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-RESEARCH ARTICLE-

Assessing DNA Barcodes for Identification of Pufferfish Species (Tetraodontidae) in Turkish Marine Waters

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Abstract

In Turkish marine waters, pufferfish belongs to Tetraondontidae family are represented with 8 species, *Lagocephalus lagocephalus*, *L. sceleratus*, *L. spadiceus*, *L. suezensis*, *L. guentheri*, *Sphoeroides pachygaster*, *Torquigener flavimaculosus* and *Tylerius spinosissimus*. DNA barcoding can be useful in the assessment of cryptic or morphologically similar species of identification which is widespread in marine environment. DNA barcode identification of the eight puffer species of the Tetraodontidae family in Turkish marine waters were examined by using mtDNA sequencing of the amplified partial mitochondrial cytochrome c oxidase I (COI) gene. COI contained 189 variable and 337 conservative nucleotides of which 183 were parsimony informative over 526 bp. Mean genetic diversity all species was found 0.18164. The highest and lowest nucleotide divergence was observed *L.spadiceus* (0.0022) and between *L. sceleratus*, *L. suezensis*, *L. lagocephalus*, *L. guentheri* and *S. pachygaster* (0.0000) respectively. The number of detected different haplotypes were 10 out of 23 sequences, and haplotype diversity was found to be 1.000.

Keywords:

Pufferfish, catch amount, Aegean Sea, Mediterranean Sea, Turkey

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Introduction

Pufferfishes are marine fish species that are distributed in tropical and subtropical areas of the Atlantic, Indian and Pacific Ocean. Puffers include 28 genera and approximately 184 species in all over the world marine waters within the Tetraodontidae family (Matsuura, 2015; Farrag et al., 2016), among which at least ten are found in the eastern Mediterranean (Farrag, 2014). This Lessepsian invasive species has established large populations along the coasts of many countries of the eastern basin such as Israel, Lebanon, Turkey (Mediterranean and Aegean coasts), Cyprus and Greece (Aegean and Ionian coasts), while still rapidly expanding westwards along the coasts of Egypt, Libya, and along the entire Tunisian coastline (Soussi et al. 2014). Apart from several large species used for human consumption as a delicious food in few countries, particularly in China, Korea, Japan and Taiwan (Oyaizu et al. 2000), most pufferfish species have not commercial value. Besides the small size of most species, the family is renowned for the occurrence of a powerful toxin in their skin and organs called tetrodotoxin (TTX). Tetrodotoxin is a very potent neurotoxin and one of the strongest marine paralytic toxins (El-Sayed et al., 2003; Sato et al., 2008).

In Turkish marine waters, pufferfishes are represented with 8 species, *Lagocephalus lagocephalus* (Linnaeus, 1758*), Lagocephalus sceleratus* (Gmelin, 1789), *Lagocephalus spadiceus* (Richardson, 1845), *Lagocephalus suezensis* Clark & Gohar, 1953, *Lagocephalus guentheri* Miranda Ribeiro, 1915, *Sphoeroides pachygaster* (Müller & Troschel, 1848), *Torquigener flavimaculosus* Hardy & Randall, 1983, *Tylerius spinosissimus* (Regan, 1908) (Turan et al.,2007). In this study aimed to identification DNA barcodes of pufferfishes in Turkish marine waters.

Molecular genetic studies on mtDNA have proven benefits useful for examining hypotheses about the phylogeny and phylogeography of marine species (Meyer, 1993; Avise, 1994; Turan et al. 2015a). Sequence analysis of mtDNA regions quick tool to reveal phylogenetic relationships of marine species (Avise, 1994; Turan et al. 2008; Tabata & Taniguchi, 2000). Ever since different regions of mtDNA evolve at different rates, specific mtDNA regions have been targeted for inter and intra specific variation (Hauser et al. 2001; Mohindra et al., 2007; Turan et al., 2015b). DNA barcoding is a global venture that provides a standardized and effective genetic marker to marine and freshwater biodiversity, with significant conservation applications. The DNA barcoding approach is concentrated on a single part of the mitochondrial genome, because it presents portions conserved across taxa that are appropriate for primer design, while including polymorphism between and within species (Hebert et al., 2003; Kress & Erickson, 2008). The cytochrome oxidase subunit I (COI) region of the mitochondrial genome is sufficiently diverse so as to let the specific identification of a great majority of fish species (Kochzius et al., 2008; Kochzius et al., 2010).

