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DNA Barcoding and Phylogenetic Analysis of Two Species Populations of *Diplodus* from the Eastern Mediterranean Coasts of Turkey

Makbule BAYLAN[1*], Gamze MAZI[2], Bahri Devrim ÖZCAN[3], Sedat GÜNDOĞDU[4], Dilek TEKDAL[5]

[1,2,4]Department of Basic Science, Faculty of Fisheries, Cukurova University, 01250 Adana, Turkey
[3]Department of Animal Science, Faculty of Agriculture, Cukurova University, 01250 Adana, Turkey
[5]Department of Biotechnology, Faculty of Science and Letters, Mersin University, 33343 Adana, Turkey

[1]https://orcid.org/0000-0003-0549-0662
[2]https://orcid.org/0000-0002-3160-2480
[3]https://orcid.org/0000-0002-9198-656X
[4]https://orcid.org/0000-0002-4415-2837
[5]https://orcid.org/0000-0002-4545-9005
*Corresponding author: makyan@cu.edu.tr

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ABSTRACT

Since Türkiye is surrounded by seas on three sides and has different climatic characteristics, it has an extremely rich biodiversity. Identifying fish species and varieties is of great importance for protecting them from drought conditions seen in recent years and using them as a source of the human food protein. The purpose of this study was to identify the genetic diversity of *Diplodus*, one of the fish species, the importance of which is underappreciated in Turkish seas. To this end, the polymorphism between the populations of two species (*Diplodus vulgaris* and *Diplodus sargus*) from the Sparidae (Coral fish) family was determined using the nucleotide sequence of the mitochondrial *cytochrome c oxidase I* (*COI*) gene, which is 652 base pairs long. 143 specimens of *Diplodus* were collected from the locations in the Eastern Mediterranean (İskenderun and Mersin) and Western Mediterranean (Antalya). The *COI* gene was amplified by PCR, genetic diversity analyses of these loci were carried out, and a phylogenetic tree was created to show the relationships between the isolates with a high difference. In conclusion, based on the haplotype and nucleotide diversity patterns, it was found that the *COI* gene had a low genetic variation among the populations.

Türkiye'nin Doğu Akdeniz Kıyılarında Yayılım Gösteren İki Diplodus Tür Popülasyonunda DNA Barkodlama Ve Filogenetik Analiz

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ÖZ

Türkiye üç tarafı denizlerle çevrili ve farklı iklimsel özelliklere sahip olduğundan biyoçeşitlilik açısından son derece zengin bir ülke konumundadır. Deniz ekosistemi için büyük öneme sahip balık tür ve çeşitlerinin tespiti gerek son yıllarda yadsınamaz derecede hissedilen kuraklık koşulları nedeni ile bu türlerin korunması ve gerekse de insan besin proteini açısından bu türlerin kullanılması bakımından önemlidir. Sunulan bu çalışmada, balık türleri içerisinde önemi en az bilinen türlerden olan *Diplodus* türlerinin Türkiye denizlerindeki genetik çeşitliliğinin belirlenmesi amaçlanmıştır. Bu amaçla, Sparidae (Mercan balıkları) familyasına ait iki türün (*Diplodus vulgaris* ve *Diplodus sargus*) popülasyonları arasındaki polimorfizm, 652 baz çiftine sahip mitokondriyal *sitokrom c oksidaz I* (*COI*) geninin nükleotid dizisi kullanılarak belirlenmiştir. 143 *Diplodus* örneği Doğu Akdeniz (İskenderun ve Mersin) ve

Batı Akdeniz (Antalya) lokalitelerinden toplanmıştır. Balık örneklerinden kas dokusu alınarak DNA izolasyonu yapılmıştır. PCR ile *COI* geni çoğaltılarak bu lokuslara ait genetik farklılık analizleri yapılmış ve farklılığın yüksek olduğu izolatlarda ilişkileri göstermek için filogenetik ağaç oluşturulmuştur. Sonuç olarak, *COI* geninin haplotip ve nükleotit çeşitliklerine dayalı olarak populasyonlar arasında düşük bir genetik varyasyona sahip olduğu saptanmıştır.

