

**DNA Barcoding Supports Species Delimitation in 11 Scorpion Species (Arachnida: Scorpiones) from Khuzestan Province, Southwestern Iran**

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**Abstract**

**Introduction:** Accurate identification of scorpion species distributed across different regions is crucial for public education, prevention of scorpion envenomation, and appropriate clinical management. Molecular approaches are increasingly recognized as a reliable means of validating species boundaries. To date, 24 scorpion species belonging to 13 genera and three families have been reported from Khuzestan province, southwest Iran.

**Material & Methods:** In this study, we investigated the scorpion fauna of Khuzestan Province. Mitochondrial cytochrome c oxidase subunit I (COI) sequences were generated for five species, and additional sequences, of six species, were obtained from GenBank. A total of 26 COI sequences representing 11 species, nine genera, and two families were used for the reconstruction of Maximum Likelihood and Bayesian Inference.

**Results:** Phylogenetic analyses supported the validity of 11 previously recorded species described based on morphological characters, including: *Androctonus barahoeii*, *Apistobuthus*

*susanae*, *Buthacus tadmorensis*, *Compsobuthus* cf. *matthiesseni*, *Hottentotta hatamtiorum*, *Hottentotta khoozestanus*, *Hottentotta zagrosensis*, *Mesobuthus crucittii*, *Odontobuthus bidentatus*, *Orthochirus mesopotamicus*, and *Hemiscorpius lepturus*.

**Conclusion:** Molecular study of scorpions from Khuzestan Province provides valuable data for scorpionism prevention and the development of species-specific antivenoms for medically important scorpions.

**Keywords:** Genetic distance, Khuzestan, phylogeny, species validity, taxonomy

## 1. Introduction

Southern parts of Iran provide favorable habitats for the occurrence of diverse scorpion species, as these arachnids are highly adapted to warm and humid climatic conditions [1]. The exceptionally high incidence of scorpion stings in these regions, estimated at 40,000–50,000 cases annually, reflect a big challenge. Unfortunately, scorpion envenomation remains a significant public health concern and may result in fatal outcomes, particularly among children [2,3].

A total of 24 species of scorpions belonging to 13 genera and three families have been reported from Khuzestan Province [4,5,6,7,8,9]. Among them, members of the genera *Androctonus* and *Hemiscorpius*, which include species possessing medically important venom, are widely distributed throughout the province [6,10,11,12].

Molecular phylogenetic studies have substantially refined the taxonomy of Iranian scorpions by validating previously recognized taxa and revealing considerable cryptic diversity across several genera. These studies have resulted in the description of numerous new species, including *Odontobuthus tirkari* from Razavi Khorasan Province [13], *O. baluchicus* and *O. chabaharensis* from Sistan and Baluchestan Province, and *O. kermanus* from Kerman Province [14], *O. persicus* from Hormozgan Province [15], *Hottentotta hatamtiorum* from western Iran [16], *Androctonus rostami* from Sistan and Baluchestan Province together with the molecular confirmation of *A. sistanus* [10], *Hemiscorpius jiroftensis* and *H. aratta* from Kerman Province [17,18], and *Compsobuthus yagi* from Sistan and Baluchestan Province [19]. In addition to the discovery of these taxa, molecular investigations have consistently supported the validity of previously described species in several genera, including *Odontobuthus*, *Androctonus*, and the scorpion fauna of Sistan and Baluchestan Province [10,14,20,21], demonstrating the important role of integrative molecular approaches in resolving the taxonomy and diversity of Iranian scorpions.

With the exception of *Mesobuthus crucittii* and *Hottentotta hatamtiorum*, all of the aforementioned species were originally described based on morphological evidence. Despite the remarkable diversity of scorpions in Khuzestan Province, no comprehensive molecular assessment of the scorpion fauna of this region has yet been conducted.

Considering the medical importance of scorpions and the occurrence of species with potentially dangerous venom in southern Iran, this study employed molecular data to investigate the scorpion fauna of Khuzestan Province. Specifically, we aimed to assess the taxonomic validity of the recorded species and to clarify their distributional ranges within the province.

