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Original Article

Phylogenetic and Morphological Analyses of *Androctonus crassicuda* from Khuzestan Province, Iran (Scorpiones: Buthidae)

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ABSTRACT

The *Androctonus crassicuda* is the most diverse scorpion species in the family of Buthidae, which is endemic to Khuzestan province, Iran. Investigation of the relationship of species by means of a molecular study of specimens is one of the new approaches due to the limitations of the morphological approaches. In the current study, the analysis was based on 32 morphological characteristics of *A. crassicuda* native to southwest Iran. Moreover, the DNA sequencing of two mitochondrial markers, namely cytochrome oxidase subunit I and *12sRNA* loci was performed, and the phylogenetic tree was constructed using maximum likelihood method with 1000 replications using MEGA software (version 7). Based on the results of the phylogenetic tree, *A. crassicuda* was classified into a monophyletic group. However, the genetic diversity of this species populations was not significant (0.001). The highest and lowest genetic distance of *A. crassicuda* was compared with the reports obtained in Urmia and west Azerbaijan, Iran. There was a clear divergence between the *A. crassicuda* isolated from northern and southern areas of Iran. This study showed the importance of geographical and climate features of the region and genetic distance among the populations. The phylogenetic analysis of *Androctonus* species from other regions showed the highest and lowest genetic distance with *A. gonneti* (Morocco) and *A. amoreuxi* (Portugal), respectively. The comparison of the morphological characteristics and morphometric results revealed that metasoma characteristics are important in the identification of *A. crassicuda*. The results of the analysis of the morphometric values of *A. crassicuda* were mainly compatible with the phylogenetic trees and supported the traditional morphological classification, thereby presenting a clearly definition of the genera of *Androctonus* species.

Keywords: *Androctonus crassicuda,* Phylogeny, Morphometric features, Cytochrome oxidase subunit I, 12sRNA

Analyses Phylogénétiques et Morphologiques d'*Androctonus crassicuda* de la Province du Khuzestan, Iran (Scorpiones: Buthidae)

Résumé: L'*Androctonus crassicuda* est l'espèce de scorpion la plus diversifiée de la famille des Buthidae, endémique de la province du Khuzestan, Iran. L'étude moléculaire des relations entre les espèces est l'une des nouvelles approches utilisées en raison des limites des approches morphologiques. Dans l'étude actuelle, l'analyse était basée sur 32 caractéristiques morphologiques d'*A. crassicuda* originaire du sud-ouest de l'Iran. De plus, le séquençage de l'ADN de deux marqueurs mitochondriaux, à savoir la sous-unité du cytochrome oxydase I et du loci *12sRNA* a été réalisée, et l'arbre phylogénétique a été construit en utilisant la méthode du maximum de vraisemblance avec 1000 réplications en utilisant le logiciel MEGA (version 7). *A. crassicuda* a été classé

dans un groupe monophylétique à partir des résultats de l'analyse phylogénétique. Cependant, la diversité génétique des populations de cette espèce n'était pas significative (0,001). La distance génétique la plus élevée et la plus faible d'*A. crassicuda* a été comparée aux rapports obtenus dans les régions iraniennes d'Urmia et de l'ouest Azerbaïdjan. Il y avait une nette divergence entre les spécimens d'*A. crassicuda* isolés des régions du nord et du sud de l'Iran. Cette étude a montré l'importance des caractéristiques géographiques et climatiques des régions sur la distance génétique entre les populations. Selon l'analyse phylogénétique des espèces d'*Androctonus* d'autres régions, la distance génétique la plus élevée et la plus faible a été respectivement observée avec A. gonneti (Maroc) et *A. amoreuxi* (Portugal). La comparaison des caractéristiques morphologiques et des résultats morphométriques a révélé que les caractéristiques des métasomes sont importantes pour l'identification d'*A. crassicuda*. Les résultats de l'analyse des valeurs morphométriques d'*A. crassicuda* étaient en grande partie compatibles avec les arbres phylogénétiques et soutenaient la classification morphologique traditionnelle, présentant ainsi une définition claire des espèces d'*Androctonus*.

