Original Article

Genetic Diversity within *Scorpio* Genus (Scorpion: Scorpionidae) from Iran: Preliminary Evidence based on 16srRNA Sequence

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ABSTRACT

The yellow digger scorpion, *Scorpio maurus*, is a medically important scorpion for which little is known about its genetic diversity. Polymerase chain reaction products of 16srRNA gene fragments were generated from scorpion specimens named SmKh1 and SmKh2. These sequences showed high similarity with the only partial sequence of *S. maurus* isolate SCA1 large subunit ribosomal RNA gene available in the Genbank database. The drawing of the phylogeny tree showed two clusters, A and B. The two specimens (SmKh1 and SmKh2), which are placed in sub-cluster A2, were provided from Behbahan, Iran, and they have the closest relationship with the only sequence of *S. maurus* (MW281771), which is also collected from Behbahan. It is noteworthy that the two sequences obtained from *S. maurus* scorpions recorded from Miandoab (MK170444) and Mahabad (KU705354), which are in sub-cluster A1, are more similar to the scorpions isolated from the Mediterranean basin than those collected from Behbahan. This issue is probably due to the fact that patterns of genetic diversity are a reflection of variation in gene flow, which is also influenced by factors such as territorial barriers and geographical distances. We conclude that the scorpions of this study accompanied by similar scorpions in the Mediterranean basin, belong to the same species despite the insignificant differences.

Keywords: 16srRNA, Phylogeny tree, *Scorpio maurus*

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1. Introduction

The order Scorpiones includes 1,259 described species that are placed in 16 families and 155 genera (1). Iranian scorpions are considered one of the most diverse populations in the region of Southwest Asia. Therefore, conducting various studies to identify the species in the region is important.

Scorpio maurus L., 1758, is a small to medium-sized scorpion that belongs to the *Scorpionidae* family. It is found in countries such as Morocco across Northern Africa, through the Arabian Peninsula and the Middle East, including Israel, Syria, Jordan, and Turkey, to as far East as Iraq and Iran. This scorpion is also known as "yellow digger scorpion" or "broad fork scorpion". Most species of *S. maurus* have a yellow to red-brown color. Dark brown to black color has also been reported in some subspecies. This type of scorpion is mostly found in desert and dry areas, although it has also been observed in thin forests. This scorpion is a digger and digs different holes based on the type of habitat. Birula first examined the entire population of *S. maurus* in North Africa and the Middle East. His research showed that most types of *S. maurus* are subspecies; however, he classified them all into two large groups: sectio *maurus* and sectio *propinquus*. There are many subspecies of this scorpion, 19 of which were described by Birula (2).

Classifying arthropods based on morphological characteristics has been used for many years. However, the studies using the sequence of mitochondrial 12S and 16S ribosomal DNA, cytochrome oxidase subunit 1 and nuclear 28S ribosomal DNA, along with the use of morphological characteristics, are considered to be among the best lineage identification studies in scorpions (3-5). Among the macromolecules used for phylogenic studies, 16srRNA is the most useful for establishing phylogenic distances. Ribosomal RNA genes are essential for the survival of all organisms. Therefore, the nucleotides in these genes are highly protected and have a slower evolution rate than other genes. Since this gene has the most genetic information, conservative nature, and universal distribution, it is used as a standard method for the identification and taxonomy of the border between species (6). Species diversity has been studied based on morphological characteristics and the structure of dug nests in *S. maurus palmatus* population in different geographical areas in Egypt (7). The first preliminary survey of *S. maurus* scorpion population in Turkey using a part of the cytochrome oxidase gene showed the complexity of this species (8). A preliminary analysis based on the cytochrome oxidase gene in scorpions of the genus *Scorpio* in Morocco showed a high level of genetic diversity in association with geographic cohesion (9). Using three mitochondrial markers, six species of medically important Iranian scorpions, including *S. maurus*, were surveyed (10).

The lack of systematic studies on *S. maurus* in Iran has made its taxonomic status and geographical distribution unclear. In this study, the taxonomic status of this scorpion was determined based on the sequence of the 16srRNA gene fragment.

