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DNA barcodes for identifications of two lionfish species *Pterois miles* (Bennett, 1828) and *P. volitans* (Linnaeus, 1758) in the Mediterranean

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Abstract
Two morphologically similar lionfish species *Pterois miles* and *P. volitans* (Scorpaenidae), reported in Turkish marine waters, were assess using partial mitochondrial cytochrome c oxidase I (COI) gene. As a result of sequence alignment, 609 bp of COI region were examined and it was contained 81 bp variable and 528 conservative sites of which 47 bp parsimony informative sites. Mean genetic diversity and genetic divergence between species was 0.009481 and 0.047660, respectively. 16 different haplotypes were detected in total 18 sequences, and there were no shared haplotypes between the two lionfish species. According to neighbor joining and maximum parsimony trees, *P. miles* and *P. volitans* were clustered in different clades.

Keywords: Lionfish, Pterois, Molecular identification, DNA Barcoding.
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Introduction

The Suez Canal has been led to the highest amount of the entered and settled alien species into the Mediterranean Sea (Galil et al. 2016; Turan et al. 2016; Zenetos et al. 2017). Introducing invasive alien species poses a significant threat to biodiversity, composition and function of the ecosystem as well as economic and human health consequences (Charles and Dukes 2007; Otero et al. 2013). Over the past decade, the number of reported alien species in the Mediterranean has comparatively risen (Zenetos et al. 2017).

The lionfishes are the most invasive species in the world (Morris et al. 2009; Schofield 2010; Johnston and Purkis 2014; Poursanidis 2015; Eagderi et al. 2019). The *Pterois* genus belongs to Scorpaenidae family has 10 valid species in the world marine waters (Froese and Pauly 2019) and among them, the devil firefish, *Pterois miles* (Bennett 1828) is an invasive one frequently found in the Indian Ocean and Red Sea (Froese and Pauly 2019). After twenty-two years of its first record from the Israeli coast (Golani and Sonin 1992), *P.miles* (Bennett 1828) was reported from Lebanon (Bariche et al. 2013) and Cyprus (Evripidou 2013; Oray et al. 2015) in the Mediterranean Sea. In Turkish marine waters, there are two species of lionfish were recorded (Turan et al. 2017a), including *P. miles* recorded firstly from Turkish marine waters in the Iskenderun Bay, north-eastern Mediterranean part of Turkey in 2014 (Turan et al. 2014). After its first observation from the Iskenderun Bay, a rapid expansion was occurred from Iskenderun Bay to Mersin and Antalya Bays, and Aegean Coasts of Turkey (Turan and Öztürk 2015; Yağlıoğlu and Ayas 2016). The red lionfish, *P. volitans* (Linnaeus, 1758) are found in the Pacific Ocean (North and South), the Atlantic Ocean (North and South) and the Indo-West Pacific Ocean as well (Schultz 1986; Whitfield et al. 2002; Kimball et al. 2004; Frose and Pauly 2019). *Pterois volitans* (Linnaeus 1758) was recorded firstly in the Mediterranean Sea, from the Iskenderun Bay (Gürlek et al. 2016) with a second record by Gökoğlu et al. (2017) in the coast of the Antalya Bay.

Molecular genetic studies on mtDNA have proved to be beneficial to study the phylogenetical and phylogeographical patterns of marine species (Meyer 1993; Avise 1994; Turan et al. 2015; Abolhasani et al. 2020). The DNA sequencing analysis of mtDNA regions is a rapid way for elucidating the phylogenetic status

