



Molecular phylogenetic analysis of *Odiellus lendlii* (Soerensen, 1894) from Soğuksu National Park, based on Mitochondrial Cytochrome c oxidase subunit I, 16S rRNA, 18S rRNA, and ITS1/ ITS2 gene markers

Aslı ÖZKARAKAŞOĞLU OLACAM¹, Fikriye POLAT^{*2}, İlkay ÇORAK ÖCAL³
ORCID: 0000-0003-1919-5728; 0000-0002-5414-2501; 0000-0003-1479-2697

¹Kocaeli University, Institute of Science, Department of Biology, 41380, Kocaeli, Türkiye

²Kocaeli University, Faculty of Education, Department of Mathematics and Science Education, 41380, Kocaeli, Türkiye

³Çankırı Karatekin University, Science Faculty, Biology Department, 18200, Çankırı, Türkiye

Abstract

Purpose: The study aims to characterize molecularly the species *Odiellus lendlii* (family Phalangiidae) occurring in Ankara's Soguksu National Park, using DNA barcoding-based methods.

Method: Specimens of *Odiellus lendlii* were collected between June 2022 and September 2023 for DNA extraction. PCR and DNA sequencing targeted mitochondrial COI, 18S rRNA, 16S rRNA, ITS1, and ITS2 gene regions using specific primers. FASTA sequences obtained with Chromas were compared against Phalangiidae and *Odiellus* species sequences in the NCBI BLAST database. For phylogenetic analysis, sequences were aligned and analyzed using MEGA version 11.0.13. Phylogenetic trees were constructed employing the Maximum Likelihood method with the Tamura-Nei/Jukes-Cantor model, supported by bootstrap analysis with 1000 replicates.

Findings: This study constitutes the first molecular investigation on *Odiellus lendlii* distributed in Ankara Soguksu National Park and provides a GenBank submission.

Conclusion: The molecular data obtained contribute to the genetic characterization and barcoding reference for *Odiellus lendlii* in this region, supporting future taxonomic and ecological studies.

Key words: *Odiellus lendlii*, COI, 18S rRNA, 16S rRNA, ITS1, and ITS2

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Soğuksu Ulusal Park'ta yayılış gösteren *Odiellus lendlii* (Soerensen, 1894)'nin mitokondriyal sitokrom c oksidaz altünite I, 16S rRNA, 18S rRNA, ITS1/ ITS2 gen markırları temelli moleküler filogenetik analizi

Özet

Amaç: Bu çalışma, Ankara Soğuksu Milli Parkı'nda yayılış gösteren Phalangiidae familyasına ait *Odiellus lendlii* türünün DNA barkodlama temelli yöntemlerle moleküler karakterizasyonunu amaçlar.

Metod: Haziran 2022 ile Eylül 2023 tarihleri arasında toplanan *Odiellus lendlii* örnekleriyle DNA izolasyonu gerçekleştirilmiştir. Ardından, mitokondriyal COI, 16S rRNA, 18S rRNA, ITS1 ve ITS2 genlerinin primerleri kullanılarak PCR ve DNA dizi analizi yapılmıştır. Chromas ile elde edilen FASTA dizileri, NCBI BLAST veri tabanındaki diğer Phalangiidae veya *Odiellus* türlerinin dizileriyle karşılaştırılmıştır. Filogenetik analiz için diziler MEGA version 11.0.13 programına yüklenmiş ve Maksimum Likelihood yöntemi, Tamura-Nei/Jukes Cantor Modeli, Bootstrap 1000 ile filogenetik ağaçlar oluşturulmuştur.

Bulgular: Çalışma, Ankara Soğuksu Milli Parkı'nda yayılış gösteren *Odiellus lendlii* üzerine yapılan ilk moleküler çalışmayı oluşturmaktadır ve GenBank'a bir kayıt sağlamaktadır.

Sonuç: Elde edilen moleküler veriler, bu tür için bu bölgede genetik karakterizasyon ve barkod tabanlı referans oluşturulmasına katkıda bulunmakta olup gelecekteki taksonomi ve ekoloji çalışmalarını desteklemektedir.

Anahtar kelimeler: *Odiellus lendlii*, COI, 18S rRNA, 16S rRNA, ITS1, and ITS2

* Corresponding author: Tel.: +90 262 303 24 42; Fax.: +90 262 303 24 03; E-mail: fikriyepolat@kocaeli.edu.tr

