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Geometric morphometric analysis in nine species of genus *Hottentotta* (Birula 1908) (Arachnida: Scorpiones) from Iran

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Abstract
Hottentotta Birula, 1908 is one of the most widely distributed buthid scorpions, with more than 40 described species from Africa, across the Middle East, to India. Currently, this genus is represented by ten morphological species in Iran (*H. akbarii*, *H. jayakari*, *H. juliae*, *H. khoozestanus*, *H. lorestanus*, *H. navidpouri*, *H. saulcyi*, *H. schach*, *H. sistanensis* and *H. zagrosensis*), all of which are endemic or subendemic in Iran. The members of this genus have not been properly studied from the taxonomic point of view. A tool that could contribute to scorpions' taxonomic studies is geometric morphometry, which is defined as the fusion between geometry and biology. In this study, the size and shape variations in sternocoxal structure in *Hottentotta* populations have been examined using the geometric morphometric method. The goal was to analyze the isometric size and conformation in nine species of *Hottentotta*. 100 individuals of *Hottentotta*, collected from different parts of Iran during 2018-2020, were photographed. Coordinate (x, y) configurations from landmarks were registered in sternocoxal structures. Geometric morphometric analyses were performed using R language. The results clearly showed divergence in the shape and size of sternocoxal structure among the studied taxa. However, the major shape changes were associated with *H. akbarii* which has a larger size of sternocoxal structure and a narrower sternum, shorter coxa II-III, and longer coxa IV.

Key words: *Coxae, Geometry, Hottentotta, Landmark, Shape variation, Sternum.*

INTRODUCTION

Geometric morphometrics (GM) is an alternative approach to the "traditional" method of measuring linear measurements and utilizing multivariate statistical analyses of these data. Landmark-based geometric morphometrics has been used frequently to quantify biological shape, shape variation, and covariation of shape with other variables (Torres & Miranda Esquivel, 2016; Nedeljković *et al*., 2015; Fox *et al*., 2020). The major aim of a morphometric analysis is to recover and recombine information in such a way as to obtain the most accurate biological insights on the variations in morphological structures (Navarro *et al*., 2004).

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A considerable number of studies have confirmed the significance of studies on shape variations in systematics and evolutionary biology (Pepinelli *et al*., 2013). Several studies revealed that GM is a good discriminator for the species in arthropods (Bechara & Liria, 2012; Chursina & Ruchin, 2018; Nedeljković *et al*., 2015; Torres & Miranda Esquivel, 2016). Few studies have been performed using GM for studying morphological structures of scorpions (Bechara & Liria, 2012).

Hottentotta is one of the most widely distributed genera of the family Buthidae, with several species distributed in Africa, the Arabian Peninsula, the Middle East, India, and Pakistan (Fet *et al*., 2000; Kovařík, 2007). This genus has ten taxonomically species in Iran, including: *H. akbarii* Yagmur *et al*., 2022, *H. jayakari* (Pocock, 1895), *H. juliae* Kovarik *et al*., 2019, *H. khoozestanus* Navidpour *et al*., 2008, *H. lorestanus* Navidpour *et al*., 2010, *H. navidpouri* Kovarik *et al*., 2018, *H. saulcyi* (Simon, 1880), *H. schach* (Birula, 1905), *H. sistanensis* Kovarik et al., 2018, and *H. zagrosensis* Kovarik, 1997.

The species delimitation in this taxon was mainly based on the traditional morphological characters, such as general coloration and carination of body segments (Barahoei *et al*., 2020). Members of the genus *Hottentotta* have not been so far comprehensively studied and the validity of some species, described based on singletons (e.g., *H. akbarii*, *H. khoozestanus* and *H. lorestanus*) needs further studies. Furthermore, some widely distributed species such as *H. saulcyi* shows extensive intraspecific variations in both morphological and morphometric characteristics, suggesting that this species might be a complex species. GM, with analyzing shape which reflects genetic variation more than what classic measurements do, is an approach that could contribute to scorpion taxonomy (Bookstein, 1982; Bechara & Liria, 2012). The importance of the coxosternal structure, as a useful attribute in discrimination of buthid scorpions, has been discussed by Bechara & Liria, (2012).

The aim of this study is to investigate variations in the shape and size of the sternocoxal structure for the nine species of the genus *Hottentotta* distributed in Iran including: *H. akbarii*, *H. jayakari*, *H. juliae*, *H. khoozestanus*, *H. navidpouri*, *H. saulcyi*, *H. schach*, *H. sistanensis*, and *H. zagrosensis*. The present investigation focused on using landmark-based geometric morphometrics of the sternocoxal structure as a new method to contribute to the knowledge of taxonomic studies.

