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A study on the genetic diversity of *Androctonus crassicauda* (Olivier, 1807; Scorpiones: Buthidae) from Turkey

Ozkan O (1, 2), Ahmet C (3), Zafer K (2)

(1) Refik Saydam Public Health Agency, Ankara, Turkey; (2) Department of Entomology and Protozoology, Faculty of Veterinary Medicine, Ankara University, Ankara, Turkey; (3) Turkish Accreditation Agency, Ankara, Turkey.

Abstract: Mitochondrial DNA (mtDNA) has been widely employed in phylogeographic and phylogenetic studies. In the present study, the genetic identification of the scorpion *Androctonus crassicauda* (Olivier, 1807) was carried out by using the 16S mitochondrial gene, since this scorpion represents the most important species in Turkey regarding scorpionism and antivenom production. Two genetic groups were found according to the sequence analysis results, while five different loci at the nucleotide level presented genetic variation in the 16S region when compared to a known *A. crassicauda* sequence data (GenBank, AJ277598). Nucleotide variations found in the current work constitute the first descriptive report for *A. crassicauda*. Moreover, future studies may enlighten the genetic and venom composition variations for this scorpion species.

Key words: scorpion, *Androctonus crassicauda*, PCR, genotype, Turkey.

INTRODUCTION

Scorpions can be regarded as "living fossils" because they have changed so little during the last 400 million years (1-3). They are distributed throughout the tropical, temperate, and cold zones of the Earth (4).

More than 1,500 species of scorpions have been described so far, but only 50 were stated as dangerous to humans (2). Scorpion sting is the most important type of arachnid envenomation resulting in adult morbidity and pediatric mortality (5, 6). Scorpionism remains a real health problem in developing countries, especially in tropical and subtropical regions including urban areas.

Lethal scorpions mostly belong to the family Buthidae, among which species of the genera *Androctonus*, *Leiurus*, and *Mesobuthus* are responsible for envenomation in Turkey (7, 8). *Androctonus crassicauda* is the most significant scorpion species in Turkey due to scorpionism and antivenom production (8, 9). This species can be found in Azerbaijan, Iran, Iraq, Syria, Jordan, Saudi Arabia and Yemen, besides Turkey (5, 10, 11).

The individual and geographical variability in venoms of one same species has become extremely important for the production of effective antivenoms and for the understanding of the clinical symptoms of patients. Intragenic differences can be a result of genetic and environmental factors (12).

Whittemore *et al.* (13) reported that the Turkish antivenom (antivenom produced against *A. crassicauda* venom) was more effective than homologous antivenom in neutralizing *A. australis* venom in Algeria. However, antivenom against *A. crassicauda* was found to have the same neutralization capacity as homologous antivenoms against *B. occinatus* venom in South Europe and North Africa, *T. serrulatus* and

T. bahiensis venoms in South America, and *L. quinquestriatus* venom in Anatolia.

In this study, we aimed to use nucleotide sequences to obtain information about the taxonomic status of *A. crassicauda* scorpion, which is the most important scorpion species in Turkey considering envenomation cases and antivenom production.

MATERIALS AND METHODS

Specimens

The scorpions were originally collected from Sanliurfa province (36° 40'-38° 02' W, 37° 50'-42° 12' E) in the Southeast region of Turkey. The animals were captured at night by using a UV lamp. They were kept in plastic boxes at the Department of Entomology and Protozoology, Faculty of Veterinary Medicine, Ankara University.

Extraction of Genomic DNA (gDNA)

Genomic DNA was extracted from fresh or preserved (96% ethanol) muscle tissue (a leg) using the phenol/chloroform method (14). Tissues were rapidly frozen and ground in liquid nitrogen to produce readily digestible pieces. Samples were homogenized in homogenization buffer solution [10 mM Tris-Cl (pH 8.0); 0.1 M EDTA (pH 8.0); 20 g/mL RNAse A; 10% sodium dodecyl sulfate; 0.1 mg/mL proteinase K] by agitation for ten minutes under incubation at 50°C. After homogenization, DNA was purified in standard 25:24:1 phenol/chloroform/isoamyl alcohol series and precipitated by addition of 0.2 volumes of 10 M ammonium acetate and two volumes of 100% ethanol. The DNA pellet was centrifuged, washed twice in 70% ice-cold ethanol, dried at room temperature and resuspended in TE buffer (10 mM Tris-Cl, 1 mM EDTA) until dissolved. The quality of the genomic DNA was examined on 0.8% ethidium bromide-stained agarose gels and OD_{260} was determined by using UV spectrophotometer.

