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Research Article

Successful colonization of the Red Sea Yellowspotted Puffer, *Torquigener flavimaculosus* in the Mediterranean without a genetic bottleneck

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Abstract

The Yellowspotted Puffer *Torquigener flavimaculosus* (Hardy & Randall, 1983) invaded the Mediterranean from the Red Sea via the Suez Canal. In the present study, we analyzed two mitochondrial loci, the cytochrome c oxidase 1 (COI) and the control region (D-loop), from the Mediterranean and the Red Sea populations. Both the COI and the D-loop showed no decrease of genetic variability in the Mediterranean population compared to the source population from the Red Sea. When comparing the genetic variability to two other species of the Tetraodontidae family (*Takifugu rubripes* and *Takifugu obscurus*), the mean divergence within the *T. flavimaculosus* is almost twice as large. *T. flavimaculosus* has two distinct genetic groups, similarly represented both in the Red Sea and in the Mediterranean, with similar coefficients of differentiation in COI, in D-loop, and, not surprisingly, in the two genes combined. This suggests that *T. flavimaculosus* has most likely established a sustainable population in the Suez Canal, that has gradually dispersed northward and eventually entered the Mediterranean with a large number of individuals, carrying a great deal of its genetic variability.

Key words: COI, control region, D-loop, founder effect, Lessepsian migration, speciation

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Introduction

Biological invasions constitute a major influence on global biodiversity. Exotic species, especially those that succeed in establishing sustainable populations in their new region, have been the subject of numerous studies (Roman and Darling 2007; Simberloff et al. 2013; Katsanevakis et al. 2014; Havel et al. 2015). One of the main questions that arise from these research studies is: are there characters that facilitate success in colonization, since the new environment differs from the original habitat. One hypothesis predicts a decrease in genetic variability of the invasive population as compared to its native population, due to genetic bottleneck, where the founder group of the colonizers consists of a small number of individuals, which represents a

sub-sample of the native population. The opening of the Suez Canal in 1869 caused a massive unidirectional migration of biota from the Red Sea into the Mediterranean; this phenomenon is often termed Lessepsian migration (Por 1978; Golani 2010). This ongoing migration phenomenon includes over 120 fish species (Golani et al. 2021 and unpublished data) and provides a unique opportunity to examine the hypothesis of reduction of genetic variability in the new population in the Mediterranean.

The Yellowspotted Puffer *Torquigener flavimaculosus* Hardy & Randall, 1983 (Pisces: Tetraodontidae) was first recorded in the Mediterranean in 1987 from the coast of Israel (Golani 1987). Several years later, it established a large population throughout the eastern Mediterranean (Golani et al. 2021). The family of Tetraodontidae includes 192 species worldwide in 28 genera. Most species live in marine environments while a few species inhabit brackish and freshwater (Nelson et al. 2016; Fricke et al. 2023). In the Red Sea the family is represented by 13 species (Golani and Fricke 2018), of which five species were recorded in the Mediterranean as Lessepsian migrants (Golani et al. 2021). *Torquigener flavimaculosus* is a benthic small species up to 11 cm Total Length (with one exception of a single specimen of 18.5 cm Total Length). Its native distribution includes the Red Sea, the Arabian Gulf, East Africa and Reunion (Gadenne et al. 2021). It was first recorded in the Mediterranean in 1987 (Golani 1987) and remained rare in its new region for 15 years, until it was recorded from Türkiye (Bilecenoğlu 2003) and spread to Greece (Corsini-Foka et al. 2006) and Libya (Al-Mabruk et al. 2018). Though little is known of its biology and ecology, in the Mediterranean, it is caught in large numbers as by-catch by trawlers at 20–50 m (Edelist et al. 2013). Golani and Lerner (2007) found it in large numbers in the sandy beach of Eilat (Gulf of Aqaba, Red Sea). It feeds mainly on slow moving invertebrates, such as crustaceans, mollusks and echinoderms (Bilecenoğlu 2005; Chartosia et al. 2021). In the Gulf of Suez, the reproductive period peaks in the spring and summer months. The specimens reach sexual maturity at 8–10 cm total length (Ramadan and El- Halfawy 2019).

