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Original Article

Phylogenetic Relationships of Scorpion *Compsobuthus matthiesseni* Based on Sequences of Internal Transcribed Spacer 2 Gene from Khuzestan Province, Iran

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Abstract

Buthidae family includes scorpions with highly potent venom such as *Compsobuthus matthiesseni* is important due to the prevalence of scorpion stings in Khuzestan Province, Iran. Morphometric comparison of males and females (n=5 each) showed that the body and carapas of the females were longer and wider (32.57±0.23 mm and 3.75±0.22 mm, respectively) than those of males (28.89±0.25 mm and 3.55±0.12 mm, respectively). From the seven specimens of *C. matthiesseni* scorpion, 410-bp gene fragments of ribosomal internal transcribed spacer 2 were amplified by polymerase chain reaction. The specimens of CM1 and CM2 (isolated from Baghmalek, Khuzestan) were in the same group with bootstrap values of 87%. Nevertheless, CM4 and CM3 (isolated from Shushtar and Bidroobe, Khuzestan) with bootstrap values of 73% and 62% were separated from the two specimens of Baghmalek, respectively. The two specimens CF3 and CM5 (isolated from Masjed Soleiman, Khuzestan) with bootstrap values of 88% were placed next to each other in a separate group. CF2 was separated from the rest of the specimens with a bootstrap value of 54%. Out of the seven scorpions that were examined, six specimens (CM1, CM2, CM3, CM4, CM5, and CF3) showed the greatest similarity between 1.1% and 4%. However, the genetic distance between CF2 and the rest of the specimens was at the range of 10.8-14.2%. It can be concluded that all *C. matthiesseni* scorpions from Khuzestan Province belonged to one species; nonetheless, differences were observed within the species, especially in the case of CF2, which might be intraspecies.

Keywords: Scorpion, *Compsobuthus matthiesseni*, Phylogenetic, Internal transcribed spacer 2

1. Introduction

Scorpion stings are considered a serious health problem worldwide; therefore, performing area-specific studies on scorpion species and subspecies is a key for effective treatment. The geological history and topographic and climatic characteristics of the Iranian plateau have led to the formation of a variety of habitats (1).

Iranian scorpions belong to three families of Buthidae, Hemiscorpionidae, and Scorpionidae that are widely located in the south and southwest regions of Iran. It has been shown that among the latest checklists, at least 68 valid species of scorpions have been reported in Iran belonging to these families, which are dominated by the Buthidae family with 58 species equivalent to 85.3% of total species (2, 3). The Buthidae family includes all scorpion species with highly potent toxins and medical importance. The highest prevalence of scorpion sting and resulting death has been reported from Khuzestan Province, Iran, due

to dry and mid-dry climate, which provides an appropriate environment for venomous animals.

The *Compsobuthus* species is a genus of Buthidae scorpions, containing more than 43 different species worldwide. This genus was first introduced in 1815 and new species were described in Africa and Asia (4) and Pakistan and Afghanistan (5). *Compsobuthus matthiesseni* was first identified as the species responsible for scorpion stings in the south and southwest of Iran. This species has also been reported in central parts of Iran, southeast of Turkey, and west of Iraq (6, 7).

Numerous studies have discussed the phylogeny of the Buthidae family based on nuclear, mitochondrial, and ribosomal markers (8-10). Among the genome regions used for the analysis of molecular variation, the ribosomal DNA consists of transcriptional units with each unit containing the genes for ribosomal ribonucleic acid and the internal transcribed spacers (ITS)1 and ITS2 separating gene units (11). In particular, ITS2 is generally more conserved among species than ITS1, providing an efficient phylogenetical marker for closely related species. The taxonomic status of three species of *Anderctonus* scorpion belonging to the Buthidae family in Tunisia was revised by molecular studies based on the ITS sequence (12).

The present study aimed to investigate the taxonomic status of this species using morphological and phylogenetic analyses.

