Original Article

Genetic Variability of *Hemiscorpius lepturus* in Khuzestan Province, Iran, Using ISSR-PCR and Mitochondrial Cytochrome C Oxidase Subunit I Gene Sequencing

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ABSTRACT

Background: Scorpion stings pose a serious public health concern, particularly in Khuzestan Province, Iran. The genus *Hemiscorpius* scorpions are a major cause of scorpion stings and related deaths, mostly in children.

Objectives: This study aimed to study the potential intraspecific variability of *Hemiscorpius lepturus* in Khuzestan.

Methods: We used inter-simple sequence repeat anchored-polymerase chain reaction (ISSR-PCR) and cytochrome c oxidase subunit 1 (COI) to study potential intraspecies variability of this scorpion. Twenty-two specimens of *H. lepturus* scorpions were collected from 5 geographically distinct regions of Khuzestan. Genomic DNA was extracted using the phenol/chloroform method. For phylogenetic analysis, target gene fragments were amplified using ISSR-PCR. By agarose gel electrophoresis of the PCR products, bands produced in each specimen were categorized using a zero and one system, and a dendrogram was drawn using the UPGMA algorithm.

Results: ISSR-PCR generated 5 bands ranging from 0.9 to 2.5 kb. The results showed that the specimen Hl4Ch was clearly different within its group. To validate these findings, a 637-nucleotide fragment of the *COI* gene was amplified and sequenced from 5 genetically variable specimens. Out of 5 sequences, Hl1Ba and Hl6Be have a relatively close relationship (57%) with the *H. lepturus* reference sequence (KU341987). However, Hl4Ch was placed with a relatively high distance (72%) from the rest of them, next to the other reference sequence *Hemiscorpius* sp. (OP433762.1).

Conclusion: Although the scorpions of each region were mostly placed together in the phylogeny tree, no major genetic diversity related to regional differences was observed in the province. Based on the genetic distance of Hl4Ch from other sequences (12.5%), it is definitely an intraspecies variation.

Keywords: Dendrogram, Inter simple sequence repeat (ISSR), Khuzestan, Phylogeny, Scorpion

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Introduction

Over the last two decades, the number of scorpion species, genera, and families worldwide has changed, with nearly 2231 species now described. To date, 2231 scorpion species have been classified into 208 genera and 20 families (Shahbazzadeh et al., 2003). Iran has one of the most diverse scorpion faunas in West Asia. Therefore, it is one of the best areas for studying scorpions (Dehghani et al., 2016). Despite this high species diversity, the scorpion fauna and geography have not been fully studied due to the vast size of the country. According to the latest review of scorpion classification in Iran, there are three families: Buthidae (51 species), Scorpionidae (3 species), and Hemiscorpiidae (6 species), with representatives from different parts of the country reported (Dehghani et al., 2016).

Among the existing scorpions of Khuzestan, the yellow scorpion called Gadim, with a scientific name of *Hemiscorpius lepturus*, is one of the most common and dangerous scorpions in Iran. This scorpion belongs to the family Hemiscorpiidae. Three species of this genus, *Hemiscorpius gaillardi*, *H. lepturus*, and *Hemiscorpius persicus*, have been reported in Iran. The distribution of *H. lepturus* scorpion appears to be limited to Iraq and the western and southwestern regions of Iran. However, the distribution area of this species also includes western Pakistan (Lourenco,2001). The highest mortality rate from *H. lepturus* stings has been reported in Khuzestan Province, Iran. The venom of this scorpion leads to severe hemolysis and cardiovascular disorders (Radmanesh, 1998).