## 2. Materials and methods

COI gene sequences were generated from scorpion specimens collected in Khuzestan Province to assess the taxonomic validity of the species. Genomic DNA was extracted from pedipalp tissue using the Favorgen Tissue DNA Extraction Kit (Taiwan), following the manufacturer's protocol. Amplification of the mitochondrial cytochrome c oxidase subunit I (COI) gene was performed using an Analytik Jena thermal cycler (Germany) and the universal primers of Folmer et al. [22], following the protocol described by Barahoei et al. [10]. PCR products showing high quality were sequenced at the Noor Comprehensive Medical Genetics Center (Tehran, Iran).

Sequence quality was inspected, and the obtained sequences were edited and aligned using BioEdit. Sequence characteristics and genetic distances were analyzed in MEGA12. Maximum Likelihood (ML) was conducted with 10,000 bootstrap replicates, whereas Bayesian Inference (BI) was performed with 50 million generations. All phylogenetic analyses were carried out through the CIPRES Science Gateway. The resulting phylogenetic trees were visualized and edited using FigTree v1.4.4 and Adobe Photoshop.

## 3. Results

A total of 26 COI sequences (Table 1) representing 11 species, nine genera, and two families were used for the reconstruction of Maximum Likelihood (ML) and Bayesian Inference (BI). The dataset comprised seven newly generated sequences representing five genera from two families, together with 19 sequences retrieved from GenBank representing seven genera from two families. *Hemiscorpius lepturus* was selected as the outgroup.

**Table 1.** Data of 26 scorpion specimens (seven sequenced specimens and 19 sequences from GenBank) from Khuzestan, Iran, were used for constructing a phylogenetic tree. *Hemiscorpius lepturus* was used as the outgroup.

| Species                              | Collection | Locality                    | Genbank  | Reference  |
|--------------------------------------|------------|-----------------------------|----------|------------|
|                                      |            |                             | PV725999 |            |
|                                      |            |                             | PV726002 |            |
| <i>Androctonus barahoeii</i>         | -          | Khuzestan, Izeh             | PV726003 | GenBank    |
|                                      |            |                             | PV726001 |            |
|                                      |            |                             | PV726000 |            |
|                                      | -          | Khuzestan                   | PV931979 | [26]       |
| <i>Apistobuthus susanae</i>          | T-14A      | Khuzestan, Chazabeh         | PZ870767 |            |
|                                      | T-14B      |                             | PZ870768 |            |
| <i>Buthacus tadmoresis</i>           | SK-22      | Khuzestan, Chamim           | PZ870769 | This study |
| <i>Compsobuthus cf. matthiesseni</i> | T-11A      | Khuzestan, Chazabeh         | PZ870770 |            |
|                                      | T-13A      | Khuzestan, Dezful           | PZ870771 |            |
| <i>Hottentotta hatamtiorum</i>       | -          | Khuzestan, Andimeshk        | PP133856 |            |
|                                      |            |                             | PP133857 |            |
| <i>Hottentotta khoozestanus</i>      | -          | Khuzestan, Rigsefid         | PZ037926 | [16]       |
|                                      |            | Khuzestan, Ahwaz            | PZ037927 |            |
| <i>Hottentotta zagrosensis</i>       | -          | Khuzestan, Dezful           | PZ037923 |            |
|                                      |            | Khuzestan, Izeh             | PZ037924 |            |
|                                      |            |                             | HM567388 |            |
| <i>Mesobuthus crucittii</i>          | -          | Khuzestan, Ahwaz, Baghmalek | HM567387 | [5]        |
|                                      |            |                             | HM567386 |            |
|                                      |            |                             | HM567385 |            |
|                                      |            |                             | KF701314 |            |
| <i>Odontobuthus bidentatus</i>       | -          | Khuzestan, Omidiyeh         | KF701315 | [14]       |
|                                      |            | Khuzestan, Baghmalek        | MW655776 |            |
| <i>Orthochirus mesopotamicus</i>     | RIZ-Ort-98 | Khuzestan, Izeh             | PZ870772 | This study |
| <i>Hemiscorpius lepturus</i>         | HD-42C     | Khuzestan, Ahwaz            | PZ870773 |            |

The aligned dataset consisted of 591 bp, including 396 conserved sites (67%), 195 variable sites (33%), and 159 parsimony-informative sites (27%).

Analysis of intra- and interspecific genetic distances further supported the taxonomic validity of the examined genera and species. Among the buthid genera included in this study, the lowest intergeneric genetic distance was observed between *Odontobuthus* and *Androctonus*, whereas the highest genetic distance was found between *Hottentotta* and *Apistobuthus* (Table 2). These results are consistent with the phylogenetic analyses and provide additional evidence for the distinctiveness of the recognized taxa.