2. Materials and Methods

2.1. Collection of scorpion specimens

Specimens of *S. maurus* were collected overnight using ultraviolet light (UV) from Behbahan, located east of Khuzestan Province, which is considered a habitat for this type of scorpion. Three scorpion specimens from Behbahan were recorded along with the longitude and latitude of the sampling areas using a location tracking device called Global Positioning System (GPS).

2.2. Sampling and morphological measurements

Morphological characteristics, including color, pedipalp, prosoma, metasoma, and trichobothria patterns, have been studied using a stereomicroscope (Leica MZ 7.5, Germany). The morphological measurements were taken using digital calipers.

2.3. Genomic DNA extraction

To extract genomic DNA, 0.1-0.5 g of scorpion foot tissue was crushed in the presence of liquid nitrogen; then, 600 µl of resuspension buffer (10 mM

Tris-HCl pH 7.4/10 mM NaCl/25 mM ethylenediaminetetraacetic acid 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 3M of sodium acetate.

2.4. Polymerase chain reaction (PCR) amplification and sequencing

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 mM), *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'-CGATTTGAACTCAGATCA and ITSR 5'-GTGCAAAGGTAGCATAATCA (15). The amplified PCR products were subjected to electrophoresis 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 the performance of DNA sequencing according to the dideoxy termination method using an automated Applied Biosystems 373 DNA Sequencer.

2.5. 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. The alignments of multiple sequences were obtained using the CLUSTAL_W program (11). Genetic distance and phylogenetic tree were performed using the Neighbor-Joining method with p-distance value via 1,000 replicates of bootstrapping using the MEG7 software (12).

3. Results

3.1. Collection of scorpion specimens and morphology

Specimens of *S. maurus* were collected at night using ultraviolet light from Behbahan, located in the east of Khuzestan province, with longitude and latitude 50°12'17"E and 30°14'46"N, respectively. Behbahan, the place where the specimens were collected, is shown on the map (Figure 1). After transferring the specimens to the lab, they were identified using morphological identification keys (13-14). The maximum size of these scorpions reaches 6.5 cm in maturity. The color of the body is yellow to pale brown and can be seen with bright pincers and legs. The tail is shorter than the body and covered with hairs. In the first segment of the tail, the width is greater than the length, but gradually, the length of the segments increases (Table 1).

3.2. DNA extraction

Five µg of genomic DNA was extracted from 0.5 g of foot tissue. Using spectrophotometer, the quantity of SmKh1 and SmKh2 samples were estimated to be 650 and 820 nanograms, respectively. To determine the quality of DNA and its contamination with proteins, an absorbance ratio of 260/280 was calculated, which was 1.73. Moreover, its quality was also confirmed by running the DNA extracted on 1% agarose gel.

3.3. Amplification and sequence analysis

During the PCR reaction, a 16srRNA gene fragment of approximately 410 bp was generated. 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 negative control samples where template DNA and enzyme were excluded (Figure 2).

After DNA sequencing of the 16srRNA gene fragments by aligning the forward and reverse strands, the complete sequence was determined by finding overlapping regions. In nucleotide sequencing, often about twenty nucleotides at the beginning of each

Figure 1. Collection site of the *S. maurus* scorpions. The specimens from Behbahan is shown on the map along with the location of two reference sequences with accession numbers MH170444 and KU705354 that were isolated from Miandoab and Mahabad, respectively