Amplification, Sequence Determination and Alignment

A part of the large-subunit mitochondrial ribosomal gene (16S rRNA) was amplified by using a pair of scorpion-specific 16S primers or LR-J-12887. The sequence of the forward primer was 5'-CGA TTT GAA CTC AGA TCA-3' and that of the reverse primer was 5'-GTG CAA AGG TAG CAT AAT CA-3', as reported by Gantenbein *et al.* (15). These primers corresponded to the positions 12.867–12.887 and 13.218–13.310 in the *Drosophila yakuba* mitochondrial genome (15). In a final volume of 25 µL, amplification mixtures contained 1.25U of Taq DNA polymerase (Promega, USA), 2.0 mM of $MgCl_2$, 0.2 µM of dNTPs, 0.3 pmol of each primer (alpha DNA) and 100 ng of genomic DNA. The thermal cycling program started with an initial denaturing step at 94°C for five minutes, followed by 45 amplification cycles (94°C for one minute denaturation, 48°C for one minute annealing, 72°C for 1.5 minute extension), and a final extension at 72°C for ten minutes in a gradient PCR (Biometra T® Gradient, USA). Finally, the amplification products were verified on 1.5% agarose electrophoretic gel and purified by using the QIAquick PCR® Purification kit (Qiagen, Germany).

The sequencing reaction was carried out with the forward and reverse primers using the gel-purified PCR product as template. The purified templates were sequenced with BigDye®Terminator v.3.1 (Applied Biosystems, USA), run on an ABI Prism® 3100 Genetic Analyzer (RefGen, Turkey). The accuracy of data was confirmed by two-directional sequencing with the forward and reverse primers. Chromatograms and sequences were analyzed by using FinchTv program®, version 1.4.0 (Geospiza, Inc.; USA; http://www.geospiza.com). The obtained DNA sequences were edited and aligned by using Basic Local Alignment Search Tool software [BLAST, version 2.2.22 (http://blast.ncbi.nlm.nih.gov)] (16). All new DNA sequences reported in this paper were deposited in GenBank sequence database provided by the National Center for Biotechnology Information (accession numbers: FJ217732-FJ217739).

Phylogenetic Analyses

The neighbor-joining tree (17) was constructed from the distances by using MEGA4 software (http://www.megasoftware.net) (18, 19). As outgroup, we selected *Pseudochactas ovchinnikovi* Gromov, 1998 (20), the relict taxon of the monotypic family Pseudochactidae, which is considered the basal group for Buthidae (21).

RESULTS

All scorpions were examined under stereomicroscope. Most of them were reddish-brown, varying from brown to black. The patellae of pedipalps did not present ventral trichobothria. All scorpions were identified as *A. crassicauda* according to morphological data.

Eight samples were analyzed and amplified to ca. 400-base pair (bp) fragment of the 16S rRNA