**Table 2.** Genetic distances (average Kimura 2-parameter) of *COI* gene among and within (bold) ten species of scorpions from Khuzestan province. *Hemiscorpius lepturus* was used as the outgroup.

|                                         | 1            | 2            | 3     | 4            | 5            | 6            | 7            | 8            | 9            | 10    |
|-----------------------------------------|--------------|--------------|-------|--------------|--------------|--------------|--------------|--------------|--------------|-------|
| 1. <i>Androctonus sumericus</i>         | <b>0.015</b> |              |       |              |              |              |              |              |              |       |
| 2. <i>Apistobuthus susanae</i>          | 0.119        | <b>0.045</b> |       |              |              |              |              |              |              |       |
| 3. <i>Buthacus tadmorensis</i>          | 0.142        | 0.142        | NA    |              |              |              |              |              |              |       |
| 4. <i>Compsobuthus cf. matthiesseni</i> | 0.154        | 0.154        | 0.166 | <b>0.001</b> |              |              |              |              |              |       |
| 5. <i>Hottentotta hatamtiorum</i>       | 0.160        | 0.182        | 0.150 | 0.148        | <b>0.073</b> |              |              |              |              |       |
| 6. <i>Hottentotta khoozestanus</i>      | 0.144        | 0.171        | 0.162 | 0.145        | 0.118        | <b>0.068</b> |              |              |              |       |
| 7. <i>Hottentotta zagrosensis</i>       | 0.156        | 0.160        | 0.170 | 0.156        | 0.110        | 0.092        | <b>0.001</b> |              |              |       |
| 8. <i>Mesobuthus crucittii</i>          | 0.159        | 0.157        | 0.152 | 0.148        | 0.164        | 0.164        | 0.151        | <b>0.029</b> |              |       |
| 9. <i>Odontobuthus bidentatus</i>       | 0.107        | 0.128        | 0.128 | 0.162        | 0.148        | 0.157        | 0.150        | 0.150        | <b>0.013</b> |       |
| 10. <i>Orthochirus mesopotamicus</i>    | 0.118        | 0.126        | 0.146 | 0.166        | 0.162        | 0.149        | 0.140        | 0.140        | 0.108        | NA    |
| 11. <i>Hemiscorpius lepturus</i>        | 0.220        | 0.233        | 0.212 | 0.239        | 0.219        | 0.209        | 0.214        | 0.214        | 0.217        | 0.199 |

The ML recovered members of *Hottentotta* as the basal lineage, representing the earliest-diverging group within the dataset. They were followed successively by members of *Compsobuthus*, *Mesobuthus*, *Buthacus*, *Androctonus*, *Apistobuthus*, *Orthochirus*, and *Odontobuthus* (Figure 1). All genera and species formed distinct and well-supported monophyletic clades, confirming their taxonomic validity.

The BI showed a somewhat different topology. Members of *Mesobuthus* occupied a distinct basal clade (Clade A), whereas all remaining genera were grouped within a second major clade (Clade B). Within Clade B, members of *Hottentotta* and *Compsobuthus* formed a sister-group relationship and were clustered together in Subclade B1. The remaining genera were placed within Subclade B2, where *Buthacus* formed the basal lineage, followed successively by *Odontobuthus*, *Orthochirus*, *Apistobuthus*, and *Androctonus* (Figure 2). Similar to the ML, the BI supported the monophyly and taxonomic validity of all examined genera and species.