Mots clés: *Androctonus crassicuda*, Phylogénie, Caractéristiques morphométriques, Sous-unité I du cytochrome oxydase, 12sARN

INTRODUCTION

Scorpion is a venomous arthropod of the Arachnida class which is widespread globally. The members of this family are found mainly in tropical and subtropical regions, and partly in temperate habitats (Fet and Braunwalder, 2000). Iranian scorpion includes three families, namely Buthidae, Scorpionidae, and Hemiscorpiidae. The epidemiological studies of scorpion sting have shown that the incidence of scorpionism is higher in Iran than the global average (Chippaux and Goyffon, 2008; Dehghani et al., 2009). The Buthidae family with 85 genera and 895 species is the largest of the scorpion families (Sousa et al., 2017). Buthidae, with 44 species, is the most diverse family of scorpions in Iran. The first documented report of Iranian scorpions was regarding *Androctonus crassicauda*, which was observed in Kashan, by Olivier in 1807 (Farzanpay, 1987). *Androctonus crassicauda* is the most significant scorpion species widely distributed and found in the majority of Iran provinces, especially at the southern Zagros mountain with a height of 2,000 meters, as well as the coastline of the Persian Gulf and Oman Sea (at the height of 5 meters). Moreover, this species can be found in Azerbaijan, India, Afghanistan, Iraq, Syria, Jordan, Saudi Arabia, Yemen, Turkey, and North Africa (Kovařík, 1999; Fet and Braunwalder, 2000; Lourenço and Qi, 2006). The wide distribution of this scorpion in different regions indicates its adaptation to different climatic conditions. According to previous studies, 27% of the scorpionism was by *A. crassicauda* in Khuzestan province (Dehghani et al., 2009). The venom of this scorpion is used for the production of polyvalent antivenom through hyperimmunizing healthy horses by the Razi Vaccine and Serum Production and Research Institute located in Karaj, Iran. The classification of scorpions is based on the analysis of morphological and morphometric characteristics, such as pedipalp, mesosoma, metasoma, chelicerae, carapace, femur, and trichobothrium pattern, which lead to limitations in the identification of genus, species, and subspecies (Hjelle, 1990; Ochoa et al., 2013; Santibanez-Lopez et al., 2019). A solution to these challenges can be the implementation of studies on the identification of phylogenetic relationships based on both molecular and morphological characteristic methods with the purpose of determining and assessing the relationships of these species (Chippaux and Goyffon, 2008). The first report on the phylogeny of scorpions, genus *Euscorpius,* was

presented by Gantenbein et al. (1999). In phylogeographical studies on Buthidae (Gantenbein et al., 2000; Gantenbein et al., 2001; Gantenbein and Largiadèr, 2002; Gantenbein and Largiadèr, 2003; Parmakelis et al., 2006; Mirshamsi et al., 2010, 2011; Fet et al., 2018), DNA-based approaches revealed species which were not easily differentiable according to morphological characteristics. Since the comparison of morphological and molecular phylogenetic studies of *A. crassicauda* is poorly defined in Iran, extensive research is needed to review the classification. In the present study, the samples were collected from the different parts of Khuzestan province, Iran and then examined based on their morphometric and sequence variations at the cytochrome oxidase subunit I (*COXI*) gene, *12sRNA*. In addition, an investigation was conducted on the phylogenetic relationships and the partition of the genetic diversity of *Androctonus* species.

MATERIAL AND METHODS

Sampling and Morphological Measurements. *Androctonus crassicuda* were nightly collected from Khuzestan region based on their dispersion and diversity in different regions from April to September 2018 and April to October 2019. The morphological measurements were conducted using digital calipers. Several preserved specimens were measured for each species; subsequently, average measurements were calculated. In this study, the measurement was performed on the basis of morphological characteristics defined by Stahnke (1970) and Lamoral (1979). Morphological characteristics, including color, pedipalp, prosoma, metasoma, trichobothria pattern, and makeup, were investigated using a stereomicroscope (Leica MZ 7.5, Germany).

