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Phylogenetic analysis of *Alpiscorpius phrygius* (Bonacina, 1980) (Scorpiones: Euscorpiidae) distributed in Soğuksu National Park

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Abstract

Anatolian fauna is very rich in terms of scorpion diversity. New species are added to the fauna day by day in line with new studies. Some mistakes can be made during the identification process and even some different species of the *Alpiscorpius* lineage can be defined as the same species if they are characterized using solely conventional methods. The scorpion specimens were collected from Ankara Soğuksu National Park and examined using both conventional and molecular methods. As a result of molecular analysis using Mitochondrial cytochrome oxidase subunit I (COI) gene as a DNA barcoding, the scorpion species in this study were identified as being *Alpiscorpius phrygius* (Bonacina, 1980).

Keywords: *Alpiscorpius phrygius,* COI, scorpion, phylogenetic, Turkey

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Soğuksu Milli Parkı'nda yayılış gösteren *Alpiscorpius phrygius* (Bonacina, 1980) (Scorpiones: Euscorpiidae)'un filogenetik analizi

Özet

Anadolu faunası akrep çeşitliliği bakımından oldukça zengindir. Yapılan çalışmalar ile her geçen gün faunaya yeni türler eklenmektedir. Ancak teşhis sürecinde bazı hatalar yapılabilmekte ve *Alpiscorpius* cinsinde bulunan bazı farklı türler yalnızca geleneksel yöntemler ile teşhis edilmeye çalışıldığında aynı tür olarak da tanımlanabilmektedirler. Yaptığımız bu çalışmada Ankara Soğuksu Milli Parkından toplanan 10 akrep örneği hem geleneksel hem de moleküler düzeyde tanımlanmaya çalışılmıştır. Bir DNA barkod geni olarak kullanılan mitokondrial sitokrom oksidaz subunit I (COI) ile yapılan moleküler analizde akrep türlerinin *Alpiscorpius phrygius* (Bonacina, 1980) oldukları tespit edilmiştir.

Anahtar kelimeler: *Alpiscorpius phrygius ,* COI, akrep, filogenetik, Türkiye

1. Introduction

Turkey has a rich biological diversity due to its zoogeographical position and the fact that it is situated in the Palearctic zoogeography region. Turkey has great importance because of its geographical features. Changing climate, vegetation and geomorphological features at very short distances lead to diversification and enrichment of habitats for plants and animals [1]. Scorpions are important and useful components of many ecosystems and one of the oldest known terrestrial arthropods [2]. Scorpions, categorized in Arachnida at the ordo level, are carnivorous and dissolve their prey with their poisons. Scorpions are nocturnal animals. They hide under stones, tree hollows, or under the soil during the day [3]. Scorpions have special sense organs for hunting and protection. All scorpion species known today are poisonous. Moreover, some scorpion species are a danger to humans as well. According to data from the World Health Organization (WHO), approximately 5,000 people die annually due to scorpion stings. Scorpion venoms are neurotoxic or cytotoxic. Neurotoxic venoms are generally more dangerous for humans.

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Until today, 2584 species of scorpions are known in the world [4], of which 41 are found from Turkey [5, 6, 7, 8, 9]. *Alpiscorpius phrygius* (Bonacina, 1980) belongs to Euscorpiidae family. *Alpiscorpius phrygius* is endemic in Turkey. In Turkey, this species is located in Sakarya, Bolu, Karabük, Ankara, and Düzce [5].

In the genus *Alpiscorpius* (Scorpiones: Euscorpiidae), there are fifteen species [10]. However, like other species in this genus, the species in *Alpiscorpius phrygius* have usually been classified with morphological diagnostic methods. In the studies carried out previously in Soğuk Su National Park, scorpions collected from the same locality were identified as *Alpiscorpius phrygius* [11]. This demonstrates that the identification of species that are conducted with existing morphological characters may not always be accurate. Therefore, classical systematic studies should be supported by methods at the molecular level. From this point of view, in this study, it is aimed to characterize *Alpiscorpius phrygius* at species level with molecular analysis using mitochondrial cytochrome oxidase subunit I (COI) gene, a DNA barcode gene, in addition to morphological analysis.

