www.biodicon.com Biological Diversity and Conservation

ISSN 1308-8084 Online ISSN 1308-5301 Print

Research article/Araştırma makalesi
DOI: 10.46309/biodicon.2021.986130
14/3 (2021) 470-479

Assessment the *Promachus leoninus* Loew 1848 (Diptera: Asilidae) Species, with COI and $NADH_2$ Gene Regions, with New Locality Records in Anatolia

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Abstract

Promachus leoninus Loew 1848 is known to be distributed just in the western part of Anatolia for a very long time. In the present study additional information is given about the distribution of this species with a new and previous localities map. Assessment of new populations evaluated phylogenetically with the COI and $NADH_2$ gene regions. As a result of this analysis the new populations are grouped into 3 different diverging populations. Genital features and habitus of the male specimen are photographed.

Keywords: Asilidae, phylogeny, new locality, COI, $NADH_2$ Turkey

Promachus leoninus Loew 1848 (Diptera: Asilidae) Türünün COI ve $NADH_2$ Gen Bölgeleri ile Değerlendirilmesi ve Anadolu'da Yeni Lokalite Kayıtları

Özet

Promachus leoninus Loew 1848'un çok uzun zamandan beri Anadolu'nun sadece batısında yayılış gösterdiği bilinmektedir. Bu çalışmada, yeni ve eski yerleşim haritası ile bu türün yayılışları hakkında ek bilgi verilmektedir. Elde edilen yeni popülasyonlar COI ve $NADH_2$ gen bölgeleri ile birlikte filogenetik olarak değerlendirilmiştir. Analizler sonucunda yeni populasyonların birbirinden farklılaşmakta olan 3 populasyon şeklinde gruplandığı görülmüştür. Erkek bireyin genital özellikleri ve habitusu fotoğraflanmıştır.

Anahtar kelimeler: Asilidae, filogeni, yeni lokalite, COI, $NADH_2$ Türkiye

1. Introduction

Ecological balance is known as a state of dynamic equilibrium within a community of organisms. The balance should be in the number of each species in an ecosystem. Predator groups are very important because of their stabilize feature on the specimen number in a community.

Asilidae is one of the essential groups of these predators, in controlling numerous insect groups many of which are harmful. This family represents 7104 described species and 776 genera in the world [1], 1688 species in Palearctic and nearly 241 species and 9 subspecies are reported from Anatolia [2].

There are many taxonomical and ecological studies about Turkish robber flies (e.g. [3; 4; 5]). However, despite the increasing number of phylogenetic studies about Asilidae in the world ([6; 7; 8; 9]) there is no study in Anatolia about the phylogeny of this group. Therefore, it is important to study the phylogeny of taxa belonging to Asilidae in our country.

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© Copyright 2021 by Biological Diversity and Conservation Received: 18.09.2021; Published: 15.12.2021 BioDiCon. 1005-180921

Promachus genus, which has 223 species worldwide, is infrequent for Turkish Asilidae fauna since five species of this genus are known from Anatolia; *Promachus canus canus* (Wiedemann, 1818), *Promachus canus leontochlaenus* Loew, 1871, *Promachus leoninus* Loew, 1848, *Promachus microlabis* Loew, 1857, *Promachus mustela* Loew, 1854 [1]. Among these the *Promachus leoninus* is distributed in Azerbaijan, Bosnia-Herzegovina, Croatia, Greece, India, Iran, Israel, Myanmar (Burma), Romania, Russia, Slovenia, Turkey on Palearctic ([10; 11; 12]). The species is known to be distributed in the western part of Anatolia on following provinces; Balıkesir, Aydın, Muğla, Ankara, Adana, Adıyaman, Afyon, Antalya, Denizli, Isparta, Mersin, Çanakkale, Eskişehir [13].

So, in the present study we aimed; (i) to provide a new data about the distribution of *P. leoninus.* (ii) to present illustration of its morphological and genital features (iii) make phylogenetic assessment with COI and $NADH_2$ gene regions (iv) Enter the gene bank first gene region records from Anatolia about the Asilidae.