In addition to simple identification of pufferfishes by DNA barcoding, the current level of interspecific and intraspecific genetic variation at pufferfish species which distributed in Turkish waters is very important to know. In spite of the wide scientific interest given to this family because of their commercial value, there are not any studies which investigated genetic structure of these species in Turkish waters.

The goal of this study is to evaluate the practicability of DNA barcoding in the monitoring of the pufferfish species biodiversity distributed along the Turkish waters at two levels by confirming the taxonomic identification and specifying intraspecific and interspecific variations for eight species found in Turkish marine waters.

Material and Methods

Species, *Lagocephalus lagocephalus, L. sceleratus, L. spadiceus, L. suezensis, L. guentheri and Torquigener flavimaculosus*, were collected from Iskenderun Bay, and the others *Sphoeroides pachygaster* and *Tylerius spinosissimus* sequences taken from GenBank (S. *pachygaster*: JQ681814.1, JF494545.1, KJ709636.1- *T. spinosissimus:* JQ681847.1, KP266781.1, JQ681456.1).All species showed that Figure.1.All samples were put in plastic bags individually and frozen at -20 °C till they were transported to the laboratory. All tissue samples were stored at -20 °C and 95 % ethanol till the analysis.

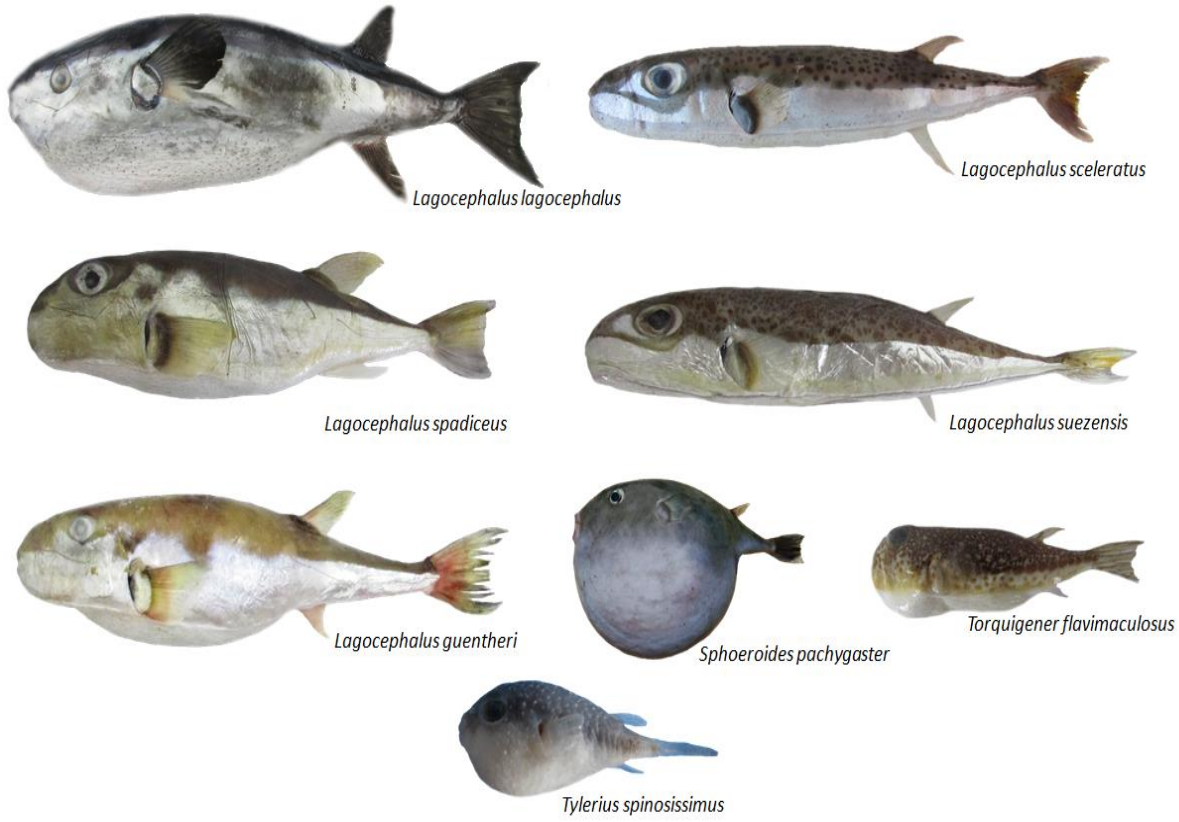

Figure 1. Pufferfish species in Turkish Marine Waters.

Total genomic DNA was extracted from the muscle and fin samples using the DNeasy Blood and Tissue Kit (Qiagen, USA). Manufacturer's protocols were used during all steps. Polymerase chain reaction (PCR) amplification was performed with following selective primers especially designed for this experiment:

COI-Forward 5'-TCAACCAACCACAAAGACATTGGCAC-3'