It is well known that, despite the close morphological similarities, it is difficult to differentiate scorpions using this feature alone (Polis et al., 1990). Therefore, a molecular taxonomic study can be an appropriate solution to this problem. In one study, species boundaries for *Mesobuthus przewalski* from northwestern China were determined using phylogenetic analysis of the mitochondrial marker cytochrome oxidase c subunit 1, ecological modeling, and morphological comparison (Zhang et al., 2019). Other studies on scorpions in Khuzestan Province using mitochondrial genes include scorpion phylogeny of *Ortoshirus iranus* (Jafari et al., 2017), *Mesobuthus eupeus* (Nikkahah et al., 2019), *Odontobuthus* genus (Mirshamsi et al., 2010), and *H. lepturus* (Jolodar, 2019). A study on *Androctonus crassicauda* in Turkey using the cytochrome oxidase gene found no evidence of intraspecific diversity (Oscan et al., 2007).

Phylogenetic studies based on nuclear and mitochondrial genes face limitations due to the need for sequencing, a relatively expensive process. Therefore, in cases where taxonomic studies require a large number of samples and access to genomic DNA is also restricted, using inter-simple sequence repeat (ISSR) markers is preferable in mammals and plants due to their simplicity and low cost (Weber et al., 1990). The technique for studying several eukaryotic species was first developed by Zietkiewicz et al. (1994). This method was used to study Arabidopsis plants by designing primers targeting repetitive CT nucleotides, using the random amplified microsatellite polymorphism (RAMP) technique (Wu et al., 1994). It has also been used to study fungal genomes (Hantula et al., 1996). The ISSR-polymerase chain reaction (PCR) was used with its concentration on the use of microsatellites that are dispersed in the genome of eukaryotes, to study the intraspecific variability of *Trypanosoma cruzi*, *Leishmania braziliensis*, and *Schistosoma mansoni*, showing that the band profiles obtained were comparable to those resulting from AP-PCR (Oliveira et al., 1997; Uras et al., 2024). A dendrogram using the UPGMA algorithm for scorpions in the *Hottentotta* genus from Khuzestan Province revealed intraspecific genetic diversity (Pirmoradi et al., 2021). Similar results were obtained with SSR-PCR and AP-PCR using isolated *T. cruzi* strains from chronic patients with Chagas disease (Gomes et al., 1998). Given the presence of unknown populations of *H. lepturus* in Khuzestan, we studied potential intraspecific variability in this scorpion.

Materials and Methods

Collection of scorpion specimens

A total of 22 scorpions *H. lepturus* were caught at night using ultraviolet (UV) light from 5 geographically different regions, including the cities of Izeh (3 specimens 49°52’56”N, 31°46’13”E), Baghmalek (7 specimens, 31°54’16”N, 49°20’14”E), Behbahan (3 specimens 30°14’46”N, 50°12’17”E), Choghazanbil (5 specimens 32°00’54”N, 48°31’03”E), and Masjedsoleyman (4 specimens 31°46’30”N, 49°00’37”E) (Figure 1). They were identified in the Razi Vaccine and Serum Research Institute in Ahvaz using the identification key (Vachon et al., 1974; Lamoral et al., 1979).

Genomic DNA extraction

To extract genomic DNA, 0.1-0.5 g of scorpion metasoma was crushed in liquid nitrogen, and then 600 µL of RSB buffer (10 mM Tris-HCl, pH 7.4 / 10 mM NaCl / 25 mM EDTA, SDS 1%) was added, and the mixture

was homogenized. Genomic DNA was extracted with the same amount of phenol/chloroform, then once with the same amount of chloroform. Finally, it was precipitated with pure ethanol and 3 M sodium acetate. DNA concentration and purity were determined by calculating the A260/280 absorbance ratio.

