



DNA Barcoding of Tetraodontidae Species from the Mediterranean Sea: Filling Knowledge Gaps for Improved Taxonomic Accuracy

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Abstract

Most of the Mediterranean members of the family Tetraodontidae are non-native to the region, and include species of both Atlantic and Indo-Pacific origin. Anthropogenic activities are synergistically causing the populations of these non-native species to expand, causing ecological and economical losses in the Mediterranean Sea. The current study evaluated the morphological and the genetic characteristics of six Tetraodontidae species collected from the Mediterranean Sea. Two mitochondrial sequences, cytochrome c oxidase subunit I (COI) and the control region were studied covering over 1400 bp from each specimen, with *Lagocephalus sceleratus* and *L. suezensis* exhibiting the lowest intraspecific divergence, while *Torquigener flavimaculosus* exhibited the largest intraspecific divergence. Comparative analyses of the current data with other publically available COI data show the need to further evaluate species diversity of Tetraodontidae not only in the Mediterranean but also in their native range. Filling knowledge gaps for improved taxonomic identification of species is essential to accurately track these species and their populations in the Mediterranean and beyond.

Keywords: DNA barcoding, Tetraodontidae, pufferfish, Mediterranean, taxonomy.

Introduction

In the Mediterranean Sea, the family Tetraodontidae is represented by four genera (Mouneimné, 1977; Golani, 1987; Golani 1996; Reina-Hervas *et al.*, 2004; Akyol *et al.*, 2005; Corsini *et al.*, 2005; Vacchi *et al.*, 2007; Froese and Pauly, 2017; Golani *et al.*, 2017). These include the native *Lagocephalus lagocephalus* (Linneus, 1758), four tropical Atlantic species [*Ephippion guttifer* (Bennett, 1831); *Sphoeroides marmoratus* (Lowe, 1838); *S. pachygaster* (Müller & Troschel, 1848); and *S. spengleri* (Bloch, 1785)], and five Indo-Pacific species [*L. guentheri* Miranda Ribeiro, 1915; *L. sceleratus* (Gmelin, 1789); *L. suezensis* Clark and Gohar, 1953; *Torquigener flavimaculosus* Hardy and Randall, 1983; and *Tylerius spinosissimus* (Regan, 1908)]. Additionally, there are records of the Indo-pacific *L. spadiceus* (Richardson, 1845) (Tuncer *et al.*, 2008; Bariche *et al.*, 2015) whose occurrence in the Mediterranean reputed to be questionable (Galil *et al.*, 2016; Zenetos *et al.*, 2017). Records of Atlantic species are mostly restricted to the Western Mediterranean, with only *S. pachygaster* being known to have expanded its range and population size into the Eastern Mediterranean (Corsini-Foka and Economidis, 2007; Lipej *et al.*, 2013; Farrag *et al.*,

2016; Golani *et al.*, 2017). On the other hand, the species of Indo-Pacific origin are mostly considered as Lessepsian migrants through the Suez Canal, which in general establish populations in the Eastern Mediterranean followed by a population expansion as they move westerly colonizing new areas along the coast of several Mediterranean countries (Ben-Abdallah *et al.*, 2011; Azzurro *et al.*, 2016).

Alien taxa in the Mediterranean Sea are continuously subject to taxonomic revisions as new data on species distributions, identification and nomenclature are updated (Galil *et al.*, 2016; Zenetos *et al.*, 2017). Within this scenario, Tetraodontidae are no exception as there is controversy over possible misidentifications of species such as *S. spengleri* with *S. marmoratus* (Vacchi *et al.*, 2007; Shao *et al.*, 2014) and within the genus *Lagocephalus* (Golani 1996; Akyol *et al.*, 2005), including the doubtful distinguishing features between the closely related *L. spadiceus* and *L. guentheri* (Zenetos *et al.*, 2017) that are both recorded in the Mediterranean Sea (Tuncer *et al.*, 2008; Bariche, 2012; Bariche *et al.*, 2015; Farrag *et al.*, 2016; Shirak *et al.*, 2016). Within this scenario, the correct taxonomic classification utilizing different identification tools is imperative, thus morphological data has to be linked and backed with molecular data through techniques, such as DNA barcoding, that

