Original Article

Molecular Survey of Mitochondrial Genes in Different Populations of the Black Fat-Tailed Scorpion, *Androctonus crassicauda*

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Abstract

Background: *Androctonus crassicauda* is the most medically relevant scorpion and understanding its genetic forms is essential for improvement of anti-venom sera, and risk management of scorpionism. Present study was designed to identify the variations of mitochondrial genes in different populations of *A. crassicauda*.

Methods: Adults of *A. crassicauda* were collected from Zanjan Province during 2016–2017. Genomic DNA of samples was extracted and fragments of mitochondrial 16S, COI and ND1 genes were amplified and some of the amplicons were sequenced. Haplotype of samples were identified by multiple alignment of sequences, then phylogenetic trees of haplotypes were constructed.

Results: Fragments of 352bp, 618bp and 680bp were amplified from 16S, COI and ND1 genes respectively. Nucleotide sequence in COI fragments was conserved, however, five haplotypes with some specific polymorphic sites were detected in 16S and ND1 fragments. Haplotype I was dominant and found in all areas. Other haplotypes were rare and limited to specific regions. Analysis of the phylogenetic trees inferred from 16S and COI genes, confirmed a strong positive correlation between geographic and genetic distance.

Conclusion: Mitochondrial COI, 16S and ND1 genes were detected suitable for identifying the population structure. Five genotypes were found using 16S and ND1 genes. To prepare and improve the anti-venoms quality, additional studies are necessary to identify the toxin electrophoretic profile and geographical/ecological niche models of these genotypes in future.

Keywords: *Androctonus crassicauda*; 16S; ND1; COI; Mitochondrial genotypes

Introduction

Scorpions are potentially fatal venomous animals whose venom consist of a variety of toxic compounds specifically target ion channels and other cellular receptors (1). Scorpion envenomation is a major global health problem and anti-venom is still widely used for its treatment because there is no vaccine or other effective agents (2). Among different families of the scorpions, Buthidae with the universal distribution is the greatest and the most dangerous scorpions in this family belong to the *Androctonus* (2).

Androctonus, is a typical Saharo-Sindian taxon that occurs frequently from the Atlantic coast of North Africa through western India. This genus includes the largest known buthid scorpions that some of them have extremely toxic venom to humans (2). The taxonomic relationship of *Androctonus* types is not well resolved. This genus was described by Vachon in 1952, but since then it has been catalogued first by Fet et al. (3) and last by Teruel et al. (4). After these revisions, many species were described as new, resurrected from synonymy, or

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raised from subspecies to species level (4–8). Nowadays there are correctly seven known species and 40 formerly valid subspecies in this genus (9). The black fat-tailed scorpion, *A. crassicauda*, is the most dangerous species of *Androctonus* due to causing several human deaths and antivenom production. This species is abundant in various natural habitats ranging from subtropical to temperate regions mostly in the Middle East and Africa (3). From 33 provinces of Iran, the black fat-tailed scorpion has been found in 28 provinces (10) Extensive distribution of this species exhibits its adaptation to different ecological conditions and probably possess different types (11). Diagnosis of taxonomic groups in this species is traditionally based mainly on morpho-sculpture and coloration characters (12). Although few comparative morphological studies have been done on the black fat-tailed scorpion, no modern precious analyses method exists to differentiate various types and the only existing key is out of date and its types remain largely unresolved. Despite the wide distribution of this species in different regions, its ecological requirement brings it into close contact with human settlements regarded as a potential threat then responsible for several deaths (11). Given its medical importance, understanding the intraspecific diversity and their spatial distribution are essential for, production of effective anti-venoms, and treatment of envenomation.

Over the last four decades, mitochondrial DNA (mtDNA) has been the most popular marker in the study of intra and interspecies variations (13). The genetic information of these markers including gene arrangement patterns, coding usage patterns, nucleotide sequence from protein-coding genes, amino acids and A+T contents (skewness) could be applied as a powerful tool for taxonomy and identification of some species/subspecies. These markers also have been efficiently applied to detect introgression between taxa, answer fundamental questions of the population structure, and resolve their phylogenetic problems (14). Moreover, they are assumed to be clock-like and could generate some signals about population history and could reflect its divergence time (13).

Recently molecular tools have been used to assess the phylogeny of *Androctonus* using nuclear DNA ITS regions (15), barcoding markers for species determination using 16S-rDNA (16) and clade identification of species with COI genes (17). In recent years also two genetic groups were found in studied populations of *A. crassicauda* from Turkey according to the sequence analysis of 16S gene (18). In addition, the phylogeographical patterns in six species of *Androctonus* were exhibited in North Africa by COI, 16s and ND1 mtDNA markers (19). Moreover, COI as a molecular tool has been used for phylogeny of different species of scorpions (20–23).