2. Materials and Methods

2.1. Collection of Scorpion Specimens

To conduct the study, ten specimens of *Compsobuthus matthiesseni*, including five females (CF) and five males (CM), were collected overnight using ultraviolet light (UV) from five different regions of Khuzestan Province, which is considered as a habitat for this type of scorpion, within June-October 2019. According to table 1, scorpion specimens from Baghmalek, Bidrobeh, Shush, Lali, and Masjedsoleiman regions, Khuzestan, Iran, were recorded along with the longitude and latitude of the sampling areas using a location tracking device called Global Positioning System. To identify scorpions, the identification key was used (5).

Table 1. Location of scorpion specimens along with latitude, longitude, and altitude of sampling areas in different regions of Khuzestan Province

Specimens	Region	Latitude	Longitude	Height (m)	Gender
CM1, CM2, CF1, CF4	Baghmalek	31° 26' 42" N	49° 58' 61" E	183	Male and Female
CM3	Bidroobeh	32° 45' 23" N	48° 14' 43" E	503	Male
CM4	Shush	32° 11' 93" N	48° 16' 63" E	73	Male
CM5	Lali	32°17' 54" N	49° 02' 67" E	325	Male
CF2, CF3	Masjed Soleyman	31° 37' 32" N53	48° 54' 62" E	53	Female

2.2. Sampling and morphological measurements

Morphological characteristics, including color, pedipalp, prosoma, metasoma, and trichobothria pattern, were performed using a stereomicroscope (Leica MZ 7.5, Germany). The morphological measurements were taken using digital calipers. Several preserved specimens were measured per species and average measurements were calculated (5, 13).

2.3. Abbreviations of Morphometric Ratios

Ca_L/W: carapace length to width; Met-I_L/W: metasomal segment I length to width; Met-I_L/H: metasomal segment I length to height; Met-II_L/W: metasomal segment II length to width; Met-II_L/H: metasomal segment II length to height; Met-III_L/W: metasomal segment III length to width; Met-III_L/H: metasomal segment III length to height; Met-IV_L/W: metasomal segment IV length to width; Met-IV_L/H:

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

2.4. Genomic DNA Extraction

In order to extract genomic DNA, 0.1-0.5 g of scorpion foot tissue was crushed in the presence of liquid nitrogen, and then, 600 µl of resuspension buffer (10 mM Tris-HCl pH 7.4/10 mM NaCl/25 mM ethylenediaminetetraacetic acid, safety data sheet 1%) was added and the mixture was homogenized. Genomic DNA was extracted with the same amount of phenol/chloroform and once with the same amount of chloroform. Finally, it was precipitated with pure ethanol and 3 M of sodium acetate.

2.5. Polymerase Chain Reaction Amplification and Sequencing

In order to amplify the target gene fragments, polymerase chain reaction (PCR) for each sample in a final volume of 25 µl containing 350 ng DNA template, 1 X PCR buffer, deoxyribonucleotide triphosphates (0.25 mM), magnesium chloride (1.5 mM), forward and reverse primer (each 0.4 µM), *Taq* DNA polymerase (0.5 U). Polymerase chain reaction thermal program was performed 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); subsequently, at 94°C for 45 sec, 51°C for 60 sec, and 72°C for 60 sec (35 cycles); and finally, at 72°C for 10 min as a final extension. The primers were ITS2F 5'-GGGTCGATGAAGAACGCAGC and ITSR 5'-ATATGCTTAAATTCAGGGGG (14).

Reverse and forward primers were selected from the 5.8S and 28S regions, respectively, to amplify the gene fragment according to schematic figure 1. 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 prior to the performance of DNA sequencing according to the dideoxy termination method using an automated Applied Biosystems 373 DNA Sequencer.