2. Materials and methods

1.1. Collection and identification of specimens

The specimens used in the study were collected in August 2018 from Ankara Soğuk Su National Park (40° 27′ 14.87 and 32° 37′ 20.34) (Figure 1). They were collected from the field with the help of forceps and stored at -80℃ in 70% ethanol. The species of the scorpions were diagnosed according to the identification key [11, 12, 13], which was defined under the stereomicroscope in the laboratory.

1.2. DNA Isolation and polymerase chain reaction

The protocol suggested in the DNA isolation kit of the Macherey-Nagel brand (Genomic DNA from insects, NucleoSpin DNA Insect) was modified and DNA isolation from the scorpions was carried out. To prepare the mixture of polymerase chain reaction, 5x FIREPol Master Mix (Solis BioDyne) was used. The specific primer sets were used for PCR amplification of mitochondrial COI gene: Forward primer (LCO): 5′-GGTCAACAAATCATAAAGATATTGG-3′; reverse primer (HCO): 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′ [14]. An initial heat activation step at 94°C for 5 minutes was followed by 35 cycles, including that of denaturation at 95°C for 60 seconds, annealing at 55°C for 60 seconds, and extension at 72°C for 60 seconds, then a final extension at 72°C for 420 seconds [14]. The amplified PCR products were then separated using gel electrophoresis on a 1 agarose gel (100V for 30-45 minutes) and visualized with a UV transilluminator by using Safe-T staining (ethidium bromide alternative) to detect the DNA bands.

1.3. DNA sequence analysis

PCR products were purified using the procedures of ExoSAP-ITTM PCR Product Cleanup Reagent (Thermo Fisher Scientific, USA) kit by BM Labosis. The purified PCR products were mixed with forward and reverse primers of the COI gene and sequence analysis was performed. Sequence analysis was carried out in the Macrogen Netherlands laboratory by using the ABI 3730XL Sanger sequencing device (Applied Biosystems, Foster City, CA) and BigDye Terminator v3.1 Cycle Sequencing Kit.

1.4. Phylogenetic Analysis

DNA sequences obtained were displayed using the Chromas (v.2.6.5.) Program. The sequences were recorded in FASTA format separately via Chromas Program. The forward and reverse complement readings of the sequences were compared by aligning them through ClustalW program. Sequences of each gene region were loaded into NCBI Nucleotide BLAST in FASTA format. Their similarities with the sequences recorded in NBC database were compared. The most similar sequences were noted for use in the phylogenetic tree along with GenBank access numbers according to their sequence similarity. All sequences that belong to the four gene regions studied were loaded into the MEGA (v.7.0) program separately. Their modeling methods and distances were determined. The phylogenetic tree was created by using Bootstrap 1000, Maximum Likelihood (ML) method, and Tamura-Nei Model (1993) [15].

2. Results

Specimens' distributions of trichobothria in pedipalps were evaluated. 4 trichobothria were detected in the external (et) part of the patella and 6 trichobothria were detected in the ventral (Pv) part. The ratio (> 1.51) of Pedipalp Chela (Lmet / Wmet) was found to be 1.54. In the light of these morphological data, the specimens were identified as *E. phrygius* (Figure 1).

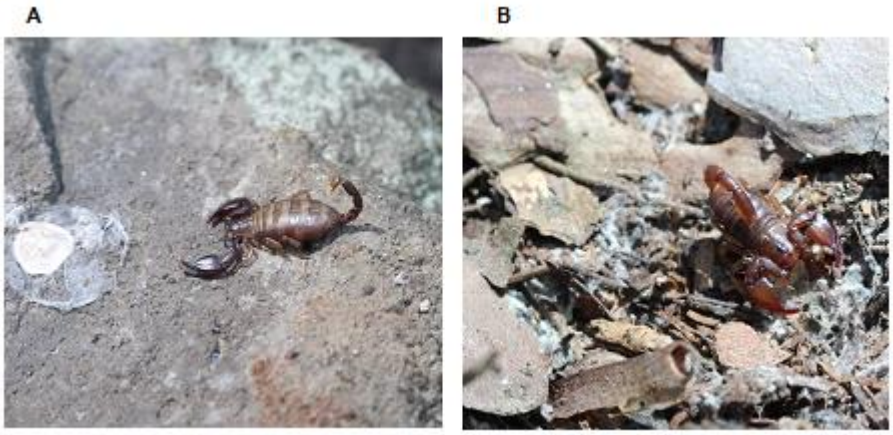

Figure 1. A-B *Alpiscorpius phrygius* habitus.

