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Original Article

Genetic Diversity of *Hottentotta sp.* Scorpions (Scorpionidae: Buthidae) in Khuzestan, Iran

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Abstract

The genus of *Hottentotta sp.* scorpion is one of the few medically important scorpions in Iran. This study assessed the genetic relationship analysis of the cytochrome c oxidase subunit I (COXI) and 12sRNA genes and morphometric parameters among the population of *Hottentotta sp* in Khuzestan. Morphological analysis using the ANOVA T-test with a significance level of *P*-value less than 0.05 showed differences between the *Hottetotta saulcyi* and *Hottetotta zagrosensis*. However, this method was not able to distinguish between members of the same species. The amplification of gene fragments was done on 12srRNA (374 bp), and cytochrome c oxidase subunit I (COXI) (624 bp) from *Hottentotta sp.* collected from Khuzestan by PCR. Based on sequence 12srRNA, all *H. saulcyi* specimens (HS4, HS6 and HS7) except HS5 were included in cluster B. While two specimens of *H. Zagrosensis* (HZ6 and HZ1) with 99% bootstrap value were placed in cluster A. By using 12srRNA sequences, the highest genetic distance between the Khuzestan specimens was related to HS5 and HS7, which was calculated to be 16.7%. However, the amount of amino acid difference between HS5 and HS7 using the COXI sequence was 9.2%. The genetic distances of HS7 and HS5 with the only scorpion reference sequence, *H. saulcyi*, were 11.8% and 9.2%, respectively. Morphological data showed the separation of the two species, consistent with molecular phylogenetic trees. On the other hand, the genetic distance of specimens HS7 and HS5 with other members of the group as well as the scorpion reference sequence using the COXI gene, confirmed the possibility of an intraspecies difference that could not be proved by the morphological data alone.

Keywords: Scorpion, phylogenetic analysis, 12srRNA, cytochrome oxidase c subunit 1, *Hottentotta sp.*

1. Introduction

Scorpion sting is one of the health problems in tropical and subtropical regions that, in some cases, have been associated with death in human societies (1). The Buthidae family of scorpions is the largest and most widespread family of scorpions in the world. However, little published information is available on phylogenetic relationships within this family (2). There are a number of molecular studies available that have examined the molecular phylogenetic relationships between members of the Buthidae family (3, 4).

The demographic composition of the Iranian scorpions is one of the most diverse in the region in western Asia. On the other hand, it has been shown that morphotaxonomies have limitations in differentiating and defining species boundaries (5). Therefore, it is clear that with molecular information, there is a greater understanding of these taxa's diversity, distribution and intraspecies evolution. According to the latest

morphometric studies, the number of Iranian scorpions includes 18 genera which belong to three families, Buthidae, Scorpionidae and Hemiscorpidae. Among them, 14 genera have been reported in Khuzestan province (6, 7).

The species diversity within the *Hottentotta sp.* genus has been divided into three lineages: the African, the Sahara-Sindian and the Indian. These lineages are only proposed based on morphological characteristics (Birula 1914), and their relationships remain mainly uncertain. The scorpion genus *Hottentotta sp.* Birula, 1908 which belongs to the Arthropoda phylum and the Arachnida class, is a widespread and diverse genus. This genus is located in the Buthidae C.L. Koch, 1837; the most prominent scorpion family contains about 35 species that mostly live in semi-arid to humid places and prairie areas. So far, the only study that tried to determine the phylogeny of the Buthidae using DNA sequence data placed *Hottentotta sp.* as the sister taxon to *Buthacus sp.* Birula, 1908 (8). It is extensively distributed in Africa (except for large parts of the Great African Desert), the Middle East, the Arabian Peninsula, and southern Turkey, Iraq, Iran, Afghanistan, Pakistan, India, and Nepal. According to the literature, in addition to Khuzestan province, *the Hottentotta sp.* genus was reported for the first time from Isfahan, central Iran (9). Also, the genetic diversity of the Maghrebian *Hottentotta* sp. using cytochrome oxidase 1 (COXI) sequences show a prominently different picture of the Maghrebian *Hottentotta sp.* taxa to that found using morphological data only (10).