Black fat-tailed scorpion consists of the main species of scorpions in Zanjan Province, northwest of Iran (24, 25). This species is the most significant scorpion species in Iran and other countries of the Middle East and includes many cases of scorpionism in Iran (26–28), Turkey (29) and Egypt (30). This paper is a part of an ongoing study of *A. crassicauda* biodiversity. We report the first pilot data based on the analysis of COI, 16S and ND1 mtDNA genes from different populations of this scorpion in Zanjan Province. The aims of the current report are identification of intraspecific variations of mitochondrial genes in populations of *A. crassicauda* and expression of relevant molecular markers in order to select of candidate groups for improving anti-venom quality.

Materials and Methods

Sampling

A total of 84 adult specimens of the black fat-tailed scorpion were collected from twenty localities of Zanjan Province, northwest of Iran, from spring 2016 to autumn 2017 (Fig. 1). The study areas have different ecological conditions and include low (below 700 meters above sea level (masl)), semi-high (700–1500masl) and

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high (above 1500masl) lands. Sample size from each locale were rather low (n< 12) because we found the scorpions in general at extremely low densities. Main morphological characters used in identifying this species include carapacial and metasomal carination and coloration, and absence of trichobothria under pedipalp patella (Fig. 2). Scorpion samples were stored at -20 °C pending molecular analysis. The sex of the scorpions was assessed by the general sexual dimorphism, male having significantly higher number of pectinal teeth than females (Fig. 2, d, e).

DNA extraction

Total DNA was extracted from muscle of dissected tissue of coxa III–IV after two washes in distilled water. To extract genomic DNA (gDNA), samples were transferred to 1.5mL Eppendorf tubes and suspended in 500mL of TENS lysis buffer (10mM Tris-HCl (pH 8), 1mM EDTA, 150mM NaCl, 0.5% SDS) in the presence of 0.1g of acid-washed glass beads (400–600µm in diameter) and two stainless steel ball bearings (5mm in diameter). The extraction mixture was centrifuged at 10,000g for 5min. The upper phase was extracted and added with 100µl of 5M potassium acetate, and an equal volume of ethanol incubated for 1h at -20 °C. After incubation the extracted suspension was centrifuged at 17,000g for 15min, washed twice with cold 70% ethanol, dried and resuspended in a 50µL nuclease-free TE buffer (SinaClon, Iran).

Evaluation of DNA quantity and quality

DNA samples were analyzed by electrophoresis in 1.2% agarose gels containing safe stain, nucleic acid gel stain (SinaClon) in Tris-borate-EDTA buffer and visualized with UV light using a Gel Doc system (Uvitec, Cambridge United Kingdom). A spectrophotometer (NanoDrop; Thermo Fisher Scientific Inc.) was also used to measure absorbance at 260, 280 and 230nm and estimate concentration and contamination with protein.

Amplification of mtDNA fragments

The 16S, COI and ND1 fragments were amplified in all samples using specific primers (Table 1). The amplifications were performed in a final volume of 25µL containing 1U *taq* DNA polymerase (SinaClon), PCR master mix, 10 pM each primer and 1µL purified DNA. All amplification conditions were slightly modified for the DNA polymerase requirements and consisted of 1min at 94 °C; followed by 40 cycles of 94 °C for 30s; 53 °C for COI and 48 °C for 16S and ND1 genes for 60s and 72 °C for 60s; and a final step of 72 °C for 10min. Amplicons from the PCR were separated by electrophoresis as described previously and a 100bp DNA ladder (Invitrogen) was used to estimate amplicon size. Great precautions were taken to minimize the risk of DNA contamination of PCR amplification. PCR amplifications, DNA extractions and electrophoresis were set up in separate areas and using specific sets of materials including gloves, pipettes, filter tips and laboratory coat. Working positions were repeatedly cleaned with 10% NaOCl to denature potential contaminating nucleic acids. Amplified products were analyzed by electrophoresis and single bands of the expected size were sequenced with both forward and reverse primers by Seqlab (www.Seqlab.dc).