2. Material and methods

2.1. Sampling and identification

This study is based on 21 specimens collected from 12 localities of 6 provinces in June and July on 2018 (Table 1; Figure 1). The specimens are identified with the [14] literature. Habitus and genital features were photographed with Leica DFC 490 camera, attached to a Leica MZ 16 microscope. Specimens are deposited in the Entomology collection, Department of Biology, Eskişehir Osmangazi University.

Table 1. Study site information of P. *leoninus* specimens

Study site	Province	Locality	Date	Coordinate		Altitude
				Latitude (N)	Longitude (E)	
1	Amasya	Yeşilöz	26.06.2018	40°33'0.06"	36°8'56.31"	855 m
2	Erzurum	Gelinalan village	29.06.2018	40° 7'59.29"	42°33'26.62"	1472 m
3	Erzurum	Horasan	29.06.2018	40° 1'56.84"	42° 7'41.07"	1560 m
4	Erzurum	Mercimekli	30.06.2018	40° 3'26.58"	41°49'33.04"	1860 m
5	Erzurum	Samikale village	30.06.2018	40°23'32.36"	41°55'58.66"	1506 m
6	Erzurum	Oltu village	30.06.2018	40°31'0.99"	41°57'49.70"	1322 m
7	Erzurum	Oltu village 2	30.06.2018	40°34'26.56"	42° 1'9.94"	1244 m
8	Erzurum	Korucuk village	30.06.2018	40°30'29.18"	41°49'53.91"	1538 m
9	Sivas	Tödürge village	03.07.2018	39°51'46.09"	37°36'56.01"	1338 m
10	Yozgat	Alicik village	03.07.2018	39°47'8.77"	35°58'35.92"	1302 m
11	Bitlis	Süphan mountain/Kıskıllı village	24.07.2018	38°54'55.59"	42°54'38.71"	2250
12	Edirne	Aslıhanlar Village	19.06.2018	41°25'0.54"	26°47'53.96"	73 m



Figure 1. General view of collecting site

2.2. Molecular taxonomy

In this study we studied the two gene regions of mitochondrial DNA; Cytochrome c oxidase subunit I (COI) gene region and NADH subunit 2 ($NADH_2$) (Table-2). Total DNA was extracted with the Thermo Scientific GeneJET Genomic DNA Purification kit.The primer sequencing was gathered from [15].

COI: 1490: 5'- GGTCAACAAATCATAAAGATATTGG-3'-
3014:5'-TCCAATGCACTAATCTGCCATATTA-3'.
$NADH_2$: N2L-J210: 5'-AATTAAGCTAATGGGTTCATACCC-3'-
TW-N1284: 5' AYAGCTTTGAARGYTATTAGTTT-3'

Table 2. Phylogenetic study information of specimens

Locality	Province	Date	COI codes	Accession codes-COI	$NADH_2$ codes	Accession codes-$NADH_2$
Yeşilöz	Amasya	26.06.2018	1CamayesPrl	OL415109	1NamayesPrl	OL462856
Gelinalan village	Erzurum	29.06.2018			3NerzgelPrl	OL462855
Gelinalan village	Erzurum	29.06.2018	4CerzgelPrl	OL415106	4NerzgelPrl	OL462854
Gelinalan village	Erzurum	29.06.2018			5NerzgelPrl	OL462853
Horasan	Erzurum	29.06.2018	6CerzhorPrl	OL415105	6NerzhorPrl	OL462852
Horasan	Erzurum	29.06.2018			7NerzhorPrl	OL462851
Mercimekli	Erzurum	30.06.2018	8CerzmerPrl	OL415104	8NerzmerPrl	OL462850
Samikale village	Erzurum	30.06.2018	9CerzsamPrl	OL415103	9NerzsamPrl	OL462849
Samikale village	Erzurum	30.06.2018	10CerzsamPrl	OL415102	10NerzsamPrl	OL462848
Oltu village	Erzurum	30.06.2018	11CerzolPrl	OL415101	11NerzolPrl	OL462847
Oltu village	Erzurum	30.06.2018	12CerzolPrl	OL415100	12NerzolPrl	OL462846
Oltu village 2	Erzurum	30.06.2018	13CerzolPrl	OL415099	13NerzolPrl	OL462845
Oltu village 2	Erzurum	30.06.2018			14NerzolPrl	OL462844
Küçük Korucuk	Erzurum	30.06.2018	15CerzkucPrl	OL415098	15NerzkucPrl	OL462843
Tödürge village	Sivas	03.07.2018			16NsivtodPrl	OL462842