The objective of this study is to better understand the mode of migration by comparing the genetic variability of the original, native populations to the migrant populations. Specifically, we compare our samples from Eilat (at the northern tip of the Gulf of Aqaba, Red Sea) with the migrant population in the Eastern Mediterranean, several decades after the first record of their invasion. This enables us to assess whether there was a decrease in genetic variability in the Mediterranean, due to a bottleneck or a founder effect. In order to support our findings, we compare the genetic variability within *T. flavimaculosus* populations to other species of the Tetraodontidae family (*Takifugu rubripes* and *Takifugu obscurus*), studied by Katamachi et al. (2015).

Materials and methods

Sample collection and DNA extraction

Twenty-nine specimens of *Torquigener flavimaculosus* were collected for this study. Fifteen Red Sea specimens were collected at a depth of 0.5–1.5 m on a sandy beach location at ca. 4 km east of Eilat (29°32'41.05"N, 34°58'21.22"E), using a 30 m experimental beach seine with decreasing mesh size from 40 mm knot to knot, to 2 mm at the center. Fourteen Mediterranean specimens were collected as a by-catch of commercial fishing trawlers at a depth of 30–60 m. All studied specimens were deposited as voucher specimens in the Fish Collection of the National Natural History Collections of the Hebrew University of Jerusalem (HUJ), Israel (Table 1).

Adult fish muscles (about 50 mg), taken from the right side of the back, were used for DNA sample preparation using the Accu-Prep genomic DNA extraction kit (Bioneer, Daejeon, Korea).

Table 1. Data on the studied specimens.

Collection date	Location (all in Israel)	Number of specimens	HUJ voucher number
09/12/2005	Red Sea, Eilat	15	21205
29/12/2008	Mediterranean	1	21207
27/04/2009	Mediterranean	1	21206
05/03/2011	Mediterranean	8	20074
10/04/2011	Mediterranean	3	20355
29/11/2012	Mediterranean	1	20373

PCR and sequencing

A segment of 729–736 bp (depending on size variation) was amplified from the 3' region of the mitochondrial DNA D-loop (control region) using the newly designed primers by YT. D-loop and the flanking tRNAs Sequences from 10 different Tetraodontidae family species were aligned, and the consensus sequences were used to design the primers:

TF-D-F TATATCGAACATTTCATAACATGCATAAC
TF-D-R GGTCCATCTTAGCATCTTCAGTA

PCR reactions were carried out in 20 µl reaction volumes containing 1× Taq PCR mix (Tiangen Biotech, Beijing), 0.5 µM of each primer, and about 100 ng of template DNA. PCR reactions were processed in a Bio-Rad C-1000 thermal cycler with the following thermal regime: an initial step of 3 min at 95 °C followed by 30 cycles of 0.5 min at 95 °C, 0.5 min at 58 °C and 0.5 min at 72 °C, followed by 3 min at 72 °C and then held at 12 °C.

A segment of 595 bp was amplified from the 5' region of the mitochondrial cytochrome c oxidase subunit I gene (COI) using the consensus FishF2 and FishR2 primers (Ward et al. 2005).

PCR reactions were carried out in 20 µl reaction volumes containing 1× Taq PCR mix (Tiangen Biotech, Beijing), 0.5 µM of each primer, and about 100 ng of template DNA. PCR reactions were processed in a Bio-Rad C-1000 thermal cycler with the following thermal regime: an initial step of 3 min at 95 °C followed by 30 cycles of 0.5 min at 95 °C, 0.5 min at 55 °C and 0.5 min at 72 °C, followed by 3 min at 72 °C and then held at 12 °C.

PCR products were visualized on 1.5% agarose gels and sequenced bidirectionally using the PCR primers on an ABI 377 DNA Sequencer (Applied Biosystems, Foster City, CA) following the manufacturer's instructions.