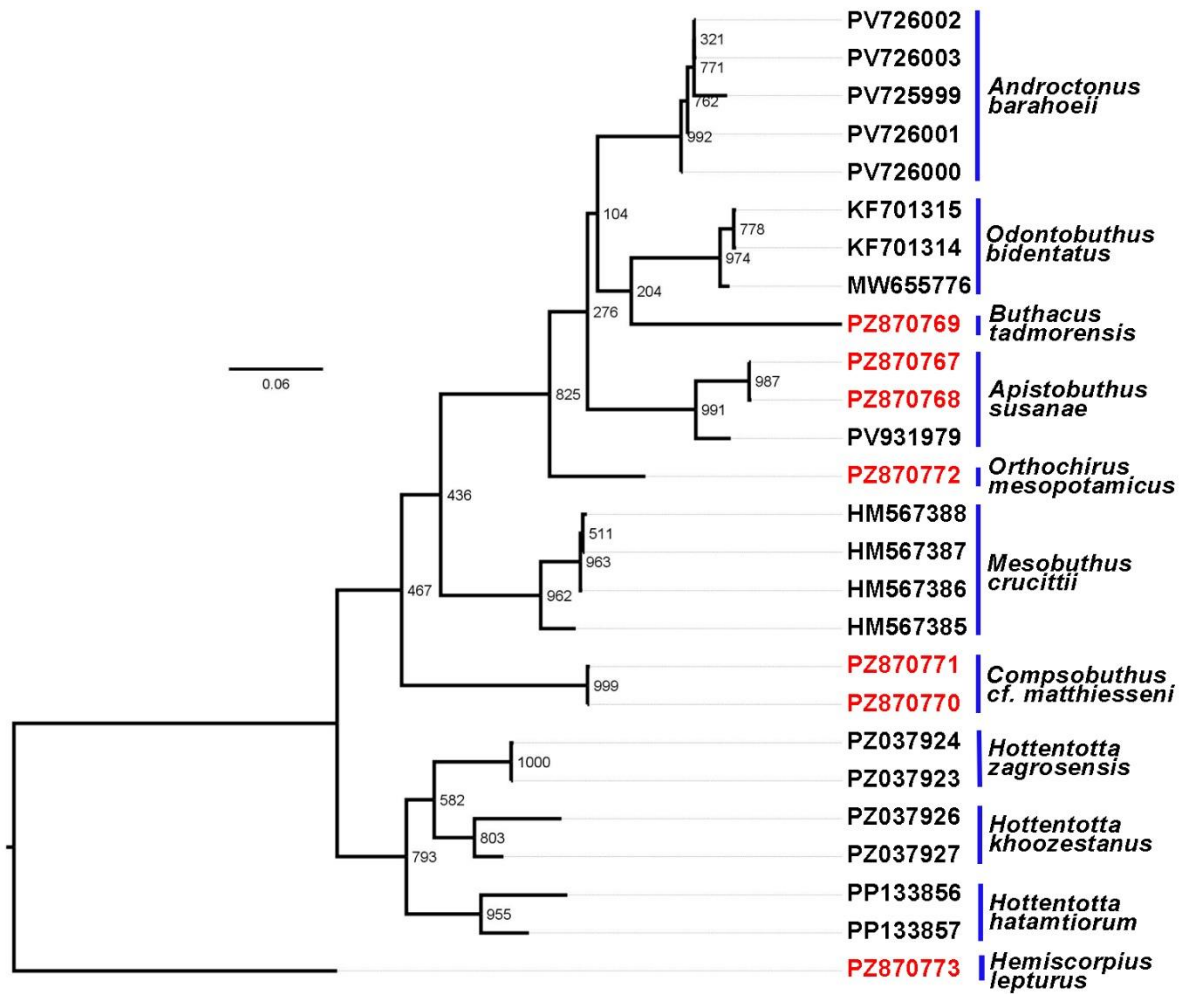


**Figure 1.** Phylogenetic relationships of scorpion species inferred from mitochondrial COI sequences using Maximum Likelihood based on 26 sequences from Khuzestan, southwest Iran. Bootstrap values indicated at nodes. *Hemiscorpius lepturus* was used as the outgroup.