1. Introduction

To date, 6545 opiliones species have been identified worldwide [1]. Turkey is located in the Mediterranean Sub-region of the Palearctic, encompassing areas of the Black Sea, Irano-Turanian, and Eastern Mediterranean regions. Additionally, Turkey occupies a strategic position as a bridge between the continents of Europe, Asia, and Africa. Currently, 105 opiliones species have been documented in Turkey. However, considering Turkey's zoogeographical position, it is believed that the actual species diversity is much higher. Most of the opiliones inhabit in agro-ecosystems of tropical and temperate regions. They act as predators of some agricultural pests, and play a significant role in biological control due to their position in the food chain [2-4]. Additionally, since opiliones cannot survive in polluted ecosystems, they can be considered indicator species [5]. Numerous studies on Opiliones have addressed various aspects of their biology, including hunting behavior, feeding ecology, morphological and taxonomic characteristics, geographical distribution, as well as anatomical and histological structures observed through light and electron microscopy. The studies are concentrated especially in fauna, systematics, taxonomy, and ecology. Globally, 22 species are defined within the genus *Odiellus* (Roewer, 1923). In Turkey, three species have been identified: *Odiellus lendli* (*O. lendli*) (Sorensen, 1894), *Odiellus zecariensis* (*O. zecariensis*) (Mkheidze, 1952), and *Odiellus ramblae* (*O. ramblae*) (Sánchez-Cuenca & Prieto, 2014). There is no information in the literature about the molecular studies done on *Odiellus* species in Turkey except for the 16S rRNA gene (GeneBank: MN192912.1). In this study, it was aimed to identify *O. lendli*, which was collected from Soğuksu National Park and analyzed morphologically, at the molecular level using mitochondrial cytochrome oxidase subunit I (COI), 16S rRNA, 18S rRNA, ITS1, and ITS2 barcode genes.

2. Material and method

2.1. Examined material and morphological identification

Thirty specimens used in the study were collected from Ankara Soğuksu National Park between June 2022 and September 2023 through field surveys conducted at two different localities. A hand aspirator and collection jars were used to collect the samples. Samples were collected from soil and rock surfaces, under stones, tree cavities, grass crevices, and grass bottoms, and then preserved in 70% ethanol and stored at -80°C . The species of the Opiliones were diagnosed according to the identification key [6], which was defined under the stereomicroscope in the Çankırı Karatekin University Zoology Research Laboratory.

2.2. Molecular analysis

DNA isolation was performed using the NucleoSpin® DNA Insect kit (Macherey-Nagel, Cat. No. 740470.50) according to the manufacturer's protocol. Prior to extraction, muscle tissue was washed in physiological saline solution for 15–20 min to remove residual ethanol and subsequently dried on filter paper. Before DNA extraction, the muscle tissue was washed in physiological saline solution for 15–20 minutes to eliminate residual ethanol, followed by drying with filter paper. DNA concentrations and purity were quantified using the Qubit 2.0 Fluorometer kit (Invitrogen, America). The PCR was performed in a final reaction volume of 20 μl containing 10 ng of genomic DNA, 10 pmol of each primer, and 5x FIREPol Master Mix (Solis BioDyne). Amplified PCR products were then run for 45 minutes at 100 volts in a 1.2% agarose gel electrophoresis and visualized with a UV transilluminator by using Safe-T staining (ethidium bromide alternative). Specific primer sets were used to amplify the COI [7], 16S rRNA [8], 18S rRNA [9], ITS1 [10], and ITS2 [11].

PCR products were purified by BMLabosis using ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific, USA) manufacturer's protocol. Sequence analysis was performed in the MacroGen Netherlands laboratory using the ABI 3730XL Sanger Sequencer (Applied Biosystems, Foster City, CA) and the forward and reverse primers of the mitochondrial COI, 16S rRNA, 18S rRNA, ITS1, and ITS2 genes.

2.3. Phylogenetic analysis

Ankara Soğuksu Ulusal Park'tan toplanan *O. lendli* örneklerinden mitokondrial COI, 16S rRNA, 18S rRNA, ITS1 ve ITS2 barkod genleri başarılı bir şekilde sekanslandı. Bu sekanslara COI, 16S rRNA ve 18S rRNA barkod genleri için NCBI veri bankasında bulunan otbiçen türlerinden outgruplar eklendi fakat ITS1 ve ITS2 için outgrup bulunamadı (Table 2, 5, 7).

DNA sequences were visualized and edited using FinchTV. The sequences were recorded separately in FASTA format via FinchTV. The forward and reverse complement sequences were compared by aligning them using the ClustalW program. Sequences of each gene region were loaded into NCBI BLAST in FASTA format. Their similarities with the sequences recorded in the NCBI genome database were compared. All sequences belonging to the three gene regions studied were loaded into the MEGA version 11.0.13 program [12] separately. Their modeling methods and distances were determined. The phylogenetic tree was created by using Bootstrap 1000, the Maximum Likelihood (ML) method, and the Tamura-Nei (1993) /Jukes-Cantor (1969) Model.

3. Results

Examined material: 30 specimens, Türkiye Ankara Soğuksu National Park (40.2755, 32.3717, 1030 m above sea level), 18♀ and 12♂.



Figure 1. The habitus of Opiliones



Figure 2. Dorsal view of *O. lendli* (Stereo Microscope)

3.1. Morphological identification

O. lendli (Sorensen, 1894) belongs to the subfamily Oligolophinae within the family Phalangidae (suborder Eupnoi). This species has a wide global distribution. The body coloration ranges from dark yellowish-gray to grayish-brown, with a distinct saddle marking. The ocular area is small relative to the body size and covered with indistinct tubercles. The tridents are prominent, characterized by a 10-degree angle between their branches. The prosoma bears scattered tubercles (Figures 1 and 2).