Data analysis

D-loop

We identified the D-loop haplotype of 29 specimens of our study, GenBank accession numbers OQ354349–OQ354377. In addition to the 29 specimens of our study, mitochondrial DNA D-loop (control region) haplotypes of two *Torquigener flavimaculosus* specimens from Malta (GenBank numbers MG559807 and MG559808) were added to the analysis.

For comparing the diversity of *Torquigener flavimaculosus* of our study to the diversity of a few species of *Takifugu* – a genus that belongs to the same sub-family (Tetra-

odontinae) as *T. flavimaculosus* – we used data by Katamachi et al. (2015) pertaining to the same 3' region of the mitochondrial DNA (control region). Thus, we included 41 specimens of *Takifugu rubripes*, 38 *T. obscurus*, 2 *T. orbimaculatus* and 1 *T. fasciatus*.

COI

We identified the COI haplotype of 26 out of 29 specimens of our study, GenBank accession numbers OQ345528–OQ345553. In addition, haplotypes of 12 *Torquigener flavimaculosus* specimens were obtained from the GenBank and added to our analysis: Four from the Mediterranean Sea, Israel (GenBank numbers KM538604-07), one from an unspecified Mediterranean location (KR861566), five from Türkyie (KY176669-73) and two from the Arabian (Persian) Gulf (KU499713 and KU499785).

For both markers, sequence alignment, neighbor-joining analysis and tree construction were carried out using the Molecular Evolution Genetics Analysis (MEGA 7) software (Kumar et al. 2018). Distances were calculated by the Kimura 2-parameter model, and the neighbor-joining trees were each tested by 1000 bootstrap replications.

Results

D-loop

Comparison of all 31 sequences showed 73 polymorphic sites – 54 transitions, 11 transversions and 8 indels. These polymorphic sites enabled the phylogenetic analysis based on genetic distance, using the Kimura 2-parameter model.

The neighbor-joining tree displays a distinct separation into two major branches (See Suppl. material 1). Each of the two branches is composed of Mediterranean as well as Red Sea and Arabian Gulf specimens. One of the branches is further split into two minor branches, both consisting of specimens from the two locations.

Mean genetic distances within and between the two main branches, and the coefficient of differentiation are given in Table 2 (Block (A), blue shading). Table 3 (Block (A), blue shading) displays the mean genetic distances within each of the two locations, the Mediterranean and the Red Sea.

Mean divergence within *T. flavimaculosus* is almost twice as large as the mean divergence within *Takifugu rubripes* or within *Takifugu obscurus*, and is comparable to the mean divergence of all 82 *Takifugu* specimens of Katamachi et al. (2015) together (Table 4). Note, moreover, that for the *Takifugu* samples the segment contained 825 bp (of which 127 were polymorphic), compared to 736 bp (73 polymorphic) in the *Torquigener* sample.

COI

Comparison of all 38 sequences showed 23 polymorphic sites – 21 transitions and 2 transversions. These polymorphic sites enabled the phylogenetic analysis based on genetic distance, using the Kimura 2-parameter model.

The neighbor-joining tree displays a distinct separation into two major branches (See Suppl. material 2). Each of the two branches is composed of Mediterranean as well as Red Sea and Arabian Gulf specimens. Moreover, each major branch of the COI tree consists of the same specimens as in the corresponding branch of the D-loop tree (see Suppl. material 1, with only the 26 relevant specimens).

Mean genetic distances within and between the two main branches, and the coefficient of differentiation are given in Table 2 (Block (B), yellow shading). Table 3

Table 2. Mean genetic distances within and between the two main branches: (A) with respect to the D-loop marker (see also Suppl. material 1). (B) with respect to the COI marker (see also Suppl. material 2). (C) with respect to the both the D-loop and the COI markers (see also Figure 1). The left column in each block represents the number of specimens with the corresponding number of pairs (in parentheses). The last row of the table presents the coefficient of differentiation, which is the proportion of the inter-branch diversity out of the entire diversity. Standard errors (s.e.) were estimated by 1000 bootstrap replications.