COI-Reserved 5'- ACTTCAGGGTGACCGAAGAATCAGAA-3'

The PCRs were conducted in a 50 ml total volume with 0.4 uM of each primer, 0.2 mM of dNTP and 1.25U of Taq DNA polymerase in a PCR buffer that included 20mM of Tris–HCl (pH 8.0), 1.5mM of MgCl2, 15 mM of KCl and 1-2 µl template DNA. Denaturation step 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. PCR products were visualized using electrophoresis on 1.5 % agarose gel. The DNA sequencing was attempted to determine the order of the nucleotides of mtDNA COI region. The chain termination method by Sanger et al. (1977) was applied with Bigdye Cycle Sequencing Kit V3.1 and ABI 3130 XL genetic analyzer. The initial alignments of partial COI sequences were performed with Clustal W program (Thompson et al., 1994) and final alignment was completed manually with BioEdit (Hall, 1999).

After sequence alignment, sequence divergences were calculated using the Kimura two parameter (K2P) distance model (Kimura, 1980). The molecular phylogenetic tree was constructed using Mega7 (Kumar et al. 2016). A distance-based method as neighbour joining (NJ) (Nei & Kumar, 2000) and a cladistics phylogenetic tree as maximum parsimony (MP) criterion were used. The reliability of the inferred phylogenies was evaluated using the bootstrap method (Felsenstein, 1985) with 1000 replicates.

Results

There were 189 variable and 337 conservative nucleotides of which 183 were parsimony informative over 526 bp sequences. The average nucleotide composition was 21.3% A, 28.8% T, 18% G and 31.9% C. Twelve haplotypes were found out of 27 sequences, and it was not found common haplotypes between species (Table 1.). Minimum spanning tree that shows the relationships among the haplotypes (Figure 2.) Variable nucleotide positions and frequencies of COI DNA barcode in pufferfish species show that Figure 3.