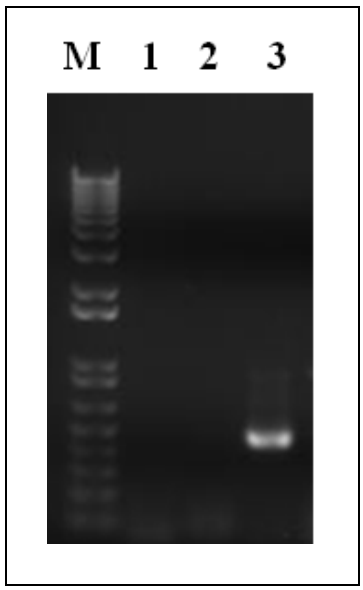

Figure 2. Agarose gel electrophoresis of 16srRNA (SmKh) PCR amplification. Lane M: 100DNA size marker, Line 1 and 2 were negative controls, which lacked DNA and enzyme, respectively. Lane 3: PCR amplification products

sequenced strand cannot be read due to binding the primers. After trimming and removing the binding site of the primers, two sequences of SmKh1 and SmKh2 were identified with 381 and 380 nucleotides, respectively. The two studied sequences, SmKh1 and SmKh2, were used in the next step for alignment and phylogeny. Two nucleotide sequences obtained from *S. maurus* specimens named SmKh1 and SmKh2 were compared using Clastal_w program. In this alignment, it was observed that the two sequences are similar with 99.7% similarity and E-Value equal to 6.6e-51. The SmKh1 sequence has an extra T nucleotide at position 63 (Figure 3).

To compare the nucleotide sequence of SmKh1 and

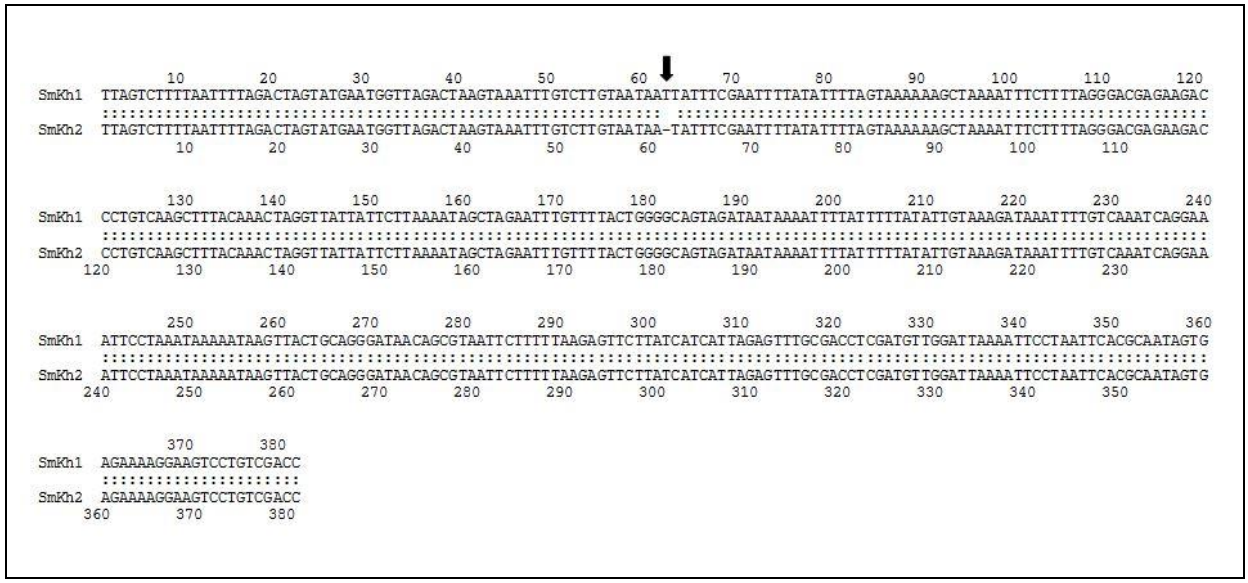

Figure 3. SmKh1 and SmKh2 sequence alignment. The location of an additional T at position 63 of the SmKh1 gene fragment is indicated by an arrow

SmKh2 with the sequences available in the Genbank database, the BLASTn program available in the NCBI website was applied using the "Somewhat similar sequences" program. These two sequences showed 100% homology with the only partial sequence of *S. maurus* isolate SCA1 large subunit ribosomal RNA gene (MW281771).

