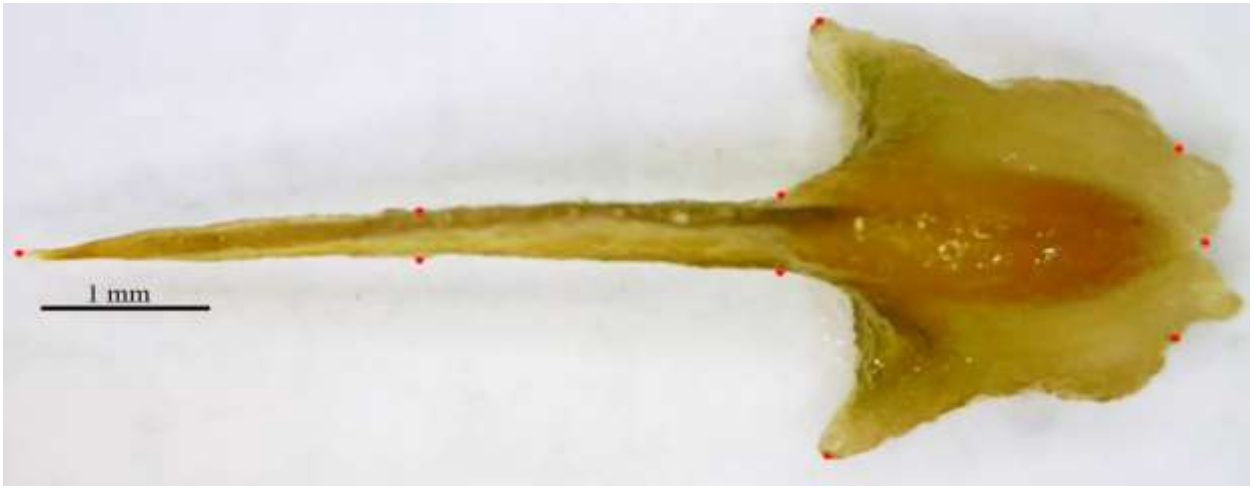

Fig. 2. Position of the 10 achene semilandmarks.

Table 1. Details of voucher specimens examined in geometry morphometric study. Abbreviation: ALUH: Alzahra University Herbarium, TARI: Herbarium of Research Institute of Forests and Rangelands of Iran, T: Kharazmi University Herbarium.

Species	Location	Herburium Number	Herbarium acronym	Traditional morphometry	Geometric morphometry
Ceratocephala falcata (L.) Pers. var. *incurva*	Lorestan: Zorujerd	11781	FAR	+	+
	Khorassan: Neishabur	26177	FAR	+	+
	Hamedan: Tuyserkan	11983	FAR	+	+
	Golestanan	55386	TARI	+	+
	Esfahan: Daran Chahis	340	TARI	+	+
C. falcata var. *falcata*	Tehran: Rudehen	37777	ALUH	+	+
	Lorestan: Borujerd	34443	FAR	+	+
	Kurdestan: Sanandaj	46213	FAR	+	+
	Ghazvin	11352	FAR	+	
	Kermanshah	17303	FAR	+	+
	Esfahan- Borujen to Lordegan	2666	TARI	+	+
	Esfahan: Meymeh	72920	TARI	+	+
C .testiculata (Crantz) Besser	Khorassan: Kashmar	12520	FAR	+	+
	Tehran: Abali	11987	FAR	+	+
	Mazandaran: Siahbisheh	11364	FAR	+	+
	Tehran: Firuzkuh road	11338	FAR	+	+
	Khorassan: Gonabad	46198	FAR	+	+
	Tehran: Darband	11339	FAR	+	+
	Tehran: Darakeh	11962	FAR	+	+
	Tehran: Damavand	35095	FAR	+	+
	Lorestan	32390	FAR	+	+
	Hormozgan: Bandarabbas	52458	TARI	+	+

Morphological study

Nineteen quantitative characters (including leaf and fruit traits) of 80 plant samples from different parts of Iran were measured and used for the morphological studies (Table 2). *Ceratocephala falcata* var. *falcata* and *C. testiculata* have very wide distribution area but var. *incurva* has a narrower distribution. Our specimen was collected from a location close to the locus classicus. To realize a model for dataset, all taxa were subjected to multivariate analysis of canonical discriminant analysis (CVA). Also, the collected data were analyzed by multivariate analysis of variance using cluster analysis (CA) to study relationships among the taxa by using SPSS statistics software (SPSS 25). Then Cluster dendrogram and scatter plot were created in order to visualize the relationships of studied groups. In both parts of the study, morphological and geometric morphometric analyses were conducted on the specimens and the results were interpreted separately.