	(A) D-loop		(B) COI		(C) D-loop & COI	
	Specimens (pairs)	**Mean ± s.e.**	**Specimens (pairs)**	**Mean ± s.e.**	**Specimens (pairs)**	**Mean ± s.e.**
Within Branch I	17 (136)	0.013 ± 0.002	22 (231)	0.004 ± 0.001	15 (105)	0.008 ± 0.001
Within Branch II	14 (91)	0.014 ± 0.003	16 (120)	0.008 ± 0.003	11 (55)	0.011 ± 0.002
Between Branches	31 (238)	0.041 ± 0.007	38 (352)	0.020 ± 0.005	26 (165)	0.032 ± 0.004
Coefficient of Differentiation		0.522 ± 0.055		0.519 ± 0.097		0.534 ± 0.049

Table 3. Mean genetic distances within the Mediterranean and the Red Sea: (A) D-loop; (B) COI; (C) D-loop and COI combined. The left column in each block represents the number of specimens with the corresponding number of pairs (in parentheses). Standard errors (s.e.) were estimated by 1000 bootstrap replications.

	(A) D-loop		(B) COI		(C) D-loop & COI	
	Specimens (pairs)	**Mean ± s.e.**	**Specimens (pairs)**	**Mean ± s.e.**	**Specimens (pairs)**	**Mean ± s.e.**
Within Mediterranean	16 (120)	0.027 ± 0.004	23 (253)	0.014 ± 0.003	13 (78)	0.022 ± 0.003
Within Red Sea*	15 (105)	0.026 ± 0.004	15 (105)	0.010 ± 0.003	13 (78)	0.017 ± 0.002

* Remark: COI contained also two specimens from the Arabian Gulf.

Table 4. D-loop: Mean divergence within various Tetraodontinae taxons. Standard errors (s.e.) were estimated by 1000 bootstrap replications.

	Number of specimens	**Mean divergence ± s.e.**
Takifugu obscurus	38	0.014 ± 0.002
Takifugu rubripes	41	0.014 ± 0.002
All *Takifugu* specimens	82	0.032 ± 0.004
Torquigener flavimaculosus	31	0.027 ± 0.004

(Block (B), yellow shading) displays the mean genetic distances within the two body waters, (1) the Mediterranean Sea and (2) the Red Sea and the Arabian Gulf.

D-loop and COI together

For the 26 specimens for which we have both the COI and the D-loop haplotypes, we can consider a concatenated joint haplotype for each specimen – a sequence consisting of its COI sequence, followed by its D-loop sequence.

Using the Kimura 2-parameter model, we constructed a neighbor-joining tree (tested by 1000 bootstrap replications). The tree displayed a distinct separation into two major branches (Figure 1). Each of the two branches is composed of Mediterranean as well as Red Sea specimens.

Mean genetic distances within and between the two main branches, and the coefficient of differentiation are given in Table 2 (Block (C), green shading). Table 3 (Block (C), green shading) displays the mean genetic distances within each of the two locations, the Mediterranean and the Red Sea.

Thus, we can conclude that (1) the genetic variability within the invading Mediterranean population of *T. flavimaculosus* is not smaller than the variability within the source population of the Red Sea; (2) mean divergence within *T. flavimaculosus* is large, compared to divergence within two species of *Takifugu*, and (3) *T. flavimaculosus* displays a genetic separation into two distinct clades.