3.2. Mitochondrial COI gene

A total of 10 specimens underwent COI gene sequence (658 base pair-fragment) analysis, revealing their genetic variation. When the G-C ratios of our specimens were examined (Table 1), the average values were estimated as 33.3%. The nucleotide frequencies used in the calculations were 30.2% for A, 36.4% for T, 18.4% for C, and 14.9% for G. When looking at the NCBI-Nucleotide gene bank, there was no COI barcode gene study in the species belonging to the genus *Odiellus*. Therefore, other species from the Phalangidae family registered in NCBI were evaluated as outgroups. These species are registered ones from Canada, Finland, France, Germany, and Turkey on NCBI (Table 1).

Comparison of the *O. lendli* specimens revealed 6 nucleotide variations (2.47%) across the 658 bp COI region. Transition/Transversion rates (R), Parsim-info sites, and singleton sites are shown in Table 2.

Table 1. Nucleotide composition (%) and length of base pairs of the region of the COI gene and GenBank sequence accession numbers of the species used in this study

Species-Number (Accession number) Country	T(U)	C	A	G	G-C	A-T	Total bp
<i>O. lendlii</i> 3 (ON669271)	36.5	18.4	30.2	14.9	33.3	66.7	658.0
<i>O. lendlii</i> 4 (ON669272)	36.5	18.4	30.2	14.9	33.3	66.7	658.0
<i>O. lendlii</i> 14 (ON669273)	36.5	18.4	30.2	14.9	33.3	66.7	658.0
<i>O. lendlii</i> 15 (ON669274)	36.5	18.4	30.2	14.9	33.3	66.7	658.0
<i>O. lendlii</i> 16 (ON669275)	36.5	18.4	30.2	14.9	33.3	66.7	658.0
<i>O. lendlii</i> 17 (ON669276)	36.5	18.4	30.2	14.9	33.3	66.7	658.0
<i>O. lendlii</i> 19 (ON669277)	36.5	18.4	30.2	14.9	33.3	66.7	658.0
<i>O. lendlii</i> 20 (ON669278)	36.5	18.4	30.2	14.9	33.3	66.7	658.0
<i>O. lendlii</i> 21 (ON669279)	36.5	18.4	30.2	14.9	33.3	66.7	658.0
<i>O. lendlii</i> 22 (ON669280)	36.3	18.5	30.1	15.0	33.5	66.4	658.0
<i>Phalangium opilio</i> (KM840602.1) Canada	34.7	19.5	30.4	15.5	35.0	65.1	658.0
<i>Mitopus morio</i> (MZ630130.1) Finland	36.8	17.3	31.0	14.9	32.2	67.8	658.0
<i>Rilaena sp.</i> (KR098100.1) Canada	32.8	21.7	29.0	16.5	38.2	61.8	631.0
<i>Egaenus turcicus</i> (MK947100.1) Türkiye	32.6	23.6	27.7	16.1	39.7	60.3	607.0
<i>Opilo orocastaneus</i> (MN182965.1) France	38.3	16.1	29.6	16.0	32.1	67.9	658.0
<i>Opilio canestrinii</i> (KY269417.1) Germany	36.9	17.3	29.9	15.8	33.1	66.8	658.0
Avg.	36.1	18.7	30.0	15.2	34.6	65.4	652.8

Table 2. Nucleotide pair frequencies for the COI gene in *O. lendli*

	From O3 to O22
Conserved sites	652/658 (99.08%)
Variable sites	6/658 (2.47%)
Parsim-info sites	none
Singleton sites	6/658 (0.91)
Identical Pairs (ii)	657
Transitional Pairs (si)	1
Transversional Pairs (sv)	0
R (si/sv)	2.0

Substitution rates were estimated using the maximum likelihood method with the Tamura-Nei model (1993) (Table 3). The substitution rates represent the possibility of a substitution transition from one base to another. Transitional substitution rates were calculated as 18.1 for A/G, 15.08 for T/C, 36.7 for G/A, and 29.87 for C/T. Transversional rates are given in Table 4. In light of these results, the ratio of transition/ transversion (R) was calculated as 2 (Table 3).

Table 3. Nucleotide substitution matrix* for the COI gene in *O. lendli*

	A	T	C	G
A	-	<i>0.04</i>	<i>0.02</i>	18.1
T	<i>0.04</i>	-	15.08	<i>0.02</i>
C	<i>0.04</i>	29.87	-	<i>0.02</i>
G	36.7	<i>0.04</i>	<i>0.02</i>	-

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

An examination of evolutionary divergence, calculated using the Kimura 2-parameter model (Table 4), revealed that the *O. lendli* from the current study exhibited a close genetic affinity with *Mitopus morio* (MZ630130.1), with a mean interspecific divergence of 4.4%.

Table 4. Estimates of Evolutionary Divergence over COI Sequence Pairs between Groups. Shown here are the mean genetic distances calculated using the Kimura 2-parameter model. Standard error estimate(s) are shown above the diagonal

		1	2	3	4	5	6	7
1	<i>Phalangium opilio</i> (KM840602.1) Canada		0.320	0.488	0.482	0.757	0.305	0.366
2	<i>Mitopus morio</i> (MZ630130.1) Finland	0.055		0.467	0.483	0.714	0.266	0.258
3	<i>Rilaena sp.</i> (KR098100.1) Canada	0.092	0.096		0.481	0.989	0.398	0.412
4	<i>Egaenus turcicus</i> (MK947100.1) Türkiye	0.095	0.086	0.088		1.174	0.483	0.532
5	<i>Opilo orocastaneus</i> (MN182965.1) France	0.139	0.128	0.201	0.273		0.634	0.767
6	<i>Opilio canestrinii</i> (KY269417.1) Germany	0.051	0.046	0.076	0.097	0.120		0.326
7	<i>O. lendli</i>	0.061	0.044	0.075	0.101	0.144	0.057	

When examining the phylogenetic tree, it is observed that the species *O. lendli* clusters closely with *Opilio canestrinii* (KY269417.1), *Mitopus morio* (MZ630130.1), and *Rilaena sp.* (KR098100.1), while the other outgroups, *Phalangium punctipes* and *Phalangium opilio*, are clustered separately (Figure 3).