Alicik village	Yozgat	03.07.2018	17CyozaliPrl	OL415097	17NyozaliPrl	OL462841
Aslıhanlar village	Edirne	19.06.2018	20CedasPrl	OL415096	20NedasPrl	OL462840
Aslıhanlar village	Edirne	19.06.2018	21CedasPrl	OL415095	21NedasPrl	OL462839

Polymerase chain reaction (PCR) was made in 50 µl volume; 0,2 µl from primers (100 pm), 1 µl dNTP mix (10 mM), 4 µl l 50 mM MgCl2 (25 mM), 5 ml Standard Taq reaction Buffer 10X [containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl], 0.25 TaqDNA polymerase (New England Bio labs), and 3 µl of sample DNA. PCR cycling parameters; denaturation at 95˚C for 30 sec, 35 cycles of 95˚C for 20 sec., annealing at 41˚C for 30 sec, elongation 72˚C for 1min 40 sec. and final extension 72˚C 5 min. Results were viewed with agarose gel electrophoresis by running 3 µl of PCR product. DNA purification step and Sanger sequence analysis were carried out by Macrogen Europe (Amsterdam, the Netherlands).

2.3. Data analysis

We used 13 COI and 18 $NADH_2$ sequences of *Promachus leoninus* for analyses. Two outgroups are used for COI dataset; *Leptogaster cylindrica (*Accession code: OL415107) and *Dysmachus bifurcus (*Accession code: OL415108) (our own samples) and one outgroup is gathered from NCBI for $NADH_2$ dataset (Accession code: EF216223.1) *Drosophila guanche.* All of the sequences were checked manually with SEQUENCER v. 4.1 (Gene Codes Corporation). The alignment was made with MEGA v.7 [15] and the program was used for calculating the conservative, variable and parsimony-informative sites. Haplotypes and their frequency were determined with DnaSP v.5 [17]. Sequences are deposited in the Genbank database (accession number can be seen at Table 2). The best fit evolutionary model for the data matrix was estimated by jModelTest v.0.1.1 [18].

Sequences were analyzed with three different phylogenetic methods; (i) Maximum parsimony (MP) by PAUP Version 4.0b10 [19], with 100 random additions, with nearest neighbor interchange (NNI) algorithm and heuristic search approach. (ii) Maximum Likelihood (ML) with raxmlGUIversion 1.5 [20] with ML-rapid 1000 bootstrap option. (iii) Bayesian phylogenetic inference (BI) analyses with MrBayes v.3.1.2 [21] program, four simulations of Markov chains, 10 M generations and sampling every 100^{th} generations and with the program the 1000 trees were discarded as burn-in.

We applied to the COI matrix two different analyses to test species limits. DNA sequence-based analysis TCS [22] and Automatic Barcode Gap Discovery (ABGD) programs were applied to the dataset to make species delimitation test.

3. Results

3.1. Plot summary of morphology

Body; slender with a dense hair, the color of the body is dark metallic which is bright to pale, with yellow to orange bands, with these colors the species reminds bees. Thorax; pronotum with hairs. Wings are transparent and clear (Figure 2b), legs are strong and also covered with striped with black and yellow dense hair (Figure 2a). Head; typically slender with big black dichoptic eyes (varies to green blue), antenna's 3rd segment is elongated thinning at the end (Figure 2c). Mouthparts are black and very strong. The aedeagus is conspicuously long and can be seen at the end of the abdomen (Figure 2d-f). Gonocoxites are not fused. Dististylus broad, curved with a pointed end and with setae at the ventral margin. Hypandrium short, with rounded sides and laterally short setae.