utilizes cytochrome c oxidase subunit 1 gene (COI) as a marker to distinguish between species (Ratnasingham and Hebert 2007). However, to better trace the species' phylogeographic connections and population structures, markers that show a higher degree of intraspecific divergence are more useful to better track each species' expansion pattern and be able to prioritize on specific management measures for these invasive species. Such markers include the mtDNA control region that has been extensively used in population studies of several fish species (McMillan and Palumbi, 1997; Quattro et al., 2006; Chen et al., 2015; Vella and Vella, 2017). However, in Tetraodontidae this marker has only been applied to the genus *Takifugu* (Katamachi et al., 2015; Takahashi et al., 2017), and never utilized on other pufferfish species, not even in the Mediterranean Sea, to evaluate the species connectivity within the region as they expand in population size and range.

Materials and Methods

In this study, 37 specimens representing five species of the Family Tetraodontidae were collected from fisheries landings in the Mediterranean Sea (*L. guentheri* n = 5; *L. lagocephalus* n = 1; *L. scleratus* n = 12; *L. suezensis* n = 8; *S. pachygaster* n = 9; *T. flavimaculosus* n = 2) (Figure 1). Specimens were preliminarily identified using diagnostic features (Smith and Heemstra, 1986; Bariche, 2012; Psomadakis et al., 2015; Froese and Pauly, 2017) and measured to the nearest 0.1 mm. DNA was then extracted from 10 mg muscle tissue using the proteinase K, phenol-chloroform extraction as described in Milligan, (1998). The COI gene was amplified using the FISH-F1 and FISH-R1 primers following the protocol described by Ward et al., (2005). The control region (CR) together with flanking genes was amplified using two new primers

that were specifically designed for Tetraodontidae species using conserved regions in tRNA-Thr and the 12S rRNA genes (TetraCR-F AGAGCGCCGGTCTTGTAAC and TetraCR-R GGTGCGGATACTTGCATGTG). The latter was amplified in a 25 µL reaction volume using ~ 50 ng DNA template, 1x FIREPol® Master Mix (Solis BioDyne, Estonia), and 0.5 µM of each primer, through a temperature profile of 95°C for 5 min; followed by 35 cycles of 95°C for 45 s, 53°C for 45 s, 72°C for 45 s; and a final extension at 72°C for 10 min. PCR products were sequenced in both directions using the forward and reverse primers through ABI3730XL. Sequences were assembled using Geneious R10 (<http://www.geneious.com>, Kearse et al., 2012). The sequences were deposited in GenBank under accession numbers MG559735-808.

The COI sequences were submitted to the BOLD Species Level Barcode Records Identification Engine (<http://www.boldsystems.org>) and to GenBank via Blastn (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The Barcode Index Number (BIN) and the Tree Based Identification approach within BOLD was adopted to evaluate each specimen's hierarchical placement vis-à-vis the already available genetic data. CR data were separately submitted to GenBank via Blastn to further corroborate COI results.

Molecular diversity indices (haplotype diversity and nucleotide diversity) within the species were estimated via Arlequin v3 (Excoffier and Lischer, 2010). Interspecific and intraspecific *p*-distance were measured using MEGA v7 (Kumar et al., 2016). Additionally, the currently generated data were integrated with publically accessible COI data for Tetraodontidae specimens collected from the Mediterranean for tree-based phylogenetic analyses to evaluate the genetic differences between the various haplotypes. The *p*-distance model (Collins et al.,



Figure 1. Photos showing the six Tetraodontidae species analysed in this study. [a. *L. guentheri*; b. *L. lagocephalus*; c. *L. scleratus*; d. *L. suezensis*; e. *S. pachygaster*; f. *T. flavimaculosus*].

Table 1. Morphometric measurements of the Tetraodontidae specimens analysed in this study.