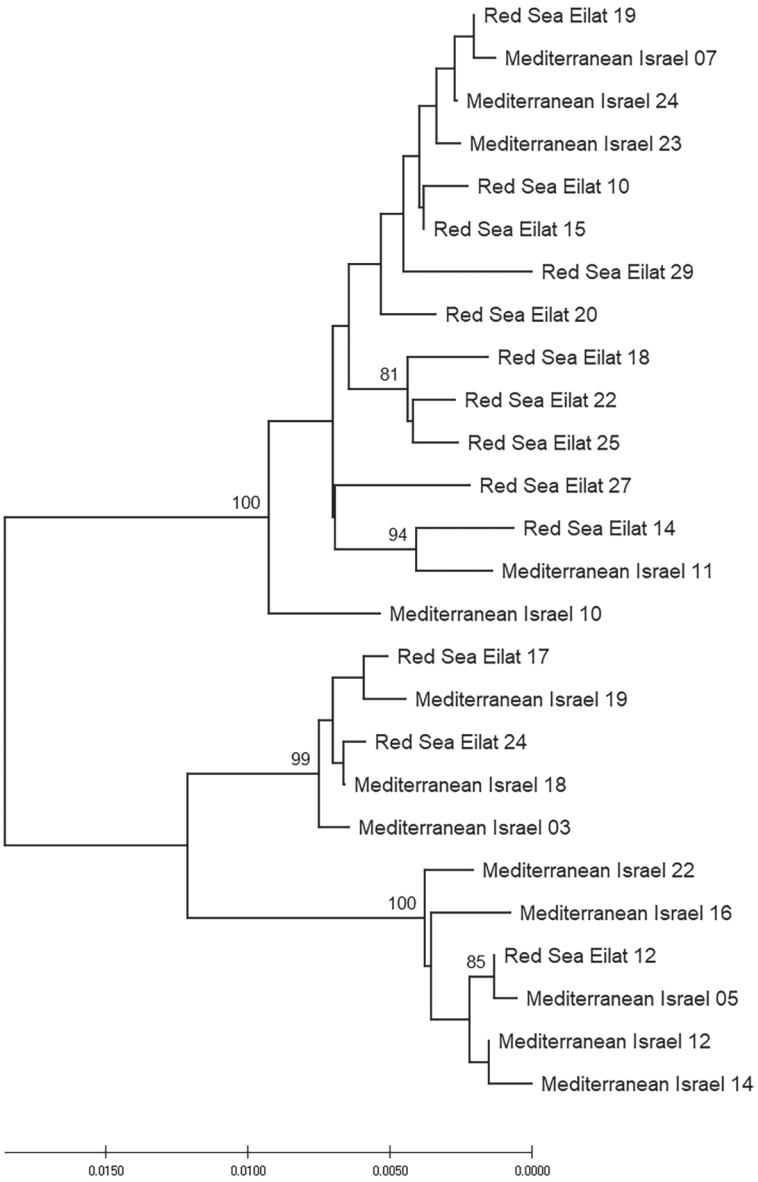

Figure 1. D-loop and COI together: Neighbor-joining phylogenetic tree for *Torquigener flavimaculosus* of our study. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown only if larger than 80.

Discussion

The initial objective of this study was to determine the nature of *Torquigener flavimaculosus* colonization into the Mediterranean Sea. Studying two mitochondrial markers, the cytochrome c oxidase 1 (COI) and the control region (D-loop), we found no evidence of a bottleneck type migration. The genetic variability within the migrant population of the Mediterranean is by no means smaller than that of the source population of the Red Sea.

This finding is compatible with previous studies, e.g., *Upeneus moluccensis* and *U. pori* by Golani and Ritte (1999), *Atherinomorus forskali* by Bucciarelli et al. (2002), and *Siganus luridus* and *S. rivulatus* by Hassan et al. (2003). However, in two Lessepsian migrants, *Fistularia commersoni* and *Nemipterus randalli*, the genetic variability of the Mediterranean population was distinctly lower (Golani et al. 2007; Tikochinski et al. 2019). These authors explained that the difference between these species, as compared to those that did not show genetic reduction

in the colonizing populations, was that they were sampled relatively shortly after their introduction in the new environment, by relatively small number of founder individuals. However, *Torquigener flavimaculosus* was also sampled quite soon after its introduction, but the lack of bottleneck effect may be due to their mode of introduction, by a large number of individuals in the founding group.

Golani (1993) and Golani and Lerner (2007) showed that *Torquigener flavimaculosus* is very abundant in its native range in shallow waters (0.5–1.5 m) on sandy beaches with calm sea. The similar condition in the Suez Canal allowed this species to establish a sustainable population within the Canal, where it was recorded already as *Tetrodon poecilonotus* by Gruvel and Chabanaud (1937) half a century prior to its invasion. This population gradually dispersed northward, eventually entering the Mediterranean with large number of individuals, representing much of the genetic variability of the source population.