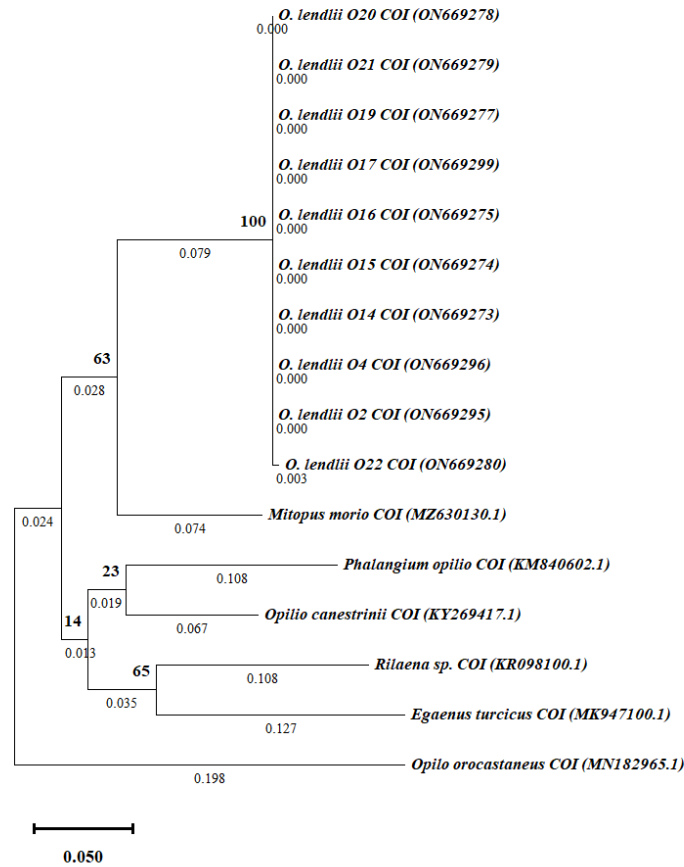


Figure 3. Phylogenetic tree created by the Maximum Likelihood method for the mitochondrial COI gene region of *O. lendli*

3.3. 18S rRNA gene

The genetic differences of the 10 specimens with the best results in DNA sequencing for the 18S rRNA region are shown in Table 5. It was determined that the G-C ratios of the *O. lendli* samples varied between 50.4 - 50.7%. The nucleotide frequencies used in the calculations were found as 25.0% for A, 24.4% for T, 23.1 % for C, and 27.4% for G (Table 5).

Table 5. Nucleotide composition (%) and length of base pairs of the region of the 18S rRNA gene and GenBank sequence accession numbers of the species used in this study

Species-Number-Accession number	T(U)	C	A	G	G-C	A-T	Total bp
<i>O. lendli</i> 2 (ON669295)	24.5	23.2	25.0	27.3	50.5	49.5	1702.0
<i>O. lendli</i> 4 (ON669296)	24.5	23.1	25.0	27.4	50.5	49.5	1702.0
<i>O. lendli</i> 7 (ON669297)	24.6	23.1	25.0	27.3	50.4	49.6	1702.0
<i>O. lendli</i> 13 (ON669298)	24.5	23.1	25.0	27.4	50.5	49.5	1702.0
<i>O. lendli</i> 17 (ON669299)	24,3	23.3	25.0	27.4	50.7	49.3	1702.0
<i>O. lendli</i> 18 (ON669304)	24.4	23.1	25.1	27.4	50.5	49.5	1702.0
<i>O. lendli</i> 20 (ON669300)	24.4	23.1	25.1	27.4	50.5	49.5	1702.0
<i>O. lendli</i> 21 (ON669301)	24.4	23.1	25.1	27.4	50.5	49.5	1702.0
<i>O. lendli</i> 22 (ON669302)	24.4	23.1	25.1	27.4	50.5	49.5	1702.0
<i>O. lendli</i> 23 (ON669303)	24.4	23.1	25.0	27.4	50.5	49.4	1702.0
<i>O. troguloides</i> (X81441.1)	24.4	23.2	25.0	27.4	50.6	49.4	1702.0
Avg.	24.4	23.1	25.0	27.4	50.5	49.5	1702.0

Regarding to 18S rRNA sequence, when the 10 samples that we studied were examined within themselves, it was observed that there were 2 nucleotide changes (0.1%) in 1702 nucleotides. When examined together with *O. trogluoides* (X81441.1) selected as the outgroup, it was found that there were also variations in 28 more nucleotides, and this situation might be considered as normal among different species belonging to the same genus. Transition/Transversion ratios (R), Parsim-info sites, and singleton sites are given in Table 6.