Due to the inadequacy of morphometric methods in detecting *Hottentotta sp.*, mitochondrial genes were used. In different studies using mitochondrial DNA, the phylogenetic relationships of Scorpion *Orthochirus iranus* (11), *Mesobuthus eupeus* (12) and *Hemiscorpius lepturus* (13) scorpions in Khuzestan province were investigated. In another phylogenetic study of the genus *Odentobothus sp.* in the province of Khorasan, at least five species of this genus have been reported in Iran, which statistical morphometric analyses and molecular evidence confirm the separation of these species (14).

This study investigates the morphometry and genetic diversity of two species of scorpions, *H. saulcyi* and *H. zagrosensis*, in the Khuzestan province using mitochondrial genes.

2. Materials and Methods

2.1. Scorpion Specimen Collection

Six scorpion specimens of *the Hottentotta sp.* genus were collected from the Khuzestan province in the southeast of Iran in the summer of 2019 (Table 1). All scorpions were adults. They are collected by UV light at night. They were transported in glass jars containing ethanol 70% and glycerin (95:5 ml). For DNA extraction, specimens were kept in 95% ethanol and frozen at -20°C.

Table 1. Location of scorpion specimens with longitude and latitude in Khuzestan province

Longitude	Latitude	Region	Specimens
49° 00′ 38″	31° 58′ 55″	Masjedsoleiman	HZ6
49° 32′ 03″	31° 15′ 07″	Ramhormoz	HZ1
49° 52′ 56″	31° 46′ 13″	Izeh	HS6
48° 33′ 36″	32°36′41″	Dezful	HS4, HS5
48° 31′ 46″	32° 12′45″	Chogha Zanbil	HS7

2.2. Morphological Measurements

Morphological examination and diagnosis of scorpions were made by the taxonomic keys of Polis and Stanford (15), (16). Proportion ratios of 18 morphometric features under a microscope using digital callipers were measured. The Abbreviations of morphometric features were as follows. Ca_L/AW: carapace length to anterior width; Ca_AW/PW: carapace anterior width to posterior width; Ch_L/W: pedipalp chela length to width; Met-I_L/W: metasomal segment I length to width; Met-I_L/H: metasomal segment I length to a height; Met-II_L/W: metasomal segment II length to width; Met-II_L/H: metasomal segment II length to a height; MetIII_L/W: metasomal segment III lengths to width; MetIII_L/H: metasomal segment III lengths to a height; MetIV_L/W: metasomal segment IV length to width; MetIV_L/H:

metasomal segment IV length to a height; MetV_L/W: metasomal segment V length to width; Met-V_L/H: metasomal segment V length to height.

2.3. DNA Extraction

Six genomic DNA was extracted from the metasoma of individual scorpion tissue (0.5-1.0 g) by DNA extraction kit (CinnaGen, Iran) according to the manufacturer's instructions. DNA concentration and purity were determined by calculating the absorbance ratio A260/280.

2.4. PCR Amplification and Sequencing

PCR amplification was carried out in a final volume of 25 µl containing 350 ng genomic DNA template, 1 X PCR buffer, dNTPs (0.25 mM), magnesium chloride (1.5 mM), forward and reverse primer (each of 0.4 µM), *Taq* DNA polymerase (0.5 U). The primers were 12SF (5'-CGATTTGAACTCAGATCA) and 12SR (5'-CAAAGGTAGCATAATCA) (17). PCR thermal program at 95°C for 3 min (one cycle), 94°C for 45 sec, 45°C for 45 sec and 72°C for 60 sec (5 cycles), then 94°C for 45 sec, 51°C for 60 sec and 72°C for 60 sec (35 cycles), finally 72°C for 10 min as a final extension was performed. In order to amplify the target gene fragments of COXI, the primers were COXI-F 5'-GGTCAACAAATCATAAAGATATTGG and COXI-R 5'- TAAACTTCAGGGTGACCAAAAAATCA (18). PCR was carried out with initial denaturation for 5 min at 95°C, followed by 35 cycles of 45 s at 94°C, 1 min at 48°C, and 1 min at 72°C and, finally, 7 min of incubation at 72°C. The Amplified PCR products were electrophoresed through a 1% agarose gel and stained with DNA Safe Stain (Sinaclon, Iran) before detection by UV transillumination. The amplified DNA fragments were extracted from agarose gel before performing DNA sequencing according to the dideoxy termination method using an automated Applied Biosystems 373 DNA Sequencer. All 6 specimens were sequenced with 12srRNA primers from both directions. Moreover, two specimens (HS5 and HS7) were sequenced with COXI primers.

