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Original research

Phylogenetic analysis of *Nelima pontica* (Opiliones: Sclerosomatidae) based on mitochondrial COI and 16S rRNA genes

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Abstract: *Nelima pontica* is a species of Opiliones belonging to the family Sclerosomatidae. In this study, two mitochondrial genes, namely, cytochrome c oxidase subunit I (COI) and 16S were used to determine the phylogenetic analysis of *Nelima pontica*. The gene regions of this species and the genes sequences were identified. Obtained sequences were assembled using Codon Code Aligner V.8.0: 2. The phylogenetic tree was constructed by Neighbor-joining (NJ) method with 1000 boostrap replicates using MEGA 7.0.26 package. It was determined that morphological data and the data obtained from the COI gene region supported each other.

Keywords: COI, *Nelima pontica*, phylogenetic analysis, 16S rRNA

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Introduction

The Opiliones which is the third largest order of Arachnida class is known to have 6500 taxa around the world, while in Turkey they are represented by approximately 105 taxa. (Kury 2013; Kurt 2014).

Nelima pontica Charitonov,1941 a member of *Nelima* genus from the family Sclerosomatidae and distributed in Abkhazia, Bulgaria, Georgia, Russia and Turkey (Snegovaya and Marusik 2012; Kurt 2015).

In recent years, DNA barcoding has been one of the most important molecular methods used to identify the classification of living beings. This method enables the identification of organisms using a short and standard fragment of genomic DNA. It is a fast, accurate and automatic way of identifying species that is mainly based on the use of 600-700 base pairs of a standard region of mitochondrial DNA (Hebert and Gregory 2005).

Sequence-level identification is performed via the DNA barcoding technique by sequencing a short portion of the mitochondrial cytochrome c oxidase subunit I (COI) gene sequence of the taxonomically unknown sample. The data obtained is evaluated against the reference library that contains the previously identified sequence of known species (Wilson, 2012).

The objective of this study was to determine the phylogenetic relationships of *Nelima pontica* using the mitochondrial genes COI and 16S rRNA.

Materials and Methods

Sample collection

Sample used in this study were collected by hand and using forceps in Trabzon province of Turkey in 2014. Totally of 20 samples were collected and 10 samples were used for molecular studies. The samples examined were preserved in 70% ethanol and were kept in the collection of the Arachnological Laboratory of Şiran Vocational School, Gümüşhane University (GUSAL), Gümüşhane, Turkey. The diagnosis of the specimens was used on the genital structure of the male organ. The specimens were photographed by using a Leica EZ4 stereomicroscope.

DNA extraction

Genomic DNA was extracted from the legs of one adult individual from each specimen using the GeneAll Exgene Tissue Kit (Korea) according to the protocol of the manufacturer.

PCR amplification and sequencing

Primers used to amplify regions of COI, and 16S genes are given in Table 1. PCR was performed in a total volume of 20 µl containing: 3 µl DNA template, 8 µl Mastermix (2x) (Mastermix:10X Tampon, 2,5 mM dNTP, 25 mM $MgCl_2$, Taq polymerase), 1 µl from each primer, and 7 µl sterile distilled H_2O.

Amplification conditions for both genes consisted of an initial denaturation for 5 min at 95°C, followed by 40 cycles of denaturation for 30 sec at 95°C, annealing for 30s at 49- 50°C, elongation for 30 sec at 72°C and a final extension step at 72°C for 5 min (GeneAll, Seoul, Korea).

PCR products were evaluated for successful amplification using gel electrophoresis in 1% agarose and purified using DNA gel extraction kit (GeneAll Gel SV; Cat no:102-150).

The purified PCR products were then directly subjected to Sanger sequencing using the amplification primers at Applied Biosystems. Genetic Analyser device was used for sequencing. The newly obtained sequences were confirmed via BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 1. List of primer sequences used for amplification and sequencing

Gene	Primers	Sequence	References
COI	LCO1490	5'-GGTCAACAAATCATAAAGATATTGG-3'	
	HCO2198	5'-TAAACTTCAGGGTGACCAAAAAATCA-3	Folmer et al.1994
16S rRNA	16Sa	5'-CGCCTGTTTATCAAAAACAT-3'	Xiong and Kocher1991
	16Sb	5'-CTCCGGTTTGAACTCAGATCA-3'	Edgecombe et al.2000

Data analysis

Finch TV 1.4 program was used to visualize chromatograms of the nucleotide sequences that were determined by the Sanger-Coulson method. Sequences were analyzed with Codon Code Aligner V.8.0: 2 program and the sequences were saved in the Fasta format. A phylogenetic tree was constructed using the MEGA V.7.0.26 (Molecular Evolutionary Genetic Analysis) program.