Table 6. Nucleotide pair frequencies for the 18S rRNA gene in *O. lendli*

	From O2 to O23	All species
Conserved sites	1681/1702 (98,76%)	1672/1702
Variable sites	2/1702 (0.1%)	30/1702
Parsim-info sites	2/1702	2-1702
Singleton sites	19/1702 (1.11)	28/1702
Identical Pairs (ii)	1697	1696
Transitional Pairs (si)	3	3
Transversional Pairs (sv)	2	3
R (si/sv)	1.2	1.07

The Tamura-Nei Model (1993) was used to determine the substitution rates using the maximum likelihood method. Transitional substitution rates were calculated as 4.05 for A/G, 12.46 for T/C, 13.18 for C/T, and 3.71 for G/A. According to the Jukes-Cantor model, when the Pairwise genetic distance matrix was analyzed, the lowest genetic distance, including outgroup, was found as 0.001. The highest genetic distance was found as 0.011 (1.1%). These are the normal values of variation that can be seen among individuals representing the same species/genus for the 18S rRNA sequence. The Genetic distance results were calculated according to the pairwise analysis of the DNA sequences of 11 *O. lendli* specimens, and it was benefited from 1000 repetitive bootstrap tests to calculate standard errors (These data were not shown on tables).

In our study, the Maximum Likelihood (ML) method was used to calculate the evolutionary relationships of the *O. lendli* samples and to construct the phylogenetic tree, the Jukes-Cantor Model was used to determine the genetic distances between species, and the 1000 repetitive bootstrap test was used to determine the genetic distances between species, and the 1000 repetitive bootstrap test was used to ensure the reliability of the tree. The phylogenetic tree created at 0.0005 scale is shown in Figure 4.

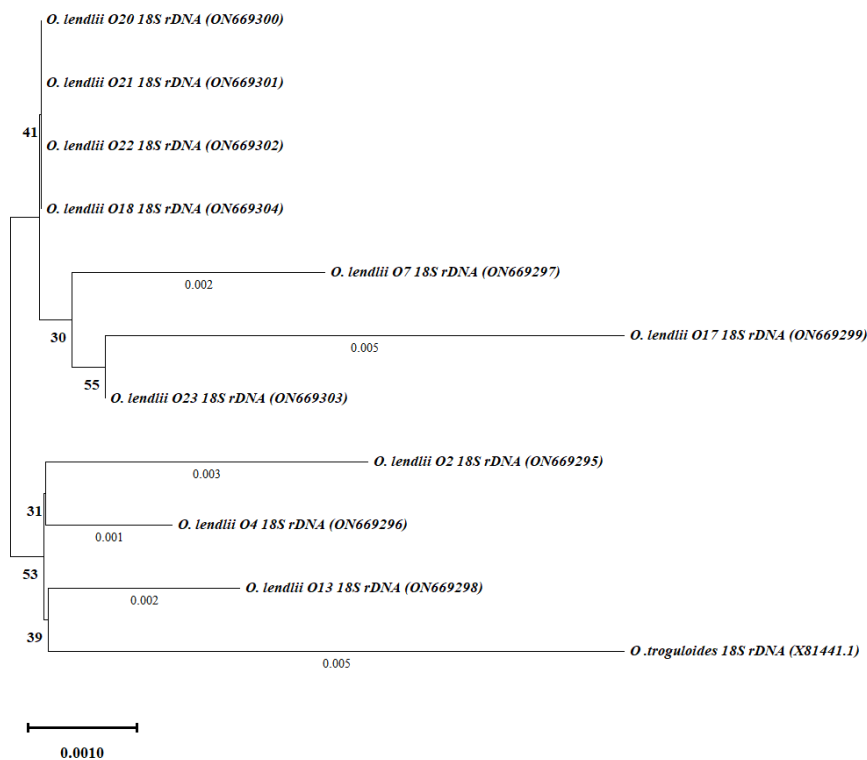


Figure 4. Molecular phylogenetic tree created by the Maximum Likelihood method for the 18S rRNA gene region of *O. lendli*

3.4. 16S rRNA

We obtained the best DNA analysis results in only two of our samples amplified by PCR using the 16S rRNA gene region. These samples are *O. lendlii* O24 with access number OR611712.1 and *O. lendlii* O21 with access number OR611711.1. The nucleotide composition of the 16S rRNA gene region for *O. lendlii* belonging to O21 and O24 can be seen in Table 7. When examining Table 7, the nucleotide frequencies for our O21 and O24 samples were detected as follows: Adenine base at 38.2%, Thymine base at 35.5%, Cytosine base at 10.4%, and Guanine base at 16.6%.

When comparing the molecular analysis data obtained for the 16S rRNA gene region of *O. lendlii* species, the variation rate between bases was determined to be 2% for the 509 base pair region. The Data is not shown here. Upon examination of the NCBI-Nucleotide gene bank, *O. lendlii* with access numbers MN217231.1 and MN192912.1 from Turkey, and another harvestman species *Mitopus morio* with access number JX049150.1 from the Phalangiidae family in Austria were evaluated as the outgroup. The Maximum Likelihood (ML) method, using the Jukes-Cantor model to determine genetic distances between species, and a phylogenetic tree constructed with 1000 bootstrap replicates to ensure the tree's reliability, is shown in Figure 5.