ISSR-PCR amplification

For the ISSR primer 5'-CAA(CT)6, the protocol described by Wu et al. (1994) was followed, except that the annealing temperature was 42 ˚C. A schematic representation of simple sequence repeat and inter-simple sequence repeat regions is shown in Figure 2. The ISSR-PCR technique relies on primer anchoring at the 3' or 5' ends of microsatellites. To perform the ISSR-PCR reaction, each PCR sample contains 25 µL of 5 ng genomic DNA, 1X PCR buffer, dNTPs (0.25 mM), magnesium chloride (1.5 mM), primer (1 µM), and Taq DNA polymerase (0.5 U). The thermal program for PCR was performed at 95 ˚C for 3 minutes (one cycle), 94 ˚C for 45 seconds, 42 ˚C for 45 seconds, and 72 ˚C for 60 seconds, with 30 repetitions, and finally 72 ˚C for 5 minutes. By electrophoresis of the PCR product, gene fragments produced in each specimen were counted and categorized using a zero and one system. DendroUPGMA program (Garcia-Vallve et al., 1999) was used to calculate a similarity index, the Dice coefficient. It performed clustering using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). The created data was used to draw a dendrogram tree with the help of the program ETAToolkit (Huerta-Cepas et al., 2016).

PCR amplification of cytochrome c oxidase subunit 1 (COI) was carried out on aliquots of the *H. lepturus* genomic DNA as template with initial denaturation for 5 min at 95 ˚C, followed by 35 cycles of 45 s at 94 ˚C, 1 min at 48 ˚C, and 1 min at 72 ˚C and, finally, 7 min of incubation at 72 ˚C in a final reaction volume of 25 mL containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.2 µM of each primer, deoxynucleotides (each at 220 mM), 1.5 mM $MgCl_2$, 100 ng genomic DNA, and 1 U Taq polymerase. The primers were COI-F 5'-GGTCAACAAATCATAAAGATATTGG and COI-R 5'- TAAACTTCAGGGTGACCAAAAAATCA (Folmer et al., 1994). The amplified PCR products were electrophoresed on a 1% agarose gel and stained with DNA Safe Stain (Sinaclon, Iran) before detection under UV transillumination. The amplified fragments were sequenced in both strands using a dideoxy termination method and run on an Applied Biosystems 373 DNA sequencer. The phylogenetic analysis was performed using the neighbor-joining method with 1000 bootstrap replicates in MEGA7.

Results

ISSR-PCR

The quantity and quality of the extracted genomic DNA were estimated to be between 1.56 and 1.73. The DNA concentration of the samples ranged from 8 to 55 ng per microliter. The agarose gel electrophoresis profiles were obtained by ISSR-PCR using 22 *H. lepturus* specimens and the primer CAA(CT)6. The gel electrophoresis profile of a representative set of amplification specimens is shown in Figure 3. In this technique, the DNA sequence is amplified between two microsatellite inverted repeats located at a suitable distance from each other. The bands produced in each lane were compared with those in all other lanes of the same gel. The most easily distinguishable bands were considered for analysis. They were counted and categorized using the binary system (0 and 1). A Dendrogram was constructed based on the presence/absence of each band. Almost all specimens produced bands in the molecular weight range of 0.9-2.5 kb. Amplification with CAA(CT)6 primer in all *H. lepturus* specimens showed a certain pattern, producing a polymorphic band in at least one region. It was the production of a band around 1 kb, which probably indicates a protected band.

A dendrogram of 22 *H. lepturus* scorpion specimens from Khuzestan, based on comparisons of amplified gene fragments, was drawn (Figure 4). The three specimens Hl2Ba, Hl10Iz, Hl3Ch, and Hl4Ch were distinct, so they showed the greatest separation from the other specimens in their own group. *M. eupeus* formed a separate branch as an out group.

Phylogeny based on COI sequence data

After removing the primer sites, the 637-nucleotide gene fragments named H1Ba, Hl2Ba, Hl3Ch, Hl4Ch, and Hl6Be were amplified. To compare the amplified nucleotide sequence with sequences in GenBank, a BLASTn search was performed using the highly similar sequences (megablast) option. The target sequence was similar to 12 nucleotide sequences from the Scorpionoidea family, all belonging to the genus *Hemiscorpius*. Among them, three sequences were *H. lepturus* (Table 1).