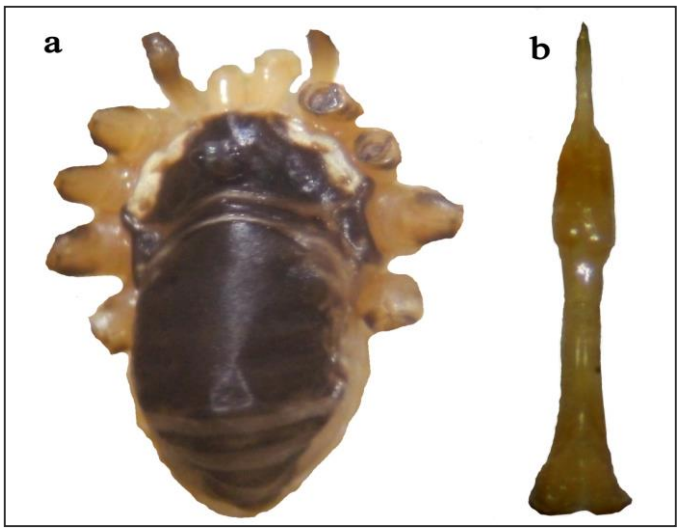

Figure 1. *Nelima pontica*: a. Dorsal view; b. Penis

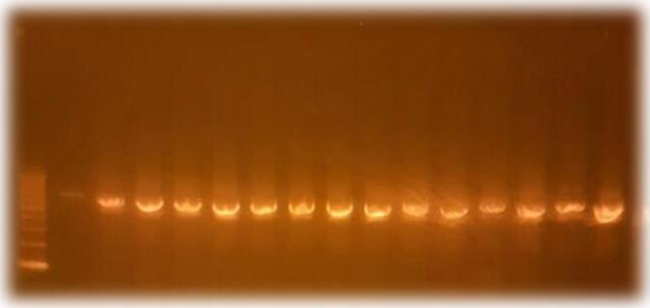

Figure 2. Agarose gel of PCR product of 16S rRNA gene amplified in *Nelima pontica*

Results and Discussion

Some morphological characters used in taxonomy have very slight differences between species and may be insufficient as a sole means for species identification (larvae, immature individuals, twin species). Therefore, taxonomic studies based on morphological characters should be supported by the use of different gene regions.

One of the most common methods used in molecular taxonomy is DNA barcoding. Mitochondrial genes (COI and 16S rRNA) and nuclear genes (18S, ITS) are suitable gene regions for DNA barcoding. In some animal groups (for example frogs, salions, and pholcidae family spiders)

16S rRNA gives reliable results, while in some spider groups, COI is more reliable (Wang et. al 2017). Today, the combination of both gene regions is more widely used (Astrin et al. 2006).

A fragment of 1258 bp from the COI gene was sequenced. The average nucleotide composition of the coding strand was 32,8% A; 29,3% T,; 19,0% C and 18,9% G. A fragment of 520 bp from the 16S rRNA gene was sequenced. The average nucleotide composition was 32,5% A, 38,9% T, 8,5% C and 20,2% G (Mega 7).

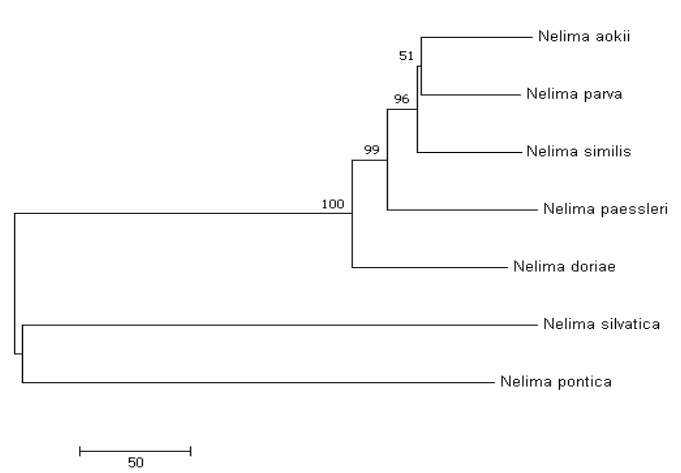

Figure 3. Neighbor-joining tree of genus *Nelima* based on COI gene.

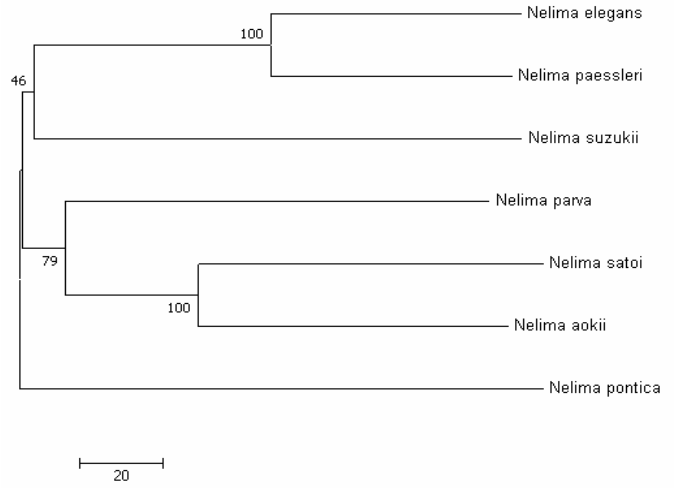

Figure 4. Neighbor-joining tree of genus *Nelima* species based on 16S gene.

The evolutionary history was inferred using the Neighbor-Joining method (Fig. 3; 4) and indicated that *Nelima pontica* is phylogenetically closer to different species of the *Nelima* genus.

Nelima pontica is mophologically similar to *Nelima silvatica.* In the phylogenetic tree based on the COI gene region two species are close. This show that COI data support moprhological data. 16S rRNA gene region data belonging to *N. silvatica* species are not available in the NCBI database. Therefore, *N. pontica* species differs from other species of the genus *Nelima* in the NCBI database. In order to establish the relations of kinship, recording molecular data of all individuals of this genus in the system will increase the reliability of the other studies.

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