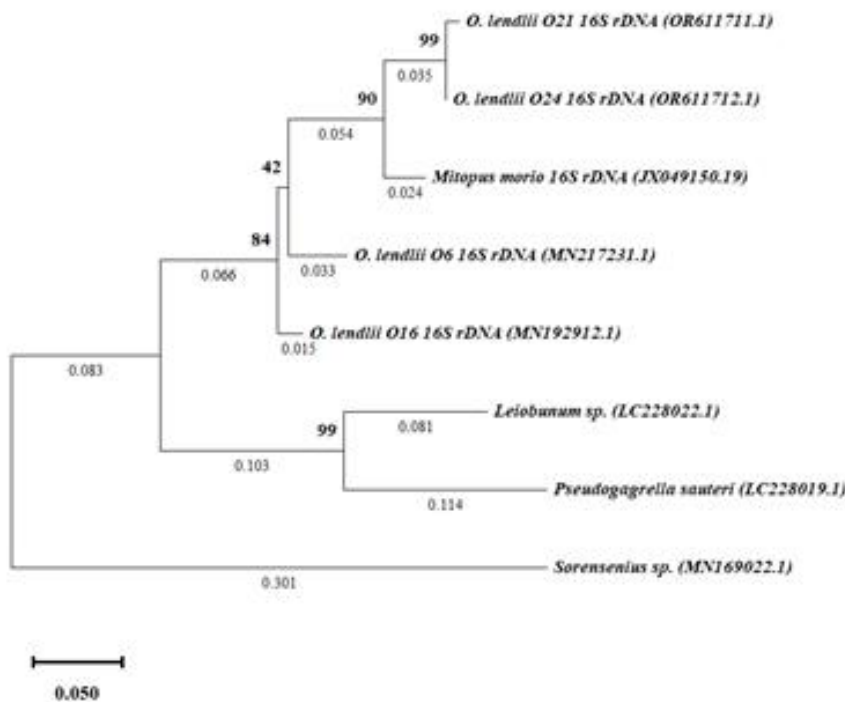


Figure 5. Phylogenetic tree created by the Maximum Likelihood method for the 16S rRNA gene region of *O. lendlii*

3.5. ITS1

Of DNAs isolated from the *O. lendlii* species, only samples coded with O3 (MZ338325.1), O4 (MZ338326.1), O11 (PQ488629.1), O18 (PQ488631.1), O19 (PQ488630.1), O28 (OR612788.1), and O30 (OR612789.1) regarding to ITS1 gene region gave quality sequence results. When examining the ITS1 gene sequences of our samples, which were registered in the NCBI-Nucleotide Genome Data Bank, it was determined that the G-C ratios were 51.9% and the A-T ratios were 48% (Table 7). Since there was no other study belonging to the genus *Odiellus* to be registered in the NCBI, an outgroup could not be formed for this gene, and a phylogenetic tree could not be made.

When our 7 samples were examined in terms of the ITS1 sequence, it was observed that 245 nucleotides were preserved out of 250 nucleotides (98%) with a 2% variation. Additionally, Transition/Transversion ratios (R=1.9), the Parsimony significant region (3), and the singleton region (2) values were determined. The pairwise genetic distance matrix was constructed according to the Jukes-Cantor model. The T92+G value was selected as the Gamma parameter for calculating genetic distance. Among the seven analyzed *O. lendlii* species, the minimum genetic distance was 0.000, and the maximum was 0.039. The genetic distance results were calculated based on pairwise analyses of *O. lendlii* sequences, and 1000 bootstrap replicates were used to estimate standard errors, but the data are not presented in the tables.

3.6. ITS2

From the DNA isolated from *O. lendlii* individuals, high-quality sequencing results were obtained only for samples coded O5 and O28 in terms of the ITS2 gene region. When the NCBI-Nucleotide database was searched, it was observed that, similar to ITS1, there were no studies related to the ITS2 gene region for the Opiliones family. The ITS2 gene sequences of our samples, registered in the NCBI-Nucleotide Genome Database with accession numbers PQ488633.1 and PQ488634.1, were analyzed using the MEGA version 11.0.13 program. The analysis revealed an average G-C ratio of 50.9% and an A-T ratio of 49.1%. The nucleotide frequencies were 22.6% for Adenine, 26.5% for Thymine, 23.3% for Cytosine, and 27.6% for Guanine. The nucleotide frequencies for all samples are shown in Table 7. To examine the ITS2 gene region, two *O. lendlii* DNA sequences were compared with each other, and no variation was observed in the 566-base-pair region. Since only two samples were compared, Parsim-info and singleton site values could not be obtained.