Analysis of sequences and identification of genotypes

Chromatograms of nucleotide sequences were checked by eye using Chromas Pro software (http://www.technelysium.com.au) and multiple sequence alignment was done with ClustralW. All sequences were aligned and edited by using BioEdit software (https:// bioedit.software.informer.com) and checked indels and single nucleotide polymorphisms within homologous groups. Some of the sequences of this study have been deposited in GenBank. The resulting alignments were checked by eye and phylogenetical relationships were performed using Maximum Likelihood (ML), Neighbor Joining (NJ) and Minimum Evolution (ME) and

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Maximum Parsimony (MP) methods with MEGA X software by using Bootstrap with 1000 replications and Kimura-2 parameter model (33). The alignments were also applied to construct haplotype networks using the transitive consistency score (TCS) method (34). Additionally, the alignments were checked against COI, ND1 and 16S sequences available in genetic data banks and consensus of nucleotide and amino acid sequences were achieved by BLAST software. *Androctonus australis* was included in phylogenetic analysis as an outgroup. This species was the most appropriate species in the buthid taxa phylogeny and has been repeatedly used in previous studies (14).

Results

The gDNA was extracted from 86 samples of adult black fat-tailed scorpions. The quality of purified DNA was an A260:A280 ratio of 1.8–2.1 and A260:A230 ratio of 1.3–2.0 for scorpion DNA extracts. The concentration of nucleic acids ranged between 50 and 100ng/µl. Fragments of 325bp, 618bp, and 680bp were produced by PCR from 16S, COI and ND1 mitochondrial genes, respectively.

Nucleotide contents

A total of 80 high-quality amplicons of the 16S, COI and ND1 fragments were subjected to sequencing. The representative sequences of each group were deposited in GenBank with accession numbers MH352581- MH352611.

From twenty-five subjected sequences of 16S rRNA fragments, 16 samples were selected for analysis. These sequences were deposited in GenBank with accession numbers of MH352581- MH352596. In these samples the PCR product size was 325bp and the A+T content reached a range of 72.84-73.54%. The nucleotide alignment of these samples showed five haplotypes that differed in eight single nucleotide polymorphic sites (SNPs) (Fig. 3). Haplotype I, distributed in all regions, was the dominance form and comprised 82% of samples (Fig. 4). BLAST analysis of this haplotype along with sequences available in GenBank databases showed 100% identity with the sample of West Azerbaijan (Sardasht, accession no AJ277598) and 93.4% with the sample of south Anatolia from Turkey (accession no EJ217735).

Like the 16S gene, 25 samples of PCR products of the ND1 gene which showed a sharp band were selected randomly for sequencing. The nucleotide sequences of six samples of these products were submitted in the GenBank with accession numbers MH352597 to MH352602. Multiple alignments of the sequences showed a length of 680 base pairs (bp) with 17 SNPs (Fig. 5). The A+T content in these sequences were in the range of 70.91–71.18%. In the alignments of the deduced sequences 415 nucleotides decode ND1 and the remaining (265 nucleotides) involve tRNA genes BLAST analysis of these sequences showed 89% identity with ND1 gene of *A. australis* (KJ538181) and 87% similarity with *Buthus occitanus* ND1 gene. Five haplotypes were obtained from TCS network tree of the ND1 gene sequences (Fig. 6). Haplotype I was a dominant group which contained 75% of samples. This haplotype is distributed in all areas. From 17 SNP sites of ND1 fragments, 2 sites were parsimonious informative including one transversion and one transition substitution. In the 15 remaining sites, 4 mutations were transition substitutions (G↔A, and T↔C) and 11 mutations were transversion substitutions (A↔T, A↔C, G↔C, and G↔T). Multiple alignment of decoded amino acids in ND1 peptide showed the study populations clustered in 3 isoforms.

Nucleotide sequencing of 20 randomly selected COI PCR products showed the products with 618bp. The deduced nucleotides in this fragment decode 206 amino acids. Data analysis revealed that all sequences have unique nucleotide arrangement, without any variation. The A+T content in these sequences was the lowest amount (59.51%) in the mtDNA fragment. Nine representative samples of these sequences were deposited in GenBank with ac-

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cession numbers of MH352603–MH352611. Analysis of the BLAST showed 99.4% identity with the *A. crassicauda* sample of Makoo (accession no MH814933) and 98.1% with sample of Sardasht (accession no MK814934) West Azerbaijan, northwest of Iran. These sequences also shared 99.4% and 98.1% similarity with COI fragments of *A. crassicauda* from Iraq (accession no MT2298940) and Egypt (accession no MT636858), respectively (Fig. 4).

