Phylogenetic relationships of the scorpion *Apistobuthus susanae* from Khuzestan province based on mitochondrial DNA cytochrome oxidase I (COXI) gene sequences

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Abstract

This study aims to analyze the morphometric and phylogeny of *Apistobuthus susanae* scorpions using mitochondrial DNA sequences. Information regarding the morphology and biology of scorpions is very limited, and the foundation of this information is the identification of habitats, morphology, and morphometric of scorpions in different regions. In this study, the morphometric study based on 32 morphological characteristics of *A. susanae* and DNA sequencing of COXI was performed. Phylogenetic tree done by wing MEGA 10 software. Both male and female specimens of *A. susanae* were collected from four cities: Hamidiyeh, Masjed Soleyman, Ramhormoz, and Andimeshk in Khuzestan province. According to the results, in all cases, the average sizes of female specimens were larger than those of the male specimens. Except for the traits Cl, CHL, ML, TIW, MTIL, MTIILL, MTIVL, MTIIW, MTIVW, and MTIH, all other features studied were identical in both male and female species ($P > 0.05$). The results of the analysis of morphometric values of the *A. susanae* were compatible with the phylogenetic tree and supported the morphometric classification. Out of 614 nucleotides of the COXI gene amplified for 10 *Apistobuthus* samples and one sample of *Androctonus crassicauda* as outgroup, 558 sites were conserved (90.97%), 41 sites were variable (6.76%), and 15 sites (2.25%) had parsimony-informative sites. The analysis of the average genetic distance within species showed that two specimens from Hamidiyeh had the least divergence (0%) and two specimens from Ramhormoz and Andimeshk had the greatest inter-species genetic divergence (0.1%). The greatest intraspecific divergence was observed in the specimens from Andimeshk and Masjed Soleyman, while the least genetic divergence was found in the Ramhormoz samples.

Keywords: *Apistobuthus susanae*, Cytochrome oxidase gene, Iran, Phylogeny.

1. Introduction

Understanding the evolutionary origins of scorpions, one of the most important and unique groups of arthropods, has long been shrouded in mystery, with numerous disagreements existing on this topic. The Buthidae family of scorpions is the largest and most widespread scorpion family in the world (1). However, limited information is available regarding phylogenetic relationships within this family. The population composition of Iranian scorpions is one of the most diverse populations in the region of West Asia (2). On the other hand, morphotaxonomy is limited in its ability to accurately define species boundaries and distinguish between species. It is well-established that molecular data allow for understanding diversity, distribution, and intraspecific evolution in scorpions (3). According to the latest morphometric studies, there are 18 scorpion genera in Iran, belonging to three families: Buthidae, Scorpionidae, and Hemiscorpidae. Of these, 14 genera have been reported in Khuzestan province (4).

Apistobuthus susanae (Lourenço 1998), a scorpion species from the Buthidae family, is an invasive species commonly found in hot, dry, desert, and sandy regions, and is easily recognizable by its rounded second tail segment. This yellow-colored scorpion has a narrow tail and long legs with hair to facilitate movement over sand. The venom of this creature may have effects upon stinging, but the likelihood of death is very low (5).

Molecular data have provided much information regarding the phylogeographic histories of many animal species. The mitochondrial DNA cytochrome oxidase I (COXI) gene sequences are one of the most widely used molecular markers for species identification. This enzyme is key in aerobic metabolism. The evolution of this gene is so rapid that it can identify phylogenetic changes not only among closely related species but also within a specific species (6). Phylogenetic analysis using COXI gene sequences has been widely performed by various research groups across different animal groups, including the *Puntius sp.* genus in the Cyprinidae family (7), 42 Culicoides species (Diptera, Ceratopogonidae) across three continents (8). This gene is widely accepted as a DNA barcode for precise and easy species identification. In this study, mitochondrial DNA sequences were used for the phylogenetic analysis of *Apistobuthus susanae* scorpions.