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1. Introduction

Diplodus, which is globally widespread, has a high economic importance for the whole world (Gordoa and Moli, 1997; Pajuelo and Lorenzo, 2004; Soykan et al., 2015). It is possible to see these species in different marine ecosystems ranging from rocky to dune habitats. There are 21 known species of this genus in the world's seas and oceans (Froese and Pauly, 2017). The main distribution areas of these 21 species are the Mediterranean and the Atlantic Ocean, but they are also distributed in the Caribbean, Gulf of Mexico, Indian Ocean, Red Sea, and Persian Gulf (Sala and Ballesteros, 1997; Summerer et al., 2001; Froese and Pauly, 2017). As well as being the main target species for small-scale, semi-industrial fishing, and sport angling, one or two members of this genus are also important for aquaculture (Reina et al., 1994; Summerer et al., 2001). The species from the genus *Diplodus* show homogeneity in terms of shape, tooth structure, and many other morphological features, except coloration (Summerer et al., 2001). All its species are carnivorous. These features make *Diplodus* a very important fish species.

With the developments in the field of molecular biology today, DNA has been the subject of many research studies on complex eukaryotic genomes. Only some of these studies were on gene functions, breeding, and degree of kinship. Molecular genetic methods are used to determine the differences or similarities between populations or individuals of a species and to solve taxonomic problems.

DNA barcoding, one of these methods, can be used as a powerful tool to examine cryptic species. Cryptic species are those that are morphologically similar but genetically different. Using morphology for species characterization and identifying all the diversity in the world is both time-consuming and challenging due to insufficient number of taxonomists (Godfray, 2002). Having an essential use in taxonomy (Ward et al., 2005; Ali et al., 2014), DNA barcoding is designed to provide precise and automated data for species. This method relies on using a small portion of mtDNA from a standard location in the genome that can be searched and matched with the sequences in the databases such as the National Center for Biotechnology Information (NCBI) GenBank and the Barcode of Life Databases (BOLD). DNA fragment with a length of approximately 655 bp from the 5' region of the mitochondrial *cytochrome c oxidase subunit I* (*COI*) gene has been accepted as the standard region for DNA barcoding of animal species (Hebert et al., 2003a, b). DNA barcoding is a fast, precise, and effective way of identifying species (Li et al., 2018). Any fish specimen (egg, larva, or carcass

fragment) and even morphologically impractical larval stages can be accurately identified using the COI barcode database.

In recent years, molecular methods have been used extensively in phylogenetic studies to analyze sequence differences in mtDNA (Bardeleben et al., 2005). Discussing species of aquaculture in mitochondrial DNA research will bring many benefits. Mitochondrial DNA, a widely used marker in evolutionary and phylogenetic studies, is sensitive to genetic drift and shows a great variation. mtDNA analysis identifies the differences between species and populations and provides highly reliable data in both systematic and population genetic studies. It is successfully used for these purposes in fishing (Bernatchez et al., 1992; Magoulas et al., 1996). Moreover, mitochondrial DNA can also be used to distinguish and organize the stocks of fish species (Grewe and Hebert, 1988; Billington et al., 1992).

In previous studies, 98-100% of many living species, including aquatic organisms, have been successfully identified using a small part of the *cytochrome c oxidase subunit I* (*COI*) gene (Aravind et al., 2007). Although some studies in Turkey in which *COI* was used in fishery products (Keskin et al., 2012; Utuk et al., 2012; Parmaksız et al., 2017), there are scarcely any studies on the species from the genus *Diplodus*.

This study was carried out to examine the genetic diversity of two fish species (*Diplodus sargus* Linnaeus, 1758 and *Diplodus vulgaris* Geoffroy Saint-Hilaire, 1817) from the Sparidae family collected from different coastal regions of the Eastern and Western Mediterranean in Turkey. DNA barcoding was performed by sequence analysis of the *COI* gene of mtDNA, and the phylogenetic relationship between two fish species collected from three coastal regions in Turkey (Iskenderun, Mersin, and Antalya) was clarified.

2. Material and Methods

2.1. Collection of fish specimens

The common two-banded sea bream *(Diplodus vulgaris*) and white seabream (*Diplodus sargus*), the fish material of the research, were collected from 3 locations along the Eastern and Western Mediterranean coasts of Turkey. These locations are the Bays of Mersin, İskenderun, and Antalya. 143 fish specimens were purchased from the fishermen in these locations (73 *Diplodus vulgaris* specimens, 24 from Mersin, 24 from İskenderun, and 25 from Antalya; 70 *Diplodus sargus* specimens, 25 from Mersin, 24 from İskenderun, and 21 from Antalya).