Species (sample size)	<i>L. lagocephalus</i>		<i>L. sceleratus</i>		<i>L. guentheri</i>		<i>L. suezensis</i>		<i>S. pachygastr</i>		<i>T. flavimaculosus</i>	
	Measure (mm)	% TL	Range (mean±SD)	% TL (mean±SD)	Range (mean±SD)	% TL (mean±SD)	Range (mean±SD)	% TL (mean±SD)	Range (mean±SD)	% TL (mean±SD)	Range (mean±SD)	% TL (mean±SD)
Total length	570.0		220.0-355.0 (262.3±37.3)		167.0-194.0 (180.8±8.7)		121.0-161.0 (137.1±125.1)		266.0-465.0 (383.9±60.3)		87.0-96.0 (91.5±4.5)	
Fork length	532.0	93.3	207.0-337.0 (249.1±35.4)	93.5-97.2 (94.9±0.9)	158.0-185.0 (171.6±8.9)	94.4-95.7 (94.9±0.5)	110.0-150.0 (127.6±11.9)	90.9-94.3 (93.1±1.0)	261.0-456.0 (375.1±58.4)	97.0-98.3 (97.7±0.5)		
Standard length	470.0	82.5	185.0-299.0 (221.5±31.6)	82.9-85.3 (84.4±0.7)	135.0-159.0 (148.2±8.0)	80.8-83.2 (81.9±0.7)	102.0-133.0 (114.0±9.7)	81.7-84.3 (82.2±0.9)	226.0-390.0 (322.0±50.1)	82.6-85.5 (83.9±0.9)	70.0-77.0 (73.5±3.5)	80.2-80.5 (80.3±0.1)
Pre-orbital length	52.1	9.1	25.9-47.0 (32.4±5.3)	11.3-13.2 (12.3±0.6)	17.1-20.7 (18.6±1.2)	9.6-11.5 (10.3±0.6)	11.7-15.6 (14.0±1.3)	9.6-10.9 (10.2±0.5)	33.0-67.0 (55.8±9.8)	12.4-16.5 (14.5±1.1)	8.0-11.0 (9.5±1.5)	9.2-11.5 (10.3±1.1)
Eye width	21.5	3.8	17.4-23.1 (20.2±1.8)	6.5-8.4 (7.8±0.5)	13.0-15.2 (14.0±0.8)	7.6-8.1 (7.8±0.2)	11.5-15.1 (13.4±1.0)	8.9-10.8 (9.8±0.7)	20.1-30.5 (25.1±3.5)	5.7-7.6 (6.6±0.5)	6.4-6.5 (6.4±0.1)	6.8-7.3 (7.0±0.3)
Head depth	80.2	14.1	23.2-42.0 (30.5±4.9)	10.5-12.5 (11.6±0.7)	26.1-34.7 (31.2±3.1)	15.6-18.9 (17.2±1.1)	12.6-18.6 (15.9±2.0)	9.3-13.6 (11.6±1.2)	49.0-80.0 (66.5±9.1)	14.2-19.3 (17.5±1.4)	12.6-16.1 (14.4±1.7)	14.5-16.7 (15.6±1.1)
Maximum body depth	93.5	16.4	26.7-45.8 (32.1±5.0)	11.0-13.3 (12.2±0.6)	31.4-36.4 (33.5±2.0)	17.5-19.8 (18.6±0.8)	13.6-19.6 (16.5±1.9)	10.0-13.4 (12.1±1.1)	51.0-90.0 (75.0±11.1)	15.5-22.2 (19.7±2.1)	12.1-19.0 (15.5±3.4)	13.9-19.8 (16.8±2.9)
Minimum body depth	31.1	5.5	5.4-8.9 (6.7±1.1)	2.4-3.0 (2.6±0.2)	8.0-9.3 (8.9±0.5)	4.7-5.2 (4.9±0.2)	3.6-5.4 (4.4±0.6)	2.9-3.8 (3.2±0.3)	12.1-23.1 (18.6±3.3)	4.3-5.5 (4.8±0.4)	5.7-5.9 (5.8±0.1)	6.1-6.6 (6.4±0.2)
Post-dorsal fin length	374.0	65.7	135.7-219.6 (164.4±25.0)	59.3-71.5 (62.7±2.9)	103.8-117.8 (112.5±5.2)	60.7-64.0 (62.5±1.0)	74.6-96.5 (83.1±6.7)	59.7-62.7 (60.7±1.0)	173.0-307.0 (252.4±39.7)	61.2-69.9 (65.8±2.3)	53.5-60.7 (57.1±3.6)	61.4-63.3 (62.4±0.9)
Pre-dorsal fin length	325.3	57.1	126.2-200.7 (149.6±20.2)	55.4-59.0 (57.1±1.1)	96.3-107.4 (101.2±4.6)	54.1-57.6 (56.0±1.4)	67.0-91.3 (77.2±7.6)	53.9-58.0 (56.3±1.2)	164.0-296.0 (238.9±38.3)	58.2-65.1 (62.2±2.0)	46.9-57.5 (52.2±5.3)	53.9-59.9 (56.9±3.0)
Head length	144.8	25.4	69.7-101.6 (79.5±9.2)	28.6-32.0 (30.4±1.1)	48.8-55.6 (52.6±2.3)	28.7-29.7 (29.1±0.4)	34.4-45.2 (39.6±3.8)	26.5-31.5 (28.9±1.4)	94.0-157.0 (135.7±18.4)	32.5-38.1 (35.5±1.5)	24.9-27.2 (26.1±1.1)	28.3-28.7 (28.5±0.2)
Pre-anal fin length	318.0	55.8	119.3-198.3 (147.8±21.2)	53.9-58.6 (56.3±1.3)	93.7-105.9 (100.5±4.2)	54.6-56.3 (55.6±0.8)	65.5-87.0 (74.8±6.6)	53.7-55.5 (54.6±0.6)	190.0-315.0 (255.4±37.8)	62.5-71.4 (66.7±2.7)	49.4-54.6 (52.0±2.6)	56.8-56.9 (56.9±0.0)
Pre-pectoral fin length	130.2	22.8	62.1-85.6 (70.6±6.6)	24.1-30.3 (27.1±1.4)	43.7-47.8 (46.0±1.3)	24.4-27.5 (25.5±1.1)	29.9-41.4 (34.1±3.5)	23.2-26.1 (24.8±1.0)	92.0-152.0 (122.9±17.4)	27.2-34.6 (32.2±2.2)	19.7-23.7 (21.7±2.0)	22.7-24.6 (23.7±1.0)
Weight (g)	2025.0		120.2-527.8 (223.5±107.2)		87.7-130.4 (113.0±15.0)		22.8-47.9 (32.9±7.6)		385.0-2275.0 (1333.0±575.0)		14.7-25.3 (20.0±5.3)	