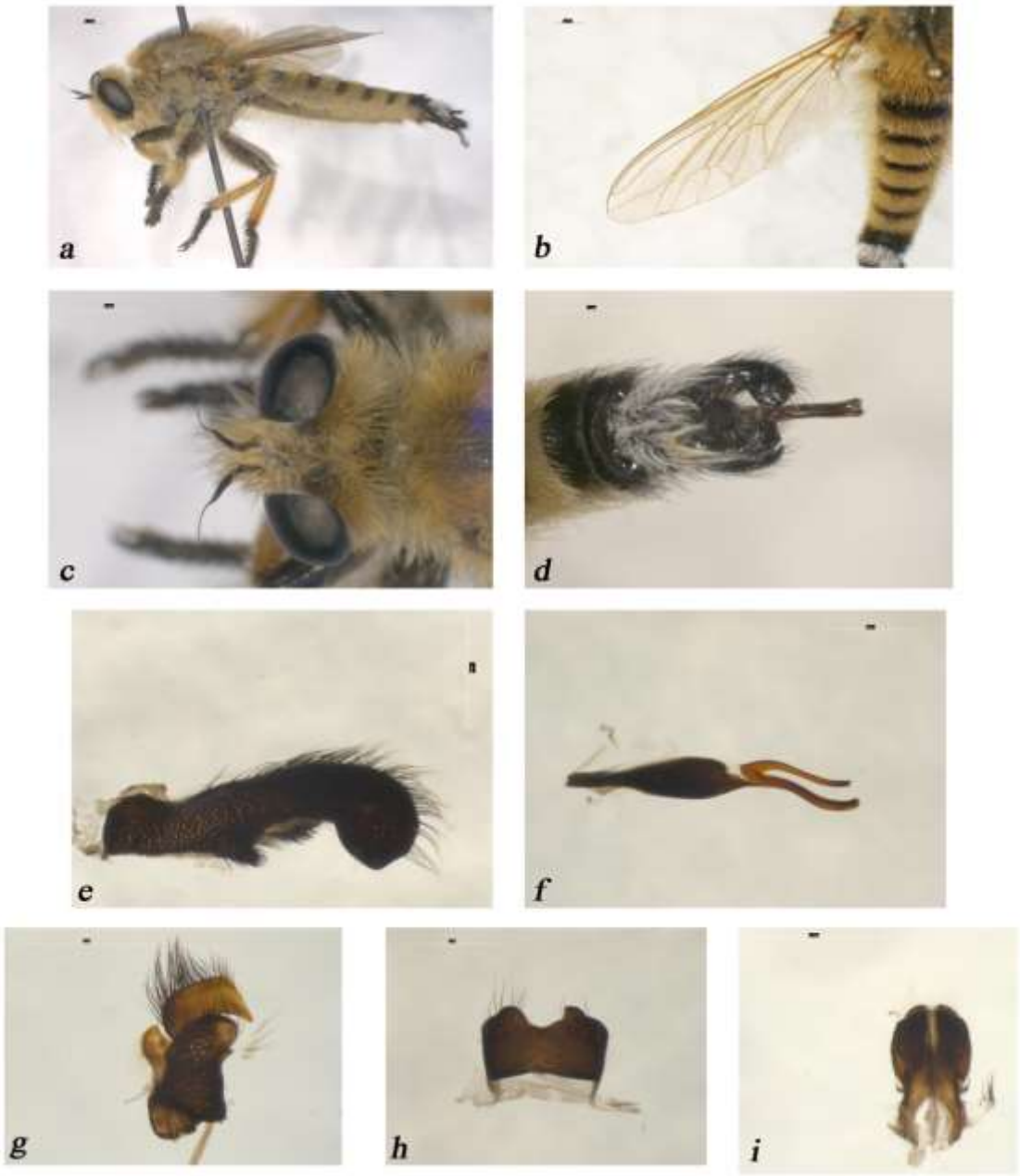


Figure 2. a-i. A male *Promachus leoninus* Loew 1848. a-lateral view of specimen b-wing c-dorsal sight of the head with antennae d-last abdominal segment with aedeagus e-epandrium f-lateral view of aedeagus g-gonocoxite and dististylus h-hypandrium i- proctiger

3.2. Molecular assessment with mtDNA gene regions

The sequences of *Promachus leoninus* (13 COI and 18 $NADH_2$) were checked with BLAST program after alignment. Data set for COI with 15 sequences (with two outgroups) and 1222 base pairs and for *$NADH_2$* with 19 sequences (with one outgroup) and 1001 base pairs were obtained. The tables can be checked for detailed information (Table-3 about data sets and Table-4 for haplotypes for two gene regions).