To compare the target nucleotide sequences, an alignment was performed using the ClustalW program. As shown in Figure 5, the highest number of gaps was observed in the area 33-102. The rest of the areas are almost without gaps. The highest amount of similarity is seen in nucleotide sequences 390-544. The similarity of

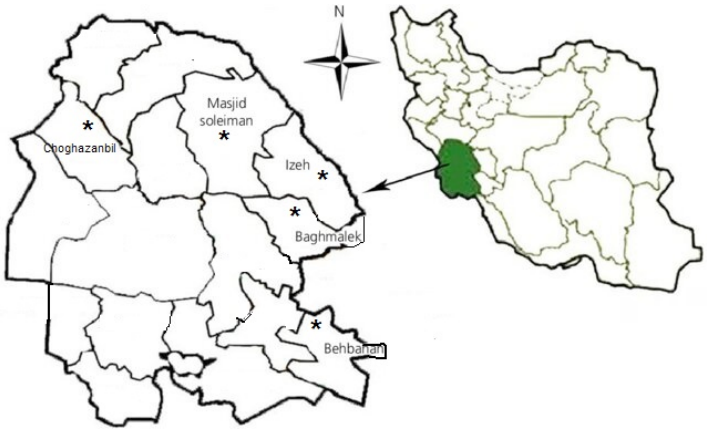

Figure 1. The collecting location of the studied scorpions on a map of Khuzestan Province

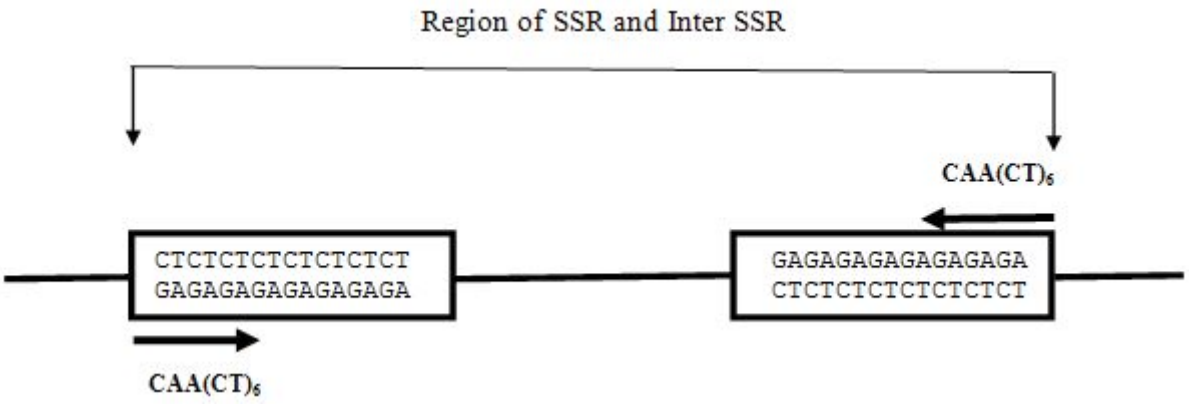

Figure 2. Schematic representation of simple sequence repeat and inter-simple sequence repeats region primer CAA(CT)6

Hl4Ch with the other 4 sequences in the aligned region ranged from 71% to 77%, whereas the similarity among those 4 sequences ranged from 86% to 94%.

Phylogeny based on the *COI* gene

The phylogenetic tree of *H. lepturus* scorpions from Khuzestan was analyzed based on protein and nucleotide sequences of the *COI* gene. The relevant sequences were retrieved from the NCBI database using the BLASTn program. Then, a phylogenetic tree was constructed from *COI* gene sequences of *H. lepturus* scorpions from Khuzestan using the Neighbor-Joining method in MEGA7. In Figure 6, of the 5 sequences, Hl1Ba and Hl6Be have a relatively close relationship (57%) with the *H. lepturus* reference sequence (KU341987). Hl2Ba and Hl3Ch were placed next to each other with a correlation of 89%-90%. Hl4Ch was placed with a certain distance (72%) from the rest of them, next to another reference sequence *Hemiscorpius* sp. (OP433762.1). A human sequence (NC012920) is shown as an out-group (Figure 6a).