2.5. DNA Analysis and Phylogenetic Tree

The DNA sequence comparisons were made using the Blastn algorithms programs in the National Center for Biotechnology Information (NCBI) GenBank database (19). Multiple alignment sequences were performed via the CLUSTAL_W program, and the resulting sequences were adjusted by visual analysis using BioEdit version 7.0.2. Sequence editing was done by BOXSHADE software (ch.embnet.org/software/BOX_form.html). William Pearson's LALIGN program is used for matching subsegments in two sequences (20). Genetic distance and phylogenetic tree were performed using the neighbour-joining method with 1000 replicates of bootstrapping using the MEG7 software. In the morphometric study, an ANOVA test was used. The confidence of the neighbour-joining (NJ) tree with a *P*-distance value was assessed via bootstrap 1000 to estimate the stability of the tree topologies. This study used an outgroup of *Rhipicephalus sanguineus* to root the phylogenetic tree.

3. Results

3.1. Morphometric Analysis

Statistical analyzes were determined on 18 morphometric ratios for 6 genetic specimens of *Hottentotta sp.* (Table 1). Analysis of the ANOVA T-test showed no significant differences between each morphometric trait measured in the two groups. Intraspecies differences were not significant. After morphological studies, the results were obtained in table 2, which were determined by performing an ANOVA test with a significance level of *P*-value less than 0.05. Based on these findings, this test could not determine whether there is an intraspecies difference between the *H. saulcyi and H. zagrosensis* species in Khuzestan province. Therefore, molecular methods were used to determine such differences. The dorsal and ventral surfaces of *H. saulcyi and H. zagrosensis* scorpions are shown in figure 1.

Table 2. Calculation of the mean of standard error, statistical analysis of ANOVA results in comparison with the mean of 18 morphometric traits related to *H. Zagrosensis* and *H. saulcyi* in Khuzestan province in pairs. *P*-value less than 0.05; Ns means, no significant difference

Items	Abbreviations	*H. zagrosensis* N=2	*H. saulcyi* N=4	*P*-value<0/05
		MEAN±SE	MEAN±SE	Ns
1	CL/AW	1.60±0.03	1.45±0.01	Ns
2	CL/PW	0.96±0.01	1.02±0.03	Ns
3	CAW/PW	0.6±0.01	0.70±0.03	Ns
4	X/Y	0.91±0.02	0.84±0.01	Ns
5	Mt(I)L/H	1.45±0.03	1.35±0.02	Ns
6	Mt(II)L/H	1.75±0.01	1.78±0.03	Ns
7	Mt(III)L/H	2.01±0.02	1.88±0.01	Ns
8	Mt(IV)L/H	1.99±0.04	2.22±0.04	Ns
9	Mt(V)L/H	2.94±0.03	2.49±0.03	Ns
10	Mt(I)L/W	1.33±0.03	1.33±0.01	Ns
11	Mt(II)L/W	1.45±0.01	1.72±0.02	Ns
12	Mt(III)L/W	1.49±0.02	1.90±0.04	Ns
13	Mt(IV)L/W	1.66±0.04	2.22±0.02	Ns
14	Mt(V)L/W	2.64±0.03	2.35±0.04	Ns
15	CHL/ML	2.39±0.01	3.33±0.03	Ns
16	MFL/ML	1.57±0.01	2.33±0.03	Ns
17	TlL/H	3.57±0.04	2.37±0.01	Ns
18	TlL/W	2.80±0.03	2.58±0.02	Ns