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of marine species (Meyer 1993; Avise 1994; Turan et al. 2015). Due to the evolution at distinct rates of different mtDNA regions, specialized mtDNA regions were aimed at inter and intra-specific variation (Hauser et al. 2001; Mohindra et al. 2007; Turan et al. 2015). The DNA barcoding is a global enterprise that offers a standardized and effective genetic marker for marine and freshwater biodiversity with significant conservation applications. DNA barcoding is a method that focuses on a single part of the mitochondrial genome, as it presents portions conserved across taxa that are suitable for the primer design, while including polymorphism between and within species (Hebert et al. 2003; Kress and Erickson, 2008). The cytochrome oxidase subunit I (COI) region of the mitochondrial genome is used for barcoding region in fishes and sufficiently diverse to allow the specific identification of alien fish species (Kochzius et al. 2003; Kochzius et al. 2010; Stern et al. 2017; Turan et al. 2017b; Karan et al. 2019). Identification of lionfish species of *P. miles* and *P. volitans* by DNA barcoding and current level of interspecific and intraspecific genetic variation at the species level which distributed in Turkish waters are very important to know. Hence, this study aimed to study DNA barcoding of two lionfish species viz. *P. miles* and *P. volitans* to minimize future taxonomic misidentifications in Turkish marine waters.

Material and Methods

The lionfish specimensof *P. miles* and *P. volitans* were collected from the Iskenderun Bay and identify according to Schultz (1986), Allen and Erdman (2008) and Turan et al. (2017a). The total DNA was extracted from the specimens using either a phenol–chloroform (Sambrook et al. 1989) or proteinase K–chelex extraction (Estoup et al. 1996) methods. The Polymerase Chain Reaction (PCR) was conducted with primers of FishF1-5'TCAACCAACCACAAAGACATTGGCAC3'and FishR2-5'ACTTCAGGGTGACCGAAGAATCAGAA3' (Ward et al. 2005). The PCRs were carried out in a 50 µl total volume with 0.4 mM of each primer, 0.2 mM of dNTP and 1.25U of Taq DNA polymerase in a PCR buffer that containing 20 mM of Tris–HCl (pH 8.0), 1.5 mM of $MgCl_2$, 15 mM of KCl and 1-2 µl template DNA. Denaturation step were at 94°C for 30 s, 50°C for 30 s, and 72°C for 45 s for 30 cycles and followed by a final extension for 7 min at 72°C. Visualization of amplified COI region was checked in 1.5% agarose gel. Spectrophotometer was used for Quantitation of the PCR product. The mtDNA sequence analysis was performed with chain termination method by Sanger et al. (1977) which was applied with Bigdye Cycle Sequencing Kit V3.1 and ABI 3130 XL genetic analyser.

Partial COI sequences were initially aligned with the ClustalW program (Thompson et al. 1994) and final alignment was accomplished with BioEdit (Hall 1999). The best NT substitution model for sequence divergences were determined using MEGA V7, and the molecular phylogenetic tree was also constructed using MEGA V7 (Tamura et al. 2011). Jukes-Cantor parameter method (Jukes and Cantor 1969) was selected as a best method for intra and interspecific variations. A distance-based method as Neighbour Joining (NJ) (Saitou and Nei 1987) and Maximum Parsimony (MP) criterion was applied as cladistic approach. *Chelidonichthys lucerna* was selected as an out group taken from GenBank (KJ204788.1). The statistical robustness in the nodes of the resulting tree was determined with bootstrap method with 1000 replicates (Felsenstein 1985).

Results

After alignment, the COI region was consisted of 609 bp fragments which were contained 81 bp variable and 528 conservative sites of which 47 bp parsimony informative sites. The mean composition of nucleotides for thymine (T), cytosine (C), adenine (A) and guanine (G) were as 30.4, 28.1, 22.2 and 19.3%, respectively. Sixteen haplotypes were found out of 18 sequences, and there was not shared haplotype. Hap_1-Hap_9 were found to belong to *P. miles*, Hap_10-Hap_16 to *P. volitans* (Table 1). Mean haplotype diversity was 0.9869. Variable nucleotide positions of COI DNA barcode in lionfish species show Table 2.

Table 1. Haplotype number and its distribution between the species.