2. Materials and Methods

2.1. Sample Collection

Scorpion specimens were collected from four cities Hamidiyeh, Masjed Soleyman, Ramhormoz, and Andimeshk located in Khuzestan province with a hot and dry climate used Geographic Information System (GIS) (Table 1, Figure 1). Since scorpions are nocturnal, sampling was done at night using ultraviolet light flashlights and long-handled forceps. The specimens were collected from the tail region and placed in containers containing ethanol. To preserve the specimens' appearance and prevent structural changes, 96% ethanol was injected into the scorpion's abdomen, and the specimens were stored in sealed glass containers containing 70% ethanol and 5% glycerin. A total of 8 male and 8 female specimens were

collected. Due to the scorpion's specific habitat in the psammophilic climate, there were limitations in sampling.This paper is based on the academic ethics of Department of Environmental Science and Desert Area Management Faculty of Engineering and Agriculture, Islamic Azad University, Arak Branch.

Table 1- Geographic coordinates of sampling sites

CITY	LONGITUDE	LATITUDE
HAMIDIYEH	31°26´56´´	48°30´17´´
MASJED SOLEYMAN	31°35´45´´	48°57´18´´
RAMHORMOZ	31°13´54´´	49°15´26´´
ANDIMESHK	31°21´44´´	48°37´36´´

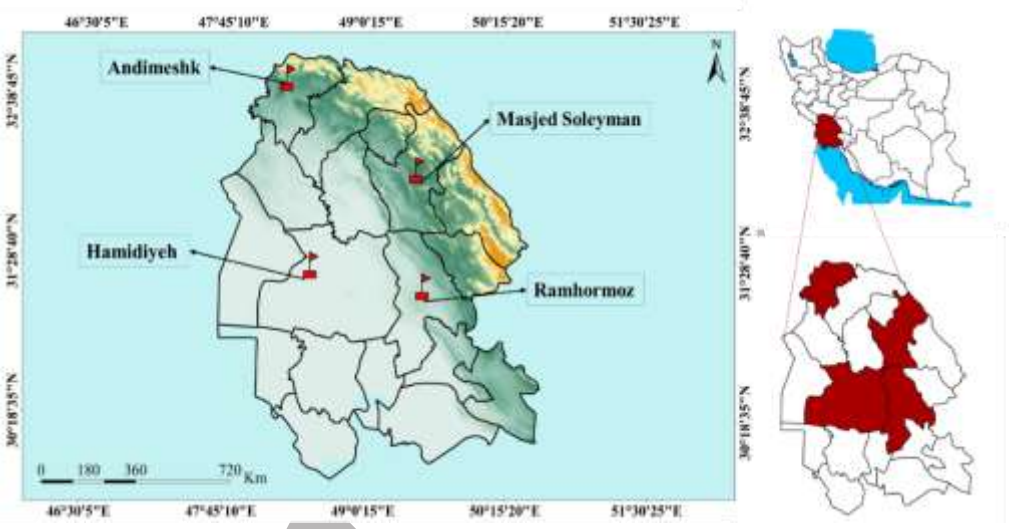

Figure 1- Geographic distribution of sampling points in Khuzestan province

2.2. Morphology

In the laboratory, each sample was placed separately on a grid paper, and morphological characteristics were examined using an Olympus SZ-CTV stereomicroscope, with the help of a caliper accurate to one-tenth of a centimeter. In this study, morphological and morphometric indices were calculated based on the indices defined by Lamoral (1979). A summary of the morphometric ratios is provided in Table 2. Statistical analysis of the data was performed in SPSS 23 using the Independent T-test, with a significance level of less than 0.05.

Table 2- Studied Morphometric Ratios

Morphometric Ratio	Description
CL/CAW	The length of the carapace to the width of the anterior part of the carapace
CL/CPW	The length of the carapace to the width of the posterior part of the carapace
CAW/PW	The width of the anterior section to the posterior section of the carapace
X/Y	The distance from the middle eye to the anterior and posterior edges of the carapace
Mt(I)l/W	The ratio of the length of segment I to the width of the segment
Mt(I)l/H	The ratio of the length of segment I to the height of the segmen
Mt(II)L/W	The ratio of the length of segment II to the width of the segment
Mt(II)L/H	The ratio of the length of segment II to the height of the segment
Mt(III)L/W	The ratio of the length of segment III to the width of the segment
Mt(IV)L/W	The ratio of the length of segment IV to the width of the segment
Mt(IV)L/H	The ratio of the length of segment IV to the height of the segment
Mt(V)L/W	The ratio of the length of segment V to the width of the segment
Mt(V)L/H	The ratio of the length of segment V to the height of the segment
CHL/ML	The length of the pincer to the length of the hand
MFL/ML	The length of the movable finger to the length of the hand
TIL/W	The length to the width of the telson