Table 1. The number of haplotype and its distribution among species

	L. lagocephalus	***L. sceleratus***	***L. spadiceus***	***L. suezensis***	***L. guentheri***	***S. pachygaster***	***T. flavimaculosus***	***T. spinosissimus***	**Total**
Hap1	-	**5**	-	-	-	-	-	-	**5**
Hap2	-	-	**4**	-	-	-	-	-	**4**
Hap3	-	-	**1**	-	-	-	-	-	**1**
Hap4	-	-	-	**5**	-	-	-	-	**5**
Hap5	**2**	-	-	-	-	-	-	-	**2**
Hap6	-	-	-	-	**2**	-	-	-	**2**
Hap7	-	-	-	-	-	-	**1**	-	**1**
Hap8	-	-	-	-	-	-	**1**	-	**1**
Hap9	-	-	-	-	-	-	-	**1**	**1**

Hap10	-	-	-	-	-	-	-	**1**	**1**
Hap11	-	-	-	-	-	-	-	**1**	**1**
Hap12	-	-	-	-	-	**3**	-	-	**3**
Total	**2**	**5**	**5**	**5**	**2**	**3**	**2**	**3**	**27**

Figure 2. Minimum spanning tree that shows the relationships among the haplotypes.

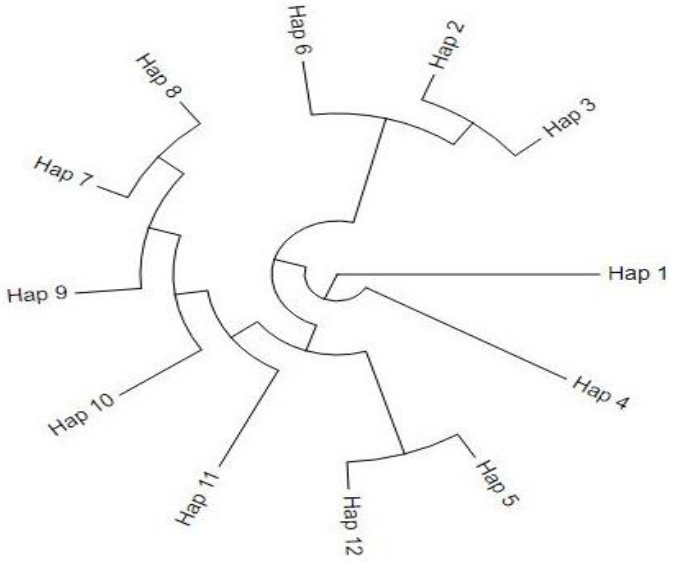

Figure 3. Variable nucleotide positions and frequencies of COI DNA barcode in pufferfish species. For all DNA barcode variable nucleotides are indicated, while identity is shown by dashes.

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            10        20        30        40        50        60        70        80        90       100
             *         *         *         *         *         *         *         *         *         *
Hap_1   CCCCACAATAATGTCTATACAGACAAGTGATCGAGCCACATCCTCCCTTTGCTTCCACTCGAGCTAAGCCTCCAGCCACTATACCTCGCTCGTTACCACC
Hap_2   TTA.G....G.C.CTC.C.TGAT..TAC.G.T.CA..GT.C.TC.TA.CCT...T.G.G.A..T.GCAT..G....TC..CA..T....C.A.AT.....
Hap_3   TTA.G....G.C.CTC.C.TGAT..TAC.G.T.CA..GT.C.TC.TA.CCT...T.G.G.A..T.GCAT..G....TC..CA..T....C.A.AT.....
Hap_4   ..........C....C.G...TTGT.CA.C.............CC.....TT....A.....CT..CA.CA..G.C..C...TAT....GG...T.
Hap_5   GTA........C..TC.C...AT..TACA...ACA........C.TA.CCT...TAGTG.A..T.GCTT.CG....TC...G..A..ATC.ACGT.A...
Hap_6   GTA.G....G.C.CTC.C.TGAT..TAC.G.T.CA..GT.C.TC.TA.CCT...T.G.G.A..T.GCAT..G....TC..CA..T....C.A.AT.....
Hap_7   ...T..TG...CACA..C.T..TTTTA..CC..CA...T...TC.TAC.CC..AGTG.GGTGTTC.CT..CAT.AA.T.C.CCTAC.C.CTACATA.C.T
Hap_8   ...T..TG...CACA..C.T..TTTTA..CC..CA...T...TC.TAC.CC..AGTG.GGTGTTC.CT.GCAT.AA.T.C.CCTAC.C.CTACATA.C.T
Hap_9   .T.T.T...G.CA..C.C.T..TTTTA..CC..CAT...G.TGC...CGC...AAA.TG.TG.A..CC..CG.CAA.T...GTTTC.C.C.T.ATT.T..
Hap_10  .T.T.T...G.CA..C.C.T..TTTTA..CC..CAT...G.TGC...CGC...AAA.TG.TG.A..CC..CG.CAA.T...GTTTC.C.C.T.AT..T..
Hap_11  .T.T.T...G.CA..C.C.T.ATTTTA..CC..CAT...G.TGC...CGC...AAA.TG.TG.A..CC..CG.CAA.T...GTTTC.C.C.T.AT..T..
Hap_12  ...T.T..C.CC..TC...T.ATATTACACC.A.A.TG..CT.CT.T..CATG..A.TATTG.T..C.T...TGCATGAC.GT.ACTA..TT.GT.GG..
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Continued