The nucleotide sequence of SmKh1 and SmKh2 showed similarity with 116 nucleotide sequences from the family *Scorpionidae*, of which 49 sequences belonged to the genus *Scorpio*. It was also observed that these sequences are similar to the sequences of 6 species of scorpions, namely *S. maurus, S. fuscus, S. kruglovi, S. palmatus, S. propinquus, and S. fuliginosus.* It is worth noting that *S. palmatus*, with 19 sequences has the most similarity in this family, while only three sequences were related to *S. maurus* species.

3.4. Phylogenetic analysis

The phylogenic tree was drawn using two nucleotide sequence of the 16srRNA gene fragment obtained from *S. maurus* Behbahan scorpions (SmKh1 and SmKh2). For this purpose, after aligning with similar sequences from the Genbank database, a phylogenetic tree was drawn in comparison with the only two sequences of this scorpion species available in the Genbank database. These two sequences with accession numbers MH170444 and KU705354 were isolated from Miandoab and Mahabad, respectively. The relevant sequences were also retrieved from NCBI GenBank using BLAST program. Then, the studied sequences of *S. maurus* from Behbahan, along with the related sequences, were aligned using MEGA7 software after sorting in FASTA format. The results showed that the phylogenetic tree has two clusters, A and B (Figure 4). The sequences in these two clusters showed 59% similarity. Cluster A is also divided into two sub-clusters, A1 and A2. In sub-cluster A2, SmKh1 and SmKh2 showed 99% similarity with the only sequence registered in the Genbank, which was also isolated from Behbahan (MW281771). It is noteworthy that the two sequences obtained from *S. maurus* scorpions recorded from Miandoab (MK170444) and Mahabad (KU705354) which are located in sub-cluster A1, are more similar to the scorpions isolated from the Mediterranean basin (*S. kruglovi*; KT188203 *and S. fuscus*; KT188192) than the studied scorpions from Behbahan (SmKh1