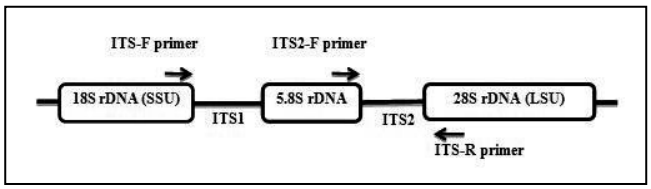

Figure 1. Schematic representation of the ribosomal gene with the location of the reverse and forward primers

2.6. Genetic Distance and Phylogenetic Tree

The comparisons of DNA sequences were conducted using the BLAST algorithms programs in the National Center for Biotechnology Information (NCBI) GenBank database (15). The alignments of multiple sequences were obtained using the CLUSTAL_W program (16) and edited with the BOXSHADE software (ch.embnet.org/software/BOX_form.html). Genetic distance and phylogenetic tree were performed using the Neighbor-Joining method with p-distance value via 1,000 replicates of bootstrapping using the MEGA software 7.0.18. In the morphometric study, a t-test was used to compare the forewing length of male and female scorpions. Statistical analyses were performed using the computer software SPSS 18.0.

3. Results

3.1. Collection of Scorpion Specimens and Morphology

Table 1 presents seven *C. matthiesseni* scorpions collected from five regions of Khuzestan Province, the collection point and latitude and longitude of the sampling areas. After transferring the specimens to the lab, the identification keys were used to identify them.

3.2. Morphological Characteristics

All collected *C. matthiesseni* were examined under a stereomicroscope, which revealed that they had slender bodies, elongated metasomas with light yellow color, black pigment surrounding median and lateral eyes, and half of IV and V (17). They also had a body size of 40-50 mm. Metasomal segments Carapace slender, almost parallel-sided. All segments elongated, and in segments I-IV, dorsolateral and lateral supra median carinae were strong and finely with irregularly serrate. Trichobothrial pattern Type A, orthobothriotaxic;

dorsal trichobothria of femur were arranged in beta-configuration.Telson has ventral aspect with median and paired lateral rows of rounded granules; subacular tubercle indicated by an elevated, rounded area when viewed from lateral aspect; aculeus gently curved and relatively short, movable finger with 4 distal granules preceding first granular row. Fixed finger trichobothria was located on opposite extreme distal end of the fourth granular row, opposite enlarged granule at the base of the fifth row. Morphometric comparison of males (n=5) and females (n=5) showed that the body and carapas of females were longer and wider (32.57±0.23 mm and 3.75±0.22 mm, respectively) than those of males (28.89±0.25 mm and 3.55±0.12 mm, respectively). Sexual dimorphism demonstrated a significant difference (P<0.05), and the details of the different parameters between male and female scorpions are listed in table 2.

Table 2. Morphometric measurements of male and female *Compsobuthus matthiesseni* scorpions (Statistically significant, P<0.05)

Parameter	Female (mm) n=5	Male (mm) n=5
Ca_L/W	3.75±0.22/3.25±0.15	3.55±0.12/3.05±0.25
Met-I_L/W/H	2.91±0.23/1.80±0.01/1.45±0.03	2.89±0.56/1.60±0.05/1.24±0.04
Met-II_L/W/H	3.60±0.05/1.86±0.06/1.63±0.15	3.58±0.05/1.65±0.06/1.45±0.15
Met-III_L/W/H	4.02±0.05/1.23±0.14/1.76±0.23	3.72±0.03/1.19±0.14/1.55±0.32
Met-IV_L/W/H	3.84±0.15/1.18±0.20/1.61±0.18	3.61±0.25/1.15±0.25/1.43±0.14
Met-V_L/W/H	4.77±0.15/1.19±0.07/1.54±0.12	4.46±0.13/1.15±0.05/1.34±0.05
Telson L/W/H	3.74±0.05/1.28±0.10/1.10±0.10	3.69±0.05/1.16±0.09/1.03±0.08
Femur L/W	4.2±0.03/1.20±0.04	3.8±0.05/1.02±0.05
Tibia L/W	5.5±0.21/1.4±0.04	5.3±0.25/1.2±0.05
Total length	32.57±0.23	28.89±0.25

3.3. DNA extraction

In the first step, 5 µg of genomic DNA was extracted from 0.5 g of foot tissue. The quantity and quality of the extracted DNA were evaluated using a spectrophotometer by recording the amount of light absorption at 260 and 280 nm. In order to determine the quality of DNA and its contamination with proteins, a ratio of 260/280 was calculated, which was in a relatively suitable range of 1.5-1.7 in all samples. Moreover, its quality was confirmed by running the DNA extracted on 1% agarose gel.