Table 1. Nucleotide sequences for eight samples of various sizes

Sample number	GenBank accession number	Sequence	Length (bp)
1	**FJ217732**	AATGAATTTTACTTTTTAGTAAAAAGGCTAAAATACATCTTTTAGACAAGAAGACCCTATAAAAC TTTATATTTTTTTTGTTTTTGATTAAAGGGAAAAATATTACACTGGGGCGGTGAAGAAATTAGG CTTTCTTTGAAAAAATAAACGAACTTGTTTTAATACCTAGAAATTTTAAAATAGAAATAAGTTAC TTTAGGGATAACAGCTTAATTCACTTAGAAAGTTCTAATTGACAAGTGAGTTTATGACCTCGAT GTTGAATTAAAATACCTTTTGTTGCGCAAAAGCTTAAAAAGGAGGTCTGTTCGACCTTTAAAAT TTTCCATGATCTGAGTTCAAATCGA	**347**
2	**FJ217733**	GAGACTGGAATGAAAGAGTAAACCAGGAGGATTTTTTTTTAGTTGAAGAAATGAATTTTACTTT TTAGTAAAAAGGCTAAAATACATCTTTTAGACAAGAAGACCCTATAAAACTTTATATTTTTTTTG TTTTTGATTAAAGGGAAAAATATTACACTGGGGCGGTGAAGAAATTAGGCTTTCTTTGAAAAA ATAAACGAACTTGTTTTAATACCTAGAAATTTTAAAATAGAAATAAGTTACTTTAGGGATAACAG CTTAATTCACTTAGAAAGTTCTAATTGACAAGTGAGTTTATGACCTCGATGTTGAATTAAAATAC CTTTTGTTGCGCAAAAGCTTAAAAA	**347**
3	**FJ217734**	AGTTGAAGAAATGAATTTTACTTTTTAGTAAAAAGGCTAAAATACATCTTTTAGACAAGAAGAC CCTATAAAACTTTATATTTTTTTGTTTTTGATTAAAGGGAAAAATATTACACTGGGGCGGTGAAG AAATTAGGCTTTCTTTGAAAAAATAAACGAACTTGTTTTAATACCTAGAAATTTTAAAATAGAAA TAAGTTACTTTAGGGATAACAGCTTAATTCACTTAGAAAGTTCTAATTGACAAGTGAGTTTATG ACCTCGATGTTGAATTAAAATACCTTTTGTTGCGCAAAAGCTTAAAAAGGAGGTCTGTTCGACC TTTAAA	**328**
4	**FJ217735**	TTTTAATTTGAGACTGGAATGAAAGAGTAAACCAGGAGGATTTTTTTTTAGTTGAAGAAATGAA TTTTACTTTTTAGTAAAAAGGCTAAAATACATCTTTTAGACAAGAAGACCCTATAAAACTTTATA TTTTTTTGTTTTTGATTAAAGGGAAAAATATTACACTGGGGCGGTGAAGAAATTAGGCTTTCTTT GAAAAAATAAACGAACTTGTTTTAATACCTAGAAATTTTAAAATAGAAATAAGTTACTTTAGGG ATAACAGCTTAATTCACTTAGAAAGTTCTAATTGACAAGTGAGTTTATGACCTCGATGTTGAATT AAAATACCTTTTGTTGCGCAAAAGCTTAAAAAGGAGGTCTGTTCGACCTT	**373**
5	**FJ217736**	AGTTGAAGAAATGAATTTTACTTTTTAGTAAAAAGGCTAAAATACATCTTTTAGACAAGAAGAC CCTATAAAACTTTATATTTTTTTGTTTTTGATTAAAGGGAAAAATATTACACTGGGGCGGTGAAG AAATTAGGCTTTCTTTGAAAAAATAAACGAACTTGTTTTAATACCTAGAAATTTTAAAATAGAAA TAAGTTACTTTAGGGATAACAGCTTAATTCACTTAGAAAGTTCTAATTGACAAGTGAGTTTATG ACCTCGATGTTGAATTAAAATACCTTTTGTTGCGCAAAAGCTTAAAAAGGAGGT	**312**
6	**FJ217737**	AGTTGAAGAAATGAATTTTACTTTTTAGTAAAAAGGCTAAAATACATCTTTTAGACAAGAAGAC CCTATAAAACTTTATATTTTTTTTGTTTTTGATTAAAGGGAAAAATATTACACTGGGGCGGTGAA GAAATTAGGCTTTCTTTGAAAAAATAAACGAACTTGTTTTAATACCTAGAAATTTTAAAATAGAA ATAAGTTACTTTAGGGATAACAGCTTAATTCACTTAGAAAGTTCTAATTGACAAGTGAGTTTAT GACCTCGATGTTGAATTAAAATACCTTTTGTTGCGCAAAAGCTTAAAAAGGAGGTCTGTTCGAC CTTTAAAATTT	**333**
7	**FJ217738**	TTTGAGACTGGAATGAAAGAGTAAACCAGGAGGATTTTTTTTTAGTTGAAGAAATGAATTTTAC TTTTTAGTAAAAAGGCTAAAATACATCTTTTAGACAAGAAGACCCTATAAAACTTTATATTTTTTT GTTTTTGATTAAAGGGAAAAATATTACACTGGGGCGGTGAAGAAATTAGGCTTTCTTTGAAAAA ATAAACGAACTTGTTTTAATACCTAGAAATTTTAAAATAGAAATAAGTTACTTTAGGGATAAC AGCTTAATTCACTTAGAAAGTTCTAATTGACAAGTGAGTTTATGACCTCGATGTTGAATTAAAAT ACCTTTTGTTGCGCAAAAGCTTAAAAAGGAGGT	**355**
8	**FJ217739**	AATTTGAGACTGGAATGAAAGAGTAAACCAGGAGGATTTTTTTTTAGTTGAAGAAATGAATTT TACTTTTTAGTAAAAAGGCTAAAATACATCTTTTAGACAAGAAGACCCTATAAAACTTTATATTTT TTTGTTTTTGATTAAAGGGAAAAATATTACACTGGGGCGGTGAAGAAATTAGGCTTTCTTTGAA AAAATAAACGAACTTGTTTTAATACCTAGAAATTTTAAAATAGAAATAAGTTACTTTAGGGATA ACAGCTTAATTCACTTAGAAAGTTCTAATTGACAAGTGAGTTTATGACCTCGATGTTGAATTAAA ATACCTTTTGTTGCGCAAAAGCTTAAAAAGGAGGTCT	**359**

