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RESEARCH PAPER

Molecular Phylogenetic Analysis of *Nemoura flexuosa* Aubert, 1949 Based on Cytochrome Oxidase C Subunit-I Gene from Türkiye

Famil Yusufoğlu [1,*], Fevzi Uçkan[1]

[1]Kocaeli University, Biology Department, İzmit, Kocaeli, Turkey

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Corresponding Author
Tel.: +905511911812
E-mail: lukarinkita@gmail.com

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Abstract

Plecoptera species are valuable both as freshwater indicators and as key role in the food chain of river ecosystems. Moreover, they constitute an order with a high speciation capacity. Therefore, identification at the species level by morphological analysis leads to reliability problems. Studies on the phylogeny of *Nemoura flexuosa* which belongs to this order are scarce in the literature. Samples were collected from Kocaeli-Türkiye and then morphological and phylogenetic descriptions of the specimens were made. The *Cytochrome Oxidase C Subunit-I* gene region in mitochondrial DNA was amplified and sequenced. Molecular analyses revealed that the samples belong to the *N. flexuosa* species. Phylogenetic analyses were carried out by comparing the gene sequences of *N. flexuosa* haplotypes in Europe with gene sequences of the same basepair length and with the gene sequence of Amphinemura borealis which was used as an outgroup. The two phylogenetic trees were consistent and the collected specimens were evolutionarily closer to the OK316196.1, OK316261.1, OK316397.1, MZ608304.1, KY261370.1 and JX905853.1 haplotype. The presence of *N. flexuosa* in Kocaeli and the phylogenetic status of the Türkiye haplotype were reported. Clarifying the phylogenetic status of ecologically important species provides basic information for biosecurity studies for possible future conservation and control programs.

Introduction

Members of the order Plecoptera, also known as stoneflies, are hemimetabolous. They are represented by about 2000 species worldwide and are known as representatives of the most primitive insects that evolved in the Carboniferous (Illies, 1965). Most species can reproduce in clean and cold streams and lakes. For this reason, they are used as freshwater indicators, especially in streams. They also play a key role in the food chain of mountain river ecosystems. They are usually pale in colour and move slowly. Many endemic species have emerged due to their slow movement. In mating, males are known to search for females with drumming behaviour. They have rapid speciation capacities due to their interesting reproductive behaviour and slow movement (Kazancı, 2008).

Nemoura flexuosa is characterized by the oddly shaped cercus, paraproct and internal sclerites of epiproct. *N. flexuosa* members show long periods of development during cold seasons. They become adult during periods of low temperature, i.e. winter and early morning (Benedetto, 1973). *N. flexuosa* larvae are present in places where oxygen concentration is reduced, such as the undersides of rocks in rivers and dried leaf piles in streams (Madsen, 1968). Adults are distributed in woodland areas far from the coast. Males have the ability to spread over larger distances than

females. There is a bias in the sex distribution of all Plecoptera species except *N. flexuosa*. In some Nemoura species, the ratio of males is higher, whereas in the rest of the Plecoptera species the ratio of females is higher (Kuusela and Huusko, 1996).

The genus Nemoura has over a hundred species known from the Holarctic and Oriental regions. It is widely distributed in Europe (Fochetti and Vincon, 2009). The presence of *N. flexuosa* has been recorded in European countries such as Austria, Bulgaria, Denmark, Estonia, Finland, France, Germany, Hungary, Italy, Luxembourg, Norway, Romania, Slovakia, Slovenia, Sweden and Switzerland. In Türkiye, it is reported to be distributed in Ankara, Bolu, Bursa, Çankırı, İzmir, Kastamonu, Ordu, Zonguldak (Darılmaz et al., 2016; Figure 1).

The utilization of morphological features in the identification of members of taxonomic groups with high speciation capacity such as Plecoptera at the species level prevents obtaining reliable and accurate results. Thus, identification by performing molecular analyses provides accurate results. Phylogenetic studies constitute the basis for many programs developed to ensure global biosecurity, such as Integrated Pest Management launched by Food and Agriculture Organization in 2013; EnviroDNA launched in Australia in 2016; and Corporate Ecosystem Services Review developed by the World Resources Institute, World Business Council for Sustainable Development and the Meridian Institute. In Türkiye, various national parks, nature parks, natural protected areas and gene banks have been built to promote and maintain biosecurity.

Mitochondrial DNA isolated from specimens and main barcode region *COI* genes are amplified. Samples were identified as *N. flexuosa* species by molecular phylogenetic analysis. The presence of *N. flexuosa* in Kocaeli was reported and its phylogenetic relationships based on *COI* gene were compared with other gene sequences that are recorded in the literature.

Materials and Methods

In 2021, 15 specimens observed and collected in Serindere Kanyonu (40º38'04''N; 30º00'06''D (DMS)) in Kartepe district of Kocaeli province. Samples were identified by examining their morphological characteristics under a stereo microscope and using the identification key (Bouchard, 2004). Afterwards, they were homogenized in a mortar and pestle, transferred to Eppendorf tubes and lysed. DNA isolation was performed by Phenol: Chloroform: Isoamyl Alcohol method (Sambrook and Russell, 2006). DNA amounts and purities were measured by spectrophotometer (SPECTROstar Nano, BMG LABTECH).