Table 3. Sequence and haplotype informations of COI and $NADH_2$ gene regions

	Number of Sequence	Total Number of Sites	Conserved	Variable	Pars-inf	Number of Haplotypes	Haplotype Diversity
COI	15	1222	901	321	117	13	0,9810
$NADH_2$	19	1001	715	286	31	10	0,7836

Table 4. Detailed information about Haplotypes

COI gene region		$NADH_2$ gene region	
Number of haplotypes	h: 13	Number of haplotypes	h:10
Haplotype diversity	Hd: 0,9810	Haplotype diversity	Hd: 0,7836
Hap_1: 1	21CedasPrl	Hap_1: 1	21NedasPrl
Hap_2: 1	20CedasPrl	Hap_2: 1	20NedasPrl
Hap_3: 1	17CyozaliPrl	Hap_3: 1	17NyozaliPrl
Hap_4: 2	15CerzkucPrl 13CerzolPrl	Hap_4: 1	16NsivtodPrl
Hap_5: 1	12CerzolPrl	Hap_5: 9	15NerzkucPrl 13NerzolPrl 9NerzsamPrl 8NerzmerPrl 7NerzhorPrl 6NerzhorPrl 5NerzgelPrl 4NerzgelPrl 3NerzgelPrl
Hap_6: 1	11CerzolPrl	Hap_6: 2	14NerzolPrl 11NerzolPrl
Hap_7: 1	10CerzsamPrl	Hap_7: 1	12NerzolPrl
Hap_8: 2	9CerzsamPrl 6CerzhorPrl	Hap_8: 1	EF216223.1_Drosophil *Outgroup*
Hap_9: 1	8CerzmerPrl	Hap_9: 1	10NerzsamPrl
Hap_10: 1	4CerzgelPrl	Hap_10: 1	1NamayesPrl
Hap_11: 1	1lckirder *Outgroup*		
Hap_12: 1	1dbkirkof *Outgroup*		
Hap_13: 1	1CamayesPrl		

3.3. COI gene region results

The dataset file was converted into different formats with Mesquite and Alter programs for use in phylogenetic analysis. Haplotype diversity was calculated as 0, 9810 for COI with DnaSP v.5. There is no haplotype sharing; all the haplotypes are unique sequences except haplotypes 4 and 8 (Table-4). The jModelTest v.0.1.1 suggested the evolutionary model as GTR+G according to AIC (Akaike Information Criterion), with p-inv = 0.2571 gamma shape = 0.4450.

Maximum likelihood, Maximum parsimony, Bayesian Phylogenetic inference analyses are result in a resembling tree topology, therefore all results are shown on BI tree (Figure 3). Haplotypes of individuals were grouped (with strong branch node values) according to population structure as expected. The Edirne, Erzurum and Yozgat-Amasya populations are branched as ingroups. The network analysis is also consistent with the phylogenetic analysis results (Figure 4). We can also observe same the population ingroups in this analysis. TCS (%90) Species delimitation test suggested two different species while TCS (%95) test suggested three populations as three different species, but morphological differences were not observed among the individuals of the population. Also, ABGD test is suggesting that all of the haplotypes belong to single species. Therefore, these populations can be considered as diverging populations and not as separate species.