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      110       120       130       140       150       160       170       180
        *         *         *         *         *         *         *         *
TTCCCCATCCCCGACTACTTAAACACTCTCGACACGCTCTCTCCCCCCGCGCCGCCCACTCCACAACCCGAGAGCGCTCACCACCTGTT

CG.T.T.C.A.TA.TCCTC.C.G.....GT.G.C......T.....TTAGC...T....CTT.TTT...AC...TAAC....G......

CG.T.T.C.A.TA.TCCTC.C.G.....GT.G.C......T.....TTAGC...T.A..CTTTTTT...AC...TAAC.T..G......

AG.T.T.C..T..G...T............AG.G.A..TCT.TTTGT.AT.T..TT..TA.........A..G.TA....T........

CG...T.C.ATTA.TCTT..C..T....GT.GTTTATCAAT.GAA.ATAGC.A.T...TCTT.TTT...AC.G.TAAC........C..

CG.T.T.C.A.TA.TCCTC.C.G.....GT.G.C......T.....TTAGC...T....CTT.TTT...AC...TAAC....G......

AGG...T.AATTA.AA.TC.C...GA....A.TC.AT.TA..G..TATAA.TA..A.C.A..T..T.TT..AGAGA.CA..G.AGGTCC

AGG...T.AATTA.AA.T.CCG..GA....A.TC.AT.TA..G..TATAA.TA..A.C.A..T..T.TT..AGAGA.CA.....GGTCC

CGAT....AA....AG.TC.....GAA.GT...GTAT.TCT.GT..A.ATC.TAT..C.C..CT.C.T.A..G..ATCT.T.....A..

CGAT....AA....AG.TC......AA.GT...GTAT.TCT.GT..A.ATC.TAT..C....CT.T.T.A..G...TCT.T.....A..

CGAT....AA....AG.T.......AA.GT...GTAT.TCT.GT..A.ATC.TAT..C....CT.T.T.A..G...TCT.T.....A..