MATERIAL AND METHODS

Specimens were collected by ultraviolet (UV) light detection at night between 2018-2020 from different localities in Iran (Fig. 1). Newly sampled material**,** were transferred to 75–96% ethanol, and deposited in the Zoological Museum of Ferdowsi University of Mashhad (ZMFUM). A total of 100 adult individuals belonging to nine species of *Hottentotta* from Iran were studied (Table 1).

Sternocoxal area (coxae II, III, and IV and sternum) was photographed using a Canon SX150 camera on a stand with a scale close to each specimen and at the same level as the sternocoxal structure to standardize the size digitization. Thirteen two-dimensional homologous landmarks (Bechara & Liria, 2012) were determined on the surface of the structure (Fig. 2), using Tpsdig 2.32 program (Rohlf, 2018). Landmarks configurations were scaled to unit centroid size and superimposed using the partial procrustes generalized least-squares method (Gower, 1975; Rohlf, 1990) using R language (R Development Core Team, 2019) following Claude, (2008). Two replicates of all individuals were photographed and digitized to calculate the measurement error. The mean of the two replicates of each individual was used in the subsequent analyses.

The size was computed as the centroid size, which is the square root of the sum of squares of the distances between each landmark and the centroid of the configuration of landmarks (Bookstein, 1991). The coordinates of the superimposed configurations were projected on the Euclidean tangent space and used as shape data for subsequent analyses. A one-way analysis of variance (ANOVA) using the specimen as a factor was performed on the centroid size of both replicates to estimate the measurement error in size (Yezerinac *et al*., 1992). Similarly, a Procrustes ANOVA (Klingenberg *et al*., 2002) was performed on the shape data taking the specimen effect into account to calculate the percent of measurement error relative to individual variation (Claude, 2008; Siahsarvie *et al*., 2012). To examine the interspecific and inter-sexual size differences, a type-II two-way analysis of variance (ANOVA) was

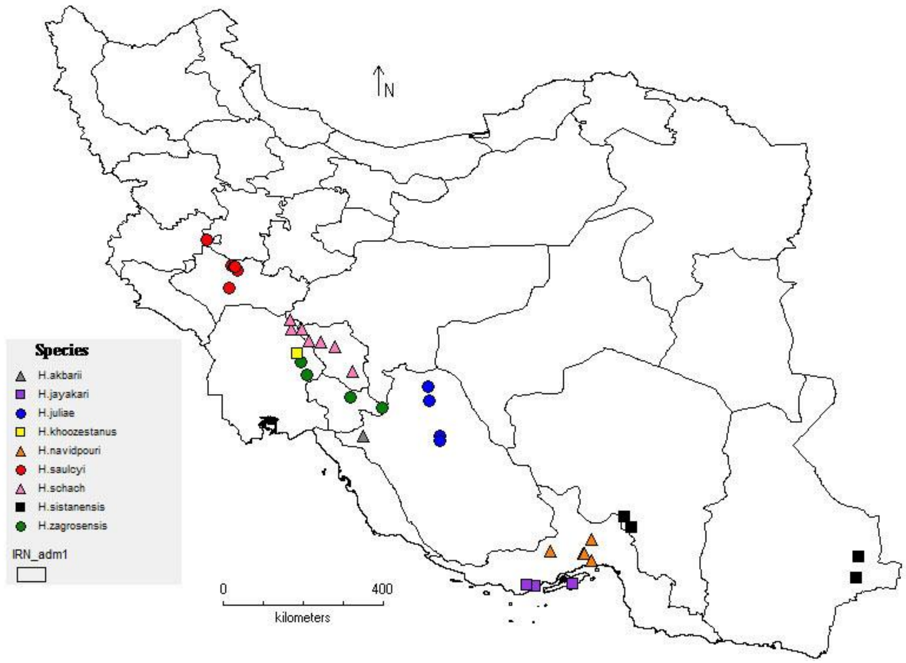

FIGURE 1. Map representing the localities of the Specimens studied in Iran.