With JJ primers (Aksöyek et al., 2017; Table 1) *Cytochrome Oxidase C Subunit-I* (*COI*) genes from the mitochondrial DNA of the specimens were amplified by polymerase chain reactions (CFX Connect Real-Time System, BIO-RAD; Folmer et al., 1994; Table 2). Amplified DNA was electrophoresed on agarose gel (1%) and the amplification success was observed in a UV illuminator (Vilbert-Lourmat UV Transilluminator). Pairwise sequencing of amplified DNA samples according to the Sanger sequencing method done by BMLabosis.

Amplified DNA sequences were edited by removing non-sense regions in BioEdit (Hall, 1999) and aligned to 658 base pairs in MEGA 7 (Kumar et al., 2016). The edited sequence was BLAST analysed (Ratnasingham and Hebert, 2007). Sequence data of *Nemoura flexuosa* haplotypes were downloaded from GenBank and added to the *COI* gene-based sequence dataset.

For genetic diversity analysis, DNA Polymorphism analysis was run in DnaSP program (Rozas et al., 2017) and Pairwise Distance analysis was run in MEGA program. Optimal base substitution model analysis was done for Maximum Likelihood tree (Felsenstein, 1985; Kumar et al., 2018).

Table 1. Sequences of primers used for amplification.

Primer	Direction	5'-3' primer sequence	Reference
LCO1490-JJ	Forward	CHACWAAYCATAAAGATATYGG	Astrin & Stüben 2008
HCO2198-JJ	Reverse	AWACTTCVGGRTGVCCAAARAATCA	Astrin & Stüben 2008

Table 2. PCR cycles for LCO1490-JJ and HCO2198-JJ primer pairs of *COI* gene.

Step	Temperature (ºC)	Duration (s)	Number of Cycles
Initial Denaturation	95	120	1
Denaturation	95	30	
Primer Annealing	46	60	5
Extension	72	60	
Denaturation	95	30	
Primer Annealing	51	60	30
Extension	72	60	
Termination	72	600	1

Phylogenetic analyses were carried out by constructing Neighbor Joining and Maximum Likelihood trees in MEGA 7 program (Kimura, 1980). The Amphinemura borealis sequence which will be used as an outgroup was added to the data set and their phylogenetic relationships were compared.

Results

In the morphological identification, it was determined that the specimens to be molecularly identified belong to the Nemouridae family. In the spectrophotometer, the DNA amount of the isolates was found in the range of 50-100 ng and the DNA purity was found in the range of 1.8-2 values at 260/280 nm. BLAST analysis of the edited sequence (OQ243281) matched 99.85% with *Nemoura flexuosa* haplotypes (JX905856.1 and JF884156.1).

In DNA Polymorphism analysis these results are found: number of polymorphic sites (S); 30, total number of mutations (Eta); 31, number of haplotypes (h); 7, haplotype diversity (Hd); 0.795, variance of haplotype diversity; 0.01191, standard deviation of haplotype diversity; 0.109, nucleotide diversity (Pi); 0.01508, mean of nucleotide diversity (k); 9.923. The mean genetic distance in Pairwise analysis was calculated as 0.015. The genetic distance between the OK316196.1, OK316261.1, OK316397.1, MZ608304.1, KY261370.1 and JX905853.1 haplotype and also between JF884158.1 and JF884159.1 haplotype was 0. The maximum genetic distance was observed between JX905856.1 and JF884160.1 with 0.041 (Table 3).

The most appropriate model was determined as T92+G+I as a result of base substitution model analysis. Bases ratios was calculated; Adenine ratio as 31%, Thymine ratio as 31%, Guanine ratio as 19%, and Cytosine ratio as 19%.

Phylogenetic analyses indicated that our specimen was evolutionarily closer to the OK316196.1, OK316261.1, OK316397.1, MZ608304.1, KY261370.1 and JX905853.1 haplotype. Neighbor Joining and Maximum Likelihood trees showed the same topology and similar results and they proved to be consistent (Figure 2, Figure 3).

Discussion

Although the presence of *Nemoura flexuosa* has been recorded in different countries of Europe and in Türkiye, studies on its phylogenetic status are limited in the literature. In the study reported by Hlebec et al. (2022), only Croatian Plecoptera species were phylogenetically studied. Vitecek et al. (2017) compared the phylogenetic status of Trichoptera and Plecoptera species in Austria. Determining the phylogenetic status of an organism provides an important pool of information for biosecurity studies. *N. flexuosa* is a species of the order Plecoptera that provides important information on water quality and plays a role in the food chain of freshwater ecosystems. If the phylogeny of *N. flexuosa* is fully clarified, basic information will be supplied to the studies to be carried out in order to protect the balance of freshwater ecosystems.