Table 2. Morphological characters measured in the current study.

Leaf blade length	Peduncle length
Petiole length	Fruit head length
Leaf width	Leaf blade length /Leaf width
Calyx length	Leaf blade length /Petiole length
Calyx width	Calyx length /Calyx width
Petal length	Petal length /Petal width
Petal width	Beak length /Achene length
Beak length	Achene length /Achene width
Achene length	Petal length /Calyx length
Achene width	Peduncle length

Table 3. The list of quantitative morphological characters ranked by CVA analysis.

Component Matrix[a]							
	Component						
	1	2	3	4	5	6	7
petiole length	0.585	-0.077	0.002	-0.610	0.387	0.105	0.211
leaf width	0.827	0.046	-0.017	-0.086	-0.137	0.037	-0.148
peduncle length	0.583	-0.077	-0.137	0.065	0.522	-0.112	0.218
calyx length	0.559	-0.338	0.519	0.342	0.139	-0.303	0.025
calyx width	-0.091	-0.221	0.746	0.051	0.317	0.027	-0.476
petal length	-0.307	-0.193	0.576	0.137	0.379	0.109	0.375
petal width	-0.059	-0.124	0.712	-0.263	-0.483	0.030	0.346
beak length	0.162	0.957	0.008	-0.078	0.042	0.008	0.054
achene length/achene width	0.484	0.217	-0.093	0.223	-0.114	0.745	0.048
beak length/achene length	-0.043	0.684	0.120	0.370	-0.069	-0.246	0.146
petal length/petal width	-0.107	0.027	-0.437	0.351	0.720	0.032	-0.213
petal length/calyx length	-0.771	0.178	-0.042	-0.207	0.163	0.312	0.239
calyx length/calyx width	0.663	-0.146	-0.175	0.340	-0.111	-0.321	0.449
leaf blade length/petiole length	-0.141	0.425	0.310	0.658	-0.207	0.001	-0.217

RESULTS

Traditional morphology

CVA analysis determined most important quantitative characters (Table 3). Among them leaf width, petiole length, peduncle length, calyx length, showed the highest correlation in the first function. Beak length, ratio of beak length/achene and leaf blade/petiole length showed the largest absolute correlation with second discriminant function. In the third function, calyx width, petal width and petal length showed the highest absolute correlation.

ANOVA analysis (Table 4 & 5) revealed that five morphometric characters are different among these taxa (sig.<0.05) which are as follows: Achene width, achene length, calyx length/calyx width, fruit head length and beak length. In order to verify if these quantitative morphological characters are suitable for varieties discrimination or not the principle component analysis was used. Two components comprise 35.78% of total variance. Results showed that these characters cannot separate these varieties from each other properly (Fig.3).

According to canonical analysis (Fig. 4) function 1 with 56.6% variance could separate *C. falcata* var. *falcata* from the other two taxa (*C. falcata* var. *incurva* and *C. testiculata*). Based on function 2 with 43.4% of variance the two variates of *C. falcata* group together but this function is not significant.

Cluster analysis based on similarity and dissimilarity of traditional morphometric revealed that *C. testiculata* is more similar to *C. falcata* var. *incurva* rather than to the *C. falcata* var. *falcata* (Figs. 5 & 6).

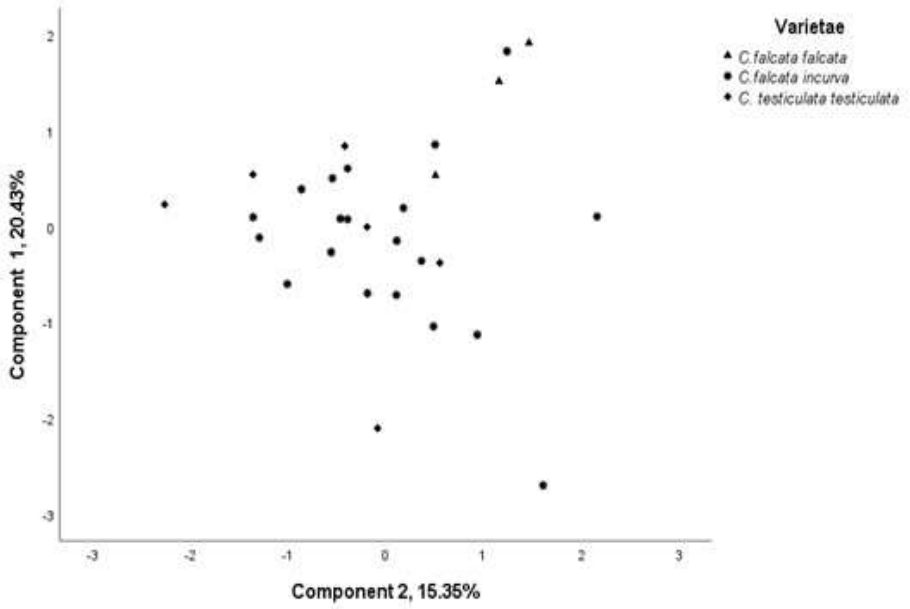

Fig. 3. Principle component analysis of quantitative characters of *Ceratocephala*.