mitochondrial (mt) DNA by PCR. The DNA sequences of the mtDNA 16 S gene are listed in Table 1.

Eight 16S DNA sequences were blasted and aligned with interspecies. The sequences were divided into two genetic groups due to a T nucleotide Indel. Genetic group I (GI) was found to be 99.7% homologous with Genetic group II (GII), according to the alignment result. There are eight T nucleotides between positions 81 and 88 in the 315 base pairs in GI. However, GII has seven T nucleotides, as shown in Table 2. According to the alignment results, FJ217732, FJ217733 and FJ217737 make up GI, while FJ217734, FJ217735, FJ217736, FJ217738 and FJ217739 are GII. Genetic GI (Table 3) and GII (Table 4) were also blasted with a known *A. crassicauda* sequence data.

All results are based on the pairwise analysis of eight sequences. Analyses were conducted by using the maximum composite likelihood method in MEGA4 (18, 19). All positions containing gaps and missing data were eliminated from the data set (complete deletion option). There were 297 positions in the final data set.

The evolutionary history was inferred by using the neighbor-joining method (17). The percentage of replicate trees, in which the associated taxa clustered together in the bootstrap test (1000 replicates), are shown next to the branches (22). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed by using the maximum composite likelihood method and are in the units of the number of base substitutions per site (18). All positions containing gaps and missing data were eliminated from the data set (complete deletion option). There were 286 positions in the final data set. Phylogenetic analyses were conducted in MEGA4 (19). Our sequence data are closely related (84%) to *P. ovchinnikovi* (GenBank accession number AY226167) (Figure 1).