2.2. DNA isolation and PCR

The tissue samples taken from the dorsal fin of the fish were put into 1.5 ml centrifuge tubes containing 95% ethanol and stored in the refrigerator at +4°C. GeneJET Genomic DNA (Thermo scientific) purification kit was used to isolate DNA from the fish tissues, and the protocol recommended by the company was followed.

In this study, the primer pair (FishF1-5'TCAACCAACCACAAAGACATTGGCAC3' and FishR1-5'TAGACTTCTGGGTGGCCAAAGAATCA3') of the *COI* mitochondrial marker gene with a length of 652 bp (Ward et al., 2005) was used.

The isolated DNA was prepared by measuring the amount and purity in a spectrophotometer and then used as a template in the PCR reaction. PCR was performed on an Applied Biosystems verity 96 Well Fast thermal cycler. The total reaction volume was 25 µl: 2 µl template DNA (approximately 20 ng), 12.5 µl PCR master mix (Promega), 1.0 µl (10 µmol) forward primer, 1.0 µl (10 µmol) reverse primer, 0.5 µl Taq, and 8.0 µl water (dH_2O). The PCR reaction was performed as follows: initial denaturation at 94°C for 5 minutes, denaturation at 94°C for 30 seconds, binding at 66.3°C for 30 seconds, elongation at 72°C for 2 minutes, and final elongation at 72°C for 7 minutes with 40 amplification cycles.

2.3. DNA sequence analysis

After a successful amplification, the DNA bands (~652 bp) purified from agarose gel were sent to Medsantek, Ankara, Turkey (http://www.medsantek.com.tr/) for Sanger sequencing, together with the primers used in the amplification.

In order to identify the genetic differences and phylogenetic relationships between the *Diplodus* species, the sequences provided by Medsantek after bidirectional reading were assembled using ChromasPro (http://technelysium.com.au/wp/chromaspro/) and aligned using BioEdit V.2 (https://bioedit.software.informer.com/versions/) (Hall, 1999).

Using MEGA7 (https://www.megasoftware.net/), a maximum likelihood phylogenetic tree was created for the *D. sargus* and *D. vulgaris* sequences among other *Diplodus* species taken from the NCBI GenBank database (Kumar et al., 2016).

3. Results and Discussion

3.1. PCR amplification of the mtDNA *COI* gene of the DNAs of *Diplodus vulgaris* and *Diplodus sargus*

After the *D. vulgaris* and *D. sargus* genomic DNA samples were isolated, they were used as templates, and the *COI* gene in the genome was amplified by PCR using oligonucleotide primers. The *COI* mtDNA genes of all 73 *D. vulgaris* and 70 *D. sargus* individuals obtained from the Bays of Antalya, İskenderun, and Mersin were amplified by PCR. Gel electrophoresis was performed to verify the presence of the amplified *COI* barcode gene with a length of 652 bp. Figure 1 and Figure 2 show the images of the *D. vulgaris* and *D. sargus* collected from Antalya, İskenderun, and Mersin, respectively.

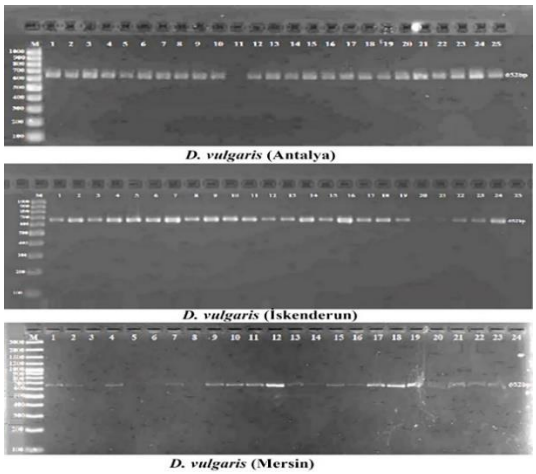

Figure 1. The images of the genomic DNAs of the *D. vulgaris* specimens collected from the Bays of Antalya, İskenderun, and Mersin in 1.5% agarose gel after the PCR analysis with COI primers (M: Marker; numbers: individual fish specimens).