Haplotype	*Pterois miles*	*Pterois volitans*	Total
Hap_1	1	-	1
Hap_2	1	-	1
Hap_3	1	-	1
Hap_4	1	-	1
Hap_5	1	-	1
Hap_6	1	-	1
Hap_7	1	-	1
Hap_8	1	-	1
Hap_9	1	-	1
Hap_10	-	1	1
Hap_11	-	2	2
Hap_12	-	2	2
Hap_13	-	1	1
Hap_14	-	1	1
Hap_15	-	1	1
Hap_16	-	1	1
Total	9	9	18

Table 2. Variable nucleotide positions of COI DNA barcode in lionfish species. The DNA barcode variable nucleotides are indicated, while identity is shown by dashes.

	10*	20*	30*	40*	50*	60*	70*	80*
Hap_1	CTGACTCGACTTCTCGGGGCGTGCACATTCCTTAATTTTTATTTGGGGGGGGCTCTCACCATGTATCTAAGCCGCCCCGCC							
Hap_2	.....							
Hap_3	.....							
Hap_4	.....							
Hap_5	.....							
Hap_6	.....							
Hap_7	.....							
Hap_8	.....							
Hap_9	.....							
Hap_10	AGTTACGACGACACT.TACACGC...TAGTA.AG.CAC..TCGGTCACC.....							
Hap_11	AGTTACGACGACACT.TACACGC...TAATA.AG.CAC..TCGGTCACC.....A...T.....T.....							
Hap_12	AGTTACGACGACACT.TACACGC...TAATA.AG.CAC..TCGGTCACC....C.....G.....T							
Hap_13	AGTTACGACGACACT.TACACGC...TAATA.AG.CAC..TCGGTCACC.....T							
Hap_14	AGTTACGACGACACT.TACACGC...TAATA.AG.CAC..TCGGTCACC....C.....G.....A...T..T							
Hap_15	AGTTACGACGACACT.TACACGC...TAATA.AG.CAC..TCGGTCACC.C.....T							
Hap_16	AGTTACGACGACACT.TACACGC...TAATA.AG.CACG.TCGGTCACC.....A...T.....T.....							

Species specific DNA barcodes were detected whereas common DNA barcode was not detected for all species. The average genetic diversity was found to be 0.009481. The intraspecific genetic diversity within *P. miles* and *P. volitans* was found to be 0.012172 and 0.0006791, respectively. The interspecific genetic diversity between two species was found to be 0.038178. The average genetic divergence between species was 0.047660.

The results of Neighbor Joining (NJ) and Maximum Parsimony (MP) phylogenetic trees indicated similar tree topologies. Two main phylogenetic nodes were determined; *P. miles* was in the first main node and *P. volitans* was in the second main node in NJ and MP tree (Figs. 1, 2). According to NJ and MP trees, *P. miles* and *P. volitans* were clustered in different clades.