It is interesting to note, that in the Mediterranean *Torquigener flavimaculosus* changed its habitat to much deeper waters (20–60 m). The shores of the Mediterranean experience much rougher seas which probably prevent establishing a population in sandy beaches in this region. In the past three decades, over 300 hauls were conducted on the shallow beaches along the Mediterranean coast of Israel with the same experimental beach seine used in the Red Sea, yet not a single specimen of *Torquigener flavimaculosus* was collected (Golani, unpublished).

Surprisingly, we found in our sample that both the native and the colonizing populations are divided into two distinct phylogenetic tree branches, suggesting a division of the sampled population into two clades. This branching into two subpopulations, found in both the source population and the migrant population, raises the question of a possible speciation process. Obviously, the accumulating mutations in the mtDNA are not the cause of speciation – they only serve as markers for the general mutation rate, which can vary greatly in different taxonomic levels. Therefore, we compared the D-loop variation within *T. flavimaculosus* to that of a few other species of the same family. It turned out that genetic variability within our branched *Torquigener flavimaculosus* database is quite high – the mean divergence within each of two different species of *Takifugu*, *T. obscurus* and *T. rubripes*, that were randomly chosen, were significantly lower than the mean divergence within *T. flavimaculosus*, which in turn resembles the total divergence within a group of several *Takifugu* species.

Based on the mitochondrial COI gene, Turan et al. (2017) described eight distinct pufferfish species in the Mediterranean waters of Türkiye. However, their description of *T. flavimaculosus* as a distinct species was based on merely two individuals. Recently, Bilecenoğlu and Yokeş (2022) proposed that *Torquigener flavimaculosus* is a junior synonym of *Torquigener hypselogeneion* (Bleeker, 1852). However, these authors discovered the taxonomic complexity of the genus *Torquigener* and concluded that it is yet to be solved. In our study, *T. flavimaculosus* displays a mean inter-clade COI distance of only 2%. COI is considered as the most common gene for fish barcoding and population analysis. According to Mabragaña et al. (2011), the mean COI divergence within the 125 Argentine fish species of their study is 0.23%, compared to 4.04% within genera. Bagley et al. (2019), who studied the COI divergence of 39 freshwater fish species in Brazil, report a mean distance of 1.3% within species, and 1.8% within genera. In their study of the COI divergence within and among 66 fish species from Zhoushan coastal waters in China, Wang et al. (2023) report a mean intraspecific distance of 0.16% (ranging from 0.00 to 1.86%), compared to a considerably larger mean distance of 16.36% between congeneric species. Hence, we hesitate to define the two clades in our *T. flavimaculosus* study as two distinct operational taxonomic units (OTUs). More research, molecular as well as morphological, should be carried out to clarify the taxonomy of this genus.

Author Contribution

Research conceptualization by DG and YT. Sample design and methodology, investigation and data collection, data analysis and interpretation, writing, review and editing by all authors.

Ethics and permits

With submission of this article the authors have complied with the institutional and/or national policies governing the humane and ethical treatment of the experimental subjects, and that they are willing to share the original data and materials if so requested.

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Supplementary material 1

D-loop: Neighbor-joining phylogenetic tree for *Torquigener flavimaculosus*

Authors: Yaron Tikochinski, Talya Ohana, Uzi Motro, Daniel Golani

Data type: jpg

Explanation note: The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown only if larger than 80.

Link: https://doi.org/10.3391/ai.2024.19.1.113978.suppl1

Supplementary material 2

COI: Neighbor-joining phylogenetic tree for *Torquigener flavimaculosus*

Authors: Yaron Tikochinski, Talya Ohana, Uzi Motro, Daniel Golani

Data type: jpg

Explanation note: The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown only if larger than 80.

Link: https://doi.org/10.3391/ai.2024.19.1.113978.suppl2