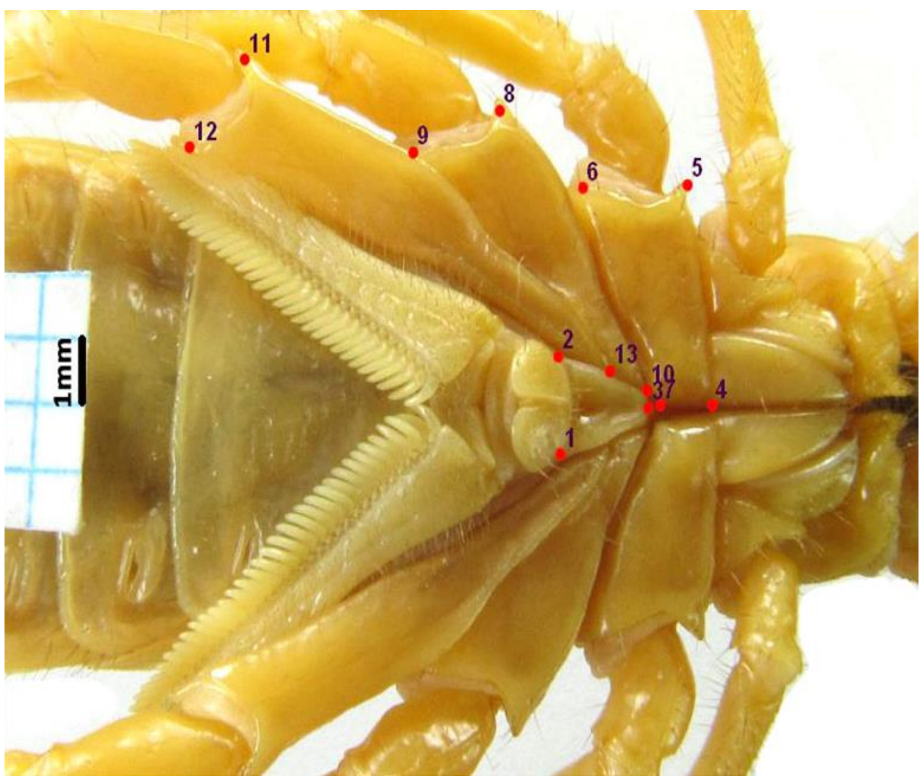

FIGURE 2. Position of 13 landmarks captured on the coxae and sternum.

carried out on the size based on two factors, sex and species, followed by Tukey's Post-Hoc Honestly Significant Difference (HSD) for pairwise comparisons. A box plot was performed on the centroid size to illustrate the size difference among species. For shape, the interspecific and inter-sexual variation was evaluated using two-way multivariate analysis of variance (MANOVA) on shape data using factors of species and sex. The analysis was followed by a pairwise MANOVA to determine the species that differ in the shape of the sternocoxal structure, significantly. As the sexual dimorphism was significant for shape, the procrustes data were orthogonally projected on the sex discriminant space, extending the Burnaby method (Burnaby, 1966). The data, hereafter called sex-corrected, were used for subsequent shape analyses. A linear discriminant analysis (LDA) was performed on the sex-corrected shape data. Species with less than five specimens were omitted from the discriminant analyze, and were, a posteriori, projected onto the discriminant axes. A neighbor-joining (NJ) tree based on Mahalanobis distance among species was estimated using the sex-corrected shape data to show the magnitude of the interspecific shape differences.

TABLE 1. Specimens, populations and provenance data (country, province or governate and abbreviated locality) of *Hottentotta* species deposited in the collections of the Zoological Museum of Ferdowsi University of Mashhad (ZMFUM), Iran, used for geometric morphometrics analysis.