2.3. Molecular Analysis

2.3.1. Tissue Collection

2 grams of muscle tissue from the scorpion's tail region were collected using sterile tools and preserved in 90% ethanol. To extract DNA, the tissue was mixed with deionized water and transferred into 1.5 ml tubes. The tubes containing the samples were centrifuged at 2000 rpm for 10 minutes at 4°C. DNA was extracted from the pellet.

2.3.2. DNA Extraction

The pellet was mixed with 600 µl of RSB buffer and 60 µl of 10% SDS solution. It was then centrifuged at 1000 rpm for 5 minutes. The supernatant was transferred to a new microtube, 500 µl of chloroform was added, and the tube was centrifuged again at 10000 rpm for 5 minutes. The supernatant was transferred to a new microtube, and 10 µl of 5 M NaCl and 1200 µl of cold ethanol were added. After centrifuging at 10000 rpm for 5 minutes, the supernatant was discarded, and the pellet was dried. Then, 50 µl of sterile deionized water was added to the pellet.

2.2.3. Polymerase Chain Reaction (PCR)

PCR was performed to amplify the cytochrome oxidase I gene fragment using primers designed by Gantenbein et al. (1999) (9). The reaction was carried out in a final volume of 25 µl containing xPCR buffer, 1 mM dNTPs, 1.5 mM magnesium chloride, forward and reverse primers at 1 µM each, and 1 ng of Taq polymerase. The thermal program included an initial denaturation at 94°C for 1 minute, followed by 30 seconds at 94°C, 30 seconds at 50–60°C, and 50 seconds at 72°C for 30 cycles. The final extension was at 72°C for 5 minutes. The PCR product was visualized by electrophoresis on a 1% agarose gel, and sequencing was done with the specific primers.

2.3.4. Genetic Diversity Analysis

The obtained sequences were first reviewed and corrected manually using SeqScape software. Then, the sequences were aligned using the Clustalw algorithm in BioEdit software. The number of haplotypes, polymorphic sites, haplotype diversity, and nucleotide diversity were determined using DNAsp software and BLAST.

2.4. BLAST Analysis

The BLAST results from NCBI indicated that the obtained gene sequence from *Apistobuthus susanae* showed 100% similarity with *Apistobuthus pterygocercus* and 89-90% similarity with other sequences. The sample presented in the BLAST program for various base pairs with the same number and a total of 627 base pairs showed zero percent gap with complete similarity to the *Apistobuthus pterygocercus* scorpion sample (pairwise alignment (BLAST) of the input gene with the different gene sequences, their number, and the percentage of overlap, which is 100%). Gene alignment in the BLAST program for the most similar samples to the target sample, showing 100 gene lines, is displayed (the solid red lines represent complete alignment of the input gene with the available and aligned genes. The comparison is made between the input gene and the aligned genes).

2.5. Statistical Analysis

The means ± standard error were calculated, and univariate and multivariate statistical analyses were performed in SPSS Inc. Illinois, USA version 23 at a significance level of less than 0.05%.

3. Results

3.1. Morphology Result

The *Apistobuthus susanae* is typically yellowish, the prosoma (carapace), mesosoma, metasoma segments, and venter, is generally yellowish. The aculeus is blackish, and the pedipalps are reddish. There are also some dark longitudinal spots on the mesosoma. Considering that the color of the male specimens was darker than that of the female. The mean and standard deviation of each of the measured features by gender of the species are presented in Table 2. In all cases, the mean measurements of the female species were higher than those of the male species. Except for the traits CL, CHL, ML, TIW, MTIL, MTIILL, MTIVL, MTIIW, MTIVW, and MTIH, the other features examined were identical between the male and female species ($P > 0.05$).

