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GENETIC DISTANCE REVEALS SYNONYMY AND NEW FISH SPECIES IN BALIKESİR STREAMS, TÜRKİYE

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Abstract

The Mediterranean-Aegean region play a significant role in the context of conserving biodiversity. The protection of endemic and local species becomes attainable only with a thorough understanding of their biology. Specifically, aquatic species dwelling in inland waters may be more vulnerable due to the irregular composition of their habitats, characterized by natural barriers. It becomes imperative to initiate the conservation process by identifying the fauna and flora of ecosystems, thereby facilitating the development of comprehensive conservation plans. In this context, the primary objective of the present study is to identify freshwater fish species inhabiting Madra and Havran Streams in Balıkesir, Türkiye using the DNA barcoding method. The procedure involves DNA isolation through the Chelex protocol, followed by the amplification of the mitochondrial CO1 region using various primer combinations. The results obtained from the gene sequences of 29 individuals in total provide valuable information on species diversity, genetic relationships, and variations. This research emphasizes the importance of DNA barcoding as a valuable tool for species identification, genetic exploration, and conservation plans. The acquired outcomes establish a foundation for the effective management of aquatic biodiversity, particularly within these vulnerable ecosystems.

Keywords: Species diversity, irregular natural barriers, endemic species, inland water basins, stream ecology

GENETİK MESAFE, TÜRKİYE BALIKESİR DERELERİNDE SİNONİM VE YENİ BALIK TÜRLERİNİ ORTAYA ÇIKARIYOR

Özet

Akdeniz-Ege bölgesi, biyoçeşitliliği koruma konsepti açısından değerlendirildiğinde önemli bir rol oynamaktadır. Endemik ve yerel türlerin korunması, ancak biyolojilerinin tam anlamıyla bilinmesiyle mümkün olabilmektedir. Özellikle, iç sularda yaşayan sucul türler, doğal engellerle karakterize edilen habitatlarının düzensiz yapısı nedeniyle daha savunmasız hale gelebilmektedirler. Ekosistemlerin fauna ve florasını tanımlayarak, kapsamlı koruma planlarının geliştirilmesini sağlamak koruma sürecini başlatmak için hayati öneme sahiptir. Bu bağlamda, mevcut çalışmanın temel amacı, Balıkesir, Türkiye'de bulunan Madra ve Havran Dereleri'nde yaşayan tatlısu balık türlerini DNA barkodlama yöntemi kullanarak belirlemektir. Süreç, Chelex protokolü kullanılarak DNA izolasyonunu ve ardından çeşitli primer kombinasyonları kullanılarak mitokondriyal CO1 bölgesinin amplifikasyonunu içermektedir. Toplamda 29 bireyin gen dizilerinden elde edilen sonuçlar, tür çeşitliliği, genetik ilişkiler ve varyasyonlar konusunda değerli bilgiler sunmaktadır. Bu araştırma, DNA barkodlamanın tür tanıma, genetik keşif ve koruma planları için değerli bir araç olarak önemini vurgulamaktadır. Elde edilen sonuçlar, özellikle bu savunmasız ekosistemlerde sucul biyoçeşitliliğin etkili bir şekilde yönetilmesi için bir temel oluşturmaktadır.

Anantara Kelimeler: Tür çeşitliliği, düzensiz doğal engeller, endemik türler, iç su havzaları, akarsu ekolojisi

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1. Introduction

Lately, there has been a growing awareness of the significance of safeguarding the variety of life on our planet. Within the realms of life's existence, freshwater ecosystems such as rivers and streams stand out as remarkably diverse, yet they also face the highest degree of vulnerability [1]. The combined impact of climate change and intense human activities has led to a concerning reality: the depletion rates of aquatic life in freshwater environments are fivefold greater than those experienced by their terrestrial counterparts [2]. Conserving ecosystems susceptible to negative impacts

requires a fundamental understanding: identifying the species within these ecosystems should be the initial step. This foundational knowledge helps the way for effective protection measures. This understanding becomes even more significant in regions of remarkable biodiversity, such as the Mediterranean Biodiversity hotspot, where the freshwater environment houses approximately 500 unique fish species, with new ones continuously being discovered [1]. Among the countries within this hotspot, Türkiye stands out for its rich diversity of species and a remarkable level of endemism among freshwater fish, accounting for 50.4% of the total [3]. Additionally, recent descriptions of new fish species [4, 5] demonstrate that there might be more unexplored taxa in streams which have relatively the often-overlooked corners.