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

Polymerase Chain Reaction Amplification and Sequencing. Genomic DNA was extracted using an extraction kit (Sina Pure^{TM} DNA kit Tehran, Iran) according to the kit instruction. Polymerase chain reaction (PCR) was performed to segment about 400 nucleotides of *12sRNA* gene using two primers, namely 5'- AGAG-TGACGGGCAATATGTG and 5'- CAGCG GCTGCGGTTATAC. Afterward, the amplification of this segmentation was carried out in 25 ml reaction volumes, containing 1-5 µl of DNA template, 2.0 mM $MgCl_2$, 200 mM of each dNTP, 25 pmol of each forward and reverse primers, and 0.5 unit of Taq DNA polymerase. The cycling protocol included an initial hot start at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 sec, 58°C for 30 sec, and 72 °C for 60 sec and a final extension of 72 °C for 7 min. Cytochrome c oxidase subunit I was amplified with COXI-F/COXI-R with the primers of 5'GGTCAACAAATATATAA TAGATAT and 5'CCGGTAAAATTAAAATATAAA using a touchdown PCR protocol. To the end, the reaction mixtures were initially incubated at 94 °C for 2 min, followed by 35 cycles of amplification (at 94 °C for 30 sec, 42 °C for 50 sec, and 72 °C for 35 sec) and a final extension of 72 °C for 5 min. The amplification of *COXI* and *12sRNA* genes using PCR products with forward and reverse primers were sent for both directional DNA sequencing using the Sanger method on the ABI 3730 sequencer (Bioneer, Daejeon, South Korea).

Phylogenetic Analyses. Multiple sequence alignments were performed by means of CLUSTALW in MEGA software (version 7). Genetic distances were also calculated using Kimura's 2-parameter model in MEGA software (version 7). The validity of variant

nucleotides at highly conserved positions was confirmed. Phylogenetic analyses were performed on individual *COX1* and *12sRNA* datasets using the maximum likelihood (ML) tree method. The ML tree analysis was performed using MEGA (version7), and the nodes were tested for robustness with 10,000 bootstrap replicates (Tamura and Nei, 1993; Thompson et al., 1994; Kumar et al., 2016), with *Hottentotta zagrosensis* being used as an outgroup.

RESULTS

Morphological Characteristics. All collected scorpions were examined under a stereomicroscope condition. They were basically black to reddish-brown with no trichobothria under their pedipalp patellae and orthobothriotaxic A-β patterns. They had one terminal and three distal granules in their movable pedipalp fingers, and their chelae were slender with long fingers. The carinae of metasomal segments I-IV developed, and the distal segments extended. The morphometric comparison of the male (n=10) and female (n=10) scorpions showed that the body length of the females was longer (89.75±14.56 mm), and their carapaces were wider (10.12± 1.05 mm), compared to those of males (82.78±5.380 and 8.01±0.61 mm, respectively). On the other hand, metasoma was longer and wider in males than in females with the same total length. Additionally, the pectinal teeth in males were longer and higher in number than in females. The details of the different parameters of males and females are listed in Table 1. Pectinal organ length, pectinal tooth count, and caudal carapace width were the most important features in sexual dimorphism, which indicated a significant difference between the two genders (P<0.05).