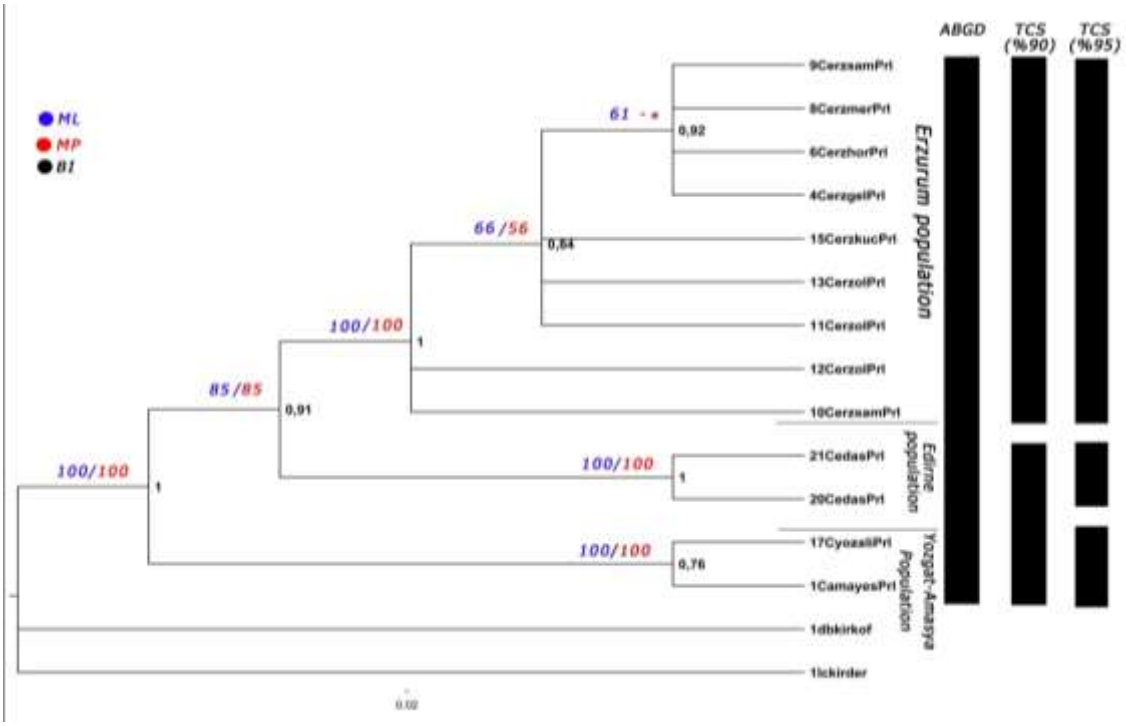


Figure 3. ML, MP, BI analyses results of COI gene region. Bootstrap values (MP-ML) and posterior probability values (BI) are shown. Respectively; Black is for BI, red is for MP and blue is for ML. (*) indicates 50% and below values or not supported by the respective analyses. ABGD and TCS Species delimitation tests are mapped on the tree.