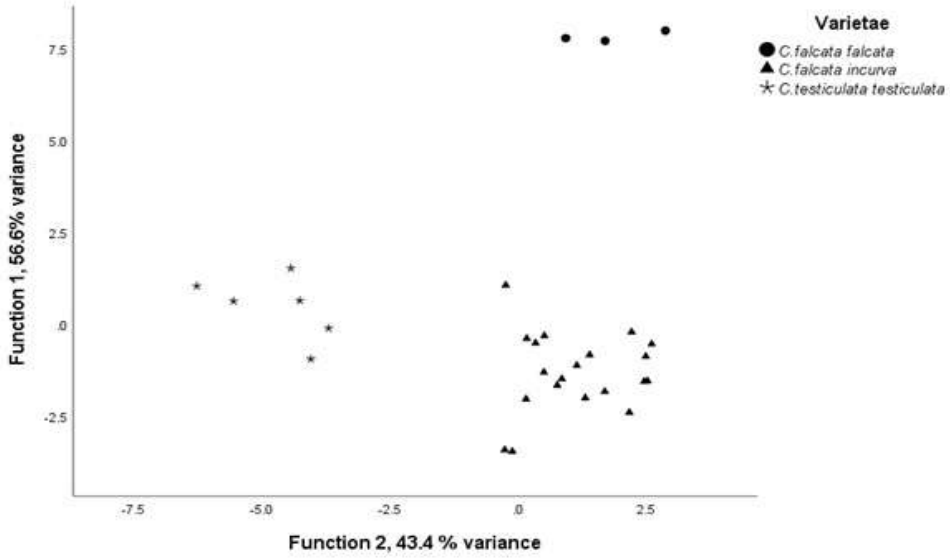

Fig. 4. Canonical variate analysis of quantitative characters of *Ceratocephala*.

Table 4. Principal component scores with corresponding variation (for morphological characters).

Component	Initial Eigenvalues		
	Total	% of Variance	Cumulative %
1	4.122	21.694	21.694
2	3.218	16.939	38.632
3	2.439	12.835	51.468

Table 5. Data for ANOVA analysis based on quantitative characters. df: degree of freedom; F: Fisher's correlation coefficient; Sig: significance.

ANOVA						
		Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**
achene length/achene width	Between Groups	1.973	4	0.493	0.175	0.950
	Within Groups	239.017	85	2.812		
	Total	240.990	89			
beak length/achene length	Between Groups	0.010	4	0.002	0.656	0.625
	Within Groups	0.315	85	0.004		
	Total	0.324	89			
petal length/petal width	Between Groups	4.353	4	1.088	1.647	0.180
	Within Groups	28.416	43	0.661		
	Total	32.769	47			
petal length/calyx length	Between Groups	1.025	4	0.256	3.366	0.018
	Within Groups	3.121	41	0.076		
	Total	4.146	45			
calyx length/calyx width	Between Groups	6.074	4	1.519	7.509	0.000
	Within Groups	11.528	57	0.202		
	Total	17.602	61			
calyx length	Between Groups	5.063	4	1.266	3.189	0.020
	Within Groups	22.621	57	0.397		
	Total	27.684	61			
calyx width	Between Groups	0.397	4	0.099	1.289	0.285
	Within Groups	4.392	57	0.077		
	Total	4.790	61			
petal length	Between Groups	1.224	4	0.306	0.387	0.817
	Within Groups	34.048	43	0.792		
	Total	35.272	47			
petal width	Between Groups	1.794	4	0.449	1.479	0.225
	Within Groups	13.043	43	0.303		
	Total	14.837	47			
beak length	Between Groups	15.602	4	3.900	11.079	0.000
	Within Groups	29.924	85	0.352		
	Total	45.526	89			
peduncle length	Between Groups	31536.287	4	7884.072	20.318	0.000
	Within Groups	38414.948	99	388.030		
	Total	69951.235	103			
petiole length	Between Groups	1508.537	4	377.134	6.405	0.000
	Within Groups	5476.333	93	58.885		
	Total	6984.871	97			
leaf width	Between Groups	136.042	4	34.011	2.242	0.070
	Within Groups	1441.291	95	15.171		
	Total	1577.333	99			
leaf blade length/petiole length	Between Groups	0.367	4	0.092	3.528	0.010
	Within Groups	2.418	93	0.026		
	Total	2.785	97			