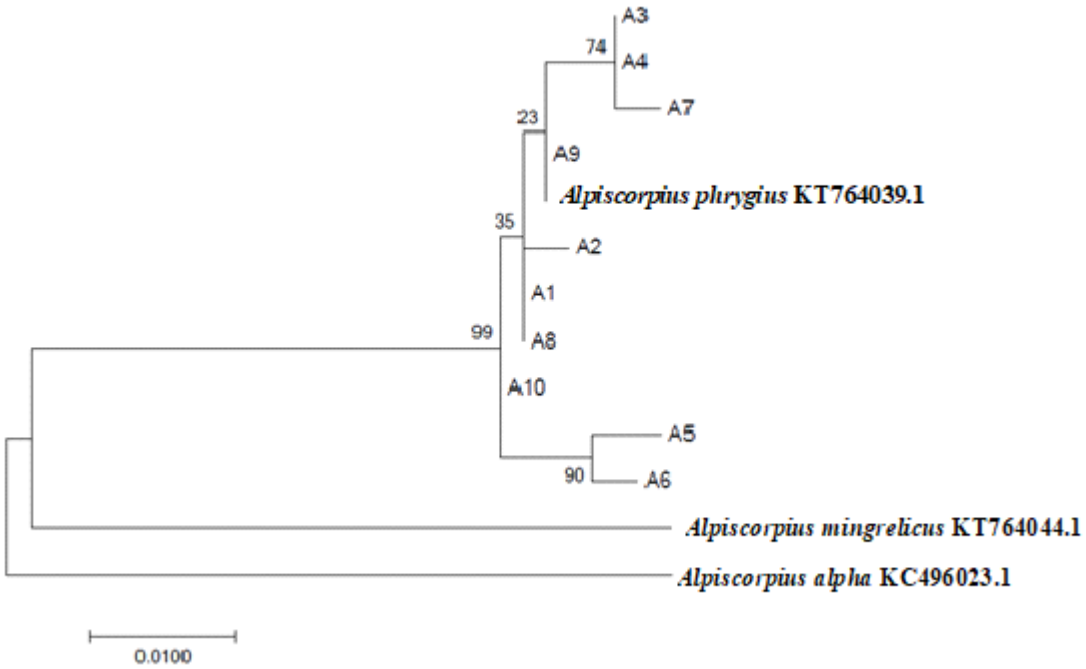

Figure 2. Molecular phylogenetic tree created by the ML method for *A. phrygius's* COI gene region

A total of 10 specimens were subjected to COI gene sequence analysis and their genetic differences were revealed. When the G-C ratios of our specimens were examined (Table 1), it was found that the values varied between 33.3-33.9%. When considered together with the external groups, it was observed that the G-C ratio was 33.6%. When our A1-A10 specimens and *A. phrygius* (KT764039.1) were taken into consideration, it was detected that there were changes in 16 nucleotides (2.47%) in 648 base farm regions. When all specimens were examined together, it was seen that there were 82 nucleotide changes in 634 (12.93%). Transition/Transversion rates (R) (Table 2), Parsim-info sites, singleton sites are given in Table 2.