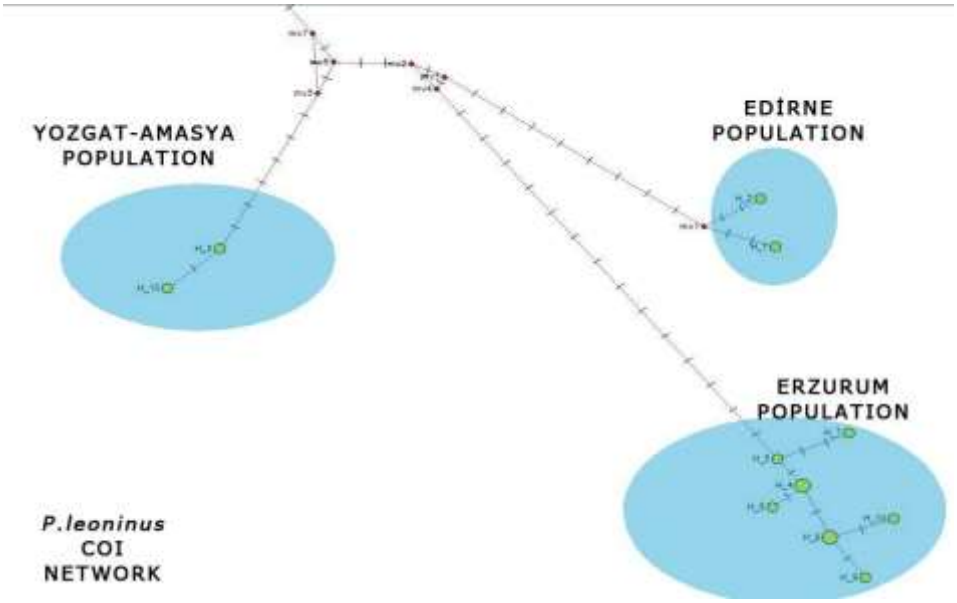


Figure 4. Network analysis results of COI gene region

3.4. $NADH_2$ gene region results

Haplotype diversity was calculated as 0, 7836 for $NADH_2$ with DnaSP v.5. There is no haplotype sharing; all the haplotypes are unique sequences except haplotype 8 (Table-4). The jModelTest v.0.1.1 suggested the evolutionary model as GTR+G according to AIC (Akaike Information Criterion), with p-inv = 0.5140 gamma shape = 100.0000.

All the analyses (ML, MP and BI) are resulted in the same tree topology, shown on MP tree (Figure 5). As seen in the phylogenetic analyses made with the COI gene region, the haplotypes are branched according to the populations, but the branch support values are lower. The branch support value (BI: ›50, ML: 54, MP: 70) at the branch node of the Edirne and Yozgat-Amasya populations seems to be quite low (Figure 5). We can observe the same result on network analysis. Network analyses resulted in same way as in phylogenetic analyses. We can see the haplotypes are grouped in three different populations (Yozgat-Amasya population, Edirne population and Erzurum population) (Figure 6).

TCS (%95) Species delimitation test suggested two different species but morphological differences were not observed among the individuals of the population. All of the haplotypes are belong to single species according to TCS (%90) and ABGD tests.

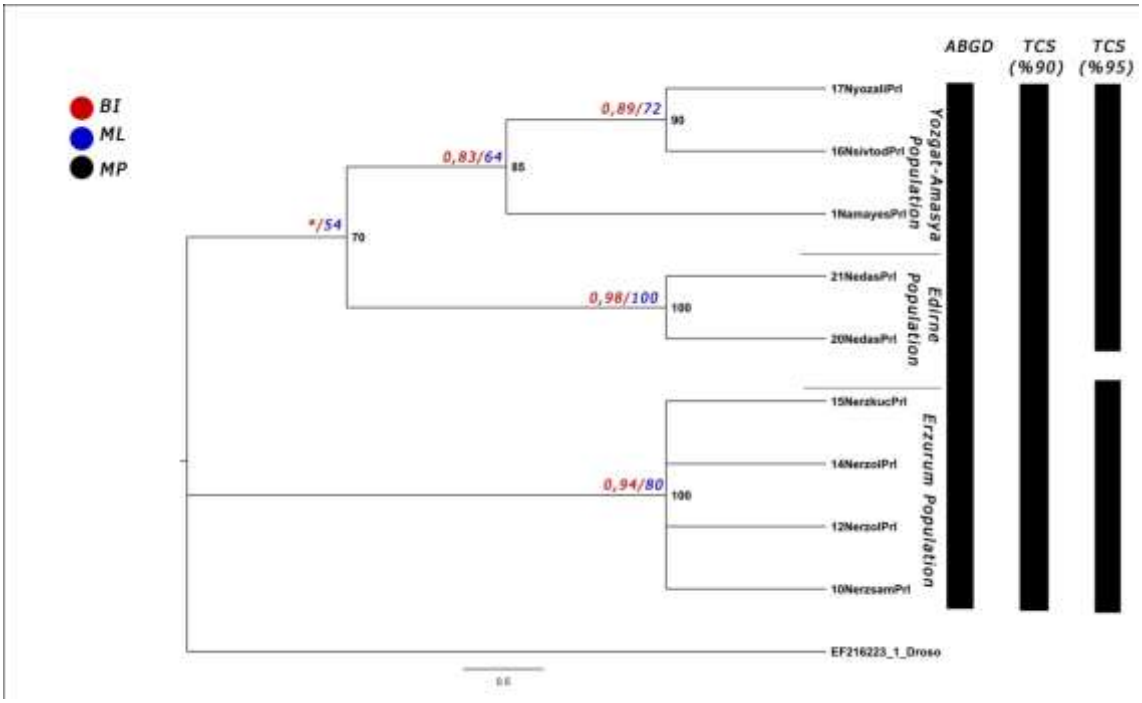


Figure 5. ML, MP, BI analyses results of $NADH_2$ gene region. Bootstrap values (MP-ML) and posterior probability values (BI) are shown. Respectively; Black is for BI, red is for MP and blue is for ML. (*) indicates 50% and below values or not supported by the respective analyses. ABGD and TCS Species delimitation tests are mapped on the tree.