Table 2: Comparison of the Mean Morphometric Traits of Male and Female *A. susanae* Scorpions Collected from Khuzestan Province

Morphometric variable	Female	Male	Significance level
Cl	10.31±1.64 [b]	7.18±0.27 [a]	P<0.05
cpw	8.90±0.75 [a]	7.54±0.70 [a]	P>0.05
Caw	5.47±0.52 [a]	4.87±0.16 [a]	P>0.05
X	5.20±1.06 [a]	3.78±0.12 [a]	P>0.05
Y	4.76± 0.72[a]	3.32±0.19 [a]	P>0.05
CHL	17.76±1.20 [b]	15.32±0.57 [a]	P<0.05
ML	5.38± 0.58 [b]	4.97±0.08 [a]	P<0.05
MFL	13.79±0.71 [a]	12.68±0.44 [a]	P>0.05
THL	8.41±0.41 [a]	7.80±0.34 [a]	P>0.05
TLH	2.95±0.24 [a]	2.45±0.16 [a]	P>0.05
TIW	2.53± 0.22 [b]	2.41±0.20 [a]	P<0.05
Mt(l)L	6.08±0.43 [b]	5.83±0.16 [a]	P<0.05
Mt(ll)L	7.26±0.51 [a]	6.79±0.16 [a]	P>0.05
Mt(llL)L	6.73±0.4 [b]	6.52±0.21[a]	P<0.05
Mt(IV)L	7.33±0.61 [b]	7.19±0.24 [b]	P<0.05
Mt(V)L	22.32±13.01 [a]	8.74±0.20 [a]	P>0.05
Mt(l)w	5.42±0.59 [a]	4.63±0.28 [a]	P<0.05
Mt(ll)w	7.19±0.65 [b]	6.75±0.36 [a]	P<0.05
Mt(llL)w	4.66±0.4 [a]	4.20±0.26 [a]	P>0.05
Mt(IV)w	3.33±0.31 [b]	3.13±0.16 [a]	P<0.05
Mt(V)w	3.34±0.28 [a]	3.06±0.16 [a]	P>0.05
Mt(l)H	3.58±0.37 [b]	2.77±0.18 [a]	P<0.05
Mt(ll)H	4.31±0.33 [a]	3.41±0.21 [a]	P>0.05
Mt(llL)H	3.75±0.28 [a]	3.02±0.15 [a]	P>0.05
Mt(IV)H	2.86±0.20 [a]	2.59±0.14 [a]	P>0.05
Mt(V)H	2.94±0.27 [a]	2.39±0.12 [a]	P>0.05

Non-matching letters indicate a significant difference between males and females ($P < 0.05$).

The difference in the dorsal and ventral views of the *A. susanae* scorpion between the male (A) and female (B) is shown in Figure 2.

Figure 2: Dorsal and ventral views of the male and female *A. susanae* scorpions collected from Khuzestan Province.

3.2. Molecular Results

To assess the polymerase chain reaction (PCR), the product obtained from the reaction was examined on an agarose gel. Samples indicating successful amplification of the target gene and optimal reaction conditions were selected for sequencing (Figure 3).

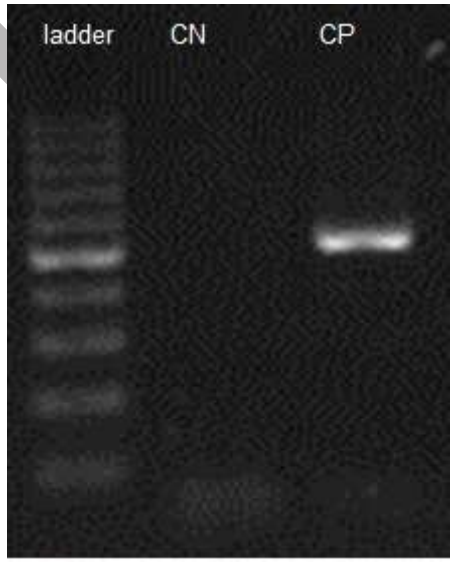

Figure 3- Electrophoresis of the PCR product for amplification of the Cytochrome Oxidase gene.