Phylogenetic analysis

Phylogenetic analysis of COI and 16S sequences of *A. crassicauda* retrieved from the GenBank database revealed a close genetic relationship among studied populations and the samples of Makoo and Sardasht, northwest of Iran, Turkey, Iraq and Egypt. Constructed trees by ML, NJ, ME and MP methods strongly support the monophyly of black fat-tailed scorpion populations in the Middle East. Analysis of the phylogeny indicates that the haplotypes of this scorpion in Zanjan and northwestern Iran, Makoo and Sardasht districts, form a clade that its sister group is the populations of Iraq, Turkey and Egypt. This analysis confirms the relationship between geographic distance and genetic diversity; as the geographical distance increases, the genetic diversity also increases (Figs. 6, 7).

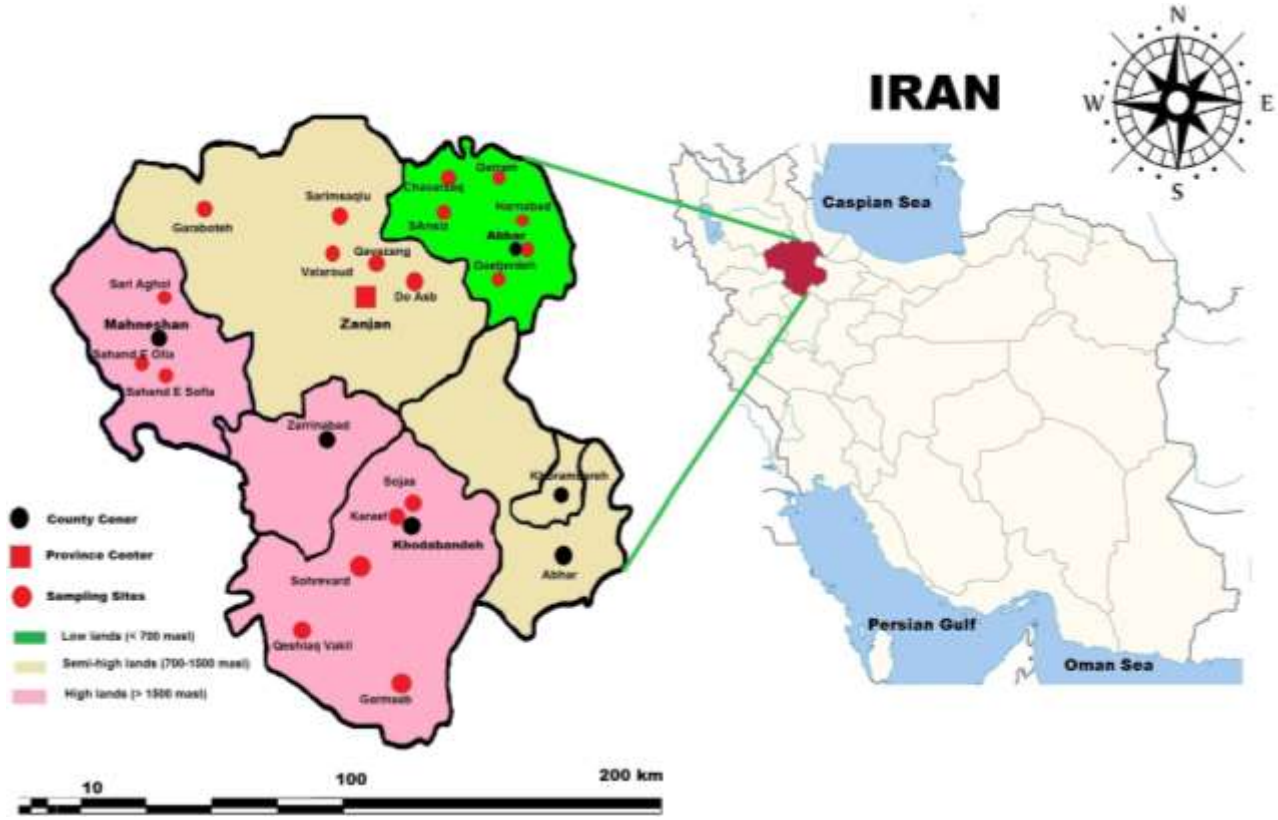

Fig. 1. Distribution map of sampling sites of *Androctonus crassicauda* in Zanjan Province

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Table 1. Details of the primers used in this study

Gene	Nucleotide sequence (5'-□3')	Reference
16S	Forward CGATTTGAACTCAGATCA	(29)
	Reverse GTGCAAAGGTAGCATAATCA	
ND1	Forward CGACCTCGATGTTTGAATTAA	(31)
	Reverse TCGTAAGAAATTATTTGAGC	
COI	Forward GGTCAACAAATCATCATAAAGATATTG	(32)
	Reverse TAAACTTCAGGGTGACCAAAAAATCA	