Phylogenetic Analyses. In total, 10 replicated fragments of *12srRNA* and *COXI* genes from 10 individual genomes (5 males and 5 females) of *A. crassicuda* were sequenced. The results of sequencing were based on a *COXI* fragment with a length of 671 nucleotides and *12srRNA* with a length of 360 nucleotides (Figure 1). All positions containing gaps and missing data were eliminated from the dataset. The intraspecific genetic distance of *A. crassicuda* isolated from Khuzestan was highly close (0.001), indicating that the genetic diversity of the species of this population was not significant. The estimates of variation among sequences were based on the sequences of *12srRNA* genes from three genera of *Androctonus* species. (Table 2). The comparison of the genetic distances of the genus *A. crassicuda* in Iran showed that the largest genetic distance was between *A. crassicuda* in Khuzestan and *A. crassicuda* in Urmia (KU705366). The genetic distance value among the species was obtained as 0.107. The smallest genetic distance was between *A. crassicuda* in Khuzestan and *A.crassicuda* in west Azerbaijan (KT972133). The comparison of genetic distance among other identified species of *Andructonus* specie in Northern Africa, Southern Europe, and Iran showed that the largest genetic distance was between *A. amoreuxi* in Portugal and *A. crassicuda* in Makoo, while the smallest genetic distance was between *A. crassicuda* in Makoo and *A. crassicuda* in Urmia. In this study, the phylogenetic tree of *A. crassicuda* genus was constructed using the ML in MEGA (version7). The ML tree revealed one group, which was not different from the samples (Figure 2). Furthermore, all sequence data were closely related to the outgroup (95%). The results showed that the constructed phylogenetic trees with *COXI* and *12sRNA* genes were mainly compatible with the classification obtained based on morphological characteristics. The *12srRNA* sequences belonging to *A. crassicuda* (Makoo, Iran,), *A. australis* (Tunisia), and *A. amurexi* (Portugal) were retrieved from GenBank. The ML tree of *Androctonus* species, which showed the same phylogenetic tree topology, was constructed based on the sequences of *12SrRNA* genes, as illustrated in Figure 3. The phylogenetic tree was divided into two branches. The first branch consisted of species of *A. crassicuda* which were collected from the western part of Iran and grouped in clade 1. The second branch was clearly divided into two subcategories, namely *A. crassicauda gonneti* species collected from Morocco and *A. australis* (Tunisia)/*A. amorexi*

(Southwest Europe, Portugal) species. The obtained findings showed a close genetic relationship among the *A. crassicuda* collected from Makoo, Iran. The highest genetic divergence was found between the species of *A. crassicauda gonneti* (Morocco) and *A. crassicuda* (Khuzestan).

DISCUSSION

Scorpions, as hunter arthropods, have significant medical importance and are widely distributed in the different regions of various climatic conditions, especially in tropical regions (Chippaux and Goyffon, 2008; Rafeeazadeh, 2009). The Buthidae family is the most diverse family of scorpions, among which *A. crassicauda* is the most geographically widespread species in Iran (Navidpour, 2015). This study assessed the genetic relationship analysis of the mitochondrial *COXI* and *12sRNA* genes and morphometric parameters among the population of *A. crassicauda* in Khuzestan. Furthermore, the phylogenetic relationship of this species with *Androctonus* species from other regions of the world was evaluated using gene *12sRNA*. The identification of scorpions and determination of their relationships are based on the analysis of morphological characteristics and morphometric studies (Fet and Braunwalder, 2000). The most common parameters for species identification included total body, metasoma, carapace, pedipalp, and telson length, as well as pectinal tooth count for the gender discrimination of scorpions. The analysis of morphological findings and morphometric ratios showed that *A. crassicauda* samples of Khuzestan are similar to those isolated from Fars province, Iran (Ebrahimi et al., 2015) and Turkey (Ozkan et al., 2006). In the present study, the total and metasoma lengths of *A. crassicauda* were 80-100 and 43-45 mm, respectively. In addition, the mean total lengths of females and males were 89.75±14.56 and 82.78±5.38 mm, respectively. The total metasoma lengths were obtained as 35.58±2.98 and 42.39±2.74 mm in males and females, respectively. However, our samples were not significantly different from those of Fars province (Ebrahimi et al., 2015) and Turkey (Ozkan et al., 2006) in terms of other morphometric ratios. Scorpions, as living fossils, have highly conserved morphology. Despite the presence of evidence regarding the inappropriateness of morphological characteristics for the detection of species and subspecies, the result of nuclear and mitochondrial markers was used in solving these challenges (Gantenbein and Largiadèr, 2002; Vignoli et al., 2005; Salomone et al., 2007). Today, the molecular study and nucleotide sequences of these genes are used to characterize the phylogenetic tree and determine the degree of affinities among the species that have a high morphological similarity. Mirshamsi et al. (2010) showed that even with morphological similarities in *Mesobuthus eupeus* species, there was a clear genetic divergence between the northern and southern clades of Iran. Therefore, the phylogeographic structure of *M. eupeus* was proposed based on the geological history of the Iranian Plateau. There is a large gap in the recognition of the diversity, distribution, and evolution of this taxon since there are no available molecular data on the black fat tail *A. crassicuda* members of Buthidae family. The sequence analysis of *COXI* and *12sRNA* genes showed no significant genetic diversity among the *A. crassicuda* populations isolated from Khuzestan (0.001). In the phylogenetic tree, *A. crassicuda* from the northwest and southwest of Iran were monophyletic groups, which formed a sister group. They indicated a more closed relationship and showed that the genetic distance is correlated with geographical distance among the populations. However, Ozkan et al. (2010) identified two genetic groups with genetic variations in the 16S region of *A. crassicuda* in Urfa, Turkey. These findings may lead to the revision of the taxonomy of samples collected from this region. The phylogenetic study of other species of *Androctonus* showed that *A. crassicauda gonneti*, *A. australis,* and *A. amorexi* were clustered together, and their relationship was in accordance with the following equation:

Table 1. Morphometric measurements of male and female *Androctonus crassicauda*

Parameter	Male (mm)	Female (mm)
Ca_L/W	8.42±0.49 /8.01±0.61	8.95±1.07 /10.12±1.05
Met-I_L/W/H	5.80±1.75 /5.98±0.97 /5.37±0.90	5.51±0.61 /5.86±0.49 /5.21±0.65
Met-II_L/W/H	6.69±0.98 /6.59±1.11 /5.83±0.94	6.36±0.78 /6.49±0.10 /5.76±0.49
Met-III_L/W/H	7.19±1.19 /6.94±1.09 /6.38±1.10	6.84±0.49 /6.79±0.68 /6.10±0.59
Met-IV_L/W/H	7.78±1.08 /6.72±1.09 /6.24±0.97	7.39 ±1.08 /6.54±0.85 /6.20±0.57
Met-V_L/W/H	8.12±1.18 /5.92±0.97 /4.57±0.95	7.91±0.48 /5.69±1.09 /4.43±0.48
Telson L/W/H	8.59±1.31 /2.97±0.49 /2.92±0.38	8.38 ±0.45 /2.80±0.54 /2.76±0.18
Pedipalp L	14.29±0.87	15.07±1.94
Trochanter L/W	3.38±1.47 /2.53±0.18	3.24±0.59 /2.71±0.45
Femur L/W	1.83±0.76 /1.94±0.25	6.74±0.68 /2.09±0.34
Tibia L/W	8.49±0.74 /2.98±0.19	8.48±1.02 /2.72±0.48
Manus L/W	3.69±0.53 /3.09 ±0.45	3.75±0.52 /3.19±0.82
Number of pectinal teeth	32.28±0.75	26.45±0.76
Pecten L	8.42 ±1.15	7.48 ±1.09
Total L	82.78±5.38	89.75±14.56

(Statistically significant at P<0.05)
H: height, L: length, W: weight

Table 2. Average genetic distance of *Androctonus crassicuda* (Makoo, Iran), *A. australis* (Tunisia), and *A. amurexi* (Portugal)