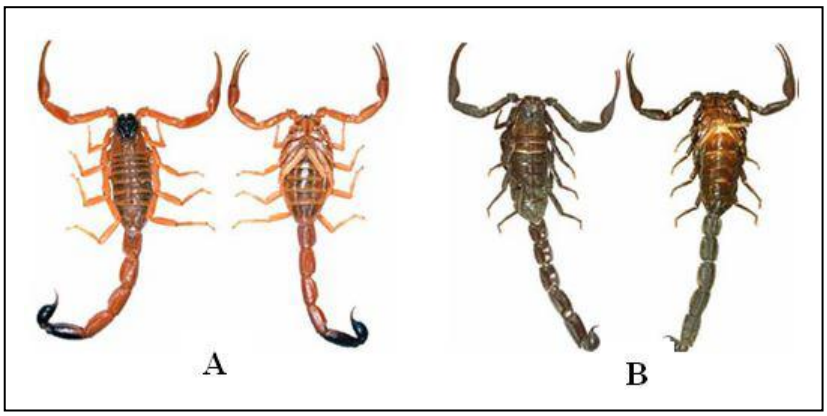

Figure 1. Dorsal and ventral surfaces of *H. saulcyi* (**A**), *H. zagrosensis* (**B**) scorpions

3.2. Amplification and Sequence Analysis

In order to obtain more DNA information about the *Hottetotta* specimens, a close examination of the nucleotide sequences of 12srRNA was conducted. It showed a relatively high number of mutations in these genetic samples. Amplification products of 374 bp were obtained for 12srRNA gene fragments. Estimates of variation among sequences were based on a fragment length of 374 nucleotides (Figure 2). In the *H. saulcyi* specimens, two sequences of HS4 (Dezful) and HS6 (Izeh) were identical. The similarity of these two sequences with sequences HS5 and HS7 was 80.3% and 78.9%, respectively. However, the similarity between sequences HS5 and HS7 was 78.9%. The highest genetic divergence was observed at 21.1% between these two specimens. In the *H. Zagrosensis* specimens, the aligned parts of HZ6 and HZ1 are more similar (92.5%) to each other. However, the first 20 nucleotides of sequence HZ1 are not available.