3.2.1. Phylogenetic Analysis of the *Apistobothus* Genus Based on COXI Gene Sequences

3.2.1.1. Sequence Characteristics and Divergence Rates

Among the 614 nucleotides of the amplified COXI gene from 10 *A. susanae* samples and one sample of *Androctonus crassicauda* as outgroup, 558 positions were conserved (97.90%), 41 positions were variable (6.76%), and 15 positions (2.52%) had informative content. The average intra-species genetic distance indicates that species 1 and 5, isolated from the Hamidiyeh region, had the lowest divergence (0%), suggesting they are genetically closer. The greatest inter-species genetic divergence (0.1%) was observed between species from the Ramhormoz (4) and Andimeshk (8) regions, indicating the largest genetic separation between these two species. Moreover, the average intra-species genetic distance across different regions shows that the greatest intra-species divergence was found in species from Andimeshk, while the samples from Masjed Soleyman and Ramhormoz had the least genetic divergence within the species (Table 2).

The nucleotide sequence numbers, number of nucleotides, haplotype counts, average nucleotide differences, nucleotide diversity, haplotype diversity, and the number of polymorphic sites for the 10 *A. susanae* species studied are summarized in Table 3.

Table 2- Average genetic distance between species and intra-species in the mitochondrial COXI gene of *A. susanae*.

Apisto1									
Apisto_2	0.002								
Apisto_3	0.002	0.002							
Apisto_4	0.003	0.003	0.002						
Apisto_5	0.000	0.002	0.002	0.003					
Apisto_6	0.005	0.005	0.003	0.005	0.005				
Apisto_7	0.002	0.002	0.000	0.002	0.002	0.003			
Apisto_8	0.008	0.008	0.008	0.010	0.008	0.008	0.008		
Apisto_9	0.002	0.002	0.000	0.002	0.002	0.003	0.000	0.008	
Apisto_10	0.002	0.002	0.000	0.002	0.002	0.003	0.000	0.008	0.000

Table 3- The number of nucleotide sequences, number of nucleotides, number of haplotypes, average nucleotide difference, nucleotide diversity, haplotype diversity, and the number of polymorphic sites within species in the ten *A. susanae*

Apistobothus susanae	
Number of nucleotide sequences	5
Number of nucleotides	614
Number of haplotypes	4
Average nucleotide difference	2.700
Nucleotide diversity	0.00897
Number of separating sites (polymorphic sites)	7

Haplotype diversity	0.900

3.2.2. Maximum Likelihood Intra-Species Phylogenetic Tree Analysis

In this study, using MEGA10 software and the Maximum Composite Likelihood model, a phylogenetic tree of *A. susanae* samples from Khuzestan province was constructed (Figure 4).

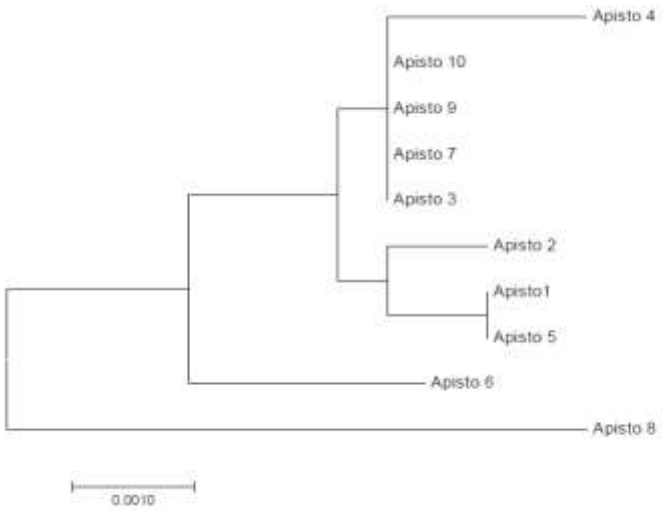

Figure 4- 50% Majority-rule Consensus Maximum Likelihood tree based on the COXI gene.