Table 1. Nucleotide composition (%)

	T(U)	C	A	G	G-C	A-T
A1 (MK784098)	44.6	13.7	21.8	19.9	33.6	66.4
A2 (MK784098)	44.5	13.9	21.6	20.0	33.9	66.1
A3 (MK784100)	44.6	13.7	22.1	19.6	33.3	66.7
A4 (MK784101)	44.6	13.7	22.1	19.6	33.3	66.7
A5 (MK784102)	45.1	13.2	21.3	20.3	33.5	66.4
A6 (MK784103)	44.3	13.7	21.5	20.5	34.2	65.8
A7 (MK784104)	44.8	13.7	21.8	19.6	33.3	66.6
A8 (MK784105)	44.6	13.7	21.8	19.9	33.6	66.4
A9 (MK784106)	44.6	13.7	21.6	20.0	33.7	66.2
A10 (MK784107)	44.6	13.7	21.6	20.0	33.7	66.2
A. phrygius (KT764039.1)	44.6	13.7	21.6	20.0	33.7	66.2
A. mingrelicus (KT764044.1)	43.2	14.4	21.1	21.3	35.7	64.3
A. alpha (KC496023.1)	43.8	13.7	20.5	21.9	35.6	64.3
Avg.	44.5	13.8	21.6	19.8	33.6	66.1

Table 2. Nucleotide pair frequencies

	From A1 to KT764039.1	All Specimens
Conserved sites	632/648 (97.22%)	552/634 (87.06%)
Variable sites	16/648 (2.47%)	82/634 (12.93%)
Parsim-info sites	10/648 (1.54%)	25/634 (3.94)
Singleton sites	6/648 (0.93)	57/634 (8.99)
Identical Pairs (ii)	643	616
Transitional Pairs (si)	3	11.00
Transversional Pairs (sv)	2	6.00
R (si/sv)	2.2	1.74

Table 3. Nucleotide substitution matrix*

	A	T	C	G
A	-	*6.75*	*2.09*	**26.16**
T	*3.27*	-	**3.68**	*3.07*
C	*3.27*	**11.89**	-	*3.07*
G	**27.91**	*6.75*	*2.09*	-

*Each entry shows the probability of substitution from one base (row) to another base (column). Rates of different transitional substitutions are given in bold and those of transversional substitutions are shown in italics.

In order to determine the substitution rates by the maximum likelihood method, the Tamura-Nei model (1993) was used (Table 3). The substitution rates represent the possibility of substitution transition from one base to another. Transitional substitution rates were calculated as 26.16 for A/G, 3.68 for T / C, 27.91 for G/A and 11.89 for C/T. Transversional rates are seen in Table 3. In light of these results, the calculated rate of transition/ transversion (R) was calculated as 2.534. The nucleotide frequencies used in the calculations were 21.56% for A, 44.48% for T, 13.76% for C, and 20.20% for G.

The pairwise genetic distance matrix was formed according to the Tamura-Nei parameter model (Table 4). The average genetic distance among the scorpions of the genus *Alpiscorpius* was 0.101 (10.1%). The lowest genetic distance of *A. phrygius* species was found to be 0.000 and the highest genetic distance was 0.016 (1.6%). These results are normal values considering the variation that can be seen among the ones representing the same species. Genetic distance results were calculated according to pairwise analysis of the sequences of 13 scorpion specimens and 1000 repetitive bootstrap tests were benefitted from to calculate standard errors (Table 4).

The ML method for the calculation of the evolutionary relationships of the scorpion specimens, Tamura-Nei model for determining genetic distances between species, 1000 repetitive bootstrap test for ensuring the reliability of the tree were used. Figure 2 shows the phylogenetic tree created on a scale of 0.01.