3.4. Amplification and Sequence Analysis

During the PCR reaction, an ITS2 gene fragment of approximately 410 bp was generated (Figure 2). Only 5 µl of the PCR products were taken for agarose gel electrophoresis, indicating the efficient amplification of the target gene and the optimal conditions of the reaction. No amplification product was obtained in the control samples where template DNA was excluded. The sequences of 7 specimens with the best quality were used for alignment and phylogeny. Figure 3 depicts the multiple alignments of ITS2 gene fragments of *C. matthiesseni* scorpion specimens from different regions of Khuzestan Province. As shown in figure 3, the changes were observed mainly in the 3'-end of the gene fragment, especially in the CF2 specimen. The two sequences CF3 (Masjed Soleyman) and CM5 (Lali) were almost identical (99.27%). However, the similarity of sequence CF2 with other specimens was calculated at the range of 83.38-85.22%.

3.5. Phylogenetic Analysis

In order to investigate the molecular diversity of nucleotide sequences of ITS2 gene fragments obtained from scorpions of *C. matthiesseni* in Khuzestan Province, the collected sequences were loaded in the MEGA7 program and after the alignment operation, the phylogenetic tree was drawn (Figure 4). Similar scorpion sequences were retrieved from the NCBI database. The specimens of CM1 and CM2 (isolated from Baghmalek region) were in the

same group with bootstrap values of 87%. However, CM4 (isolated from Shushtar region) and CM3 (isolated from Bidroobe region) with bootstrap values of 73% and 62% were separated from the two specimens of Baghmalek, respectively. The two samples CF3 and CM5, which were both isolated from Masjed Soleiman, with bootstrap values of 88%, were placed next to each other in a separate group from the rest of the specimens. While CF2 was separated from the rest of the *C. matthiesseni* specimens (bootstrap value of 54%), this specimen, along with the rest of *C. matthiesseni* specimens, were placed next to *Mesobuthus eupeus* (KU341983) and *Odontobuthus doriae* (KU341985) with a bootstrap value of 51%. It was observed that seven scorpions of *C. matthiesseni*, along with *Mesobuthus eupeus* (KU341983), *Odontobuthus doriae* (KU341985), *Hottentotta saulcyi* (KU341984), *Androctonus australis* (AJ404479), and *Androctonus crassicauda* (Ku341986), all belonged to the Buthidae family. The scorpion of *C. matthiesseni* belonging to Khuzestan Province was most closely related (bootstrap value of 51%) to the *M. eupeus* (KU341983), *O. doriae* (KU341985), while *H. saulcyi* (KU341986), *A. australis* (AJ404479), and *A. crassicauda* (Ku341986) showed less similarity than the *C. matthiesseni* specimens. The *Hemiscorpius lepturus* (KU341983) was shown separately from other specimens as an outgroup.

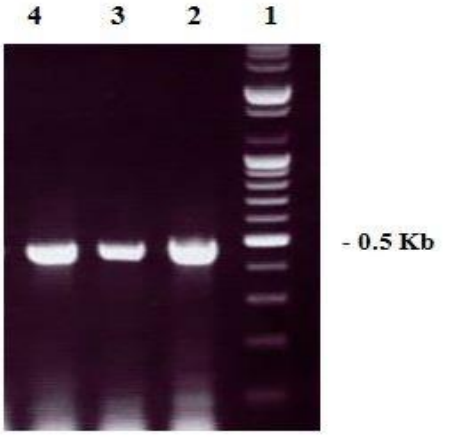

Figure 2. Electrophoresis of PCR reaction products to amplify a fragment of ITS2 gene from *Compsobuthus* scorpion on 1% agarose gel
The DNA marker is shown in the first column along with the PCR products of the *Compsobuthus* scorpion in the second to fourth columns.