2012; Srivathsan and Meier, 2012; Collins and Cruickshank, 2013) was used for the construction of the neighbour-joining tree (NJ), while the HKY+I nucleotide substitution model, which was identified as the model of best fit, was utilized for the maximum-likelihood tree (ML). Phylogenetic trees were constructed via MEGA v7 (Kumar et al., 2016) using 1500 bootstrap replicates.

Results

Lagocephalus guentheri, *L. sceleratus*, *L. suzezensis* and *T. flavimaculosus* were collected from the Eastern Mediterranean Sea and landed in Turkey, while *S. pachygaster* and *L. lagocephalus* were collected from the Southern Central Mediterranean and landed in Malta. The former five species are non-native species in the Mediterranean Sea (Golani et al., 2017), while the latter species, even though considered as native, lacks data on its occurrence in the region. The 570 mm *L. lagocephalus* specimen collected in this study (GPS: 34°32.822N 014°23.696E), constitutes one of the very rare records of this species caught by Maltese fishermen and the first specimen from Malta analysed scientifically both morphologically and genetically (Figure 1; Table 1). Morphometric measures of all the species collected is tabulated in Table 1.

Sequence-Based Classification

A 597 bp from COI representing 198 amino acids were sequenced for each specimen. No insertions, deletions or stop codons were observed on this sequence, consistent with functional protein coding genes. A total of 10 haplotypes were identified for this gene (Table 2).

The size of the studied CR analysed varied between 812 bp and 940 bp, which included most of the control region and some flanking genes, depending on the species under investigation. For each species, the smallest homologous CR sequence was chosen for further analyses, as to allow intraspecific comparison between individuals. This mtDNA region, being composed mostly of a non-coding sequence, exhibited more genetic variation than COI and consequently a total of 18 haplotypes were identified for this region (Table 2).