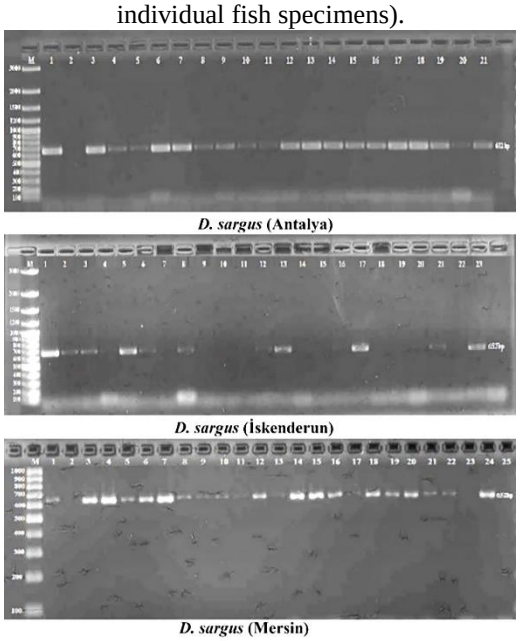

Figure 2. The images of the genomic DNAs of the *D. sargus* specimens collected from the Bays of Antalya, İskenderun, and Mersin in 1.5% agarose gel after the PCR analysis with COI primers (M: Marker; numbers: individual fish specimens).

3.2. Data analysis

The amplifications of the *D. vulgaris* and *D. sargus* specimens were sequenced by the Sanger sequencing technique. The *COI* gene sequences of 14 specimens were uploaded to the database of NCBI GenBank, Bethesda, USA, via the portal on https://www.ncbi.nlm.nih.gov/, and their accession numbers were obtained. Table 1 gives the NCBI GenBank accession numbers.

Table 1. Locations and GenBank accession numbers of the *D. sargus and D. vulgaris* samples.

Genus	Location (Turkey)	Sample code	GenBank Accession No.
Diplodus sargus	Antalya	DSA6	MZ556312
	Antalya	DSA7	MZ556313
	İskenderun	DSI5	MZ556313
	İskenderun	DSI13	MZ556315
	İskenderun	DSI17	MZ556316
	İskenderun	DSI23	MZ556317
	Mersin	DSM3	MZ556318
	Mersin	DSM4	MZ556319
	Mersin	DSM7	MZ556320
	Mersin	DSM15	MZ556321
	Mersin	DSM24	MZ556322
Diplodus vulgaris	Mersin	DVM4	MZ556323
	Mersin	DVM19	MZ556324
	Mersin	DVM21	MZ556325

Figure 3 shows the molecular phylogenetic analysis created in line with the maximum likelihood method using the sequences obtained in this study and the *COI* gene sequence data (Table 2) provided by the NCBI database for the genus *Diplodus*. This means that the *COI* gene sequences obtained by the Sanger sequencing technique were compared with those in the GenBank database.

Table 2. *COI* gene homologues provided from NCBI GenBank and used for *Diplodus* putative gene analyses and comparison of mRNA sequences.

Genus	mRNA	Sample name	Accession No	Country
Diplodus vulgaris	*COI*	Dvur13	LC203502.1	Egypt (Rashid)
	COI	Dvudm1	LC203503.1	Egypt (Damietta)
	COI	291	KC409523.1	Greece
	COI	Dvubb7	LC195195.1	Egypt (Burullus)
	COI	DV6	JX192137.1	East Atlantic
	COI	DVUM-3ALX2015	KU379681.1	Egypt (Bahari)
	COI	Dvuabk3	LC203516.1	Egypt (Abo Qir)
	COI	Dvumm2	LC203509.1	Egypt (Marsa Matrouh)
	COI	DVUL7	KJ012355.1	Western Mediterranean
	COI	CSFOM-160	KJ709520.1	Portugal
Diplodus sargus	*COI*	Dsaabk6	LC203107.1	Egypt (Abo Qir)
	COI	Dsar9	LC203120.1	Egypt (Rashid)
	COI	Dsabb1	LC203085.1	Egypt (Burullus)
	COI	Dsamm7	LC203130.1	Egypt (Marsa-Matrouh)
	COI	DS1	JX192125.1	East Atlantic
	COI	TR611EK	KC500582.1	Turkey
	COI	Dsabh4	LC203100.1	Egypt (Bahari)
	COI	BT21	JX192292.1	South Africa
Outer group-1 (*Diplodus annularis*)	*COI*	DanM1	LC152205.1	Egypt
Outer group-2 (*Diplodus cervinus*)	*COI*	EMA2014-DceM1	KU757074.1	Egypt
Outer group-2 (*Diplodus noct*)	*COI*	EMA2014-DnoR2	KP308273.1	Egypt