GG..TT..TATT.GT..TC.G..T.A.TATA.GCTAT..CTCT.A.ATAG.TA.T....A..C.CCT.T.T...T.AC..T..T..A..
```

Variable nucleotide positions and frequencies of DNA barcode are given Fig. 1. Species special DNA barcode were detected whereas it was not detected common DNA barcode between species. Kimura 2 parameter method was selected as a best method for intra and interspecific variations. Mean genetic diversity all species was found 0.18164. The matrix of pairwise distances within species is presented in Table 2. intraspecific genetic diversity within *L. sceleratus, L. suezensis, L. lagocephalus, L. guentheri* and *S. pachygaster* was observed to be zero while it was highest within *T. flavimucolosus* specimens (0.01149). The lowest genetic distance is observed between *L. guentheri* and *L.spadiceus* (0.00305) whereas the highest one is observed between T. flavimucolosus and *L.spadiceus* (0.26127). Pairwise comparisons of genetic distance revealed statistically significant differences ($P < 0.01$) between *L. suezensis* and *L. sceleratus* (Table 3.).

Table 2. The matrix of intraspecific genetic distances between species and diversity (transversal diagonal) given in bold

	1	2	3	4	5	6	7	8
L. sceleratus (1)	**0.00000**							
L.spadiceus (2)	0.19850	**0.00229**						
L. suezensis (3)	0.13133	0.22827	**0.00000**					
L. lagocephalus (4)	0.21867	0.10202	0.19204	**0.00000**				
L. guentheri (5)	0.19673	0.00305	0.22639	0.09859	**0.00000**			
T. flavimucolosus (6)	0.25622	0.26127	0.24380	0.24518	0.26042	**0.01149**		
T. spinosissimus (7)	0.21345	0.21469	0.20354	0.20343	0.21338	0.19274	**0.00896**	
S. pachygaster (8)	0.25101	0.24912	0.23208	0.21013	0.24759	0.25409	0.21512	**0.00000**

Table 3. Pairwise genetic distance between species (P<0.01**, P<0.05*).

	1	2	3	4	5	6	7
L. sceleratus (1)							
L.spadiceus (2)	0.00808**						
L. suezensis (3)	0.00769**	0.00848**					
L. lagocephalus (4)	0.04795*	0.04786*	0.04826*				
L. guentheri (5)	0.04634*	0.04876*	0.04778*	0.33431			
T. flavimucolosus (6)	0.04813*	0.04793*	0.14073	0.33824	0.33424		
T. spinosissimus (7)	0.01930*	0.01839*	0.06885	0.40532	0.40092	1.00000	