		1	2	3	4	5	6	7
1	A.crassicauda (khuzestan)							
2	A.crassicaudaKU705365.1(Iran)	0.093						
3	A.crassicauda KT972133.1(Iran)	0.045	0.080					
4	A.crassicaud_KU705367.1(makoo)	0.104	0.026	0.056				
5	A.crassicaud KU705366.1 (urmia)	0.107	0.028	0.059	0.002			
6	A.australisKJ538404.1 (Tanisia)	0.164	0.267	0.180	0.271	0.267		
7	A.amoreuxiJQ423120.1_(portugal)	0.175	0.278	0.192	0.282	0.277	0.015	
8	A. cf. gonnetiKJ538379.1 (Morocco)	0.193	0.266	0.199	0.279	0.275	0.215	0.220

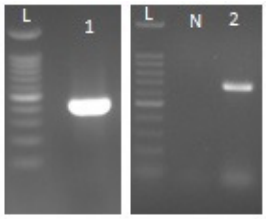

Figure 1. Polymerase chain reaction amplification with *12srRNA* (1) and *COXI* (2) genomes from *Androctonus crassicuda*
L: ladder, N: negative control

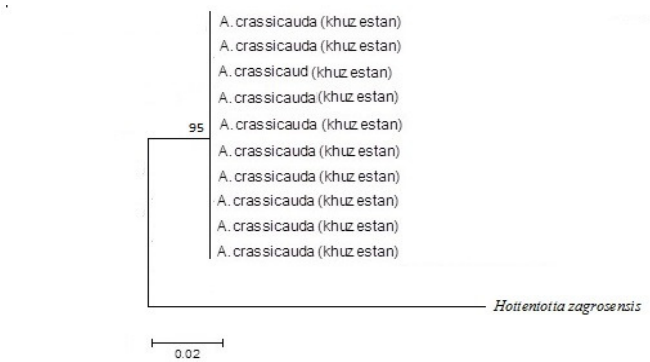

Figure 2. Maximum likelihood tree resulting from the analysis of the combined sequences of *COXI*, and *12sRNA* genes of *Androctonus crassicuda* from Khuzestan province and *Hottentotta zagrosensis* as an outgroup

(*A. australis* + *A. amorexi*) + *A. crassicauda gonneti*

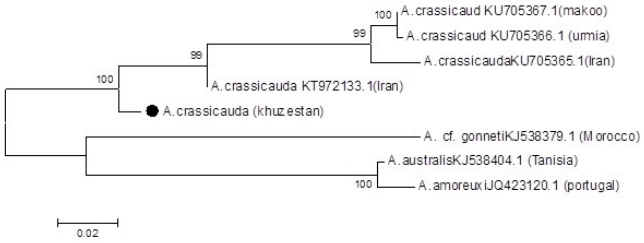

Figure 3. Maximum likelihood tree resulting from the analysis of sequences of *12SrRNA* genes from three genera of *Androctonus* species

This result supports the traditional morphological classification and clearly defines the genera of *Androctonus* species. The study of the morphology and phylogeny of this genus is highly important in the different parts of Iran due to the fact that scorpions have a high geographical distribution in Iran. Accordingly, they can be found in the fractures of stones and bricks, sandy areas, rocky deserts, semideserts, and inside houses (Navidpour, 2015). Another factor that highlights the results is that changes in environmental conditions lead to morphological changes to adapt to biological conditions (Gantenbein and Largiadèr, 2002; Salomone et al., 2007). If genetic variation exists, further studies should be carried out on scorpion toxin compounds to develop the efficiency of scorpion envenomation.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Authors' Contribution

Study concept and design: Jafari, H.
Acquisition of data: Jafari, H., Salabi, F.
Analysis and interpretation of data: Jafari, H., Salabi, F., Navidpour, Sh., Forouzan, A.
Drafting of the manuscript: Jafari, H.
Critical revision of the manuscript for important intellectual content: Jafari, H., Salabi, F.
Statistical analysis: Forouzan, A., Navidpour, Sh.
Administrative, technical, and material support: Jafari, H., Navidpour, Sh., Salabi, F., Forouzan, A.

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