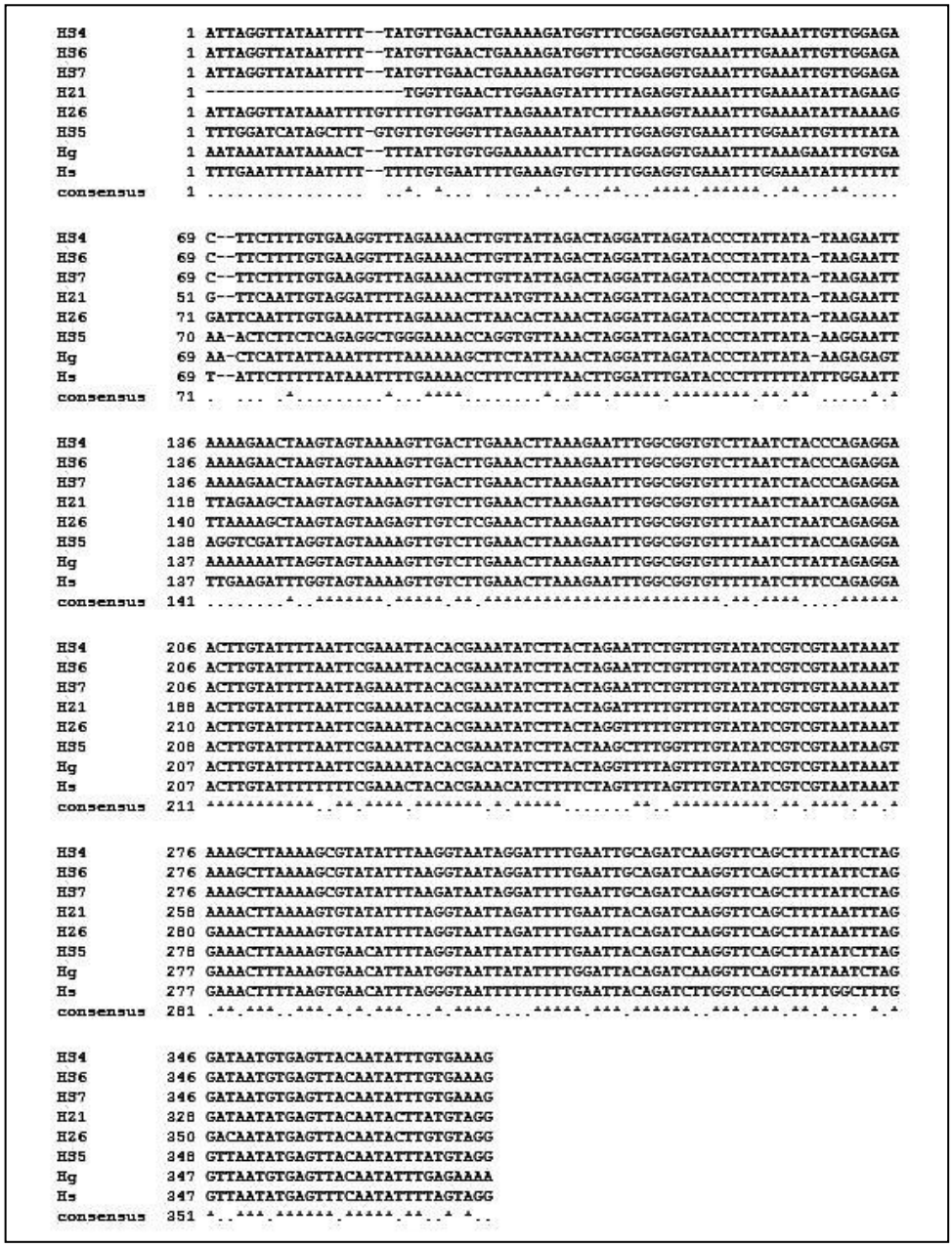

Figure 2. Comparison of the nucleotide sequence of 12srRNA gene fragments from *H. saulcyi* and *H. Zagrosensis*. The only two existing *Hottentotta* sequences belonging to *H. Gentili* (Hg; JQ423119) and *H. saulcyi* (Hs; KU705364) were retrieved from GenBank. The *H. saulcyi* (KU705364) reference sequence was isolated from Sardasht near Dezful, which was recorded as a direct submission in the gene bank. The completely identical nucleotides are shown in dark colours. Nucleotides that are similar and protected are shown in grey. Similar and protected nucleotides are observed in a separate rows as stars and dots, respectively

3.3. Phylogenetic Analysis

Using 12srRNA gene sequences, the related sequences from different scorpions were retrieved from the NCBI GenBank using the highly similar sequence program (megabases). The sequences of *the Hottentotta* scorpion from Khuzestan, along with other scorpions, were converted to FASTA format and aligned with the MEGA7 software, and the exact alignment results were used to draw the phylogenetic tree. The analysis includes 6 nucleotide sequences in which all situations containing gaps and missing data are omitted. Figure 3 shows two main clusters, A and B, were observed. Cluster A can be divided into sub-clusters A1 (HS5) and A2 (HZ1 and HZ6), and the specimens in these subclasses were related to bootstrap values of 56%. HS4, HS6 and HS7 were included in cluster B. The HS5 specimen, along with the only two *Hottentotta* reference sequences in the GenBank, *H. Gentili* (JQ423119) and *H. saulcyi* (705364KU) are located in the cluster A. while all *H. saulcyi* scorpion specimens (HS4, HS6 and HS7) except HS5 were all included in cluster B, two *H. Zagrosensis* scorpion specimens (HZ6 and HZ1) were distinguished by a correlation of 99% in sub-cluster A2.