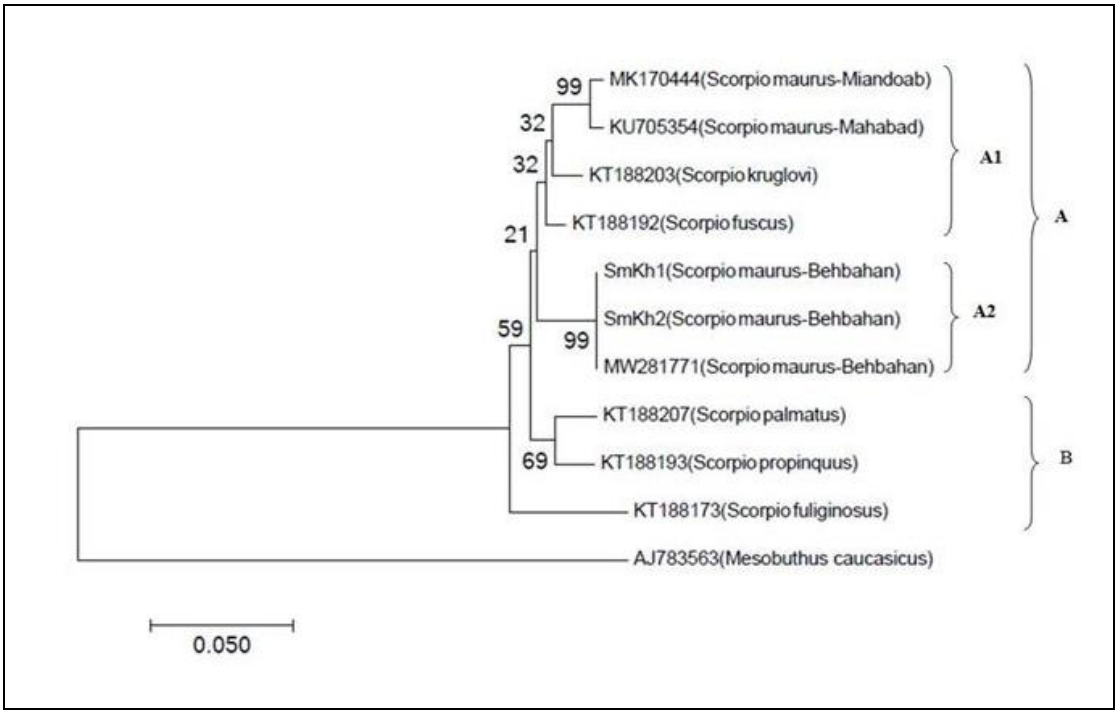

Figure 4. Phylogenetic tree of *S. maurus* scorpions from Behbahan and similar sequences of this species. This tree is based on the sequence of 16srRNA gene fragment using Neighbor -joining analysis. Bootstrap numbers are based on 1050 replicates. The numbers in front of the species are the accession numbers of the related genes in the Genbank. The numbers above the lines indicate the relationship between the groups

and SmKh2). *Mesobuthus caucasicus* scorpion with accession number AJ83563 was used as out group, which was completely isolated from the rest of the sequences.

Phylogenetic tree of *S. maurus* scorpions from Behbahan compared to related scorpion sequences showed two clusters, A and B. All sequences of *S. maurus* scorpion species with SmKh1 and SmKh2 sequences, were placed in cluster B. The two scorpions in this study from Behbahan showed the highest similarity with the only scorpion *S. maurus*, recorded in the GenBank from Behbahan (MW281771). These three samples had the closest similarity (99%) with scorpions *S. Fuliginosus* (KT188173), *S. Palmatus* (KT188207), and *S. propinquus* (KT188193). While all the scorpions in cluster B belong to the genus *Scorpio*, there is no scorpion of this genus in cluster A (Figure 5).

3.5. Genetic distances

The genetic distance of *S. maurus* scorpions from Behbahan was compared with the other scorpions of this genus using MEGA7 software. According to Table 2, the percentage of genetic difference between these sequences was estimated between 0.9-7.4%. Since the genetic distance of about 10% between the species has been used as a criterion to confirm the new species (15), it can be mentioned that all the studied specimens (Smkh1 and Smkh2) are in the same species. The outgroup scorpion specimen (*Mesobuthus caucasicus* with accession number AJ83563) showed a significant difference with the rest of the specimens (between 36.6-38.4%).