Despite their size, small streams often contain a unique assembly of aquatic organisms, adapted to the specific conditions of their localized habitat. Therefore, in this study, we focused on biodiversity of two small streams-Madra and Havran streams-located in Balıkesir province (NW, Türkiye). Our main goal here was to identify the fish species of these streams and provide new data on barcode libraries with additional samples from already studied sites [i.e., Madra stream, 6], maybe even describe new fish species that have remained hidden from scientific view. On this purpose, we used DNA barcoding approach, which has recently been employed as a tool for identifying species and unveiling the diverse array present across a broad spectrum of taxa [7, 8, 9, 10]. Through DNA sequencing and computational analyzes, we can accurately differentiate between species and uncover hidden diversity that might otherwise go unnoticed. In the present study, we aimed to explore the use of the DNA barcoding approach as a molecular technique for the identification of fish species. Furthermore, studying the genetic makeup of these organisms not only sheds light on their evolutionary history but also contributes to our understanding of ecosystem dynamics, adaptation, and potential conservation measures.

Materials and Methods

2.1. Study Area

Madra Stream is an alkaline stream that originates from the Madra Mountain mass and spans approximately 65 km in length before ultimately discharging into the Aegean Sea. This watercourse meanders diverse landscapes and ecosystems, supporting a variety of flora and fauna along its path [11]. Havran Stream is a 36 km long small stream that originates from the merging of various streams around Madra Mountain. It flows into Edremit Bay, which is located to the west of Balıkesir, Türkiye. The stream has experienced several floods in the past years, leading to the construction of the Havran Dam on the stream [12].

2.2. Sample Collection

In December 2019 and a total of 29 fish samples were caught by electrofishing from different locations from the Madra Stream and Havran Stream (Table 1). Fish were euthanized in 2-phenoxyethanol solution in accordance with ethical rules during the field and a small piece was taken from the tail fins with the help of sterile scissors and forceps. These tissue samples were stored in 2 ml vial tubes, 1.5 ml of which was filled with 96% ethanol.

2.3. DNA Extraction, Amplification, Sequencing

DNA extraction was performed on 29 individuals, using the Chelex as the extraction method [13, 14]. The ca. 650 base pair (BP) long fragment of the mitochondrial Cytochrome Oxidase 1 (Mt-CO1) gene was amplified using different primer combinations (Table 2) and polymerase chain reaction (PCR) conditions of [15]. Following the manufacturer's guidelines, the PCR products (5 µl) underwent purification using Exonuclease I (2 U, EURx Ltd., Gdańsk, Poland) and alkaline phosphatase Fast Polar-BAP (1 U, EURx Ltd., Gdańsk, Poland) treatment [16]. Subsequently, the purified products were sent to Macrogen Europe (Amsterdam, the Netherlands) for sequencing.

2.4. Sample Identification

All sample sequences were identified through a BLAST-Basic local alignment search tool- [17] search of the NCBI (National Center for Biotechnology Information) and BOLD (Barcode of Life) databases [18]. The mtCO1 sequence similarities were obtained by aligning the homologous fragment sequences from the NCBI and BOLD to evaluate the accuracy of the morphological identification. Similarly, to [19], we used a general rule that defined a best match with a sequence similarity of at least 99% to indicate a potential species identification and 1% as a relatively loose criterion. Subsequently, Geneious 10.2.6 [20] was employed to edit, align, and trim the sequences to a standardized final length. To ensure traceability, DNA sequences with pertinent voucher information were submitted and deposited into the BOLD (Table 1). All fish samples were stored as voucher samples in the permanent collection of the Department of Invertebrate Zoology and Hydrobiology, University of Łódz, Poland.

2.5. Data Analyses

In total 151 sequences (average length of 637 BP) were used for subsequent analyses, 29 of which were obtained during present study, while the remaining 142 were retrieved from BOLD. The evolutionary history (phylogenetic trees) was inferred by using the Maximum Likelihood method [18] and analyses were conducted with 1000 bootstrap replicates in MEGA version X [21]. Subsequently, through the MEGAX software, the genetic

distances in species level were calculated, using Kimura's two-parameter model and bootstrap of 1000 replicates. We calculated the mean genetic distance within and between species. Additionally, we calculated the pairwise genetic distances between the individuals of each genus. From the pairwise distances between individuals of every species, we were interested in the mean, maximum and minimum distance between species.

All identified taxa were confirmed through the implementation of the automatic partitioning approach known as "ASAP-Assemble Species by Automatic Partitioning," as proposed by [22]. This innovative species delimitation program employs genetic distance calculations to segregate and delineate distinct taxa within the dataset, thereby enabling the precise and efficient classification of species based on their genetic relatedness.