Fig. 2. Schematic presentation of external body parts in *Androctonus crassicauda*. Dorsal view of carapace (a) and metasomal segments (b), pedipalp patella without ventral trichobothria (c), pectinal area of male (d) and female (e), and lateral view of telson (f)

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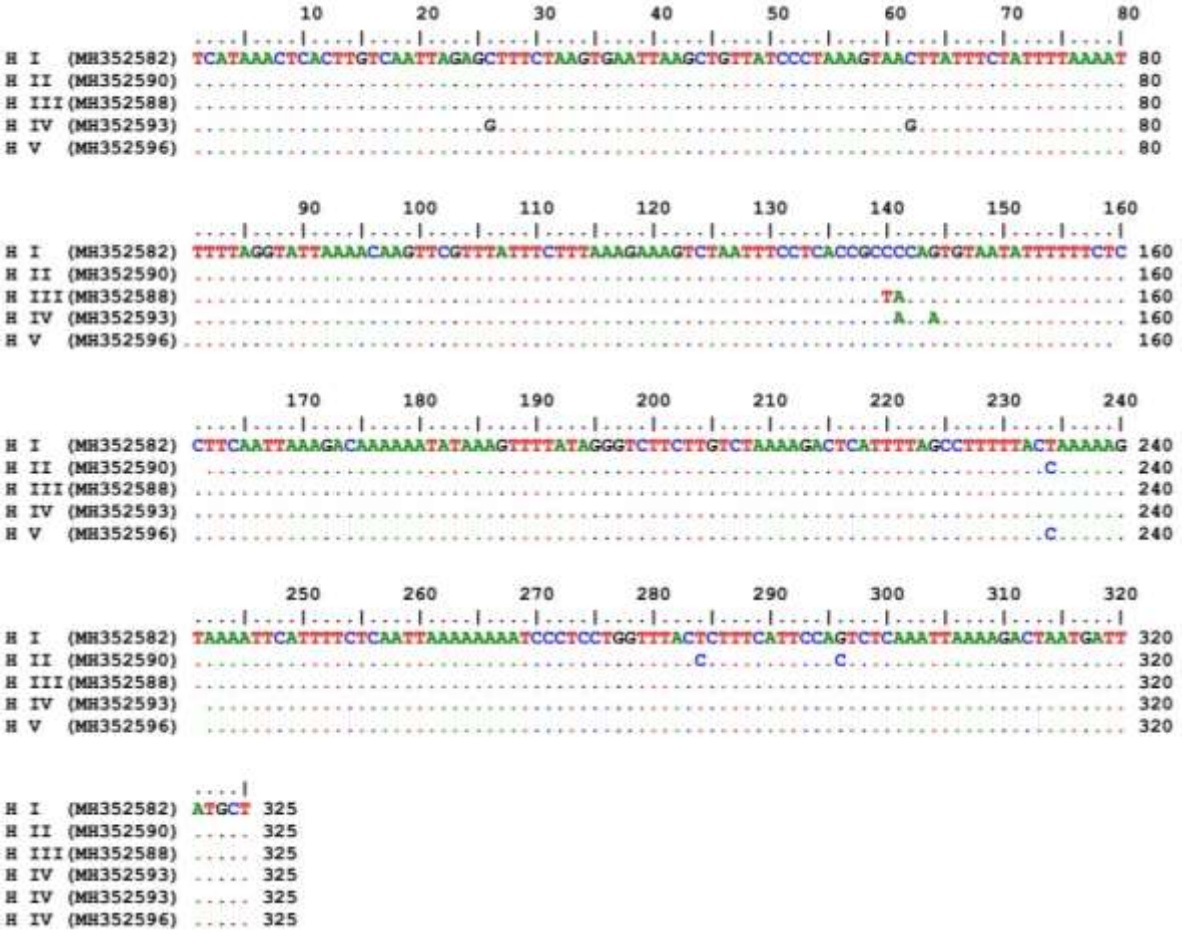

Fig. 3. Multiple alignment of 16S fragments in different haplotypes of *Androctonus crassicauda*. The numbers in the parentheses after the name of each haplotype denote the representative accession no of sample that each haplotype belongs to