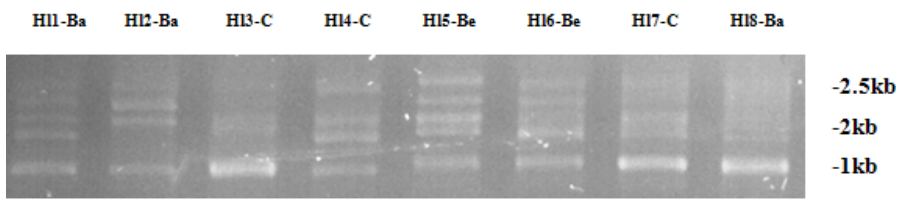

Figure 3. Agarose gel electrophoresis of some specimens as representatives of ISSR-PCR products on 1% gel

Abbreviations: Ba: Baghmalek; Be: Behbahan; Ch: Choghazanbil; Ma: Masjedsoleiman.

Note: The size of the DNA marker is shown on the right.

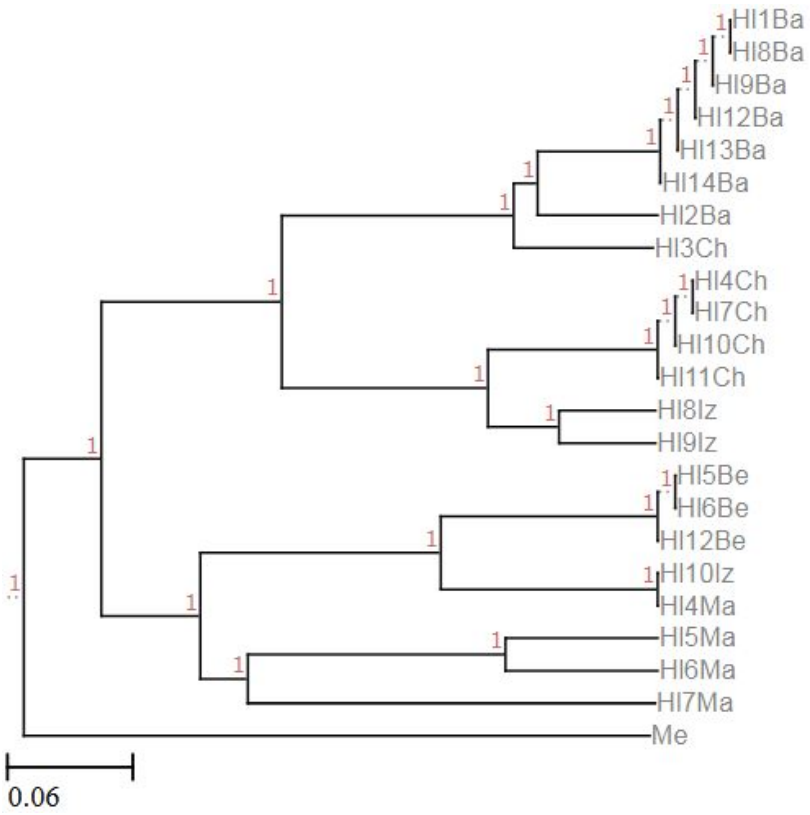

Figure 4. Dendrogram derived from cluster grouping using the band pattern of *H. lepturus* scorpion specimens using the UPGMA algorithm

Abbreviations: Ba: Baghmalek; Be: Behbahan; Ch: Choghazanbil; Ma: Masjedsoleiman.