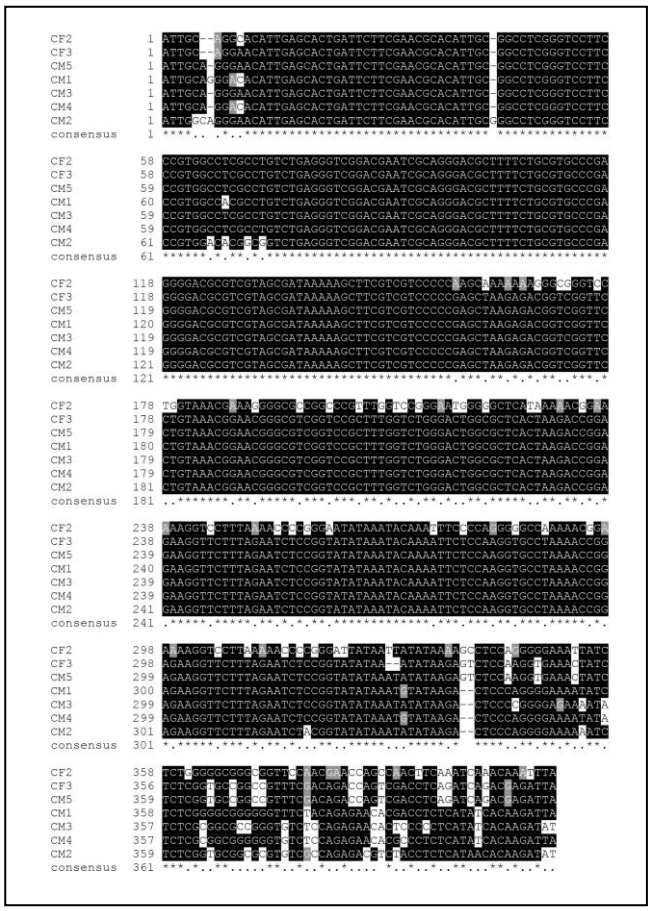

Figure 3. Comparison of the nucleotide sequence of an ITS2 gene fragment related to scorpion specimens of the genus *Compsobuthus* in Khuzestan Province
The nucleotides are shown to be exactly the same as the dark color. Nucleotides that are similar and protected are shown in gray.

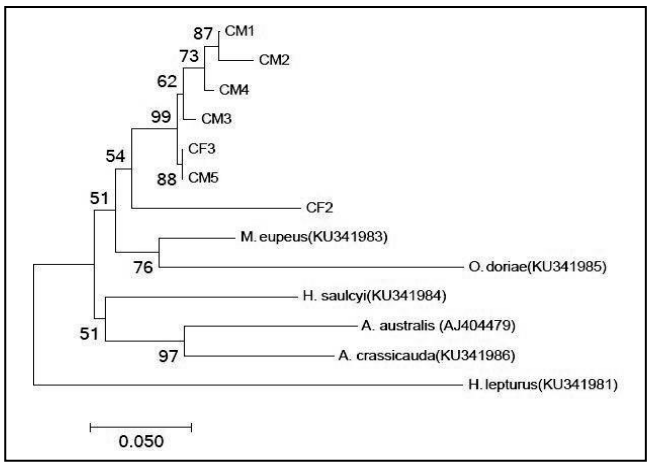

Figure 4. Neighbor-joining phylogram of seven specimens of *C. matthiesseni* scorpions in Khuzestan Province based on sequence ITS2 gene with the related scorpion sequences
The numbers at the top of the lines indicate the bootstrap value (1,000 replications).

3.6. Genetic Distances

The genetic pairwise distances of seven *C. matthiesseni* in Khuzestan Province, compared to the related scorpions, were calculated using MEGA7 software (Table 3). Out of the seven scorpions that were examined, six specimens (i.e., CM1, CM2, CM3, CM4, CM5, and CF3) showed the greatest similarity of 1.1-4% with each other. The two specimens CF3 and CM5 did not show significant differences. The percentage of nucleotide difference between CF2 and the rest of the group was in the range of 10.8-14.2%.