Comparative Genetic Analyses

All species were clustered within their respective species BOLD BINs (Table 3) and formed part of species specific clusters (Figure 2) as discussed below.

(1) *L. guentheri*: The currently studied *L. guentheri* specimens got clustered in a BOLD BIN containing both *L. guentheri* and *L. spadiceus* (Table 2). On excluding Mediterranean records (Table 4), where both species are considered as aliens, this

BIN's composition becomes solely composed of *L. guentheri* of Indo-Pacific origin (Table 4). This indicates that probably all the Mediterranean records belonging to this BIN (BOLD:ADG5739; Figure 2) are *L. guentheri*. The currently studied specimens were identified using the morphological characters as described by Smith and Heemstra (1986) and Psomadakis et al., (2015), and have been distinguished from *L. spadiceus* since all specimens had only white corners on their caudal fins (Figure 1) rather than a complete rear white margin, while the patch of spinules on the back did not extend to the dorsal fin origin. Although in freshly caught specimens the dorsal two thirds of the caudal fin had a dark yellow colouration and the rest of the caudal fin was dusky coloured (Figure 1), similar to the description of *L. spadiceus* reported Matsuura et al. (2011) and Psomadakis et al. (2015), it has to be noted that upon freezing and thawing the yellow colouration was mostly gone leading to a brown coloured caudal fin with white tips. Photographic analyses of the *L. guentheri* specimens presented on BOLD:ADG5739 show the same caudal fin colouration for all specimens within this cluster, matching the specimens investigated in this study.

In this study we identified two haplotypes for *L. spadiceus* matching the two main haplotypes of the *L. guentheri* / *L. spadiceus* group (BOLD:ADG5739) found in the Mediterranean Sea. Nonetheless, the Mediterranean Sea holds another haplotype identified as *L. spadiceus* which is at least 5.8% different from the other haplotypes of the rest of the Mediterranean *L. spadiceus* / *L. guentheri* group (Figure 2). This haplotype, which was recorded once in Turkey (HQ167726, unpublished), matches other *L. spadiceus* specimens collected from the Indo-Pacific, and fits in BOLD:AAD4510 (Table 4). These genetic results highlight the need for the correct taxonomic identification of *L. spadiceus* and *L. guentheri* in the Mediterranean Sea.

(2) *L. lagocephalus*: The COI data of the currently analysed specimen of *L. lagocephalus* genetically matched with one collected from the Eastern and Western Atlantic Ocean, however differed by 1.5% from specimens collected from the Indo-Pacific (Table 4). Although all these records belong to the same BOLD BIN (BOLD:AAI2183), sequences within this BIN are grouped into two main clusters that differ from each other (maximum *p*-distance 1.55%) indicating that *L. lagocephalus* is composed of genetically distinct lineages and that the Mediterranean population is closer to that of the Atlantic rather than to the Indo-Pacific.

(3) *L. sceleratus*: Nearly all the publically available *L. sceleratus* GenBank COI records of Mediterranean origin share the same COI haplotype (Table 4; Figure 2). However, CR data of the specimens analysed led to the formation of 6 haplotypes that were denoted by a low nucleotide diversity index ($\pi = 0.0034$), leading to a haplotype

Table 2. A list of the genetically analysed specimens in this study, including the location of capture, sample size (n), number of haplotypes (H), haplotype diversity (*h*), and nucleotide diversity (π) (including the standard error using pairwise difference), for both COI and CR

Species	Sampling location	n	COI			CR		
			H	<i>h</i>	π	H	<i>h</i>	π
<i>Lagocephalus lagocephalus</i>	Southern Central Mediterranean	1	1	-	-	1	-	-
<i>Lagocephalus guentheri</i>	North Eastern Mediterranean	5	2	0.400 ± 0.237	0.00201 ± 0.00178	3	0.700 ± 0.218	0.0017 ± 0.0014
<i>Lagocephalus scleratus</i>	North Eastern Mediterranean	12	1	0.000 ± 0.000	0.0000 ± 0.0000	6	0.879 ± 0.060	0.0034 ± 0.0021
<i>Lagocephalus suezensis</i>	North Eastern Mediterranean	8	1	0.000 ± 0.000	0.0000 ± 0.0000	3	0.464 ± 0.200	0.0015 ± 0.0012
<i>Spherooides pachygaster</i>	Southern Central Mediterranean	9	3	0.417 ± 0.191	0.00074 ± 0.00082	3	0.639 ± 0.126	0.0019 ± 0.0014
<i>Torquigener flavimaculosus</i>	North Eastern Mediterranean	2	2	1.000 ± 0.500	0.01843 ± 0.01925	2	1.000 ± 0.500	0.0329 ± 0.0335