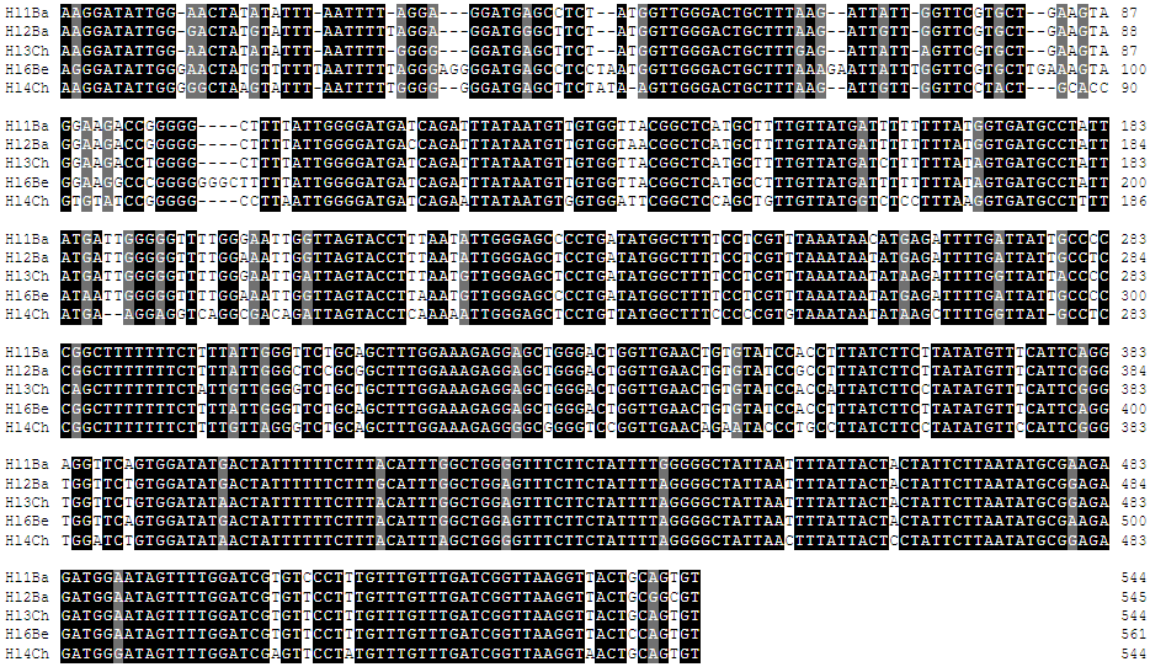

Figure 5. Nucleotide sequence comparison of 5 *H. lepturus* scorpion from Khuzestan Province based on the mitochondrial nucleotide sequence of the *COI* gene

Note: Identical nucleotides are shown in dark color. Conserved nucleotides are shown in gray.