Species	Locality	Sex	Museum code
H. akbarii	IRAN: Fars, Nurabad	1♂	ZMFUM-scr-2090
H. jayakari	IRAN: Hormozgan, Bandar Lengeh	1♂	ZMFUM-scr-1995
	IRAN: Hormozgan, Gheshm	1♂, 3♀	ZMFUM-scr-1972-1975
	IRAN: Hormozgan, Moalem	3♂, 6♀	ZMFUM-scr-1935-1942, 1951
H. juliae	IRAN: Fars, Abadeh	1♂, 1♀	ZMFUM-scr-1954-1955
	IRAN: Fars, Eghlid	5♂, 5♀	ZMFUM-scr-1956-1965
	IRAN: Fars, Shiraz, Marvdasht	1♂, 1♀	ZMFUM-scr-2037,2038
	IRAN: Fars, Shiraz, Sivand road	1♀	ZMFUM-scr-2050
H. khoozestanus	IRAN: Khuzestan, Behbahan	1♀	ZMFUM-scr-2015
H. navidpouri	IRAN: Hormozgan, Bandarabas, Chahchakor village	3♀	ZMFUM-scr-1952,1996,2043
	IRAN: Hormozgan, Bandarabas, Geno Mountain	2♂, 5♀	ZMFUM-scr-1966-1971,1997
	IRAN: Hormozgan, Bandarabas, Roydar	1♂	ZMFUM-scr-2071
	IRAN: Hormozgan, Bandarabas, Siahoo village	1♂	ZMFUM-scr-2072
H. saulcyi	IRAN: Lorestan, Aleshtar	8♂, 4♀	ZMFUM-scr-1907, 1908. 1910-1913, 1976, 2013, 2014, 2046, 2062, 2063
	IRAN: Kermanshah, Sahneh	3♂, 6♀	ZMFUM-scr-1921-1929
H. schach	IRAN: Chaharmahal and Bakhtiari, Ardal	2♀	ZMFUM-scr-1989,1990
	IRAN: Chaharmahal and Bakhtiari, Lordegan	1♂	ZMFUM-scr-1988
	IRAN: Chaharmahal and Bakhtiari, Shahrekord	6♂, 1♀	ZMFUM-scr-2031-2035, 2047,2070
H. sistanensis	IRAN: Kerman, Mourdan	4♂, 5♀	ZMFUM-scr-2073,2075, 2076,2077,2078,2080,2081,2082
	IRAN: Sistan and Baluchistan, Saravan	1♂, 5♀	ZMFUM-scr-1943-1947,1949
H. zagrosensis	IRAN: Khuzestan, Baghmalek, Baraftab village	2♂, 3♀	ZMFUM-scr-1983-1987
	IRAN: Khuzestan, Izeh	1♀	ZMFUM-scr-2036
	IRAN: Kohgiluyeh and Boyer-Ahmad, Yasouj, Cheshmeh Chenar village	2♀	ZMFUM-scr-1992,1993
	IRAN: Kohgiluyeh and Boyer-Ahmad, Yasouj, Lodab village	2♂, 1♀	ZMFUM-scr-1931,1933-1934

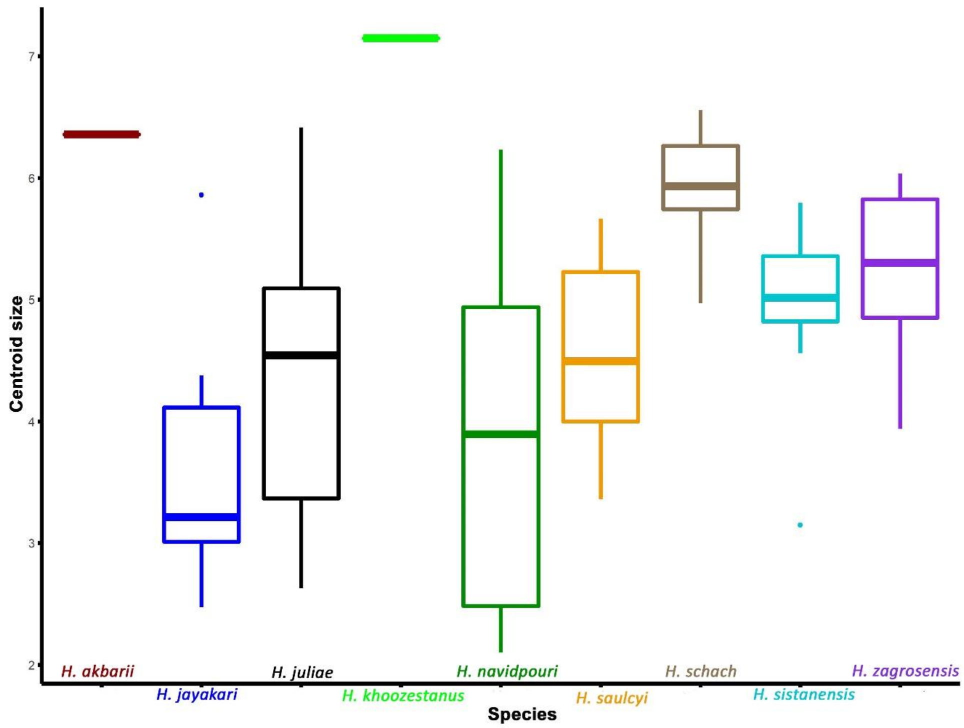

FIGURE 3. Boxplots of sex-corrected centroid size for species of *Hottentotta* occurring in Iran.