Table 3. A table including the BOLD Barcode Index Numbers (for COI data) for the currently studied specimens and the closest GenBank matches (for CR data)

	COI		CR
	BOLD BIN (species within BIN)	Maximum Distance within BIN	GenBank closest match (species with closest match)
<i>Lagocephalus guentheri</i>	BOLD:ADG5739 (<i>L. guentheri</i> ; <i>L. spadiceus</i>)	4.60%	99.6% match to KM667972 (<i>L. spadiceus</i>)
<i>Lagocephalus lagocephalus</i>	BOLD:AAI2183 (<i>L. lagocephalus</i>)	1.55%	97.6% match to AP011933 (<i>L. lagocephalus</i>)
<i>Lagocephalus scleratus</i>	BOLD:AAC5565 (<i>L. scleratus</i>)	1.69%	99.7% match to KP013618 (<i>L. scleratus</i>)
<i>Lagocephalus suezensis</i>	BOLD:ACG7296 (<i>L. suezensis</i>)	0.45%	97.6% match to KP013619 (<i>L. suezensis</i>)
<i>Spherooides pachygaster</i>	BOLD:AAB7651 (<i>S. pachygaster</i>)	2.52%	99.4% match to AP006745 (<i>S. pachygaster</i>)
<i>Torquigener flavimaculosus</i>	BOLD:AAG3690 (<i>T. flavimaculosus</i> ; <i>T. hypselogeneion</i> ; <i>T. altipinnis</i>)	2.10%	88.2% match to AP009537 (<i>T. brevipinnis</i>)

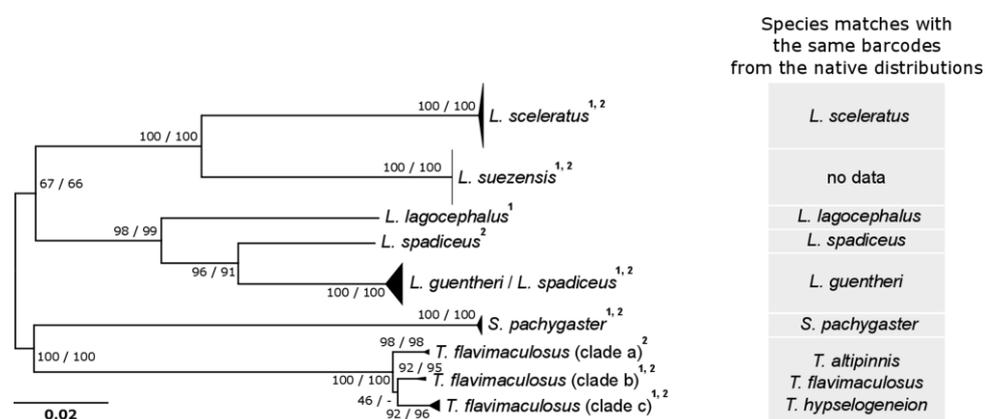


Figure 2. Neighbour-Joining tree using the *p*-distance model for the COI data of the Tetraodontidae species collected from the Mediterranean Sea. The column on the right indicates the species from the native distributions which have barcodes matching to the Mediterranean specimens of Tetraodontidae. Numbers near nodes indicate bootstrap values of the NJ and the ML trees respectively. The distance scale bar is based on NJ tree. [¹ genetic data collected from the current study; ² Mediterranean related Tetraodontidae genetic data mined from publically available sequences on GenBank].