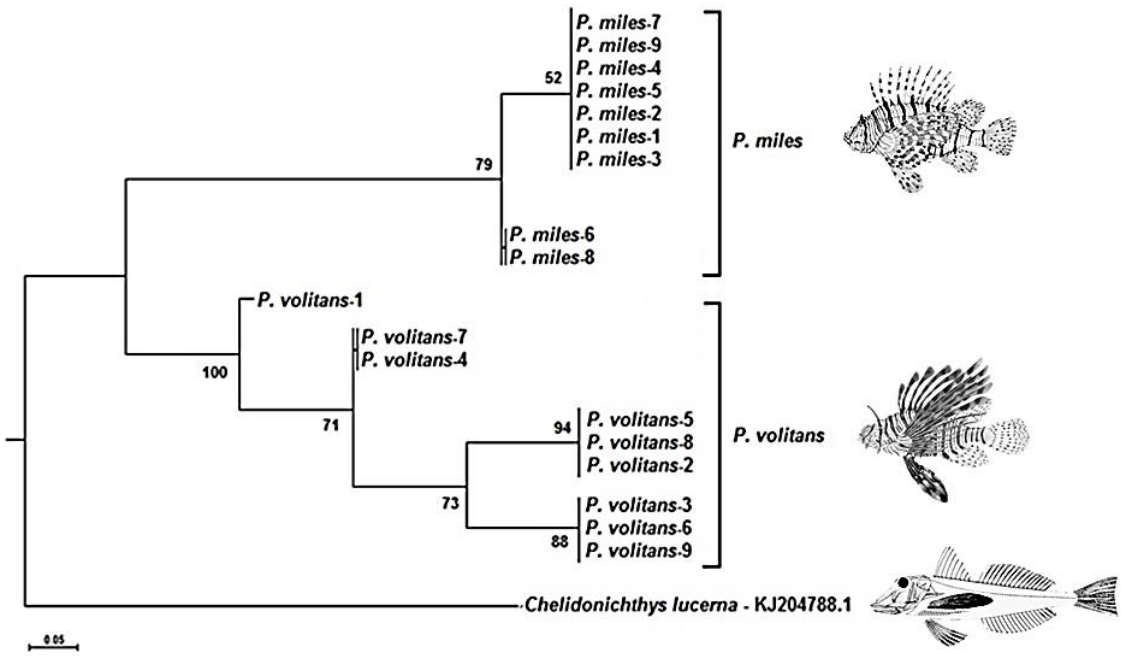

Figure 1. NJ tree on the basis of COI sequences. The tree was constructed by using the outgroup species *Chelidonichthys lucerna*. The bootstrap values were shown on nodes. Fish sketches *Pterois miles*, *P. volitans* and *C. lucerna* were given from Froese and Pauly (2019).

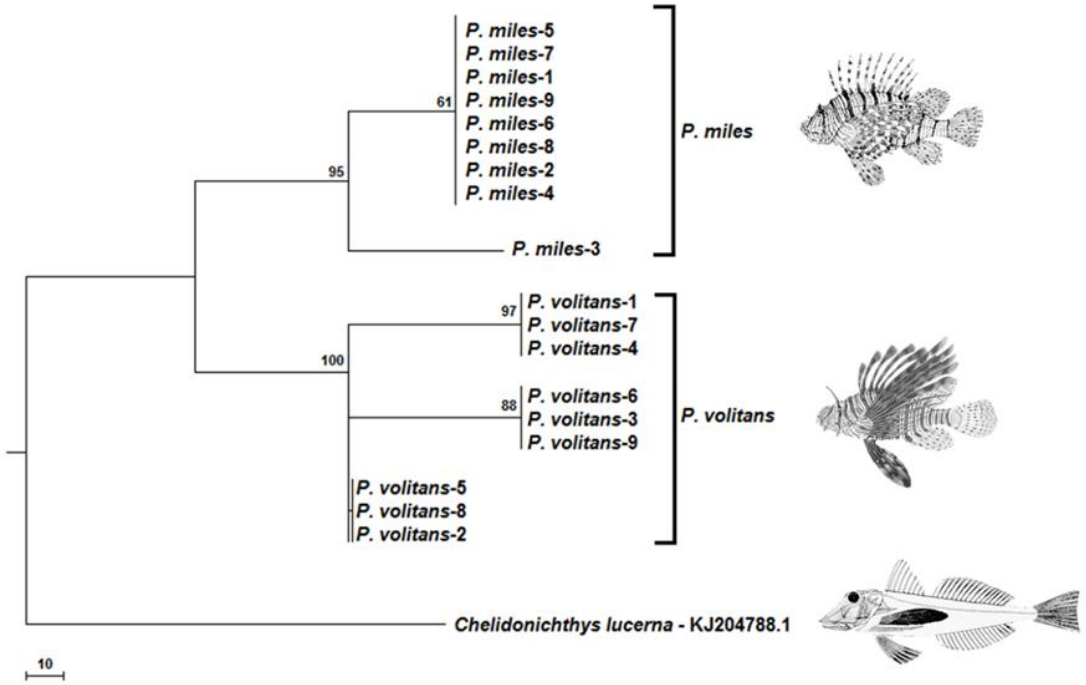

Figure 2. MP tree on the basis of COI sequences. The tree was constructed by using the outgroup species *Chelidonichthys lucerna*. The bootstrap values were shown on nodes. Fish sketches *Pterois miles*, *P. volitans* and *C. lucerna* were given from Froese and Pauly (2019).