All analyses on landmarks were conducted using R 4.2.1 (R Development Core Team, 2022). The *MASS* package (Venables & Ripley, 2002) was used for LDA, the *ape* package (Paradis & Schliep, 2019) for NJ, the *ggplot2* package (Wickham, 2016) for plots, the *RVAideMemoire* package (Hervé & Hervé, 2020) for pairwise MANOVA, the *Agricolae* package (Mendiburu, 2021) for Tukey-HSD and *Pixmap* package (Bivand, 2022) for reading image files.

RESULTS

The measurement error was 4.4% for size and 9.8% for shape variation, which is significantly lower than inter-individual variation.

Size analysis

The type II two-way ANOVA suggests the studied species are significantly different in size (F=8.16; $P<10^{-7}$), but no sexual dimorphism in size of sternocoxal structure is evidenced. (F=0.01; *P*=0.932). The interaction between species and sex was not statistically significant, either (F=0.55; *P*=0.768) (Table 2). As a result, we kept both males and females together for size analyses).

Tukey's HSD and box plot analyses on the size of species (Table 3, Fig. 3) revealed that the greatest difference was between *H. jayakari* and *H. khoozestanus*. According to (Table 3), *H. jayakari* and *H. navidpouri* were smaller than *H. khoozestanus*, *H. schach H. sistanensis* and *H. zagrosensis.* Another difference was observed between *H. schach* with *H. juliae*, *H. saulcyi*, *H. navidpouri*, that *H. schach* was larger than other species.

The biggest sternocoxal structure belongs to *H. khoozestanus*, followed by *H. akbarii*, *H. schach*, *H. zagrosensis*, *H. sistanensis*, *H. saulcyi* and *H. juliae*, and the smallest sternocoxal belongs to *H. navidpouri* and *H. jayakari* (Fig. 3). *H. jayakari*, *H. navidpouri* and *H. schach* and other species, no differences have been observed between other species.

TABLE 2. Type II analysis of variance (ANOVA) on sternocoxal structure centroid size based on two factors, sex and species in *Hottentotta* populations occurring in Iran.

	Df	Sum Sq	Mean Sq	F value	**Pr (>F)**
species	8	55.50	6.938	8.160	**4.22e-8**
sex	1	0.01	0.006	0.007	0.932
Species * sex	6	2.81	0.468	0.550	0.768
Residuals	84	71.42	0.850		

TABLE 3. Statistically significant *P* values from pairwise comparisons among species of the buthid scorpion genus *Hottentotta* occurring in Iran, for size using Tukey's Post-Hoc HSD Test (N = 100 specimens). Asterisks indicate significant *P* values: * (0.05–0.01); ** (0.01–0.001); *** (< 0.001).

Species	***P*-value**	**Result**
jayakari-khoozestanus	**	*jayakari<khoozestanus*
jayakari- schach	***	*jayakari< schach*
jayakari- sistanensis	**	*jayakari< sistanensis*
jayakari- zagrosensis	***	*jayakari< zagrosensis*
juliae- schach	**	*juliae< schach*
khoozestanus- navidpouri	*	*navidpouri< khoozestanus*
schach-navidpouri	***	*navidpouri< schach*
sistanensis -navidpouri	*	*navidpouri< sistanensis*
zagrosensis -navidpouri	*	*navidpouri< zagrosensis*
saulcyi-schach	**	*saulcyi< schach*

TABLE 4. Two-way MANOVA of shape variation using sex and species factors on sternocoxal structure of *Hottentotta* occurring in Iran.

	Df	**Pillai**	**approx F**	**num Df**	**den Df**	**Pr (<F)**
Species	8	2.83590	2.6085	128	608	<e-14
Sex	1	0.35212	2.3438	16	69	<e-2
Species * sex	6	1.38105	1.3829	96	444	<e-1
Residuals	84					

TABLE 5. Pairwise MANOVA test on sternocoxal structure among species of the buthid scorpion genus *Hottentotta* occurring in Iran, using sex corrected shape variation. Significant *P* values indicated in boldface.

Species	***akbarii***	***jayakari***	***juliae***	***khoozestanus***	***navidpouri***	***saulcyi***	***schach***	***sistanensis***
jayakari	**0.0209**							
juliae	**0.0209**	**0.0018**						
khoozestanus	0.3750	**0.0471**	**0.0209**					
navidpouri	**0.0236**	**0.0418**	0.0946	**0.0367**				
saulcyi	**0.0172**	**0.0034**	**0.0418**	**0.0172**	**0.0172**			
schach	0.0604	0.1425	**0.0347**	0.4914	**0.0418**	0.0527		
sistanensis	**0.0275**	**0.0228**	**0.0182**	0.1538	**0.0209**	**0.0172**	0.5178	
zagrosensis	**0.0262**	**0.0073**	**0.0172**	0.1534	**0.0172**	**0.0182**	0.9854	0.5901