S. pachygaster (8)	0.01810*	0.01713*	0.01625*	0.09941	0.10023	0.10558	0.09990

Neighbour Joining and Maximum Parsimony phylogenetic approaches resulted in similar tree topologies. In Neighbour joining phylogenetic tree, two phylogenetic nodes were detected; in the first node, T. *flavimucolosus* .and *T. spinosissimus* grouped together. In second node 3 branches were detected. *S. pachygaster* was in the first branch, *L. spadiceus, L. guentheri* and *L. lagocephalus* were grouped second branch which *L. guentheri* and *L.* spadiceus were grouped together as a sister group and *L. sceleratus* and *L. suezensis* were grouped third branch (Figure 4).

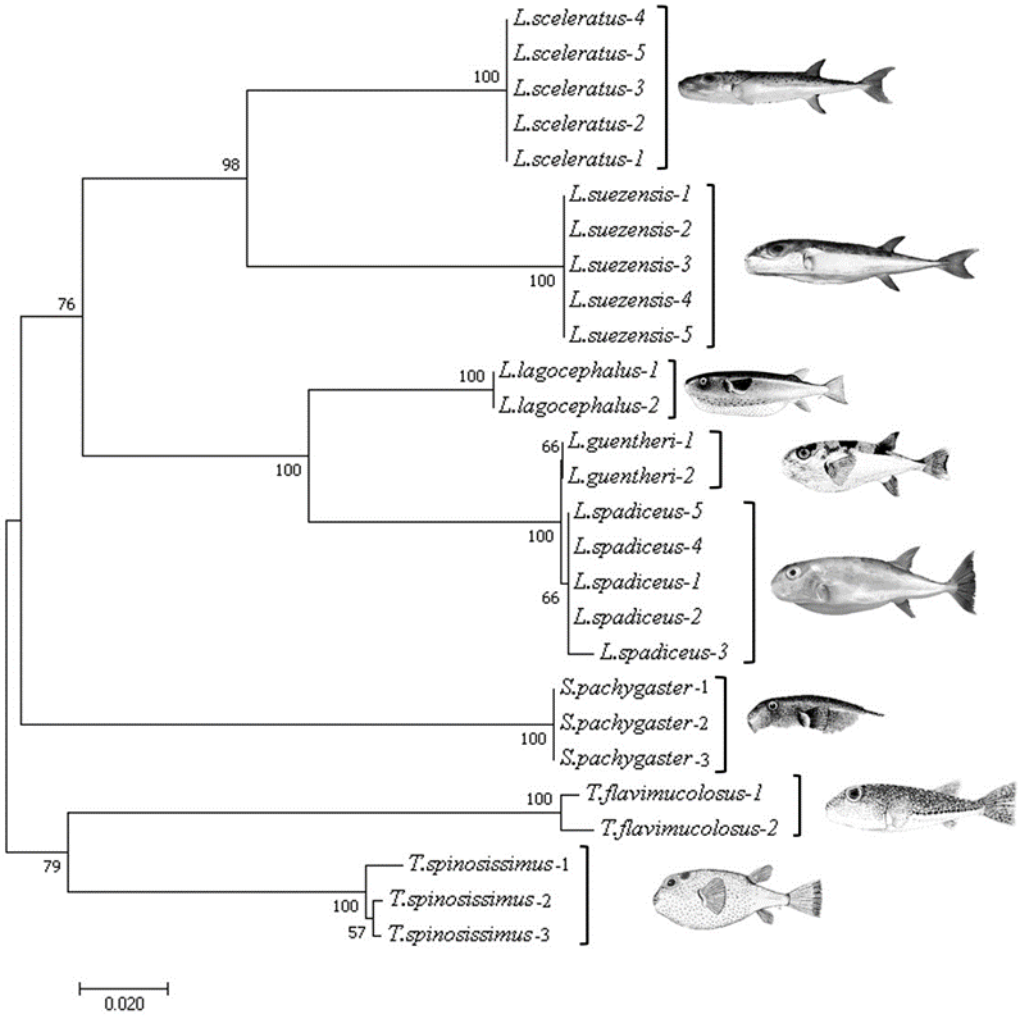

Figure 4. Neighbour joining phylogenetic tree based on COI sequences. Fish drawings from Froese & Pauly (2016)

In Maximum Parsimony phylogenetic tree, two phylogenetic nodes were detected; in the first node, T. *flavimucolosus* .and *T. spinosissimus* grouped together. In second node 3 branches were detected. *S. pachygaster* was in the first branch, *L. spadiceus, L. guentheri* and *L. lagocephalus* were grouped second branch which *L. guentheri* and *L.* spadiceus were grouped together as a sister group and *L. sceleratus and L. suezensis* were *grouped* third branch. (Figure 5).

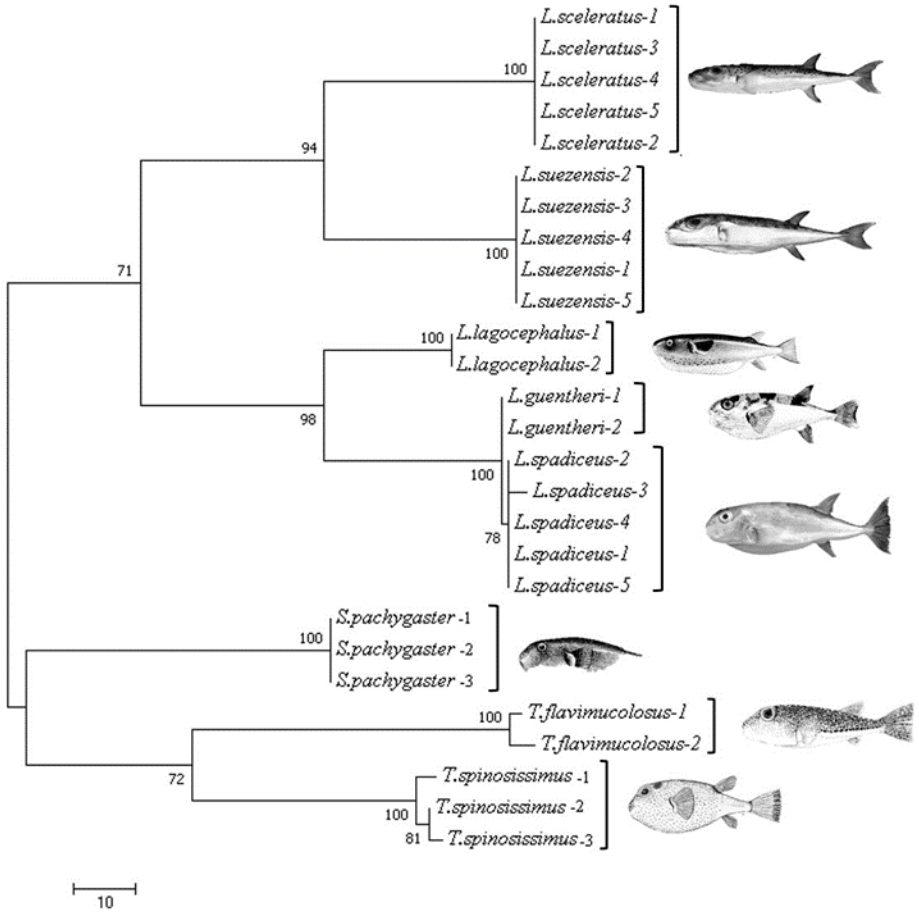

Figure 5. Maximum Parsimony phylogenetic tree based on COI sequences. Fish drawings: Froese & Pauly (2016).

Discussion

In the present study, DNA barcoding of eight pufferfish species which are distributed in the Turkish marine waters were investigated. All the species under the three genera were clearly separated by different group in the NJ and MP trees with a high bootstrap value. The universal primers amplified the target region in all species, generating 27 COI barcodes of 526 bp. Common haplotypes was not detected between species, and the DNA barcode sequences clearly discriminated taxonomic status of all pufferfish species examined.