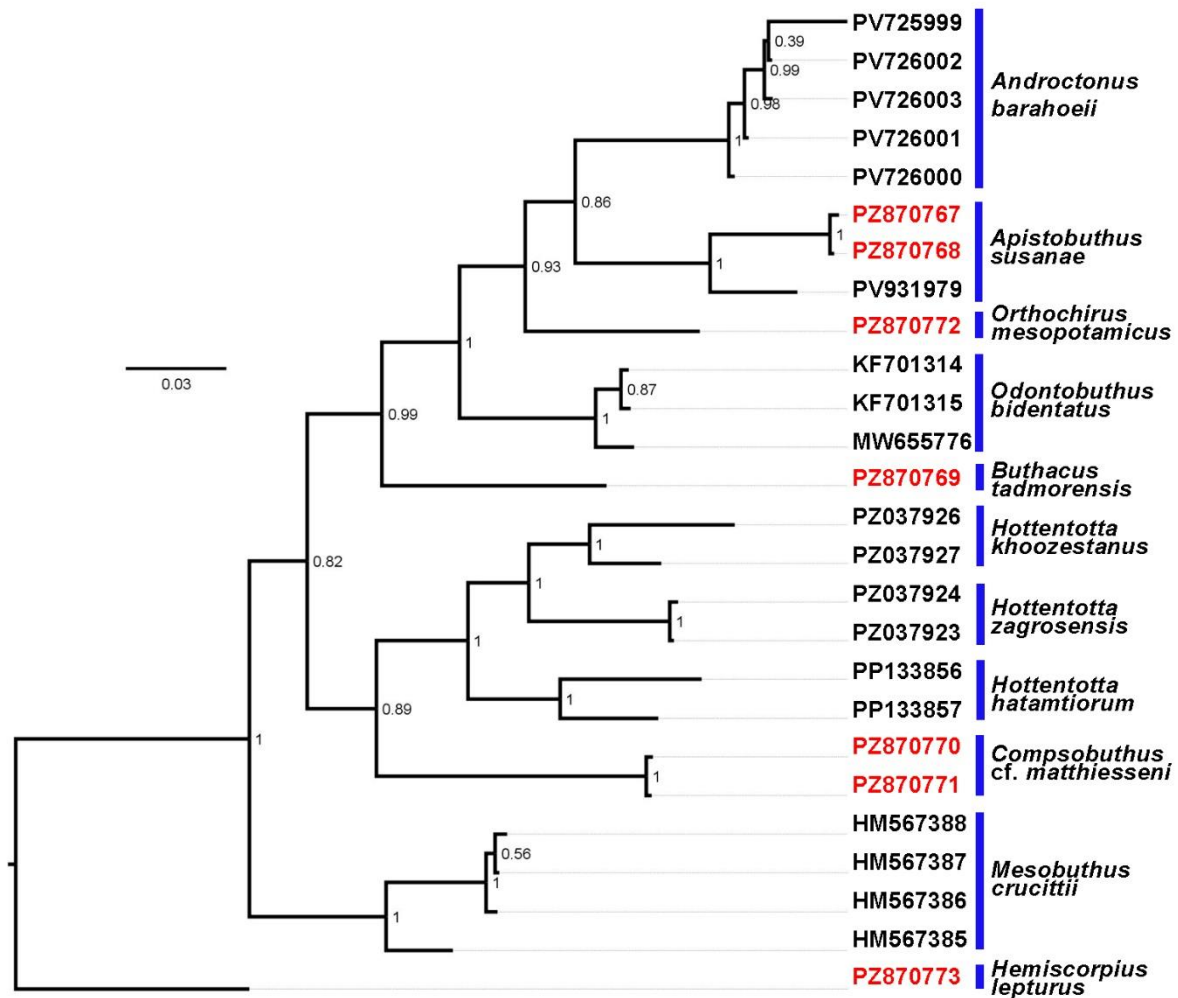


**Figure 2.** Phylogenetic relationships of scorpion species inferred from mitochondrial COI sequences using Bayesian Inference based on 26 sequences from Khuzestan, Iran. Posterior probability values indicated at nodes. *Hemiscorpius lepturus* was used as the outgroup.

#### 4. Discussion

To date, 24 scorpion species belonging to 13 genera and three families have been reported from Khuzestan Province. Several lines of evidence suggest that populations currently recognized as *Androctonus nazari* in this region are more appropriately assigned to *Androctonus sumericus*. First, the coexistence of two species of the same genus within the same habitat is generally unlikely, except in areas where distinct habitats overlap [10,14]. Second, the description of *A. nazari* was based on only two male specimens [9], including one juvenile and another specimen that may have exhibited color alteration. Furthermore, no molecular data were incorporated to support the original description. *Androctonus barahoeii* has recently been reported from the mountainous regions of Khuzestan Province [10,23]. The molecular data obtained in the present study support this interpretation. Intraspecific genetic divergence

among *A. barahoeii* populations from Khuzestan was very low (0.015), indicating that they belong to a single species. It should be noted, however, that populations inhabiting the lowland areas of western Khuzestan are most likely attributable to *A. sumericus*. Although there is no sequence of this species, morphological similarities confirm this.

The newly generated sequence of *Apistobuthus susanae* Lourenço, 1998 clustered with previously available sequences of the species (Figures 1, 2), further supporting its taxonomic validity.