Table 2. BLAST alignment between group I and group II

		Blasted of GI and GII	
GI	1	AGT TGA AGA AAT GAA TTT TAC TTT TTA GTA AAA AGG CTA AAA TAC ATC TTT TAG ACA AGA	60
		||| ||| ||| ||| ||| ||||| ||| ||| ||| ||| ||| ||||| ||||||| ||| |||	
GII	1	AGT TGA AGA AAT GAA TTT TAC TTT TTA GTA AAA AGG CTA AAA TAC ATC TTT TAG ACA AGA	60
GI	61	AGA CCC TAT AAA ACT TTA TAT TTT TTT TGT TTT TGA TTA AAG GGA AAA ATA TTA CAC TGG	120
		||| ||| ||| ||| ||| ||| || ||||| ||| ||||| ||| ||| ||| |||||| ||| |||	
GII	61	AGA CCC TAT AAA ACT TTA TA- TTT TTT TGT TTT TGA TTA AAG GGA AAA ATA TTA CAC TGG	119
GI	121	GGC GGT GAA GAA ATT AGG CTT TCT TTG AAA AAA TAA ACG AAC TTG TTT TAA TAC CTA GAA	180
		||| ||| ||| ||| ||| ||| ||| ||||| ||| ||| ||| ||| ||| ||||| ||||| ||| |||	
GII	120	GGC GGT GAA GAA ATT AGG CTT TCT TTG AAA AAA TAA ACG AAC TTG TTT TAA TAC CTA GAA	179
GI	181	ATT TTA AAA TAG AAA TAA GTT ACT TTA GGG ATA ACA GCT TAA TTC ACT TAG AAA GTT CTA	240
		|||||| ||| ||| ||| ||| ||| ||||| ||| ||| ||| ||| ||| ||||| ||| ||| |||	
GII	180	ATT TTA AAA TAG AAA TAA GTT ACT TTA GGG ATA ACA GCT TAA TTC ACT TAG AAA GTT CTA	239
GI	241	ATT GAC AAG TGA GTT TAT GAC CTC GAT GTT GAA TTA AAA TAC CTT TTG TTG CGC AAA AGC	300
		|||||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||||| ||| ||| |||	
GII	240	ATT GAC AAG TGA GTT TAT GAC CTC GAT GTT GAA TTA AAA TAC CTT TTG TTG CGC AAA AGC	299
GI	301	TTA AAA AGG AGG TCT	315
		||| ||| ||| ||| |||	
GII	300	TTA AAA AGG AGG TCT	314

Table 3. BLAST result between genetic group I (GI) DNA sequences and AcrTR (accession number AJ277598)

		Blasted of GI and AcrTR	
GI	1	AGT TGA AGA AAT GAA TTT TAC TTT TTA GTA AAA AGG CTA AAA TAC ATC TTT TAG ACA AGA	60
AcrTR	324	AGT TGA AGA AAT GAA TTT TAC TTT TTA GTA AAA AGG CTA AAA TAC ATC TTT TAG ACA AGA	265
GI	61	AGA CCC TAT AAA ACT TTA TA**T** TTT TTT TGT TTT T**G**A TTA AAG GGA AAA ATA TTA CAC TGG	120
AcrTR	264	AGA CCC TAT AAA ACT TTA TA**-** TTT TTT TGT TTT T**A**A TTA AAG GGA AAA ATA TTA CAC TGG	206
GI	121	GGC GGT GAA GAA ATT AG**G** CTT TCT TTG AAA AAA TAA ACG AAC **T**TG TTT TAA TAC CTA GAA	180
AcrTR	205	GGC GGT GAA GAA ATT AG**A** CTT TCT TTG AAA AAA TAA ACG AAC **C**TG TTT TAA TAC CTA GAA	146
GI	181	ATT TTA AAA TAG AAA TAA GTT ACT TTA GGG ATA ACA GCT TAA TTC ACT TAG AAA GTT CTA	240
AcrTR	145	ATT TTA AAA TAG AAA TAA GTT ACT TTA GGG ATA ACA GCT TAA TTC ACT TAG AAA GTT CTA	86
GI	241	ATT GAC AAG TGA GTT TAT GAC CTC GAT GTT GAA TTA AAA TAC CTT TTG TTG CGC AAA AGC	300
AcrTR	85	ATT GAC AAG TGA GTT TAT GAC CTC GAT GTT GAA TTA AAA TAC CTT TTG TTG CGC AAA AGC	26
GI	301	TTA AAA AGG AG**G** TCT	315
AcrTR	25	TTA AAA AGG AG **-** TCT	12