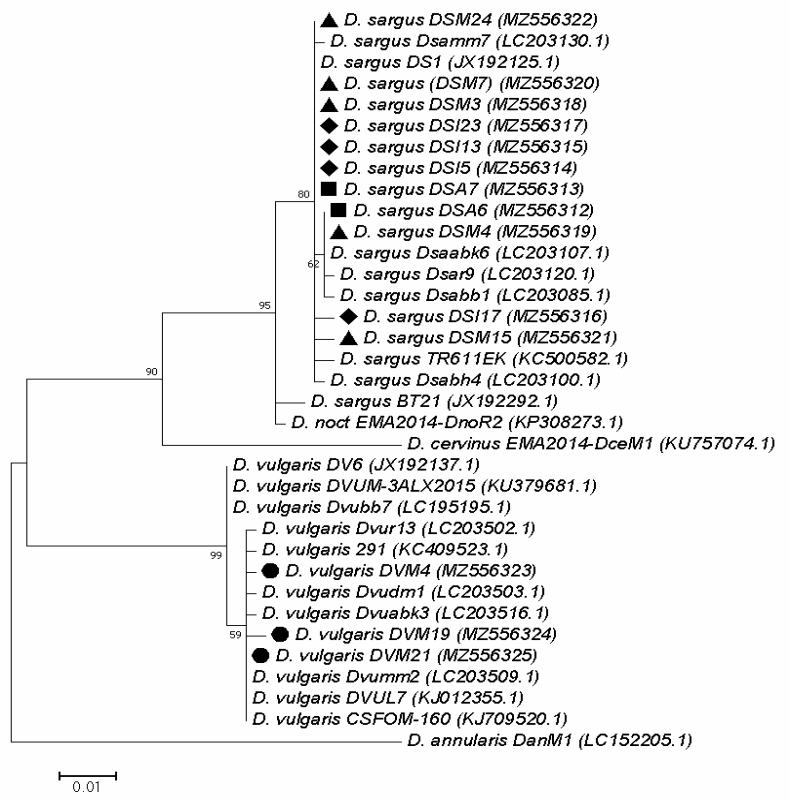

Figure 3. The molecular phylogenetic tree was created in line with Kimura's 2-parameter maximum likelihood method (Kimura, 1980) based on the partial sequences of the *COI* gene. The percentage of the replicate trees in which the associated taxa are clustered together (1000 replicates) is shown next to each branch. Triangle, square, and diamond symbols refer to the isolates. Names of the species and their accession numbers are provided as specified in NCBI GenBank.

Based on genetic differences in species whose morphological differences cannot be distinguished, molecular techniques can be used easily, especially in detecting species that are difficult to distinguish morphologically. In addition, phenotypic techniques (ecology, marking, parasites, physiology, morphometric, meristic, limestone structure) are used alone in determining the differences between species and between populations in population genetic studies. It can be misleading as it has no basis and varies according to environmental effects. Therefore, molecular genetic techniques are needed. Using nucleic or mitochondrial DNA, new information has been obtained on the genetic diversity of natural and cultural populations of various fish species (Koh et al., 1999; Sivasundar et al., 2001; Was and Wenne, 2002). The recent use of molecular tools has facilitated the identification of genetic diversity among populations with different geographical distributions. DNA barcoding is also considered a tool to determine the diversity of species within an ecosystem and investigate genetic variability within species (Rajkumar et al., 2015). Landi et al. (2014) highlight the discriminatory power of COI barcodes to identify species from different geographical origins. In addition, they emphasized that species can be identified with high sensitivity from DNA samples of different quality and origin, providing significant benefits in many areas from fishing and conservation programs to control the authenticity of fish products. Ward et al. (2005) noted that a 655-bp fragment of a single mitochondrial gene can be used to plan a phylogenetic study but is unsuitable for deep phylogenetic