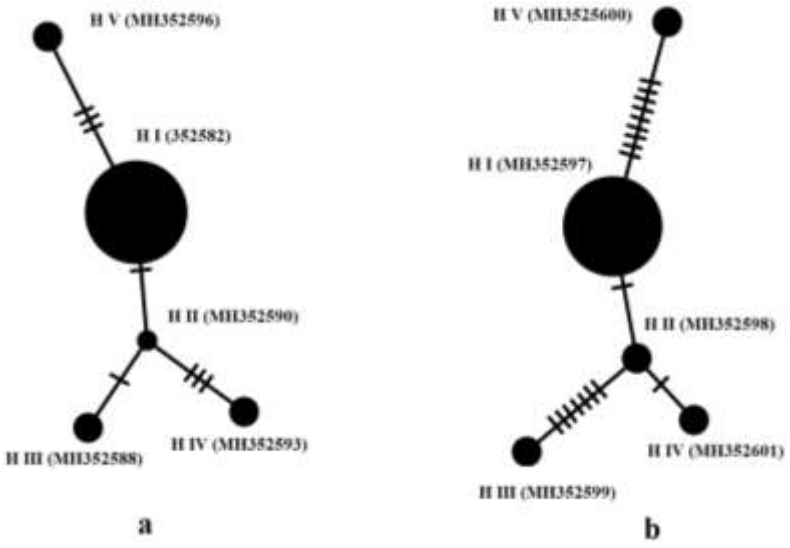

Fig. 4. TCS network tree of the 16S (a) and ND1 (b) mitochondrial gene haplotypes of *Androctonus crassicauda* in Zanjan Province. Each dash represents one single nucleotide difference between two neighboring haplotypes. The numbers in the parentheses after the name of each haplotype denote the representative accession no of sample

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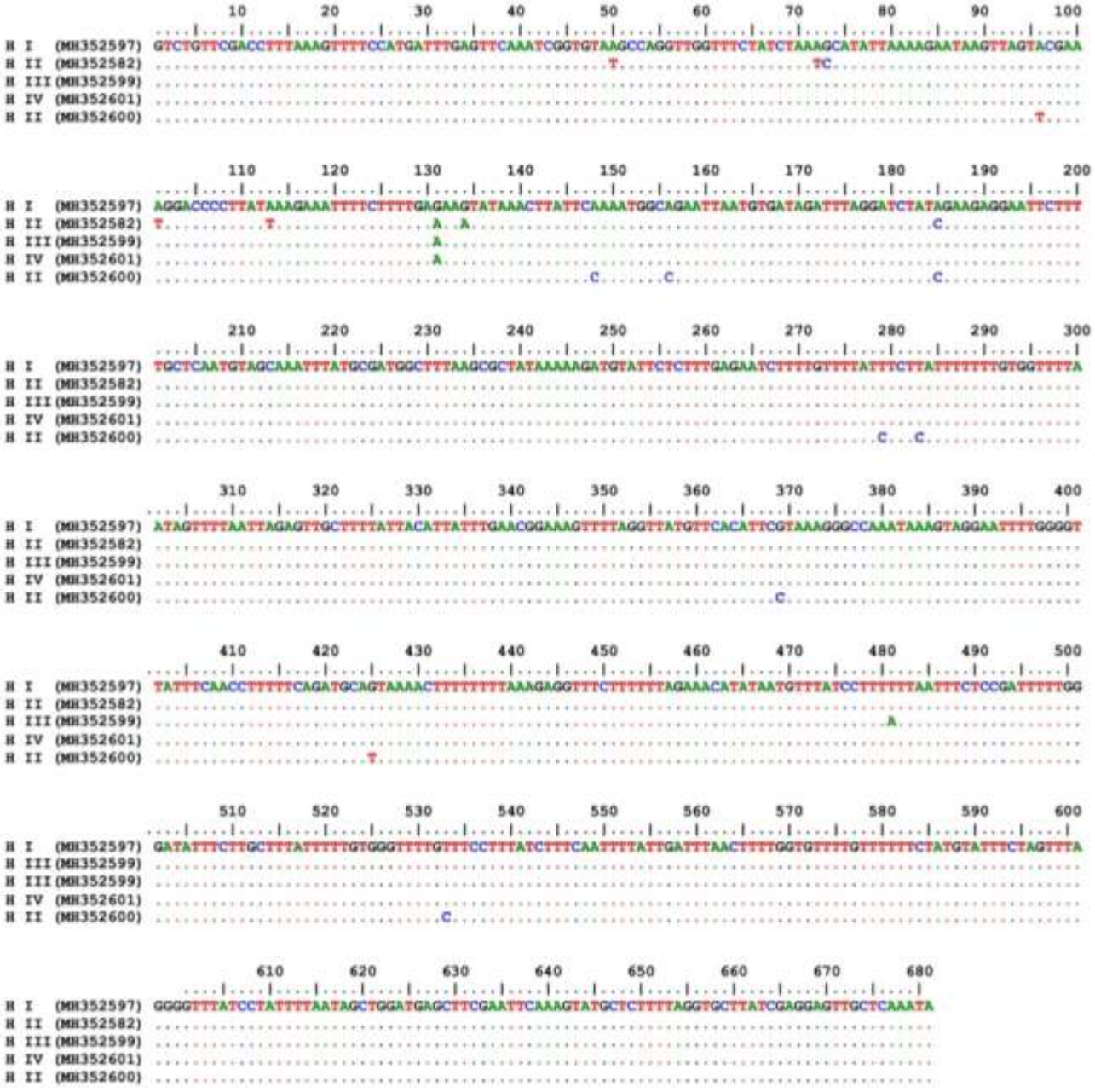