Table 4. Genetic distance matrix

	1	2	3	4	5	6	7	8	9	10	11	12	13
A1 (MK784099)		0.002	0.003	0.003	0.005	0.004	0.004	0.000	0.001	0,002	0.001	0.014	0.014
A2 (MK784098)	0.003		0.004	0.004	0.005	0.004	0.004	0.002	0.002	0.003	0.002	0.015	0.014
A3 (MK784100)	0.006	0.010		0.000	0.005	0.005	0.002	0.003	0.003	0.003	0.003	0.014	0.014
A4 (MK784101)	0.006	0.010	0.000		0.005	0.005	0.002	0.003	0.003	0.003	0.003	0.014	0.014
A5 (MK784102)	0.013	0.016	0.016	0.016		0.003	0.005	0.005	0.004	0.004	0.004	0.014	0.015
A6 (MK784103)	0.011	0.015	0.015	0.015	0.008		0.005	0.004	0.004	0.004	0.004	0.014	0.015
A7 (MK784104)	0.010	0.013	0.003	0.003	0.013	0.015		0.004	0.003	0.004	0.003	0.014	0.014
A8 (MK784105)	0.000	0.003	0.006	0.006	0.013	0.011	0.01		0.001	0.002	0.001	0.014	0.014
A9 (MK784106)	0.002	0.005	0.005	0.005	0.011	0.010	0.008	0.002		0.002	0.000	0.014	0.014
A10 (MK784107)	0.002	0.005	0.008	0.008	0.011	0.010	0.011	0.002	0.003		0.002	0.014	0.013
E. phrygius (KT764039.1)	0.002	0.005	0.005	0.005	0.011	0.010	0.008	0.002	0.000	0.003		0.014	0.014
E. mingrelicus (KT764044.1)	0.084	0,088	0.084	0.084	0.092	0.090	0.088	0.084	0.081	0.081	0.081		0.014
E. alpha (KC496023.1)	0.086	0.086	0.090	0.090	0.099	0.096	0.094	0.086	0.088	0.084	0.088	0.101	
Avg.	**0.017**												

4. Conclusions and discussion

Considering the Anatolian scorpions of the genus *Alpiscorpius*, three species have been identified in the current borders of Turkey so far [9]. *Alpiscorpius mingrelicus* (Kessler,1874), *Alpiscorpius phrygius* (Bonacina, 1980), *Alpiscorpius uludagensis* (Lacroix, 1995) belonging to the genus *Alpiscorpius* have been identified in Turkey [9].

Firstly, Fet *et al.* (2003) examined the scorpion species thought to be *E. ciliciensis* in Anatolia with 16S rRNA barcoding gene in two populations [16]. Tropea & Yağmur (2015a) also conducted their study in Anatolia, and they revised and identified four Euscorpius species with em=3. In addition, the same researchers identified two new em=3 species; namely, *E. arikani* and *E. eskisehirensis* [17, 18].

When the phylogenetic studies of Fet *et al.* (2016) with Euscorpius scorpions in 14 populations in Anatolia were examined, it was seen that they identified 5 species and they were identified as *E. eskisehirensis* (Turkey, Eskişehir), *E. ciliciensis* (Turkey, Niğde), *E. mingrelicus* (Turkey, Rize, and Bartın), *E uludagensis* (Turkey, Bursa) and *E. phyrigius* (Turkey, Bolu) [10]. It was reported that considering all these species, they were unable to obtain only *E. uludagensis* species's COI gene region and *E. mingrelicus* (Bartin)'s 16S rRNA gene region. The 8 remaining scorpions behind their study were able to be identified as only Euscorpius sp. The sample with access number KT764042.1 indicated by the same researchers in their article as *E. phyrigius* contains the 396 bp COI gene region. In the phylogenetic analysis, we used a 648 bp COI sequence with the accession number KT764039.1, which was not recorded in their articles. When COI DNA sequences of our samples and *E. phrygius* COI DNA sequence with the accession number KT764039.1 taken from Bolu-Abant Lake Nature Park location were compared with ClustalW, the similarity was found between 99.0% and 100%. When compared with *E. mingrelicus* (KT764044.1) and *E. alpha* (KC496023.1), it is seen that this rate varies between 91.14% and 92.74% [10].

In addition to these two samples, when we verified the NCBI Nucleotide database, we found another registered *E. phrygius* sample with the accession number KU987042. This scorpion sample obtained from Bolu-Abant contains a 583 bp COI gene sequence. Since the COI sequence length of *E. phyrgius* with the accession number KU987042 did not match the COI sequence lengths of our samples, we did not use it for phylogenetic analysis.

When the scorpion biodiversity in Anatolia is considered, there are scarcely any studies carried out using the DNA barcoding technique. We recommend revealing the scorpion biodiversity in our country by using both morphological and molecular methods, providing their registration to the barcode systems in order to help fill the gap in the literature of this field.

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