resolution. In this study, 143 specimens of *D. sargus* and *D. vulgaris* distributed in the Eastern and Western Mediterranean coasts of Turkey were sequenced for the *COI* gene (652 bp). The sequences were compared with those in BOLD and GenBank databases. The sequences with differences were identified, and MEGA7 was used to find a phylogenetic relationship. As a result of the sequence comparison, the sequences of the *D. vulgaris* specimens were found to be the same, and only 3 specimens collected from Mersin were found to have a different sequence. As for the *D. sargus* specimens, a different sequence was found to exist in 2 specimens collected from Antalya, 4 specimens from İskenderun, and 5 specimens from Mersin. Therefore, only those with the difference (14 sequences) were entered into the NCBI database, and the phylogenetic relationship was looked for between these sequences. The phylogenetic tree showed that the genus *Diplodus* descended from a single ancestor; it was monophyletic. Similarly, De la Herran et al. (2001) and Abbas et al. (2017) determined the *Diplodus* species as a monophyletic group. The tree created in line with the maximum likelihood method branched into two separate groups. *Diplodus annularis* was in a separate branch. *D. sargus* and *D. vulgaris* were clustered within themselves, and *D. noct* and *D. cernivus* species were closer to *D. sargus* than *D. vulgaris*. While MZ556324 and MZ556325, the isolates of *D. vulgaris*, had a high similarity, MZ556323 was on the same branch but on a different node. Interestingly, the *D. sargus* isolates (MZ556316 and MZ556321) from 2 different locations were on the same node and had a high similarity. Similarly, MZ556312 and MZ556319, and MZ556318, MZ556320, MZ556314, MZ556315, MZ556317, and MZ556313 had a high similarity and were on the same node. Among the *D. sargus* isolates, only MZ556322 was on a more distant node than the other isolates. The high similarity of the isolates from different locations with the *COI* gene suggests that these species are resistant to different habitat conditions and may have been distributed from one region to another with the movement of water. In the phylogenetic analysis, the isolates of *D. sargus* and *D. vulgaris* were clustered within themselves in terms of species; this shows that the sequence analysis was carried out successfully, and the *COI* gene was correctly sequenced.

The first class was divided into two subgroups. One of these subgroups included the *D. sargus* (MZ556312 - MZ556322) collected from different locations in Turkey and the *D. sargus* from East Atlantic (acc. No. JX192125.1), Turkey (acc. no. KC500582.1), and Egypt (acc. no. LC203107.1, LC203120.1, LC203085.1, LC203130.1, and LC203100.1); and the other subgroup included the *D. sargus* from South Africa and the *D. noct* from Egypt (acc. no. KP308273 and JX192292, respectively). On the other hand, *D. cervinus* was in a separate subclass.

The second class was also divided into two subgroups. One of these subgroups included the *D. vulgaris* collected from Egypt (Bahari), East Atlantic, and Burullus (acc. no.KU379681.1, JX192137.1, and LC195195.1, respectively), and the other group included the *D. vulgaris* sequences from Turkey (acc. no. MZ556323, MZ556324, and MZ556325) and Western Mediterranean, Abo Qir (Egypt), Rashid (Egypt), Marsa-Matrouh (Egypt), Damietta (Egypt), Portugal, and Greece (acc.No.KJ012355.1, LC203516.1, LC203502.1, LC203509.1, LC203503.1, KJ709520, KC409523.1,

respectively). The Egyptian *D. annularis* (acc. No. LC152205) constituted the third class on a single branch.

The DNA barcoding data were in line with those reported by most of the previous studies. Likewise, Abbas et al. (2017) also showed that the results of the phylogenetic tree Sparidae species are monophyletic. The tree is divided into two separate clades and some subclades. The two main clades include all species under study except *Crenidens crenidens* in a separate branch. These two studies support our study. Keskin et al. (2013) demonstrated the genetic relationship between species in the NJ tree. Each species was associated with a specific DNA barcode cluster and demonstrated the relationship between these species. As in the results, they presented a clustering model that could inform the phylogenetic relationships between conspecific, congeneric, and confamilial levels, in which species closer to genetic diversity clustered at the same nodes.

4. Conclusion

Nutritional availability, light regime, salinity, feeding, oxygen, temperature, pollutants, nutrient concentration, predator density, current speed, and intra-specific social interactions have all been demonstrated to have an impact on growth in fishes (Acarli et al., 2018). Genetics is very important parameters to identify a species. This study was carried out to examine the genetic population structure and genetic diversity of two species, *D. sargus* and *D. vulgaris*, from the Sparidae family. The isolation and sequencing were successfully performed in the study. The identification and analysis of the species, which is one of the ultimate goals of the study, was successfully carried out. It has been determined that the *COI* gene has a low genetic variation among Sparidae populations. In conclusion, the results of the current study support previous analyzes in terms of the success of barcoding in determining the genetic population structure and genetic diversity of the two species. It is thought that determining the phylogenetic tree of the two *Diplodus* species will provide a complete vision of their evolutionary relationships and enrich the genetic database with sequences.

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Statement of Conflict of Interest

The authors of the article declare that there is no conflict of interest.

Author's Contributions

The contribution of the authors is equal.

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