Discussion

Two lionfish species of *P. miles* and *P. volitans* are distributed in the Turkish marine waters were investigated based on DNA barcoding. According to the results, they were separated in the NJ and MP trees with a high bootstrap value. The universal primer amplified the target region in the species, generating sixteen COI barcodes of 609 bp. Common haplotypes was not detected between the two species, and the DNA barcode sequences clearly separated them.

For inter-specific comparison, the mean genetic distance value between *P. miles* and *P. volitans* inferred from COI sequences were found to be 0.047660. Freshwater et al. (2009) studied genetic evidence to reveal genetic differentiation of *P. miles* and *P. volitans* along the east coast of the United States and the Bahamas using Cytb gene and found interspecific genetic divergence ranged from 0.0635 to 0.0774. Despite high morphological similarity between *P. miles* and *P. volitans*, the species were found genetically distinct. Although phylogenetic relationship between two lionfish species was determined using different gene region in above cited study which were in agreement with the present study.

According to the NJ and MP phylogenetic approaches, *P. miles* and *P. volitans* were found as genetically distinct from each other. Freshwater et al. (2009) also found a clear distinction between *P. miles* and *P. volitans* in the NJ tree. Kochzius et al. (2003) investigated molecular phylogenetic relationships of the *Pterois* and *Dendrochirus* genera on the basis of mitochondrial Cyt b and 16S rRNA sequences and found that *P. miles* and *P. volitans* clearly separated species from each other according to NJ and MP phylogenetic trees as found in the present study.

Pterois miles and *P. volitans* were considered synonym to each other for many years (Dor 1984; Golani and Sonin 1992). These two species are morphologically similar that cause misidentification that the identification of these two lionfish species depends on dorsal-fin ray number, pectoral-fin length and size of spots on vertical fins. Adults have a band of tiny spines along the cheek (Kuiter and Tonozuka 2001). On the other side, interspecific sequence divergence and phylogenetic tree methods offer powerful support to identify these two species. Due to an absence of gene flow, the genetic break may reveal between two lionfish species. Although it is presumed that adult lionfish continue within a comparatively limited geographic range (Fishelson 1975, 1997), the release of *Pterois* eggs within moving mucus balls and the existence of a pelagic larval phase (Fishelson 1975; Imamura and Yabe 1996) offer a broad spreading capacity (Morris et al. 2009) and thus the potential for widespread gene flow. Kochzius and Blohm (2005) investigated genetic structure of *P. miles* populations in the Aqaba Gulf, the Red Sea and Indian Ocean and discovered panmixia among these geographically distinct locations. Divergence of an ancient *Pterois* species into two allopatric branches that divided into *P. miles* and *P. volitans* may have begun in the center to late Miocene once tectonic activity and shifts in sea level merged to displace surface water links between the Indian Ocean and the Western Pacific (Hodell and Vayavananda 1993). In general, this type of segmentation between sea levels is frequently referred to as the cause of variation of Indo-Pacific marine species including fish (McMillan and Palumbi 1995; Chenoweth et al. 1998; McCafferty et al. 2002) and was suggested by Kochzius et al. (2003) as the source of *P. miles*/*P. volitans* separation.

As conclusion, the present study proves the existence of two invasive lionfish species *P. miles* and *P. volitans* in Turkish marine waters and provide the DNA barcodes for identification of these two species. The determined clear separation between two lionfish species in different monophyletic group can be clarified by speciation which may give rise to absence of gene flow. Further studies conducted by utilizing nuclear DNA markers may be improved to determine additional species-specific identification between *P. miles* and *P. volitans* to facilitate taxonomic identifications.

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