Table 7. Nucleotide composition (%) for 16S rRNA, ITS1, and ITS2 genes in *O. lendlii* (%)

Species-Number-Accession number- Country	T(U)	C	A	G	G-C	A-T	Total bp
16S rRNA							
<i>O. lendlii</i> 21 (OR611711.1)	35.2	10.5	38.2	16.6	26.1	73.4	505
<i>O. lendlii</i> 24 (OR611712.1)	35.8	10.3	38.2	15.6	26.1	74.0	505
<i>O. lendlii</i> (MN217231.1) Turkey	35.2	8.7	39.0	17.1	25.8	74.2	310
<i>O. lendlii</i> (MN192912.1) Turkey	37.1	8.7	37.1	17.1	25.8	74.2	310
<i>Mitopus morio</i> (JX049150.1) Austria	36.6	10.3	35.9	16.8	27.5	63.4	476
<i>Leiobunum sp.</i> (LC228022.1)	36.2	11.3	29.4	23.2	34.5	65.6	453
<i>Pseudogagrella sauteri</i> (LC228019.1)	34.0	12.1	30.0	23.8	35.9	64.0	453
<i>Sorensenius sp.</i> (MN169022.1)	30.0	21.0	34.0	15.0	36.0	64.0	420
Avg.	35.1	11.7	35.0	16.6	29.7	69.1	421
ITS1							
<i>O. lendlii</i> 3 (MZ338325.1)	29.2	28.4	18.4	24.0	52.4	47.6	250
<i>O. lendlii</i> 4 (MZ338326.1)	29.2	28.4	19.6	22.8	51.2	48.8	250
<i>O. lendlii</i> 11 (PQ488629.1)	29.2	28.8	19.2	22.8	51.6	48.4	250
<i>O. lendlii</i> 18 (PQ488631.1)	29.2	28.8	18.4	23.6	52.4	47.6	250
<i>O. lendlii</i> 19 (PQ488630.1)	29.2	28.8	18.4	23.6	52.4	47.6	250
<i>O. lendlii</i> 28 (OR612788.1)	29.2	29.2	19.2	22.4	51.6	48.4	250
<i>O. lendlii</i> 30 (OR612789.1)	29.2	28.8	18.4	23.2	52.0	47.6	250
Avg.	29.2	28.7	18.8	23.2	51.9	48.0	250
ITS2							
<i>O. lendlii</i> 5(PQ488633.1)	26.5	23.3	22.6	27.6	50.9	49.1	566
<i>O. lendlii</i> 28(PQ488634.1)	26.5	23.3	22.6	27.6	50.9	49.1	566
Avg.	26.5	23.3	22.6	27.6	51.0	49.0	566

4. Conclusions and discussion

For phylogenetic analysis, five different barcode genes (COI, 16S rRNA, 18S rRNA, ITS1, and ITS2) were performed on *O. lendlii* specimens previously described morphologically. Examination of the resulting phylogenetic trees revealed weakly/moderately supported clades within three gene regions. These were identified as *Mitopus morio* (MZ630130.1) for COI (bootstrap value = 63), *O. troguloides* (X81441.1) for 18S rRNA (bootstrap value = 39), and *Mitopus morio* (JX049150.1) for 16S rRNA (bootstrap value = 90) (Figures 3–5).

O. lendlii, an opilione species belonging to the Phalangidae family, is distributed worldwide in Bulgaria, Macedonia, Romania, Russia, Ukraine, and Türkiye [13, 14]. While many morphological and systematic studies have been done on opiliones in Turkey [15-16], so far only one molecular phylogeny study has been conducted for *O. lendlii* [17]. Kurt, P., and Yildirim Dogan, N. analyzed the COI and 16S rRNA barcode genes of the *O. lendlii* species; they used *Odiellus* species collected and identified by Kurt, K. The *O. lendlii* COI sequence with accession number MN097806.1, submitted to NCBI-Nucleotide by Kurt, P., and Yildirim Dogan, N., could not be used in this study because it was unverified and does not match [18].

When the NCBI-Nucleotide genome database is examined, it is seen that while there are two 16S rRNA gene region data entries from Turkey for the *O. lendli* species, there is no data entry from other countries [19]. Therefore, no data entry was found in NCBI-Nucleotide, except for the COI, 18S rRNA, 16S rRNA, ITS1, and ITS2 barcode genes used in this study for the *O. lendli* species. When the COI gene sequences of *O. lendli* from our study were compared to those in other Phalangidae species registered as *Mitopus morio* (MZ630130.1), *Opilio parietinus* (MN097809.1), and *Phalangium opilio* (KM840602.1) in the NCBI-Nucleotide Genbank using CLUSTALW, it was found that they had similarity at the ratios of 85.25%, 84.84%, and 80.85%, respectively. A-T ratios for the COI gene region of *O. lendli* were found to be 66.7%. In the other Phalangidae species in Table 1, the lowest A-T ratio was 60.3% in *E. turcicus* (MK947100.1) registered from Turkey, whereas the highest ratio was 67.9% in *O. orocastaneus* (MN182965.1) registered from France. Among nucleotides, there was a compositional bias towards AT: 65.4%. In detail, the overall base composition was: A 30.0, C 18.7, G 15.2, T 36.1% (Table 1). Among nucleotides, there was a compositional bias towards AT: 66.1%. In detail, the overall base composition was: A 30.0, C 18.7, G 15.2, T 36.1% (Table 1).

No data entry was found for 18S rRNA and ITS2 barcode genes in the *O. lendli* species in NCBI-Nucleotide. *O. troguloides* (X81441.1), a different species of the genus *Odiellus*, registered by Giribet et al. (1999) [20], was therefore used as the only outgroup for 18S rRNA phylogenetic analysis. When Table 5 is examined, it is seen that the ratios of A, T, G, and C nucleotides between two species belonging to the genus *Odiellus* are almost the same. When both species were aligned with ClustalW, it was found that they had 99.05% similarity. The lowest genetic distance between *O. lendli* and *O. troguloides* is 0.006 (0.6%), and the highest is 0.011 (1.1%). Considering that the applicable categorical level of the nuclear 18S rRNA gene region can be up to the family level in phylogenetic analyses [21], it is normal to present such a close genetic distance between *O. lendli* and *O. troguloides* species.