Genetic diversity within species were calculated zero for *L. sceleratus, L. suezensis, L. lagocephalus, L. guentheri* and *S. pachygaster*. This low genetic diversity may be explained low number of samples sequenced probably the detected due to founder effect is something expected in lessepsian species, which form established populations starting from meager individuals. A similar result reported by Keskin & Atar (2013) using DNA barcoding to identify 89 commercially important freshwater and marine fish species found in Turkish ichthyofauna. Vinas & Tudela (2009) studied genetic identification of eight Scombrid species using mtDNA control region, mtDNA COI gene and nuclear DNA ITS1 region and reported that credibility of COI gene is questionable that also reported that COI gene is not a good marker for inferring evolutionary relationships in Thunnus species.

The present study is in accordance with many studies. Mudumala et al. (2011) studied phylogenetic relationships of *A. rochei, A. thazard, E. affinis* and *T. tonggol* species inferred from mitochondrial DNA sequences in the COI gene and reported the nucleotide compositions as A 24.0%, T 30.2%, G 18.4% and C 27.4%. Kochzius et al. (2010) aimed to evaluate the applicability of the three mitochondrial genes 16S rRNA (16S), cytochrome b (cyt b), and cytochrome oxidase subunit I (COI) for the identification of 50 European marine fish species by combining techniques of DNA barcoding and microarrays. As a result, while cyt b and COI are equally well suited for DNA barcoding of fishes. On the other hand, 16S has drawbacks in discriminating closely related species. This study, DNA barcoding on pufferfish species on Turkey. All these studies and many further have shown that genetic identification by COI barcodes can provide a useful tool to identify species and to detect possibly cryptic species, and even to describe new species.

In conclusion, in this study has strongly authenticated the efficacy of COI in identifying the pufferfish species with designated barcodes. The present results also suggest that COI barcoding can be taken up as pragmatic approach for resolving unambiguous identification of pufferfish species in marine waters of Turkey with applications in its management and conservation.

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