The *COI* gene has been shown to be reliable for species-level identification (Hebert et al., 2003a, 2003b; Hogg and Hebert, 2004; Whiteman et al., 2004; Ball et al., 2005; Schander and Willassen, 2005). The mitochondrial *COI* gene region is considered to be the main barcode region for animals (Dinca et al., 2015). Mitochondrial DNA is a product of ancestral inheritance. It also has a high evolvability capacity. Therefore, it is a highly informative marker used in phylogenetic studies. In the light of this information, the mitochondrial *COI* gene region was selected as a target in the phylogenetic analysis of *N. flexuosa*.

Based on previous studies, universal folmer primers were used for phylogenetic analyses and 658 basepairs long mitochondrial *COI* gene sequences were obtained from the specimens (Folmer et al., 1994; Hebert et al., 2003; Ratnasingham and Hebert, 2007). For the reliability of the generated sequences, the BLAST analysis score should be more than 99% (Wu et al., 2017). As a result of the BLAST analysis performed in the present study, the generated sequence showed a 99.85% match to the haplotypes registered in GenBank. In accordance with the study reported by Boumans and Brittain (2012), *Amphinemura borealis* was selected as the outgroup. In both trees generated from phylogenetic analyses, N. flexuosa haplotypes formed

Table 3. Genetic distance between haplotypes

	OK316196.1	OK316261.1	OK316397.1	MZ608304.1	KY261370.1	JX905853.1	JX905856.1	JF884156.1	JF884157.1	JF884158.1	JF884159.1	JF884160.1	Nemoura flexuosa
OK316196.1													
OK316261.1	0,000												
OK316397.1	0,000	0,000											
MZ608304.1	0,000	0,000	0,000										
KY261370.1	0,000	0,000	0,000	0,000									
JX905853.1	0,000	0,000	0,000	0,000	0,000								
JX905856.1	0,017	0,017	0,017	0,017	0,017	0,017							
JF884156.1	0,014	0,014	0,014	0,014	0,014	0,014	0,009						
JF884157.1	0,012	0,012	0,012	0,012	0,012	0,012	0,008	0,005					
JF884158.1	0,025	0,025	0,025	0,025	0,025	0,025	0,039	0,036	0,034				
JF884159.1	0,025	0,025	0,025	0,025	0,025	0,025	0,039	0,036	0,034	0,000			
JF884160.1	0,026	0,026	0,026	0,026	0,026	0,026	0,041	0,038	0,036	0,002	0,002		
Nemoura flexuosa	0,002	0,002	0,002	0,002	0,002	0,002	0,017	0,014	0,012	0,025	0,025	0,026	

three clusters. Based on the COI gene sequences, the Kocaeli haplotype was clustered with the OK316196.1, OK316261.1, OK316397.1, MZ608304.1, KY261370.1 and JX905853.1 haplotypes recorded in GenBank thus indicating evolutionary relatedness. Analyses were supported with 10,000 bootstraps to determine robustness.

Resistance to factors such as diseases, parasites, predators and ecological factors leads to intraspecific genetic diversity (Amos and Harwood, 1998). As a result of mutations caused by these factors, genetic diversity occurs among individuals of the same species. The results we obtained by genetic diversity analyses were in parallel with the genetic diversity among different *N. flexuosa* haplotypes. The main reason for this genetic diversity is the different ecological conditions. If the phylogenetic relationships of haplotypes and subspecies due to the effects of ecological conditions and other factors are fully clarified, the effectiveness of programs for the protection of biodiversity and biosafety, especially the Integrated Pest Management, will increase.

Kocaeli is one of the most industrialized cities in Türkiye. Rapidly increasing industrialization in recent years has led to a disproportionate increase in population density (Pekey et al., 2010). Industrial wastes and trashes have led to an increase in environmental pollution. Consequently, biodiversity and human health have been negatively affected. However, the presence of *N. flexuosa* species of the order Plecoptera that live in fresh waters in this region shows that biodiversity is maintained. It is considered harmless to eat fish and other aquatic organisms in the waters of this region. Furthermore, these waters can be used as a source for drinking water plants.

Conclusion

In species identification, molecular identification by phylogenetic analysis is more reliable and precise than morphological identification. In the present study, it was aimed to make a definitive species identification of the specimens collected in Kocaeli by phylogenetic analysis and to clarify their phylogenetic status to provide basic information for future biosecurity studies. As a result of the analyses, it was determined that the specimens belong to *Nemoura flexuosa* species and are evolutionarily closer to the OK316196.1, OK316261.1, OK316397.1, MZ608304.1, KY261370.1 and JX905853.1 haplotype that are registered in GenBank. In the light of these results, it was concluded that the programs to be developed in the regions where phylogenetically related haplotypes are found can be integrated into the studies in Kocaeli and nearby regions. In addition, according to the literature, the presence of *N. flexuosa* in Kocaeli was reported for the first time. In addition, a haplotype of this species in Türkiye was compared phylogenetically with haplotypes in the literature for the first time.

Ethical Statement

Not applicable.

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Author Contribution

Conceptualization: FY, FU, Data Curation: FY, FU, Formal Analysis: FY, Funding Acquisition: FU, Investigation: FY, FU, Methodology: FY, FU, Project Administration: FU, Resources: FU, Supervision: FU, Visualization: FY, Writing -original draft: FY, Writing -review and editing: FU.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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