Table 4. A list of sequences used for comparative analyses between COI haplotypes identified in this study and other publically available haplotypes present in GenBank and BOLD

L. guentheri and *L. spadiceus*:
Mediterranean (BOLD:ADG5739): Turkey [MG559735-9, current study; KY176508, unpublished]; Israel [KM538365-81, Shirak *et al.*, 2016]; Lebanon [KR861535-6, Bariche *et al.*, 2016]
Indo-Pacific (BOLD:ADG5739): South Africa [JF493720-4, unpublished]; Madagascar [SAIAD190-11]; Iran [HQ149858-9, Asgharian *et al.*, 2011]; Saudi Arabia [KU170600, unpublished]; Bangladesh [MF588654-6, unpublished]; India [KF442241, KX675919, KX758092, unpublished]
Mediterranean (BOLD:AAD4510): Turkey [HQ167726, unpublished]
Indo-Pacific (BOLD:AAD4510): Japan [ABFJ191-07]; Taiwan [GBGC6810-09]; China [FSCS302-06, FSCS610-07 - FSCS613-07]; Indonesia [FOAI121-08]

Lagocephalus lagocephalus:
Mediterranean and Atlantic: Central Mediterranean [MG559740, current study]; Portugal [KX586199, Oliveira *et al.*, 2016]; Haiti [MFLE075-12]
Indo-Pacific: California [HQ010074, unpublished], South China Sea [JF730881-2, unpublished]

Lagocephalus sceleratus:
Mediterranean: Egypt [KX017773-96, unpublished]; Israel [KM538363-4, Shirak *et al.*, 2016]; Lebanon [KR861534, Bariche *et al.*, 2016]; Turkey [MG559741-52, current study; KY176507, unpublished]
Indo-Pacific: South Africa [DSFSG691-11]; Australia (FOAH319-08, FOAH593-08, FOAI671-09, FOAI672-09)

Lagocephalus suezensis:
Mediterranean: Israel [KM538382-405, Shirak *et al.*, 2016]; Lebanon [KR861537, Bariche *et al.*, 2016]; Turkey [MG559753-60, current study; KY176509, unpublished]

Sphoeroides pachygaster:
Mediterranean: Malta [MG559761-9, current study; KJ709914-8, Landi *et al.*, 2014]; Italy [KJ709636, Landi *et al.*, 2014]; Turkey [HQ167727, unpublished]
North-Eastern Atlantic: Portugal [EU869841-3, Ward *et al.*, 2005; KJ768311, Landi *et al.*, 2014]; Cape Verde [CVERD075-13, CVERD239-13, CVERD240-13, CVERD074-13]; Angola [HVDBF493-12]
Indo-Pacific: South Africa [JF494541-5: unpublished]; China [FNSIC085-11]; Taiwan [FJ434553: unpublished; KU945243-4, Chang *et al.*, 2016]; Australia [EU869839-40, Ward *et al.*, 2005]
Western Atlantic: Uruguay [EU074596-8, Mabragana *et al.*, 2011]; United States [FWRI088-10, FWRI640-17]; Nicaragua [MOCA478-12]; Honduras [MOCA624-12]; Brazil [BARC326-16]

Torquigener flavimaculosus:
Mediterranean: Israel [KM538604-7, Shirak *et al.*, 2016]; Lebanon [KR861566, Bariche *et al.*, 2016]; Turkey [MG559770-1, current study; KY176669-73, unpublished]
Indo-Pacific (*Torquigener* species within BOLD:AAG3690): South Africa [TZMSB303-04, KZNMFO07-12, KZNMFO36-12]; Australia [FOAF382-07, AMSF209-09 - AMSF213-09]; New Zealand [FNZ219-06, FNZ225-06]

diversity of 0.879. Tree Based Identification from BOLD has shown that the Mediterranean records of *L. sceleratus* cluster more closely related with the Eastern African records, rather than with the Australian records (Table 4), further confirming that this species extended its range through the Suez Canal.

(4) *L. suezensis*: All the publically available COI data for *L. suezensis* records within BOLD:ACG7296 are from specimens of Mediterranean origin, most of which share the same haplotype (Table 4; Figure 2), while the CR data of the currently analysed specimens exhibited some nucleotide differences ($\pi = 0.0015$) between individuals leading to the formation of 3 haplotypes ($h = 0.464$). The lack of data from outside the Mediterranean makes it impossible to compare the genetic characters of the Mediterranean population against those from the native areas, again showing the need to better study this species and the phylogeographic connections at a global scale.