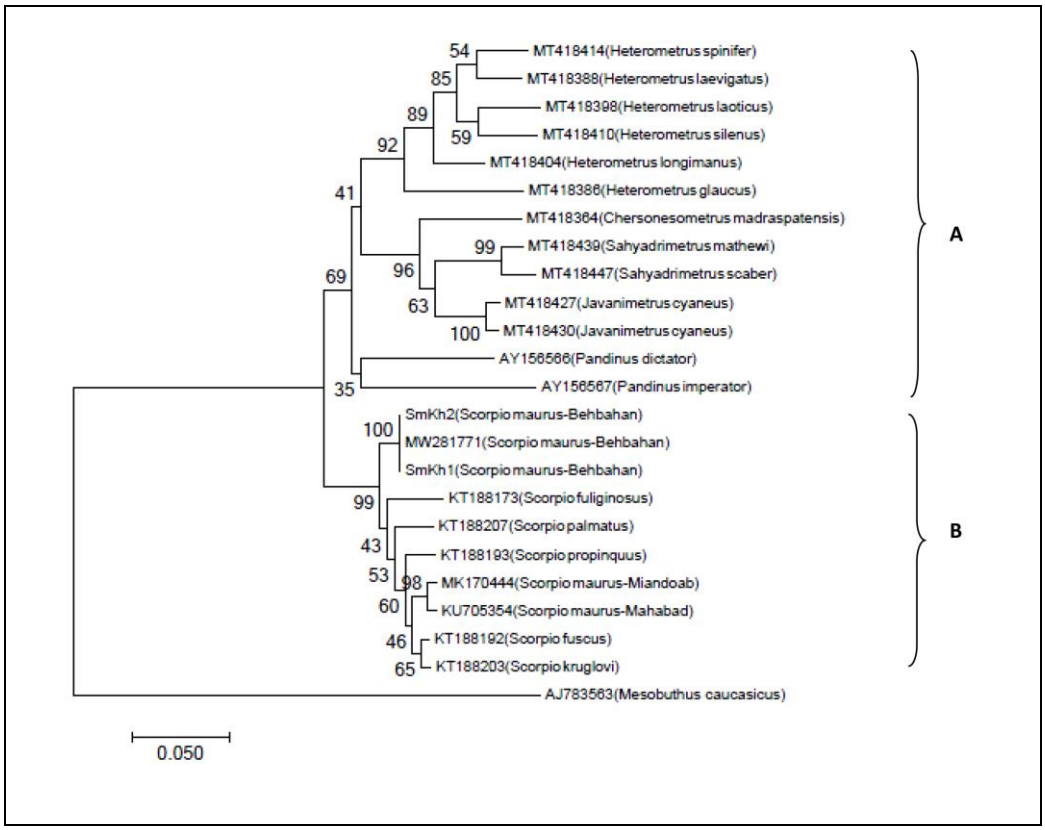

Figure 5. Phylogenetic tree of *S. maurus* scorpions from Behbahan compared to the related scorpion sequences. This tree is based on the sequence of 16srRNA gene fragment using neighbor-*joining* analysis. Bootstrap numbers are based on 1050 replicates. The numbers next to the branches are the percentage of repetition in the bootstrap test. The numbers in front of the species are the accession numbers of the related genes in the Genbank. The numbers above the lines indicate the relationship between the groups

Table 1. Taxonomy of scorpion *S. maurus* from Behbahan in Khuzestan based on BLASTn 16srRNA gene fragment

Taxonomy	Number of hits	Number of Organisms	Taxonomy
Scorpionidae	116	27	
. Scorpio	49	6	
. . Scorpio maurus	3	1	Scorpio maurus hits
. . Scorpio fuscus	18	1	Scorpio fuscus hits
. . Scorpio kruglovi	3	1	Scorpio kruglovi hits
. . Scorpio palmatus	19	1	Scorpio palmatus hits
. . Scorpio propinquus	4	1	Scorpio propinquus hits
. . Scorpio fuliginosus	2	1	Scorpio fuliginosus hits
. Heterometrinae	64	18	
. . Heterometrus	43	8	
. . . Heterometrus petersii	2	1	Heterometrus petersii hits
. . . Heterometrus longimanus	6	1	Heterometrus longimanus hits
. . . Heterometrus spinifer	7	1	Heterometrus spinifer
. . . Heterometrus silenus	5	1	Heterometrus silenus
. . . Heterometrus laoticus	8	1	Heterometrus laoticus
. . . Heterometrus thorellii	8	1	Heterometrus thorellii
. . . Heterometrus laevigatus	5	1	Heterometrus laevigatus
. . . Heterometrus glaucus	2	1	Heterometrus glaucus
. . Sahyadrimetrus	7	3	
. . . Sahyadrimetrus kanarensis	3	1	Sahyadrimetrus kanarensis
. . . Sahyadrimetrus scaber	2	1	Sahyadrimetrus scaber
. . . Sahyadrimetrus mathewi	2	1	Sahyadrimetrus mathewi

. . Deccanometrus	4	2	
. . . Deccanometrus bengalensis	1	1	Deccanometrus bengalensis
. . . Deccanometrus xanthopus	3	1	Deccanometrus xanthopus
. . Chersonesometrus	6	4	
. . . Chersonesometrus beccaloniae	1	1	Chersonesometrus beccaloniae
. . . Chersonesometrus tristis	3	1	Chersonesometrus tristis
. . . Chersonesometrus fulvipes	1	1	Chersonesometrus fulvipes
. . . Chersonesometrus madraspatensis	1	1	Chersonesometrus madraspatensis
. . Javanimetrus cyaneus	4	1	Javanimetrus cyaneus
. Pandinus	3	3	
. . Pandinus dictator	1	1	Pandinus dictator
. . Pandinus imperator	1	1	Pandinus imperator
. . Pandinus cavimanus	1	1	Pandinus cavimanus