Table 4. Genetic group II (GII) was found to be 98.7% homologous with AcrTR (accession number AJ277598)

		Blasted of GII and AcrTR	
GII	1	TTT GAG ACT GGA ATG AAA GAG TAA ACC AGG AGG ATT TTT TTT TAG TTG AAG AAA TGA ATT	60
AcrTR	367	TTT GAG ACT GGA ATG AAA GAG TAA ACC AGG AGG ATT TTT TTT TAG TTG AAG AAA TGA ATT	308
GII	61	TTA CTT TTT AGT AAA AAG GCT AAA ATA CAT CTT TTA GAC AAG AAG ACC CTA TAA AAC TTT	120
AcrTR	307	TTA CTT TTT AGT AAA AAG GCT AAA ATA CAT CTT TTA GAC AAG AAG ACC CTA TAA AAC TTT	248
GII	121	ATA TTT TTT TGT TTT TGA TTA AAG GGA AAA ATA TTA CAC TGG GGC GGT GAA GAA ATT AGG	180
AcrTR	247	ATA TTT TTT TGT TTT TAA TTA AAG GGA AAA ATA TTA CAC TGG GGC GGT GAA GAA ATT AGA	188
GII	181	CTT TCT TTG AAA AAA TAA ACG AAC TTG TTT TAA TAC CTA GAA ATT TTA AAA TAG AAA TAA	240
AcrTR	187	CTT TCT TTG AAA AAA TAA ACG AAC CTG TTT TAA TAC CTA GAA ATT TTA AAA TAG AAA TAA	128
GII	241	GTT ACT TTA GGG ATA ACA GCT TAA TTC ACT TAG AAA GTT CTA ATT GAC AAG TGA GTT TAT	300
AcrTR	127	GTT ACT TTA GGG ATA ACA GCT TAA TTC ACT TAG AAA GTT CTA ATT GAC AAG TGA GTT TAT	68
GII	301	GAC CTC GAT GTT GAA TTA AAA TAC CTT TTG TTG CGC AAA AGC TTA AAA AGG AG	353
AcrTR	67	GAC CTC GAT GTT GAA TTA AAA TAC CTT TTG TTG CGC AAA AGC TTA AAA AGG AG	15