The 12srRNA sequences of the *Hottentotta* scorpion from Khuzestan and the related sequences retrieved from GenBank were sorted into FASTA format, aligned with the MEGA7 software, and the results of the alignment were used to draw a phylogenetic tree (Figure 3). The tree consists of 18 nucleotide sequences in which all positions containing gaps and missing data are deleted. According to figure 4, three main clusters, A, B and C, were observed, of which cluster A can be divided into two sub-clusters, A1 and A2. It was observed that all Khuzestan scorpion specimens except HS5 (Dezful) were separated from the rest of the related scorpions. Specimens HS4, HS6 and HS7 (Chogha Zanbil) were placed next to other similar scorpions in cluster A1. While HS5 was separated from the other HS specimens, this specimen with 58% and 91% bootstrap values were placed next to the only two *Hottetotta* scorpion reference *H. Gentili* (JQ423119) and *H. saulcyi* (705364KU), respectively. The *H. saulcyi* (KU705364) was isolated from Sardasht near Dezful and registered as a direct submission in the GenBank. Also, HZ1 and HZ6 samples with a 99% correlation were placed next to each other and separately from HS specimens in subcluster A1, which indicates the separation between the two species.

3.4. Genetic Distances

The genetic distance of 6 scorpion specimens of the genus *Hottentotta* studied in Khuzestan province compared to each other and with the only two reference sequences registered in the NCBI GenBank was calculated using the genetic distance program of MEGA7 software. Based on table 3, it is interesting to mention that the highest difference between the Khuzestan specimens was related to HS5 and HS7, which was calculated to be 16.7%. The maximum difference with the rest of the studied specimens related to these two specimens was observed between 15.3-16.7%.

Since the phylogenetic data obtained using the 12srRNA gene showed the difference between these two specimens (HS5 and HS7) with each other (16.7%) and also with the rest of the specimens to be relatively higher than the interspecies threshold, in order to confirm the results, sequencing of partial COXI gene of the two specimens was performed. In table 4, the amount of amino acid difference between HS5 and HS7 using the COXI sequence was 9.2%. The genetic distances of HS7 and HS5 with the only scorpion reference sequence *H. saulcyi* (QFX66626; isolated from Sardasht near Dezful) in the GenBank were 11.8% and 9.2%, respectively.

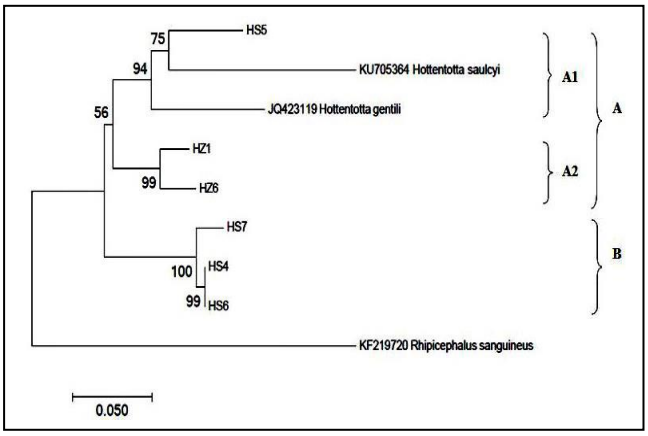

Figure 3. Phylogenetic tree of *H. saulcyi*, *H. Zagrosensis* species of Khuzestan province based on a sequence of 12srRNA gene in comparison with two *Hottentotta* reference sequences. The numbers at the top of the lines indicate the relationship between the groups. The repetition percentage is shown in the Bootstrap test resulting from 1000 repetitions at the top of the branches