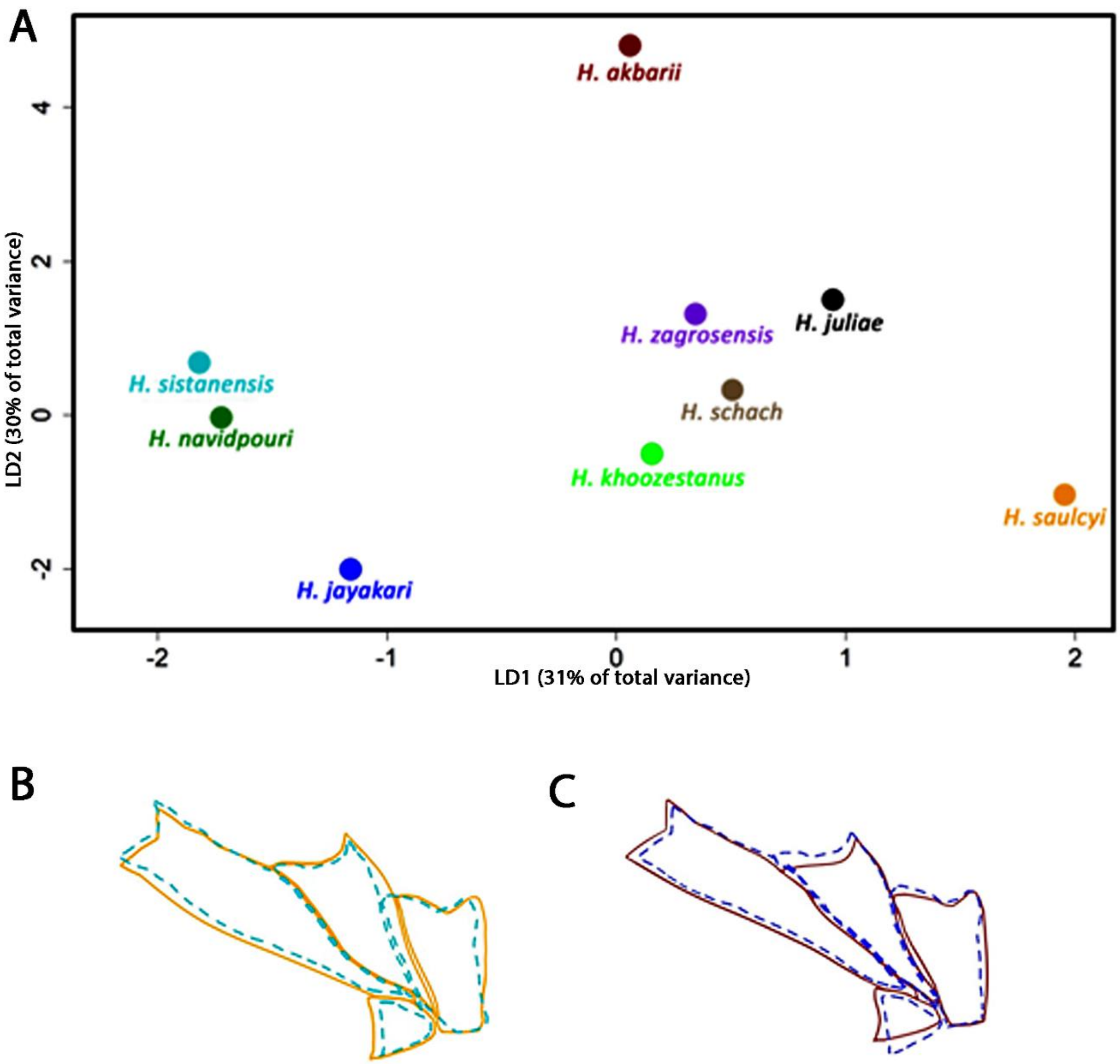

Figure 4- A) Linear discriminant analysis (LDA) of sternocoxal structure in nine species of genus *Hottentotta* in Iran based on sex corrected shape variables, B) Below of the graph is illustrated a schematic view of the sternocoxal structure and the deformation along the axes variation in the shape of the sternocoxal structure in LD1 (the orange continuous line indicates the change in the positive side and the dashed turquoise line indicates the change in the negative side of the first axes), C) Sternocoxal structural shape variation in LD2 (the brown continuous line indicates shape change on the positive side and blue dashed line indicates change on the negative side of second axes).