3.2.3. Phylogenetic Tree of the Buthidae Family Based on Nucleotide Alignment of the COXI Gene

Based on the analysis of COXI gene sequences of *A. susanae* scorpions collected from Khuzestan province which was registered in the Gene Bank with accession number (PV931979) and sequence alignment with sequences from the gene bank, the constructed phylogenetic tree shows that *Apistobothus sp.* from Khuzestan, together with *Apistobothus pterygocercus*, formed a sub-clade (0% genetic distance). These species were grouped with *Androctonus australis*, *Androctonus amoreuxi*, and *Androctonus bicolor* in a clade. Additionally, the genetic distance of each species compared to the others is shown in Figure 5.

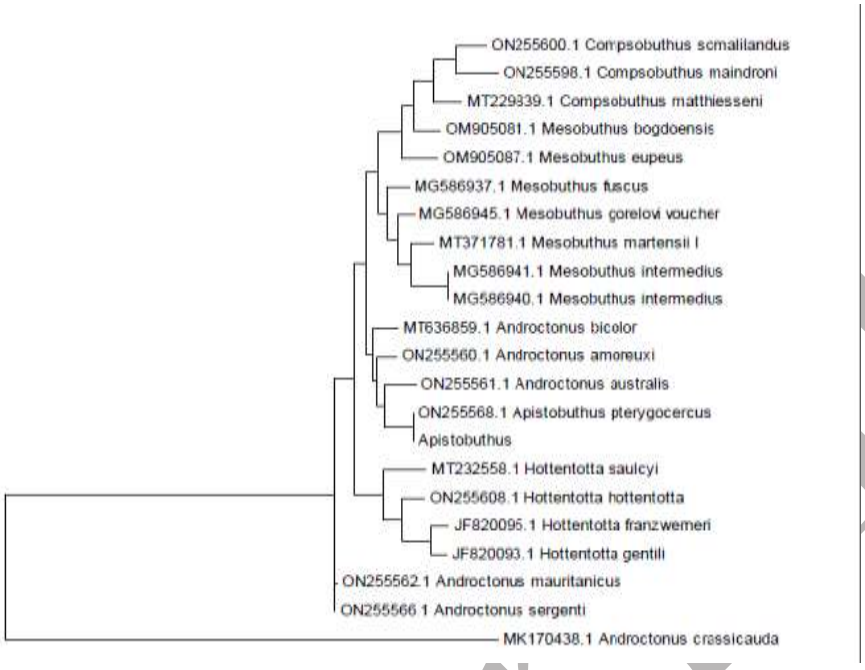

Figure 5- Genetic distance of each species compared to the other species studied.

4. Discussion

Despite extensive efforts in identifying scorpions in Iran, the information regarding their biology and morphological differences is still very limited and perhaps incomplete. In particular, data on sexual dimorphism, the expression of sexual behaviors, and the recognition of intraspecific differences are of special importance (Lourenco, 1999). Behavioral and ecomorphological adaptation has led scorpions to adjust to specific climatic conditions. In scorpions, except for a few species that exhibit sexual dimorphism, there is no significant difference between males and females due to the absence of distinct sexual organs in other species (10).

Based on the results obtained, the body length in females was significantly larger than in males, which is related to the increase in the size of the abdomen and the enlargement of the reproductive system due to the formation of embryos and the increase in the number of offspring born. Amitai (1980) reported that male bodies are narrower than those of females, which is consistent with the present study. On the other hand, the larger carapace length in females is due to the positioning of the offspring on the mother's back (12). According to the results, carapace length, chela length, and segment length played the most significant role in distinguishing males and females. In Lane's study (1996), as in the present study, the segments of the tail were used to determine sex, with males being differentiated from females (13). In Dehghani and Tirgari's study (2012), the size of the fifth abdominal segment and the number and length of the pedipalps were used to differentiate males and females, with the fifth abdominal segment size being larger in females compared to males at the dorsal surface (14).