Geometric morphometric

The first and second PCs accounted for 66.71%of the total achene shape variation (44.81 and 21.93%, respectively). In the scatter plot, three varieties formed three groups which two of them have some overlaps. PC1 and PC2 significantly discriminate *C. testiculata* form the other two varieties (Fig.7). The scatter plot of CVA analysis showed that these taxa formed distinct groups. CV1 and CV2 comprised 96.71% of total variance. CV1 with 74.95% of variance separate these three varieties. Transformation grid along the CV1 with scores of -0.15 to 0.15 also showed the differences between shapes of achene in these three taxa. The analysis indicated that these three varieties could significantly separate from each other (Fig. 8).

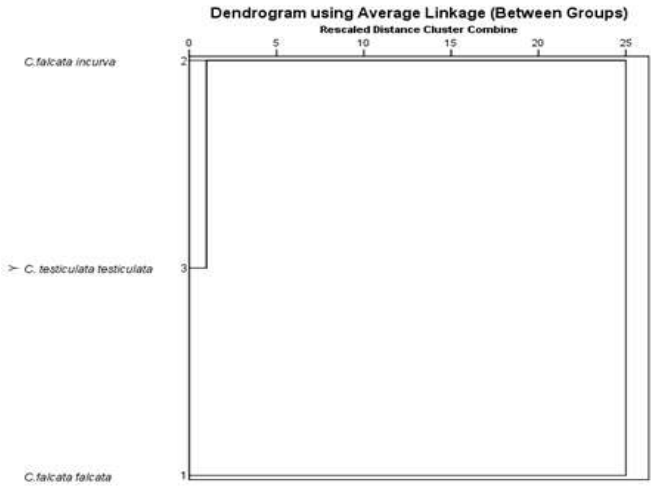

Fig. 5. Cluster analysis of quantitative characters of *Ceratocephala*.

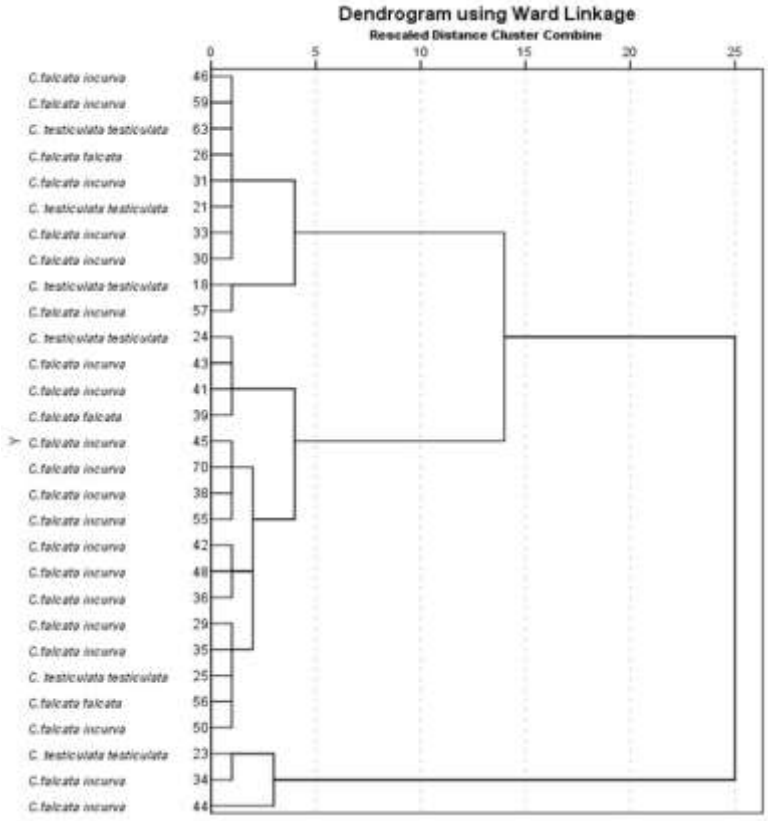

Fig. 6. Cluster analysis of quantitative characters of *Ceratocephala*.

DISCUSSION

According to Egambergieva & al. (2021), *C. testiculata* has antimicrobial and antifungal properties. If all the taxa closely related to this species are clearly identified and examined for effective compounds, it is possible to domesticate and use taxa with more effective substances.