(5) *S. pachygaster*: The specimens of *S. pachygaster* analysed in this study shared 3 COI

haplotypes and 3 CR haplotypes, with all of them being within 0.3% different from each other. Tree Based Identification through BOLD has shown that the currently analysed specimens are grouped within the same cluster containing specimens of Mediterranean origin, North-Eastern Atlantic origin and Indo-Pacific origin (Table 4). However, this cluster differed by 2% from *S. pachygaster* of Western Atlantic origin (Table 4). Such genetic divergence is higher than the 1% intraspecific divergence noted within most fish species (Ward *et al.*, 2005; Hubert *et al.*, 2008; Barman *et al.*, 2017) therefore this calls for a taxonomic revision of *S. pachygaster* as it can be composed of a species complex.

(6) *T. flavimaculosus*: The two *Torquigener flavimaculosus* specimens collected for this study had a *p*-distance of 3.3% at CR and a *p*-distance of 1.8% at COI, with the latter being well beyond the 1% intraspecific differentiation recorded in most fish taxa (Ward *et al.*, 2005; Hubert *et al.*, 2008; Barman *et al.*, 2017). Additionally, through further analyses of all the currently available genetic data for *T.*

flavimaculosus in the Mediterranean Sea (Figure 2), it became evident that there is a third more distinct lineage for this species (Table 4). Tree Based Identification of BOLD has placed these specimens in a cluster that contains *T. flavimaculosus* mostly from the Mediterranean Sea, together with *T. hypselogeneion* and *T. altipinnis* from the Indo-Pacific (Table 4). These results indicate that *T. flavimaculosus* is probably a species complex requiring further studies to better evaluate speciation. Given the large number of COI haplotypes it is evident that the Eastern Mediterranean Sea must have experienced multiple invasions by this genus, while the genetic divergence noted between the various COI sequences indicates that the Mediterranean might be hosting more than one species of *Torquigener*.

Discussions

The need for more detailed studies to improve the taxonomic classification of Tetraodontidae is evident both from results seen in this work and also from the presence of distant lineages present on BOLD for other Tetraodontidae species not evaluated here. Currently, taxonomic limitations constrain correct species identification causing confusion in classification and possible underestimations of species diversity within this family. The phylogenetic connections recorded in this study identified genetic differences that can be indicative of overlooked species (Zemlak *et al.*, 2009). Taxonomic revisions linking morphological identification keys to genetic data, where both tools need to be well managed, once achieved these are the way forward towards the correct species identification. As a taxonomic revision of Tetraodontidae may reveal that the discrete lineages are in fact new species, then the number of pufferfish species known within the Mediterranean Sea would increase further.

Clearly, the urgent need to improve Tetraodontidae identification is essential not only in areas where these species are native, but also in areas that they are invading. In this regard, the additional use of molecular markers that evolve faster than COI, can aid in better understanding of the genetic diversity within each species or species complex. One such marker is the CR, which exhibits the highest evolutionary rate as within the mtDNA (Meyer, 1993; McMillan and Palumbi, 1997), making it more diverse than COI (Quattro *et al.*, 2006; Chen *et al.*, 2015). The latter was also noted in the current study, where a total of 10 haplotypes were recorded for COI as opposed to the 18 haplotypes noted for the CR, with the mean CR intraspecific nucleotide diversity for the Tetraodontidae analysed being twice as much as that noted in COI. This higher rate of mutation that provides more intraspecific information is of great use in tackling species specific questions related to each species' connectivity and would facilitate the accurate follow-up of the genetic population structure of these

species as they expand in size and range (Gaither *et al.*, 2013; Toledo-Hernandez *et al.*, 2014; Butterfield *et al.*, 2015; Janáč *et al.*, 2017).

Reducing identification uncertainties and understanding the species' indigenous range (McGeoch *et al.*, 2012), are vital first steps to narrowing knowledge gaps. Evaluation of the complexity and the conditions that are promoting these non-native species to become invasive causing both biological and socio-economical concerns (Ünal *et al.*, 2015; Maltese fishermen pers. comm. AV and NV) depend on the achievement of precision identification methods on which management can act with greater rigour and effective results.

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