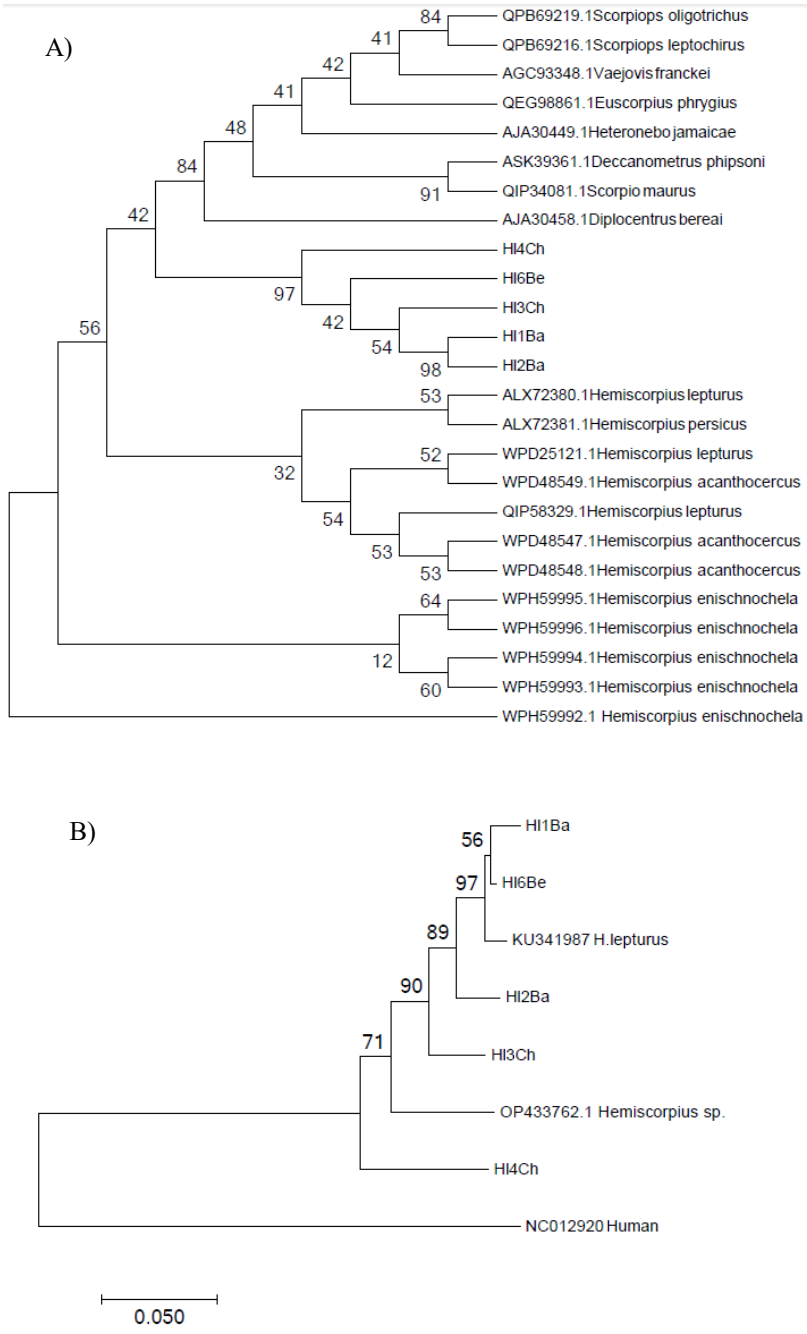

Figure 6. Phylogenetic tree of five *H. lepturus* scorpions from Khuzestan based on A) Nucleotide sequence and B) Protein sequence of the *COI* gene using neighbor-joining analysis

Note: Bootstrap numbers are based on 1000 replicates. The numbers before the species are the accession numbers of the related genes in GenBank. The numbers above the lines indicate the relationship between the groups.

A phylogeny tree of five *H. lepturus* scorpions from Khuzestan, based on the COI protein sequence, was constructed and compared with similar sequences from other scorpions (Figure 6b). The results showed that the protein sequences of Khuzestan scorpions were closely related to the scorpion branch within the Euscorpiinae and Diplocentridae families (84%). The 12 reference sequences of *Hemiscorpius* scorpion were grouped into a sub-branch with a moderate correlation (56%). Three of them are related to *H. lepturus*.

In a brief comparison of the phylogenetic trees obtained from protein and nucleotide sequences, we find that the percentage correlation between the nucleotide sequences of *H. lepturus* specimens from Khuzestan and the two sequences available in NCBI GenBank

Table 1. Taxonomy report of the *COI* gene fragment based on the BLASTn program

Organism	BLAST Name	Score	Number of Hits
Hemiscorpius	Scorpions		12
H. lepturus	Scorpions	161	3
Hemiscorpius acanthocercus	Scorpions	151	3
Hemiscorpius enischnochela	Scorpions	149	5
H. persicus	Scorpions	148	1

(KU341987) ranges from 57% to 72%. Still, this degree of similarity with the 12 protein sequences of the genus *Hemiscorpius* available in Genbank is 56%. Among the 12 reference sequences, three belong to *H. lepturus* and were isolated from Iran (ALX72380 and WPD25121) and Iraq (QIP58329).

Genetic distance

The genetic distance of five *H. lepturus* scorpion specimens from Khuzestan was calculated using the MEGA7 program, compared with each other and with only two *H. lepturus* sequences available in the gene bank (Table 2). The genetic distance of all Khuzestan scorpion specimens, except H4Ch, ranged from 1.6% to 6.6%. However, the H4Ch showed the greatest distance from other sequences (12.5%). The lowest genetic distance (1.6%) to the reference sequence (KU341987) was observed for *Hl6B*.