Shape analyzes

Type II two-way MANOVA species×sex on the shape of the sternocoxal structure (Table 4) showed significant differences both among species (F=2.83; $P<10^{-14}$), and between sexes (F=2.34; $P<10^{-2}$). The interaction between sex and species was also significant (F=1.38; $P<10^{-1}$), suggesting that shape sexual dimorphism does now show the same pattern in different species. As sexual dimorphism was significant and the sex ratios of the captured specimens were not similar in different species, the subsequent analyses were performed on corrected shape data. We could therefore analyze both sexes together.

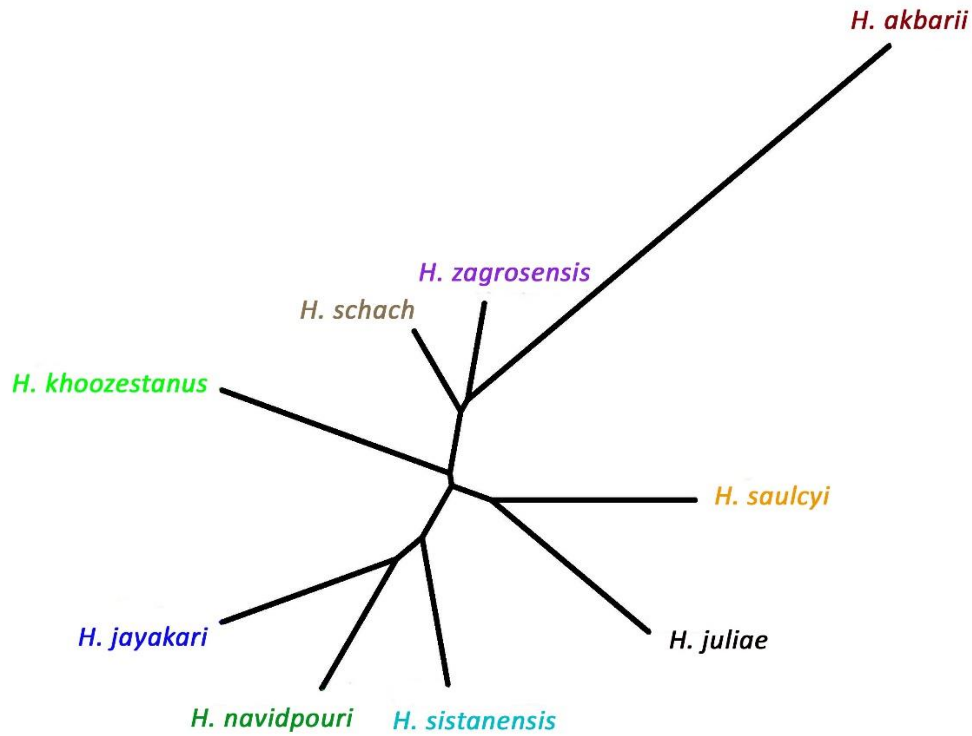

FIGURE 5. Neighbour-joining phenogram based on the Mahalanobis distance between species on the shape of sternocoxal structure in nine species of *Hottentotta*.

Pairwise MANOVA analysis (Table 5) revealed that *H. jayakari*, *H. juliae*, *H. navidpouri*, *H. saulcyi* had the most significant differences with other species. The least number of significant differences was observed between *H. schach* with other species, and it was only with *H. juliae* and *H. navidpouri*.

Figure 4 shows the pattern of shape variation among species in a linear discriminant space on species factor. The first two components of the LDA (LD1 and LD2) explained %31 and %30 of the variance of shape matrix, respectively (Fig. 4A). Figure extreme shape changes were amplified four times (Figs. 4B, C). This analysis was first performed on the species with a population of more than five samples, then the single specimen of *H. akbarii* and *H. khoozestanus* were projected in this space. The first axis discriminant (LD1) mainly distinguishes, *H. jayakari* and *H. saulcyi* from other species, and *H. khoozestanus*, *H. schach*, *H. zagrosensis*, *H. juliae* and *H. akbarii* were located together. Furthermore, *H. navidpouri* and *H. sistanensis* were in negative side of LD1. *H. akbarii* was separated from other species based on LD2.

In the analysis of the shape of the sternocoxal structure in (Fig. 4B), the continuous orange lines indicate the changes in the shape on the positive side of the LD1, and the dashed turquoise lines indicate the changes on the negative side. Based on this axis, *H. sistanensis*, *H. navidpouri* and *H. jayakari*, which are located at negative axes of LD1, the second coxae in these species are wider and the posterior apex is longer, as well as the third coxae is narrower than other species coxae. Moreover, the fourth coxa is shorter at the base and slightly longer at the apex, and in these species, the sternum is smaller than the species on the positive side of the axis (*H. saulcyi*). *H. juliae, H. khoozestanus, H. schach, H. zagrosensis* are intermediate in shape between these two groups.