Fig. 5. Nucleotide alignments of ND1 mtDNA gene in different haplotypes of *Androctonus crassicauda* in Zanjan Province. The numbers in the parentheses after the name of each haplotype denote the representative accession number of sample that each haplotype belongs to

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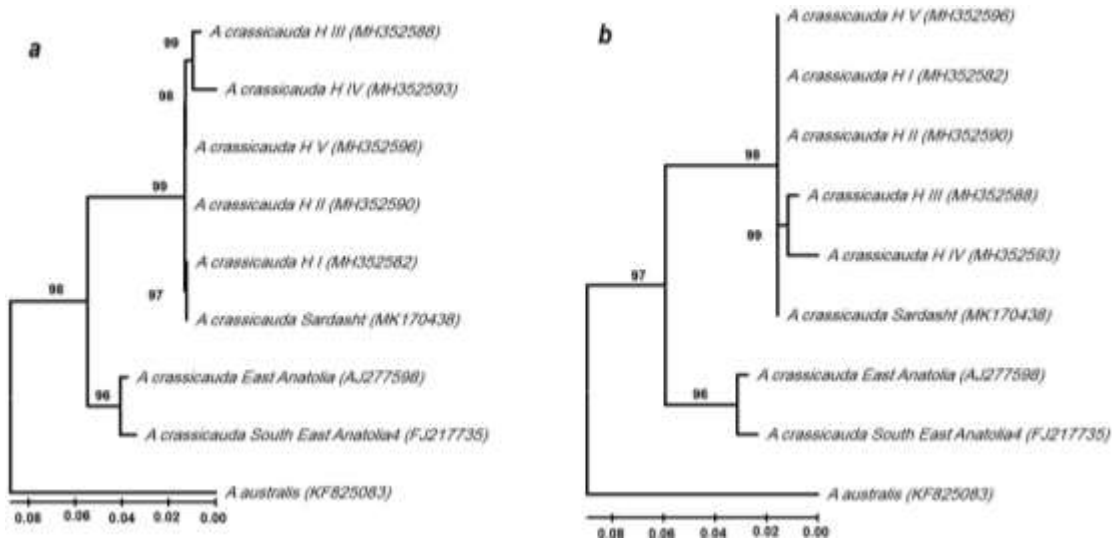

Fig. 6. Phylogenetic tree of COI haplotypes in different populations of *Androctonus crassicauda* in Zanjan Province, northwest of Iran, based on Maximum Likelihood, Neighbor-Joining, Minimum Evolution (a) and Maximum Parsimony (b) analyses methods. The numbers in the parentheses denote the accession no. The number on each branch represents percentage of tree containing that branch. The scale bar at the bottom of the trees shows the amount of genetic changes (the number of changes per 100 nucleotide sites)