Table 3. Mean pairwise genetic distances for ITS2 gene between *Compsobuthus matthiesseni* scorpions in Khuzestan in comparison with the related scorpions

	1	2	3	4	5	6	7	8	9	10	11	12
1. CF2												
2. CF3	0.108											
3. CM1	0.125	0.023										
4. CM2	0.142	0.040	0.017									
5. CM3	0.114	0.011	0.028	0.045								
6. CM4	0.125	0.023	0.011	0.028	0.017							
7. CM5	0.108	0.000	0.023	0.040	0.011	0.023						
8. *Mesobuthus eupeus* (KU341983)	0.165	0.091	0.097	0.114	0.102	0.097	0.091					
9. *Androctonus australis* (AJ404479)	0.227	0.176	0.188	0.205	0.182	0.188	0.176	0.182				
10. *Odontobuthus doriae* (KU341985)	0.250	0.210	0.222	0.239	0.216	0.227	0.210	0.188	0.307			
11. *Hottentotta saulcyi* (KU341984)	0.216	0.142	0.153	0.170	0.153	0.153	0.142	0.182	0.227	0.284		
12. *Androctonus crassicauda* (KU341986)	0.216	0.153	0.176	0.193	0.165	0.176	0.153	0.210	0.159	0.318	0.199	
13. *Hemiscorpius lepturus* (KU341981)	0.335	0.284	0.307	0.318	0.295	0.307	0.284	0.318	0.386	0.403	0.335	0.358

4. Discussion

Scorpion stings in Khuzestan Province are a serious public health problem, and the largest population in this province is children (18). In addition, the population composition of Iranian scorpions is one of the most diverse populations in the region in West Asia; in this respect, Iran has been considered one of the richest regions in this regard. Therefore, a molecular study is important to identify the species present in these areas. Since to the best of our knowledge, a comprehensive phylogenetic study for scorpion species, especially *C. matthiesseni*, has not been performed in Iran so far, this issue has left the taxonomic status and its geographical distribution unclear.

Morphological information has previously been used to determine species identity. It is clear that despite the close morphological similarities, it is difficult to identify scorpions using this feature alone (19). In this study, despite sampling from five different climate regions, morphological findings did not show a significant difference between specimens. Because the morphological findings failed to show significant differences between the seven *C. matthiesseni* scorpions from different regions of Khuzestan Province in the southwest of Iran, phylogeny was performed based on the nucleotide sequence of an ITS2 gene fragment.

All specimens of *C. matthiesseni*, along with the related scorpions, including *M. eupeus*, *Odontobuthus doriae*, *H. saulcyi*, *A. australis*, and *A. crassicauda*, were used to draw the phylogenetic tree. Although the three scorpions (i.e., *H. saulcyi*, *A. australis*, and *A. crassicauda*) are located farther away from the *C. matthiesseni* specimens, they are all located in one cluster, which belong to the Buthidae family. The CF2 scorpion specimen, which is most closely

related to *M. eupeus*, showed the greatest difference with other *C. matthiesseni* specimens, which was probably an intraspecies difference between the seven *C. matthiesseni* specimens.

In population genetics, there is an acceptable range of genetic diversity that, with genomic changes in that range, still belongs to that particular species. In invertebrates, this value is estimated at 13.4%, which is probably due to our inability to clean up similar populations in multi-species organisms. In vertebrates, this amount reaches 6% (20). Comparison of ITS2 gene sequence in *C. matthiesseni* scorpion of Khuzestan Province showed that the genetic distance of six specimens (except CF2) was between 1.1% and 4%. Therefore, they can be classified under one species. The CF2 specimen showed a genetic difference of 10.8-14.2% with the rest of the group. However, the morphometric results did not confirm this genetic difference. This amount of genetic difference was not expected due to the presence of a sexual reproductive system in scorpions. Nevertheless, it is possible that this degree of genetic difference, as suggested in the genus of Biomphalaria (21, 22), may be justified by considering other important factors, such as recombination, mutation, or gene flow.

5. Conclusion

It can be concluded that all *C. matthiesseni* scorpions in this study belonged to the same species; nonetheless, differences were observed within a species, especially in the case of CF2, which might be intraspecies.

Authors' Contribution

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

Ethics

All the procedures in this study, were performed according to the guidelines instructed by the Animal Ethics Committee of the University of Ahvaz, Ahvaz, Iran.

Conflict of Interest

The authors declare that they have no conflict of interest.

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