For the first time, *Buthacus tadmoresis* (Simon, 1892) from southwestern Iran (Khuzestan Province) was sequenced. The phylogenetic analyses confirmed the validity of the genus and species (Table 2). Considering that the type specimen of *B. tadmoresis* originates from Iraq [7], populations reported from southern Iran, particularly Bushehr and Hormozgan provinces, may belong to an undescribed species or are the result of misidentification. These populations occur under distinct environmental conditions and are geographically separated from the Iraqi populations by major barriers, including the Zagros Mountains. Such geographic isolation and environmental differentiation promote population divergence and, potentially, speciation in scorpions [10,14]. Similarly, because the type locality of *Compsobuthus jakesi* is in Najaf, Iraq, populations reported from Bushehr and Hormozgan provinces may also belong to undescribed taxa.

No molecular sequence is currently available from the for *Compsobuthus matthiesseni* from Arak, Iran, the type locality of the species. The available GenBank sequences originate from Iraq and may instead correspond to the recently described species *Compsobuthus hadei* [24]. The genetic distance between the Khuzestan population and Iraqi specimens was 0.078, supporting the presence of two distinct species. Specimens of South Khorasan province maybe belong *C. yagi*. Consequently, records of *C. matthiesseni* from Bushehr, Hormozgan, and Kerman provinces, as well as from Syria, Türkiye, and Yemen [6], are require extensive sampling and morphological and molecular examination. Sequencing of specimens from the Arak specimens is therefore essential for clarifying the taxonomic status of these populations. Five species of *Hottentotta* have been reported from Khuzestan Province. The taxonomic validity of *Hottentotta hatamtiorum*, *Hottentotta khoozestanus*, *Hottentotta schach*, and *Hottentotta zagrosensis* has been confirmed by molecular analyses [25]. However, the relatively high intraspecific genetic distances observed in *H. hatamtiorum* (0.073) and *H. khoozestanus* (0.068) (Table 2) suggest that further investigation is warranted. In contrast, *Hottentotta pooyani* has not been collected or reported since its original description, raising questions regarding its taxonomic status.

Molecular studies of *Mesobuthus* led to the description of *Mesobuthus crucittii* [5], while the validity of *Odontobuthus bidentatus* (Loureço & Pérez, 2002) has also been confirmed through molecular analyses [14].

*Orthochirus mesopotamicus* Birula, 1918 was sequenced for the first time in the present study. However, the validity of the other species reported from Khuzestan Province, namely *Orthochirus gantenbeini*, *O. iran*, *O. navidpour*, and *O. zagrosensis* [4], remains to be evaluated using molecular data.

The taxonomic status of *Razianus zarudnyi* and *Sassanidotus zarudnyi* requires comprehensive morphological and molecular investigation. Records of *R. zarudnyi* from Hamadan and Khuzestan provinces and *S. zarudnyi* from Fars and Khuzestan provinces appear doubtful and may represent undescribed species.

*Vachoniolus iran* is an Iranian endemic species distributed in Ilam and Khuzestan provinces. Although no molecular data are currently available, its distinctive morphological characters readily separate it from all other genera.

Within the family Hemiscorpiidae Pocock, 1893, two species, *Hemiscorpius kashkayi* and *Hemiscorpius lepturus*, are known from Khuzestan Province. A phylogenetic analysis based on COI sequences suggests the presence of an undescribed species in the lowland areas of the province (Figure 3 in Dehghan et al. [18]).

## 5. Conclusion

Accurate identification of scorpion species and determination of their distribution ranges are essential for the prevention of scorpion envenomation, accurate identification of the offending species, and timely clinical management of envenomed patients. Khuzestan Province, southwestern Iran, harbors the highest diversity of scorpion species and records the greatest annual incidence of scorpion stings in the country, making it the principal hotspot of scorpionism in Iran. In the present study, molecular analyses of scorpions collected from different regions of Khuzestan Province confirmed the validity of 11 species. These findings provide an important scientific basis for the development of species-specific antivenoms targeting medically important scorpions and may contribute to improving the clinical management of scorpion envenomation.

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## Authors' Contribution

H. Barahoei: conceptualization, software, validation, final identification of specimens, review and editing of manuscript, revising the manuscript and correspondence; H. Behdarvandi: collecting samples, first identification of specimens, writing manuscript original draft; A. Mousazadeh: collecting samples, first identification of specimens, writing manuscript original draft; K. Kiarssi Behdarvand: data curation.

## Conflict of interests

The authors declared no conflict of interest.

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## Data Availability

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request. DNA sequences generated in this study have been deposited in GenBank.

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