Table 2. The genetic pairwise distances of scorpion *S. maurus* from Behbahan in Khuzestan, compared to the related scorpions based on 16srRNA gene fragment

Specimens	1	2	3	4	5	6	7	8	9	10	11
1. SmKh1(Behbahan)											
2.SmKh2(Behbahan)	0.000										
3.MW281771(S.maurus-Behbahan)	0.000	0.000									
4.MK170444(S.maurus-Miandoab)	0.046	0.046	0.046								
5.KU705354(S.maurus-Mahabad)	0.046	0.046	0.046	0.009							
6.KT188192(S.fuscus)	0.028	0.028	0.028	0.028	0.028						
7.KT188203(S.kruglovi)	0.037	0.037	0.037	0.028	0.028	0.019					
8.KT188207(S.palmatus)	0.046	0.046	0.046	0.046	0.046	0.037	0.046				
9.KT188193(S.propinquus)	0.051	0.051	0.051	0.037	0.037	0.032	0.042	0.028			
10.KT188173(S.fuliginosus)	0.069	0.069	0.069	0.074	0.074	0.069	0.065	0.065	0.069		
11.AJ783563(M.caucasicus)	0.370	0.370	0.370	0.370	0.370	0.366	0.370	0.375	0.380	0.384	

4. Discussion

Based on the results, it was shown that SmKh1 and SmKh2 in cluster A2 have the closest relationship (99%) with the only recorded scorpion *S. maurus* (MW281771) available in the Genbank database, which is also collected from Behbahan. This means that all scorpion specimens in the sub-cluster A2 belong to the species *S. maurus*. Two *S. maurus* sequences recorded in the Genbank database from Miandoab (MK170444) and Mahabad (KU705354) were placed under sub-cluster A1 next to *S. kruglovi* and *S. fuscus*. Despite some genetic diversity observed among *S. maurus* scorpion specimens in both sub-cluster A1 and A2, they all belong to the same species. The important point is that the two sequences of *S. maurus* isolated from Mahabad and Miandoab (sub-cluster A2) were more similar to the scorpions of the Mediterranean basin than to the two specimens studied from Behbahan (SmKh1 and SmKh2). Moreover, the genetic distance results in Table 2 revealed that the genetic difference are between 0-7.4%. Therefore, it can be concluded that the differences among specimens of *Scorpio* genus in Table 2 are not significant. This issue is probably due to the fact that patterns of genetic diversity often reflect territorial barriers and geographical distances that affect gene flow. In fact, the phenomenon of gene flow and patterns of genetic diversity are mainly applied according to factors such as barriers in the territorial landscape and geographical distances (16). As can be seen, SmKh1 and SmKh2, were collected from Behbahan, one of the relatively low-altitude and plain areas. These two sequences were slightly different from the sequences obtained from similar scorpion reference from Miandoab (MK170444) and Mahabad (KU705354), which are part of the mountainous regions. In fact, these two reference scorpions have the most genetic similarity to the scorpions of the Mediterranean basin and to a lesser extent to the scorpions collected from Behbahan (Smkh1 and Smkh2), located at a lower altitude. The separation of species occurs due to distance and the occurrence of natural barriers; therefore, the territorial factor is effective on gene flow and genetic diversity that leads to

the separation of species. In addition, it has been shown that the different species of scorpions in the genus *Scorpio* are mainly subspecies, and the differences are generally intra-species (2). In line with this claim, our results show that not only all the *S.maurus* scorpions of this study can be placed in one species, but all the Mediterranean scorpions listed in Table 2 can also be placed in the same species, despite the existence of insignificant differences.

We conclude that the scorpions of this study, accompanied with similar scorpions in the Mediterranean basin, belong to the same species despite the insignificant differences.

Authors' Contribution

Study concept and design: A.J and H.J, Acquisition of data: A.J, B.P and H.J, Analysis and interpretation of data: A.J., B.P and H.J, Drafting of the manuscript: A.J and H.J, Statistical analysis: A. J, B.P and H. J.

Administrative, technical, and material support: A.J, B.P and H.J

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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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