Levy and Amitai (1980) reported that male bodies are narrower than those of females (11) Additionally, in studies such as that of NematElahi et al. (2018), other indices like pectinal length, the number of pectinal teeth, and the distance between the two bases of the pectines were used to determine sex in the species *Hottentotta saulcyi*, and they stated that the best time for sex determination is during mating (16). Booncham et al. (2007) reported that in *Heterometrus laoticus*, a wider carapace and a larger seventh mesosomal segment were key factors for distinguishing males from females. These differences can be used to better differentiate sex in future studies of *A. susanae.*

The COXI gene is a mitochondrial gene that is maternally inherited, and because it does not undergo recombination, it makes it easier to trace the maternal lineage of animal species (18). Mitochondrial genes exhibit higher mutation rates compared to nuclear genes, and their evolutionary changes occur at a faster rate. Due to the high species diversity of scorpions in Khuzestan province, scorpion stings and fatalities caused by them are of significant concern in the region. The present study investigates the phylogeny based on DNA sequences of the Cytochrome Oxidase I (COXI) gene of *Apistobuthus susanae* scorpions, an invasive species collected from four regions of Khuzestan province in southwestern Iran. The findings of this study indicate divergence between the species from Masjed Soleyman and Andimeshk. The genetic diversity observed between the populations of the Ramhormoz and Andimeshk regions can be attributed to the differing climatic conditions (temperature, relative humidity) between the two regions.

Additionally, this scorpion species was found to be closely related to *Androctonus sp.* species, as they all cluster in the same clade, suggesting a close genetic relationship between these two genera. Studies by Billington and Hebert (1991) and Quijano-Ravell et al. (2019) in Mexico, which investigated new species within the *Centruroides sp.* (Buthidae family) using mitochondrial 16S rRNA gene sequences, used a genetic distance of around 10% between species as a criterion to confirm a new species. In the current study, however, the maximum genetic divergence observed was 0.1%, which suggests that all identified scorpions, including *Apistobuthus susanae* identified in the gene bank, belong to the same species (19, 20).

In the study by Pirmoradi et al. (2023), which investigated the genetic diversity of scorpions in *Hottentotta sp.* in Khuzestan Province using cytochrome c oxidase subunit I (COXI) and 12sRNA, it was reported that based on 12sRNA, all *H. saulcyi* species (HS4, HS6, and HS7) except for species HS5 grouped in Cluster B, while two species of H. Zagrosensis (HZ6 and HZ1) were placed in Cluster A. Additionally, Jolodar et al., (2023) in their study on the genetic diversity of the genus Scorpio based on 16sRNA sequencing, constructed a phylogenetic tree with two clusters (A and B), which were closely related to *S. maurus*. Both studies indicate that gene flow is influenced by geographic barriers, which is consistent with intraspecific divergence observed in the present study. In fact, the separation of species occurs due to distance and the presence of natural barriers. Therefore, geographic factors affect gene flow and genetic diversity, leading to species divergence (3, 21).

The results of this study show that genetically, *Apistobuthus susanae* scorpions from the Masjed Soleyman and Andimeshk regions exhibit divergence.

Acknowledgment
The authors express their gratitude to the Razi Vaccine and Serum Research Institute, Southwest Branch in Ahvaz and the Karaj Branch, as well as Dr. Alireza Foruzan, for their valuable support and guidance in conducting this research. We also sincerely thank the esteemed reviewers who contributed to the completion of this study.

Ethic
This paper is based on the academic ethics of Department of Environmental Science and Desert Area Management Faculty of Engineering and Agriculture, Islamic Azad University, Arak Branch.

Author's contribution
Study concept and design: SHE.B and H.J, Acquisition of data: SHE.B, B.S, A.A and H.J, Analysis and interpretation of data: SHE.B, B.S, A.A and H.J, Drafting of the manuscript: SHE.B, B.S, A.A, Statistical analysis: SHE.B, A.A and H. J. Administrative, technical, and material support: SHE.B, B.S.

Conflict of interest
The authors declare no conflict of interest.

Data availability
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Funding
This work is based upon research funded by the Engineering and Agriculture, Islamic Azad University, Arak.

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