In figure 4C, brown lines show the shape changes on the positive side of the LD2 axis, and the blue dashed lines show the changes on the negative side of the LD2 axis. *H. akbarii*, which is located at the positive side of LD2, the second coxa in this species is shorter and narrower than the base and middle part, the third coxa is shorter, the fourth coxa is longer in the apex and base. The sternum is narrower and more elongated than other species. In addition, *H. jayakari* is on the negative side and other species are intermediate between these two groups in shape.

Neighbor-joining phenogram based on the Mahalanobis distance between the species on the shape showed that *H. akbarii* had the greatest distance. *H. khoozestanus* was separated from other species by a long distance. *H. schach* and *H. zagrosensis* were close together. *H. jayakari*, *H. navidpouri* and *H. sistanensis* were also separated by a short distance. Finally, the separation of *H. saulcyi* and *H. juliae* was observed with a greater distance. In general, discrimination is clearly shown in species for both size and shape (Fig. 5).

DISCUSSION

Sexual dimorphism is very common in arachnids as males are generally smaller than females (Prenter *et al*., 1999), which is affected by cannibalism and a higher rate of mortality (Polis, 1990). The results of this study reveal that although males may have smaller body size, but they have similar sternocoxal size as females, or in other words, bigger sternocoxal size compared to their body size. Males and females are, however, sexually dimorphic in terms of shape of this structure. Females have wider sternum and coxa than males.

The plots of LDA (Fig. 4) provide evidence of morphological differentiation in sternocoxal structure of the studied taxa, which is in line with the divergence between morphospecies of the genus *Hottentotta*. The results of geometric morphometric analysis on shape of the sternocoxal structure revealed that *H. akbarii* is distinguished from other species by the large size of the sternocoxal structure and having a narrower and more elongated sternum, shorter coxa II-III, and longer coxa IV. The LD1 axis (Fig. 4), discriminates *H. saulcyi* from other species, by having a wider and larger sternum, narrower coxa II, the wider coxa III, and shorter coxa IV at the apex. *H. jayakari* with the smallest size of the sternocoxal structure (Fig. 3), discriminates from other species by having a wider and shorter sternum, longer and narrower coxa II and III, and shorter coxa IV.

Although, *H. sistanensis* is larger than *H. navidpouri* (Fig. 3), but both species demonstrate similar shape pattern of sternocoxal structure and discriminate from other species on the first LDA axis. Morphologically, *H. navidpouri* and *H. sistanensis* are very close to *H. saulcyi*, and traditionally were considered as different forms of *H. saulcyi* (Navidpour *at al*., 2013; Kovařík *et al*., 2018). These two species can be easily distinguished from *H. saulcyi* based on the shape of chela which is significantly elongated in *H. navidpouri* and *H. sistanensis* (Kovařík *et al*., 2018). The shape of sternocoxal structure in *H. saulcyi* completely differs from other congeners by having a larger sternum and wider coaxes II-IV (Fig. 4). Additionally, movable fingers of pedipalps are longer in *H. sistanensis* than in *H. navidpouri* (Kovařík *et al*., 2018). As a result, the sternocoxal structure provides a reliable morphological feature, which could be applied to differentiating *navidpouri+sistanensis* from *H. saulcyi*.

According to the LDA plot, *H. schach* is the most similar to *H. zagrosensis*. Both species have entirely black coloration. However, *H. zagrosensis* is more densely hirsute and has deeper metasomal segments (Kovařík *et al*., 2019). The results of the current study showed that both species were similar in size (Fig. 3) and shape (Fig. 4).

The largest strenocoxal structure belongs to *H. khoozestanus* based on size (Fig. 3), which is congruent with the description of this species as the largest species in *Hottentotta* genus by Navidpour *et al*, (2008).

Neighbor-joining phenogram (Fig. 5) showed a similar result to the LDA plot (Fig. 4). species that demonstrate similar shape patterns on the LDA plot are close to each other by a short distance on the neighbor-joining phenogram.

In conclusion, the presented study showed a remarkable morphological variability in sternocoxal structure of the studied species. Our study revealed main novel insights into the importance of the interspecific variations of sternocoxal structure as diagnostic features for discrimination of different species of the genus *Hottentotta*.

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