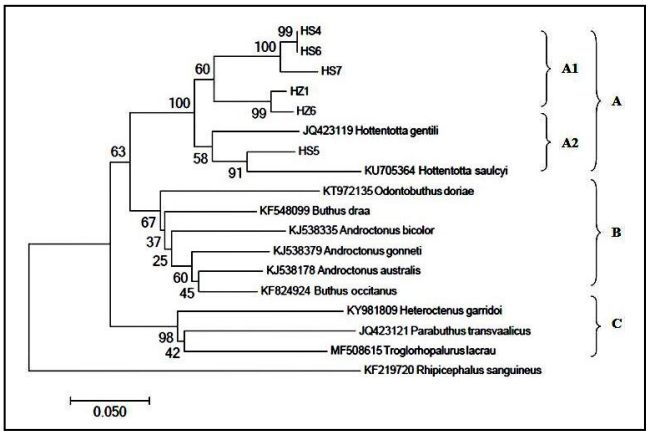

Figure 4. A neighbour-joining phylogram of 6 specimens of *Hottentotta* scorpions in Khuzestan based on sequence 12srRNA gene with the related scorpion sequences. The numbers at the top of the lines indicate the bootstrap value (1000 replications). The number above branches is the bootstrap value

Table 3. Average pairwise genetic distances for 12srRNA gene between specimens of *Hottentotta* scorpions in Khuzestan in comparison with other scorpions

Specimens	1	2	3	4	5	6	7	8	9
1.HS4									
2.HS5	0.153								
3.HS6	0.000	0.153							
4.HS7	0.022	0.167	0.022						
5.HZ1	0.109	0.120	0.109	0.124					
6.HZ6	0.120	0.127	0.120	0.135	0.040				
7.JQ423119_Hottentotta_gentili	0.160	0.131	0.160	0.175	0.153	0.145			
8.KU705364_Hottentotta_saulcyi	0.236	0.164	0.236	0.244	0.204	0.204	0.196		
9.KF219720_Rhipicephalus_sanguineus	0.316	0.338	0.316	0.320	0.302	0.313	0.349	0.396	

Table 4. Table of average pairwise genetic distances of HS5 and HS7 scorpions in Khuzestan for COXI gene at the amino acid level in comparison with related scorpions

Specimens	1	2	3	4	5	6	7	8	9	10
1. HS7										
2.HS5	0.092									
3.QFX66626_Hottentotta_saulcyi	0.118	0.092								
4.APU94128_Hottentotta_minax	0.118	0.092	0.013							
5.AEG90945_Hottentotta_gentili	0.105	0.092	0.013	0.013						
6.ANU06289_Hottentotta_rugiscutis	0.124	0.098	0.020	0.020	0.020					
7.QDH82024_Hottentotta_hottentotta	0.124	0.098	0.020	0.020	0.020	0.013				
8.ASK39373_Hottentotta_tamulus	0.124	0.098	0.020	0.020	0.020	0.013	0.026			
9.ASK39363_Hottentotta_pachyurus	0.124	0.098	0.020	0.020	0.020	0.013	0.026	0.013		
10.PYI25096_Aspergillus_indologenus	0.765	0.771	0.784	0.791	0.784	0.778	0.778	0.791	0.784	

4. Discussion

Due to the high diversity of scorpions in Iran, this region of the world is considered one of the richest scorpion fauna for studying this arthropod (6). However, the geographical distribution and biodiversity of these scorpions in Iran have yet to be thoroughly studied. One of the most important parts of Iran is Khuzestan province, one of the largest provinces in Iran. Due to its climatic conditions, it is a suitable environment for scorpions to grow.

Morphological traits have been used for many years to classify scorpions. One of the most critical challenges of systematic classification is identifying species and subspecies, which is usually the subject of most research. Using molecular methods in scorpions has made it possible to identify closely related species and subspecies with high morphological similarity (11). In this study, two genes of 12srRNA and COXI were used to investigate the genetic diversity of *Hottetotta* scorpions in Khuzestan province.