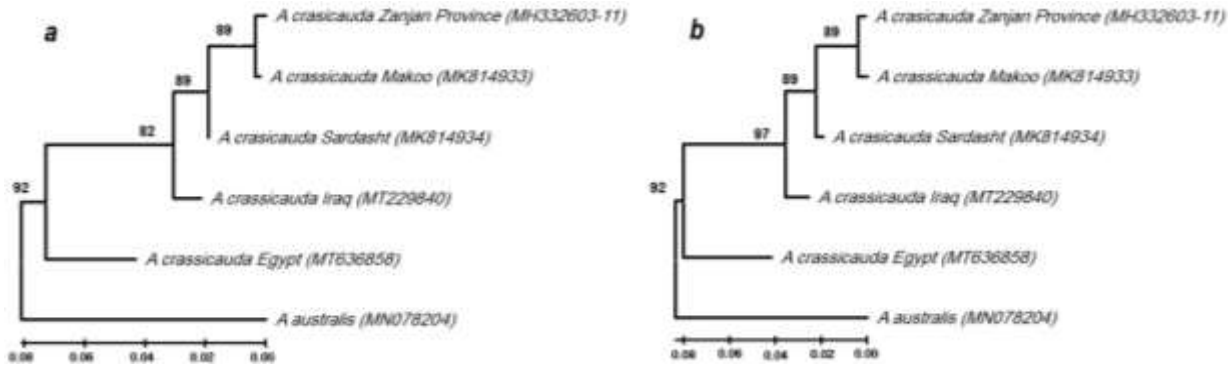

Fig. 7. Phylogenetic tree of 16S mtDNA fragments of *Androctonus crassicauda* in Zanjan Province, northwest of Iran. The constructed tree is inferred by using the Maximum Likelihood, Neighbour-Joining and Minimum Evolution (a), and Maximum Parsimony (b) analyses methods. The numbers in the parentheses denote the accession no. The value on each branch demonstrates the possibility of branching. The scale bar at the base of trees indicates the genetic diversity (the number of changes per 100 nucleotide sites)

Discussion

The most dangerous scorpion species, *A. crassicauda*, has been recorded from low to high areas with various climatic conditions in present study. These findings reflect wide range adaptation and tuning of optimal ecological conditions necessary to survive for this scorpion in different areas. The remarkable distribution of this scorpion in these areas calls for the authorities to take the necessary measures to prevent and cure envenomation.

The results of the present study showed that the A+T content in COI fragments of fat-tailed scorpion is 59.51%. It is slightly lower compared to those of other studied genes, 16S and ND1, in this species. Multiple alignments of COI fragments in study samples indicated that nucleo-

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tide sequences in this fragment are conserved. In previous studies phylogenetic analysis of COI gene in *Mesobotus eupeus* showed clear divergence between Northern and Southern clades in different areas of Iran (21). High levels of genetic diversity that represent some geographical coherences also were found in subspecies of *Scorpio mourus* from Moroco and Turkey (20, 23). In addition, gene diversity of Maghrebian *Hottentottus* is accessed by COI gene (22). Despite genetic diversity of COI gene in different species of scorpions, findings of present study suggest propriety of this molecular marker for accurate species diagnosis and interspecific taxonomic relationships of *A. crassicauda*.

Despite the stability of nucleotide sequences in the COI gene, the genetic diversity of 16S and ND1 genes was observed from different populations of fat-tailed scorpions in the study areas. These findings provide considerable detail on the diversity and valuable information about the population structure of this scorpion. Different reports have revealed diversity of 16S gene in this scorpion and other species (35–38); however, to the best of author's knowledge, this study represents the first evidence of ND1 diversity in the black fat-tailed scorpion.

Significantly genetic diversity representing 5 haplotypes have been found with 16S and ND1 gene analyses of study populations. More than half of study groups clustered in one clade. According to phylogenetic analyses, all the detected haplotypes in the present study were shared with isolates of worldwide origin, Anatolia, West Azerbaijan, and Northwest of Iran. The recorded high genetic variation within 16S sequences suggests the hypothesis that multiple introductions have occurred in populations of this species. Therefore, accurate population analysis and determination of the source of genotypes would be necessary to confirm this hypothesis in further studies.

Phylogenetic analysis results in this study showed that the haplotype I of this scorpion is a dominant group that is represented in all areas, and the other haplotypes which were separated from this haplotype distributed in special localities. Finding of this analysis suggests that one monophyletic lineage exists within study populations. Further detailed investigations should be carried out to test this hypothesis and many more populations should be tested for other data tests (toxin structure, geographic information system/ecological niche model and nuclear genes) to establish the true genetic structure of populations currently existing in this species.

Conclusion

The black fat-tailed scorpion was recorded from different altitudes in studied areas. This call for authorities to take the necessary preventive and envenomation measures for this scorpion. Among molecular markers, COI gene is suitable for determining the interspecific relationship of this scorpion. However, ND1 and 16S genes were suggested to identify the interspecies and population structures in this scorpion. Additional complementary works in further studies are needed to elucidate the facts behind the different haplotypes and population groups in this species.

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Ethical considerations

This study was approved by the Ethics Committee in Biomedical Research (ZUMS.REC. 1394.141) of Zanjan University of Medical Sciences, Zanjan, Iran.

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Conflict of interest statement

Authors declare that there is no conflict of interest.

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