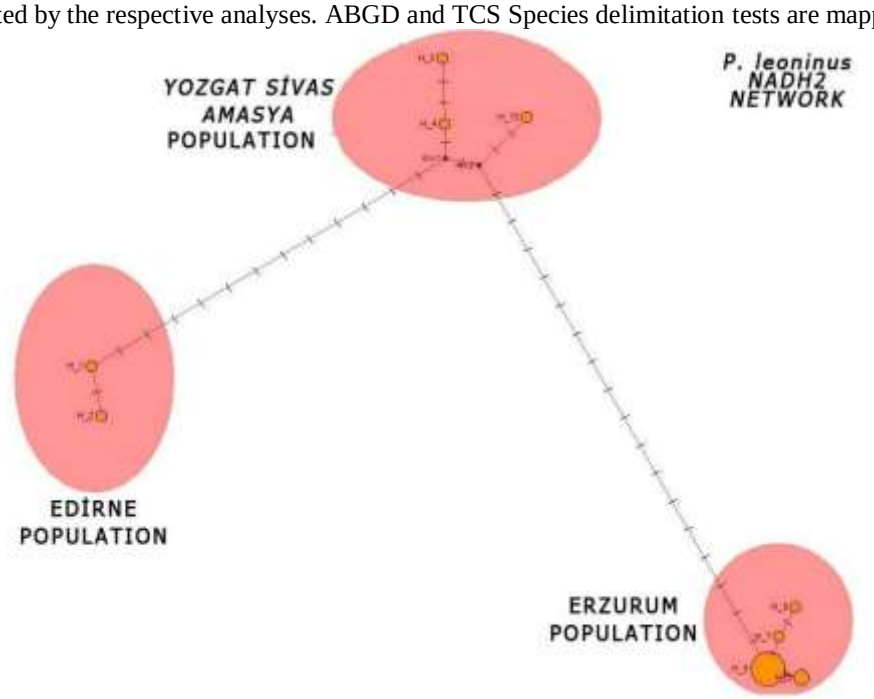


Figure 6. Network analysis results of $NADH_2$ gene region

4. Conclusions and discussions

The distributional area of *P. leoninus* was known from South and West part of Anatolia. 21 specimens (14 male and 7 female) are collected from east part of Anatolia, Middle Black Sea and the Trace region (Figure 7). The specimens examined here present a new distribution record for *P. leoninus*, which is considerably enlarging the distributional area to east part of Anatolia and the Trace region.



Figure 7. Previous and new locality records of *Promachus leoninus*

Therefore, there is known from our records that these species are active at 10:00 am in the morning to 8:00 pm on the sunset. The optimal temperature for their activity is between 21- 37 ˚C degrees, based on our observations. Further studies are needed to do for determining the host preference of *P. leoninus.*

This is the first study conducted in our country on the phylogeny of this group. The new distribution regions of *P. leoninus* are grouped into 3 different populations. Phylogenetic analyses were carried out with the COI and $NADH_2$ gene regions. The analyses performed similar results in both gene regions. Haplotypes belonging to the same populations branched together in the phylogenetic trees. While this branching in the COI gene region is supported by high branch support values, it has been observed that these values are lower in $NADH_2$.

Similar results were obtained in the haplotype Network analysis as in the phylogenetic analyses for both gene regions. In some of the species delimitation tests; populations were proposed as different species. However, individuals belonging to these populations were morphologically examined once again and it was seen that all individuals share the same morphological characters, herewith we can use the term "diverging populations" instead of "different species".

In order to reveal the systematic and phylogenetic status of the species more clearly, a broader evaluation with the Balkan and Caucasian populations is required. More gene regions or next generation sequencing methods can be studied.

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