Our samples with accession numbers O21 (OR611711.1) and O24 (OR611712.1) were individually entered into NCBI-Blast and compared with *Odiellus* species present in the database. Upon examining the species that appeared in the database, the highest similarity in the 16S rRNA gene region with *Odiellus* species was found to be 90.68% with *Mitopus morio*. A literature survey on the 16S rRNA gene region of the *Opiliones* order has listed nine studies to date. Among these, Hickerson et al. (2006) stated in their study that the COI and 16S rRNA gene regions might be limited in identifying recently evolved species, and therefore, additional gene regions should also be studied [22].

Chen and Shih (2018), in their study, conducted both morphological and molecular analyses of *Metadentobunus formosae formosae* and *Metadentobunus formosae garampiensis* species belonging to the order of harvestmen (*Opiliones*) distributed in Taiwan. They used COI and 16S rRNA gene regions for their molecular analyses [23]. According to this research, the minimum genetic distance for the studied species was found to be 6.79%. They stated that this distance between two species of the same genus was sufficient to distinguish between two different species.

In a study, nucleotide ratios for the 16S rRNA gene region were as follows: 35.2% for Thymine (Uracil), 8.7% for Cytosine, 39.0% for Adenine, and 17.1% for Guanine. The total number of base pairs for the 16S rRNA gene was found to be 310.0. In our study, we found the A-T nucleotide ratios for the 16S rRNA gene region to be an average of 35.61% for Thymine (Uracil), 10.5% for Cytosine, 38.2% for Adenine, and 15.6% for Guanine. We obtained our total base pair count as 506 as a result of DNA sequencing. When the sequence obtained in a study of the 16S rRNA gene region of *O. lendlii* with accession number MN192912.1 was compared using the ClustalW 2.1 Multiple sequence alignments program, it was found that the similarity rate with the sequence we obtained in our study was 86.46% [19]. When the literature was reviewed, no other study was found for the 16S rRNA gene region of *O. lendlii* species.

In the genome database, a phylogenetic tree could not be created because there were no ITS1 studies in the species we worked with or in closely related species. Ortiz and Francke (2016) investigated the effectiveness of COI and ITS1 gene regions in molecular classification in their study with 12 tarantulas of the *Bonnetina* genus from the *Araneae* order of the *Arachnida* class, 7 of which were unidentified. They indicated that the COI gene region is reliable in distinguishing genera because it possesses a barcoding gap. They also noted that the COI gene region shows considerable variation in different genera. They demonstrated that the ITS1 gene region does not contain a barcoding gap. They stated that ITS1 is a highly informative locus for species delimitation and phylogeny at the species level, but it performs poorly as a barcode gene, and the ITS1 gene region undergoes changes much more slowly. Therefore, they reported that the COI gene is more useful for molecular analyses [24].

When a literature search was conducted in the NCBI-PubMed database for the ITS2 gene region, no studies were found for the order *Opiliones*. Upon expanding the literature search to the class *Arachnida*, it was observed that there were 125 studies in NCBI-PubMed for the ITS2 gene region, including the species *O. lendlii* within the class *Arachnida*. The ITS2 gene region sequences of our samples from the order *Opiliones*, previously uploaded to NCBI-Nucleotide, were aligned separately with the ITS2 gene regions of *Harmonia axyridis* (*H. axyridis*) (accession number EF690220) registered in NCBI-Nucleotide using the ClustalW 2.1 Multiple Sequence Alignment program. The comparison revealed similarity rates of 98.67% between *O. lendlii* (PQ488634.1) and *H. axyridis* (EF690220) (ladybug), and 99.65% between *O. lendlii* (PQ488633.1) and *H. axyridis* (EF690220). The high similarity rate in the ITS2 gene region between *H. axyridis* and *O. lendlii*, which belong to different classes within the same phylum, indicates that this gene region has low discriminatory power. Indeed, Roy et al. (2018) reported in their study that using

ITS2 alone as a genetic marker could lead to misleading results, and therefore, at least two gene regions should be used for analysis [25].

In conclusion, in this study, morphological and molecular analyses of the *O. lendli* species collected from Soğuksu National Park were performed. Mitochondrial COI, nuclear 18S rRNA, 16S rRNA, ITS1, and ITS2 barcode genes were used for molecular analysis. The first records of the genes mentioned above for *O. lendli* were entered in the NCBI Genome Data Bank with this study. Researchers conducting a phylogenetic study for the Opiliones team can use the COI gene region, a strong barcode gene for distinguishing species. The 18S rRNA gene region can be distinctive for higher taxonomic levels, such as family and order. However, since studies related to Opiliones are limited for the five barcode genes we examined, comprehensive comparisons could not be fully made. There are many morphological studies about the biodiversity of harvestman species in Anatolian lands. However, it is necessary to reveal the harvestman biodiversity at the molecular level, provide its records in barcode systems, and close the literature gap in this field.

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