The geographical location of the sampling in Khuzestan province included mountainous (Izeh and Masjed Soleiman) and plain (Ramhormoz, Dezful and Chogha Zanbil) areas. Despite sampling from five different regions with different climatic characteristics, morphological data did not significantly differ. Even genetic data did not show a significant difference among specimens according to their location. For example, the nucleotide sequence of 12srRNA in SH4 and HS6 was the same.

Based on morphological features in Iran, three species of *H. jayakari*, *H. saulcyi* and *H. zagrosensis* have been reported in Iran, and besides *H. jayakari*, two other species have been found in Khuzestan province. In this study, the results of morphological studies showed a significant difference between the two species of scorpion, *H. saulcyi* and *H. Zagrosensis*. However, subspecies identification of specimens was not detectable by this method. Since the classification based only on the morphometric method could not give conclusive results, two mitochondrial genes, 12srRNA and COXI, were used to determine the relationships among the Hottetotta scorpions genus in Khuzestan. Mitochondrial genes have been extensively used to identify phylogenetic relationships between closely related species in vertebrates and invertebrates due to their unique characteristics. Compared to the nuclear genome, due to the high evolutionary rate and maternal inheritance, as well as the small size of and the lack of DNA recombination, all are advantages for manipulation in the laboratory (20). The classification of scorpion specimens of the genus *Hottentotta* in Khuzestan province based on the nucleotide sequence of gene 12srRNA showed a clear difference so that it can be concluded that these two species, although having the closest genetic distance from each other, but belong to two separate species. For example, the HS4 and HS6 specimens were most similar, while the HS5 and HS7 were more different. These differences, which indicate intraspecies differences, were not detectable in morphological assessments. The genetic distance of HS5 and HS7 with the only *H saulcyi* reference sequence registered in the GenBank showed 16.4% and 19.6%, respectively. It is worth noting that the range of genetic distances to separate species and subspecies boundaries in different organisms is different and should be considered in each organism. In a study in Mexico, a genetic distance of nearly 10% was used as a measure to confirm species segregation. They identify a new species of scorpion *Centruroides Marx* belonging to the *Buthidae* family using the 16SrRNA mitochondrial gene (21). In another study, a different range of genetic distance (4.4-13.2%) was reported to accept interspecies cases in *Buthidae* scorpions using mitochondrial genes (22). According to the results of determining the genetic distances of HS5 and HS7 with other HS specimens based on the 12srRNA sequence (15.3-16.7%), these two specimens are more clearly different from the other group members. Therefore, the COXI was sequenced from these two specimens to confirm the results. The percentage of genetic distances of HS7 and HS5 with only *H. saulcyi* reference sequence at the amino acid

level of the COXI gene was 11.8 % and 9.2%, respectively. As can be seen in table 4, the reduction in the genetic distance at the amino acid level could be due to codon usage bias. This is because a change in an amino acid requires a change in one or more nucleotides, especially in the first and second nucleotides of each codon. Based on the results of the COXI gene, these two specimens, along with the rest of the HS scorpion specimens, belong to *H. saulcyi*. However, due to the high morphological similarity and relatively high genetic distances, they belong to two different subspecies, and the COXI gene performed better in phylogenetic analysis.

5. Conclusion

Morphological data showed the separation of the two species, consistent with molecular phylogenetic trees. On the other hand, the genetic distance of specimens HS7 and HS5 with other group members and the scorpion reference sequence using the COXI gene confirmed the possibility of an intraspecies difference that could not be proved by the morphological data alone.

Authors' Contribution

Study concept and design: A. J., S. P. and H. J.
Acquisition of data: A. J., S. P. and H. J.
Analysis and interpretation of data: A. J., S. P. and H. J.
Drafting of the manuscript: A. J., S. P. and H. J.
Statistical analysis: A. J., S. P. and H. J.
Administrative, technical, and material support: A. J., S. P. and H. J.

Ethics

All ethical standards have been accepted by the ethics committee of the Shahid Chamran University of Ahvaz, Ahvaz, Iran.

Conflict of Interest

The authors declare that they have no conflict of interest.

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