Discussion

Scorpions of the genus *Hemiscorpius* are considered to be an important cause of scorpion stings and the resulting deaths, especially in children. The Hemiscorpionidae family, with more than 4 species, is among the deadliest scorpion families in Khuzestan Province (Shahbazzadeh et al., 2003). Today, species identification has shifted from morphology to genetic and evolutionary aspects.

Using the ISSR-PCR technique, it is possible to analyze genomic DNA sequences for the taxonomy of various organisms, including scorpions, without prior information. Therefore, the use of primers containing repetitive sequences, which are generally found in intergenic regions and introns in arthropods, has been recommended (Toth et al., 2000). Such primers are not only able to distinguish closely related species but also to distinguish between different populations (De Leon et al., 2010). In this study, molecular phylogeny was evaluated using microsatellite markers in the scorpion *H. lepturus* in Khuzestan Province.

Although the taxonomy of all 22 studied scorpions was identified as *H. lepturus* based on morphological data, genetic differences were observed in both Hl3Ch and Hl4Ch, with this variation more pronounced in Hl4Ch. This level of intraspecies diversity was unexpected given

Table 2. Genetic distance of *H. lepturus* scorpion from Khuzestan using *COI* nucleotide sequence

Organisms	1	2	3	4	5	6	7	8
Hl1Ba								
Hl2Ba	0.051							
Hl3Ch	0.066	0.055						
Hl4Ch	0.125	0.113	0.102					
Hl6Be	0.016	0.035	0.051	0.117				
KU341987_*H. lepturus*	0.023	0.039	0.059	0.121	0.016			
OP433762.1_*Hemiscorpius_sp.*	0.094	0.090	0.090	0.125	0.090	0.102		
NC012920_Human	0.414	0.422	0.406	0.406	0.414	0.414	0.398	

the presence of a sexual cycle in scorpion reproduction. Therefore, it is possible that this level of genetic diversity, as suggested in the case of the snail genus *Biomphalaria* (Paraense et al., 1957; Jarne et al., 1991), is due to other effective factors, such as recombination, mutation, or gene flow. However, this difference may also be an interspecies variation. To investigate the possibility observed among the studied scorpions, we decided to use the *COI* gene to confirm genetic differences and their geographical distribution in the province.

The genetic distance of all specimens from Khuzestan ranged from 1.6% to 6.6%, except for Hl4Ch. The results show that specimen Hl4Ch was dissimilar within its group. This specimen showed the highest distance (12.5%) among others. According to the threshold limit for interspecies genomic variation, suggested to be 13.4% in invertebrates (Kumar et al., 2017), the difference is insufficient to warrant classification as a new species, but it is clearly intraspecific variation.

Geographical and climatic features indicate that Khuzestan Province is divided into two regions: *mountainous* and plain. The low-altitude plain regions of Khuzestan include Behbahan, which accounts for approximately 60% of the entire province, while the mountainous region, mainly in the north and east of the province, including Baghmalek, covers about two-fifths of the entire province. The specimens were collected from these two regions. Considering the obvious environmental differences in Khuzestan Province, such as temperature, humidity, and altitude, it was expected that the geographic location of the specimens would be related to their genetic distance, but this was not the case. In fact, the genetic distance between the two Baghmalek (mountainous region) specimens Hl1Ba and Hl2Ba was 5.1%. Still, the genetic distance of those two Baghmalek specimens and Hl6Be from Behbahan (plain region) was 1.6% and 3.5%, respectively.

Conclusion

Although the scorpions of each region were mostly grouped in the phylogeny, no major genetic diversity associated with regional differences was observed within the province. Based on the genetic distance results, it can be concluded that Hl4Ch is definitely an intraspecies variation. Phylogeny using other genes and more specimens can achieve more accurate results.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

All authors equally contributed to preparing this article

Conflict of interest

The authors declared no conflict of interest.

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