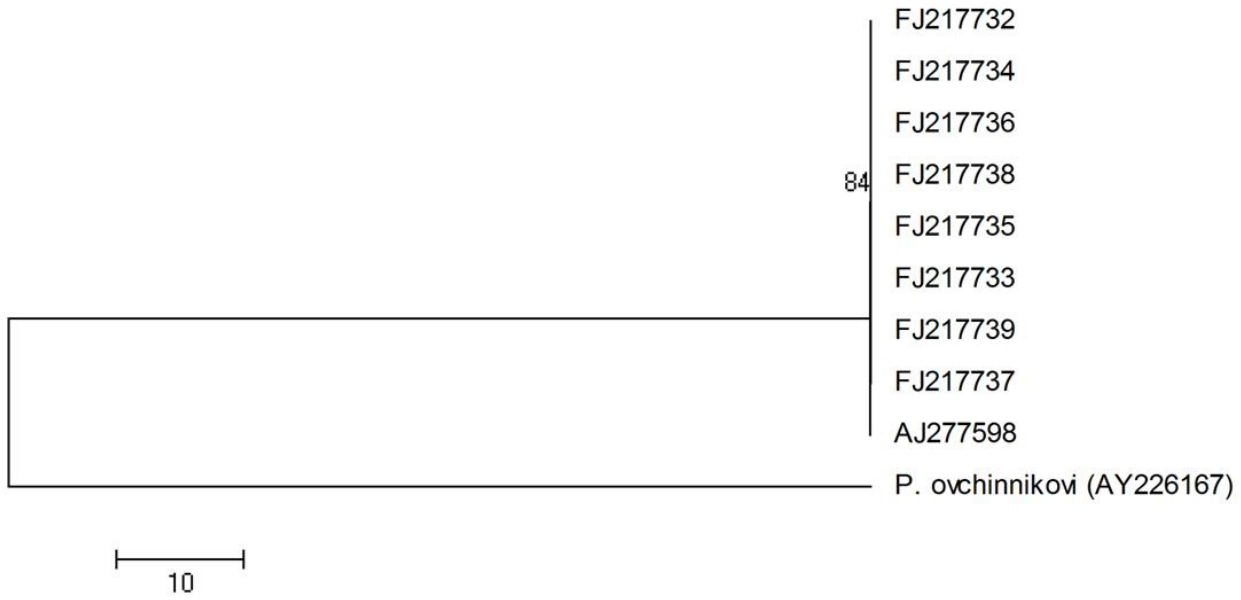

Figure 1. The neighbor-joining tree was conducted in MEGA4. *Pseudochactas ovchinnikovi* was selected as outgroup. All sequence data are closely related to the outgroup (84%).

DISCUSSION

Mitochondrial DNA (mtDNA) has been widely employed in phylogeographic and phylogenetic studies using conserved PCR primers (15). The order Scorpiones is under constant investigation regarding the taxonomic classification and phylogenetic relationships of its higher taxa (21, 23, 24). Recently, new scorpion species or subspecies have been recognized based on molecular techniques.

According to current reports, *Androctonus crassicauda* can be found in Sinai Peninsula (Egypt), across the entire Middle East (Southeastern Anatolia, Turkey), Arabian Peninsula and Armenia. In the present study, this species is reported as common in Sanlıurfa province near Syria and Iraqi borders in the Middle East. *Androctonus crassicauda* is one of the most medically important species in the Middle East and is also used for antivenom production.

The genotypes of *A. crassicauda* were determined according to the DNA sequence analysis of the 16S rRNA locus. The 16S rRNA locus in GI and GII was compared to the accession number AJ277598 (AcrTR from Urfa, Turkey).

GI was also blasted with AcrTR and was found to be 98.7% homologous with AcrTR. Differences in the first genetic group were due to a T for gap indel at position 81, a G for A substitution at positions 95 and 138, a T for C substitution at position 163, and a G for gap indel at position 312 (Table 3).

GII was blasted and was found to be 98.9% homologous with AcrTR. However, diversities in GII were due to a G for A substitution at positions 137 and 180, and a T for C substitution at position 205 (Table 4).

Comparing recent findings on *A. crassicauda* with the data from the present study, and according to the identification key, no morphological differences were detected between samples subjected to this study. However, this study determined two genetic groups according to the sequencing analysis. The genetic variation was determined in five different loci at the nucleotide level in the 16S region for the large-subunit mitochondrial ribosomal gene when compared to a known *A. crassicauda* sequence data (3). Nucleotide variations found in this study constitute the first descriptive report for *A. crasscicauda*.

Signs and symptoms of scorpionism depend on various factors including the genus, species, age, weight, feeding state and structure of scorpions, as well as the amount of venom injected, and the climate of the region. The genetic variation may

also lead to diversity in *A. crassicauda* venom collected from one same locality.

In conclusion, the current study determined two genetic groups which have genetic variation (five different loci) in the 16S region when compared to a known *A. crassicauda* sequence (GenBank accession number AJ277598).

The sequence results obtained in this paper will increase the genetic information and benefit evolutionary taxonomists who work with scorpions.

In the future, the genetic variation and the composition of scorpion venom should be investigated for the development of more efficient antivenom to be used in scorpion envenomation. A combined morphological and molecular (DNA) phylogenetic analysis approach for the samples from the neighboring countries will also clarify the relationships among scorpion species. The investigation of diverse populations could determine the exact origin and possible ways of dispersal of *A. crassicauda*. In addition, the preliminary tree presented in this study may give an indication of the relationship among/within the species in the scorpion fauna of Turkey.

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CONFLICTS OF INTEREST

There is no conflict.

CORRESPONDENCE TO

OZCAN OZKAN, Refik Saydam Public Health Agency, 06100 Ankara, Turkey. Phone: 00 90 312 498 21 50. Email: ozcanozkan_62@hotmail.com.

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