In traditional morphometric studies, 80 samples were examined. The results of Principal Component Analysis based on vegetative and reproductive traits

could not distinguish populations and varieties from each other. Using canonical analysis based on geometric morphometric of fruit size and shape, the three taxa were distinctly separated, and in this part of study 22 samples were used that have ripe achene. *Ceratocephala falcata* can be distinguished by its curved beak fruits from *C. testiculata* with straight beak. However, populations of *C. falcata* var. *falcata* and var. *incurva* cannot be identified using morphological traits. Both methods used in this study reveal *C. testiculata* separation from other taxa. The former study by Keshavarzi & al. (2017) has proved this separation using the pollen grain and fruit surface and micromorphological and anatomical traits of the stem.

The results revealed that geometric analyses were able to differentiate these varieties from each other more successfully. Therefore, these taxa could significantly separate from each other. Our results are consistent with the reports by Chen & al (2018) studies on perianth of the Delphinieae flower which showed the diagnostic value of this organ. Moreover, results of Chen & al. (2018) in a morphometry geometry study on leaf of *Asteropyrum* species confirmed the phylogenetic outcomes.

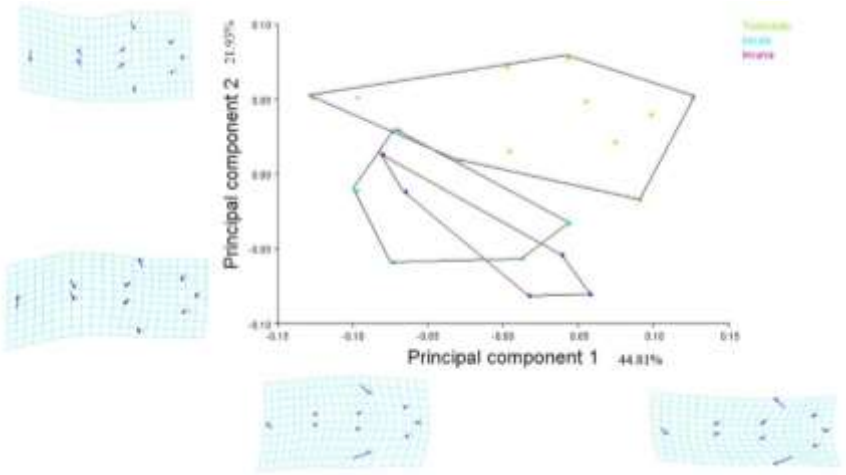

Fig. 7. Principle component analysis of achene of *Ceratocephala*. Transformation grids represent achene shapes reflecting the negative and positive extremes.

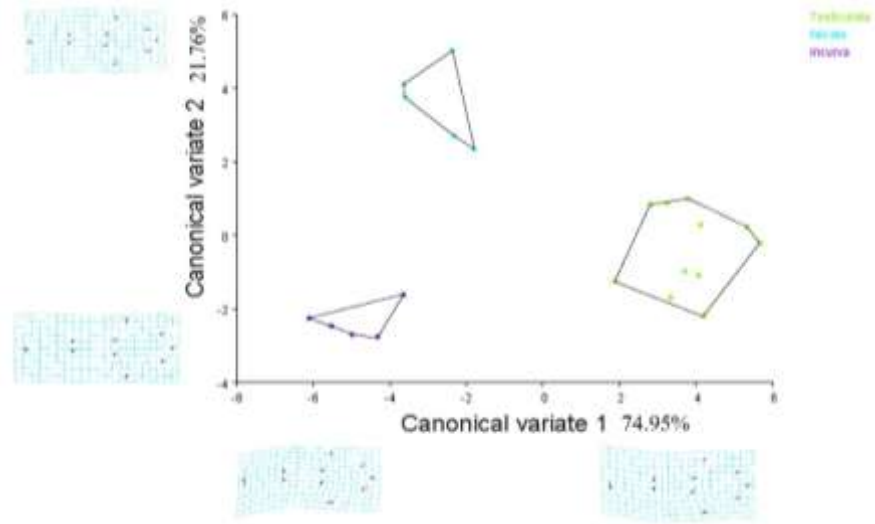

Fig. 8. Canonical variate analysis of achene of *Ceratocephala*. Transformation grids represent achene shapes reflecting the negative and positive extremes.

In an integrated approach, these morphometric methods may be accompanied with other methods such as micromorphology, anatomy or molecular data, to catch the better understanding of phylogenetic relationship of these taxa.

Declaration of competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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