Taxonomic revisions were suggested by combining the ASAP, genetic distances, and topology of the phylogenetic trees. In situations where distinct species were grouped together on the phylogenetic tree, genetic distances were used to ascertain the possibility of them constituting a single species. To be consistent for the revision suggestions, we used a mean genetic distance of 0.02 as a threshold [23, 24, 6, 10]. Groups exhibiting a mean genetic distance exceeding 0.02 were suggested as potential separate species. Conversely, for groups with a mean genetic distance below 0.02, further investigation was recommended to determine the validity of merging them into a single species.

3. Results

All the studied species were identified through DNA barcoding, resulting in 85.71% success rate of the approach. We were able to confidently identify the organisms to the species level based on the available sequences of the selected barcode region. In total seven species were identified (Table 1): *Barbus pergamonensis* Karaman, 1971, *Squalius carinus* Özuluğ & Freyhof, 2011, *S. kosswigi* (Karaman, 1972), *S.* cf. *aristotelis, Cobitis fahireae* Erk'akan, Atalay-Ekmekçi & Nalbant, 1998, *Oxynoemacheilus sp*. and *Pseudorasbora parva* (Temminck & Schlegel, 1846) through BLAST and BOLD (Table 1).

4. Discussion

DNA barcoding has emerged as a powerful tool for species identification and genetic analyses. In this study, we used DNA barcoding techniques to identify species and explore genetic relationships within the studied taxa. Among the species identified, we found intriguing insights into genetic differentiation and relationships. Notably, the use of ASAP and mean genetic distances prompted a re-evaluation of the initial species count of seven, suggesting a reduced number to six due to insufficient genetic differentiation observed between certain species (Table 3).

Our analyses revealed a lack of genetic variation (identical CO1 sequences) within *Barbus pergamonensis* and *Pseudorasbora parva*, separately. This observation signifies a notable absence of genetic ambiguity within these species in the studied DNA region. Both species are distinct within their genera, with *Barbus pergamonensis* confined to the Bakırçay, Gediz, and Madra rivers [25], while *Pseudorasbora parva*, a non-native species, is widely distributed across European and Asian freshwaters [6].

Comparative analyses of the DNA sequence of *Cobitis fahireae* against databases unveiled a perfect match (100% identity) in both BLAST and BOLD databases. This identity percentage underscores a strong similarity between the query sequence and those in the databases. Calculated pairwise distances unveiled both close genetic similarity and subtle genetic divergence within the dataset. These findings were reaffirmed through phylogenetic tree analyzes (Fig 1) and ASAP, offering a comprehensive view of genetic relationships and variation within *Cobitis fahireae*. Despite the limited sample size, the integration of additional sequences from web servers bolstered the results. Moreover, the geographical separation between our studied specimens and others within the *Cobitis* genus, as previously indicated by [26], further validated our findings.

For unidentified *Oxynoemacheilus sp*., a 91.64% match identity on BLAST was observed. Although our sequence count (*n*=2) lacked even a close match, [5] provided comprehensive insights into the rich diversity of *Oxynoemacheilus* species in Anatolian freshwaters. This recommends our specimens' classification as a new taxonomic entity, supported by phylogenetic tree analyzes (Fig 2) and mean genetic distances (Table 3). However, to support the differences with morphometric and meristic characteristics and other internal anatomical structures with further investigations are proposed with a larger sample size.

The *Squalius* genus was discerned into distinct groups using BLAST and BOLD. However, the proximity between *S. carinus* and *S.* cf. *aristotelis* (Table 3) led us to integrate *S. fellowesii* sequences from the Madra River, as described by Özuluğ and Freyhof (2011). Intriguingly, the genetic distances established a close relationship between *S. fellowesii, S. carinus*, and *S.* cf. *aristotelis,* supported by ASAP and a dedicated phylogenetic tree (Fig 3). To support this argument, we employed the analysis with additional sequences as well, including *S. cii, S. aristotelis* and *S. orpheus*. Consequently, this larger alignment prompted a suggestion that *S. fellowesii, S. carinus*, *S.* cf. *aristotelis* might be synonymous species (Fig 3). Yet, another suggestion was come up within the sequences of *S. cii, S. aristotelis and S. orpheus.* Their

genetic distances indicate their discrimination (Table 3) Moreover, ASAP scores proposed that there are three distinct groups in the dataset but it was not a significant consequence. Therefore, we propose that all these Squalius species might be considered as synonymous. However further investigation, particularly on morphometric analysis is required. Additionally, integration of sequences from a distinct geographic region for *S. kosswigi* prompted the need for a comprehensive investigation into its accurate taxonomic classification.

In summary, our study showcases the potency of DNA barcoding in unravelling genetic relationships and species identification. The findings underscore the significance of genetic differentiation and similarity within the studied taxa. While shedding light on these genetic dynamics, our study also identifies avenues for future research, including the expansion of sample sizes and deeper investigations into taxonomic relationships, further enhancing our understanding of the genetic landscape within these species.

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