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Westernmost record of the diamondback puffer, *Lagocephalus guentheri* (Tetraodontiformes: Tetraodontidae) in the Mediterranean Sea: First record from Greek waters

Short title: First record of *Lagocephalus guentheri* in Greece

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Introduction

The on-going climate change and human pressures in the Mediterranean Sea have led to substantial shifts of the species' communities, and expansion of tropical and subtropical species, particularly along the Eastern Mediterranean continental shelf which is susceptible to intoductions via the Suez Canal (Arndt et al., 2018; Givan et al., 2018). In recent years, an expansion of pufferfish species (family Tetraodontidae) across the Mediterranean has been observed; raising concerns due to the accumulation of a potentially lethal neurotoxin in their tissues, and their potential impacts to the ecosystem, fisheries and economy (Kalogirou, 2013; Giousti et al., 2018).In this respect, carefully identifying the timing of their western advance in the Mediterranean is important, in order to better understand the dynamics of their invasion.

Lagocephalus is the most speciose genus, with five species recorded so far; namely, the native *L. lagocephalus* (Linnaeus, 1758), and four Indo-Pacific Lessepsian immigrants, *L. sceleratus* (Gmelin, 1789), *L. spadiceus* (Richardson, 1845), *L. suezensis* Clark & Gohar, 1953, and L. *guentheri* Miranda Ribeiro, 1915. *Lagocephalus* identification to species level is often ambiguous, leading to misidentifications and difficulties in allowing correct distribution assessments of the species in the Mediterranean. A strong example is the taxonomic issue that occurred with the misidentification of *Lagocephalus guentheri* Miranda Ribeiro, 1915 for its congeneric, *L. spadiceus* (Richardson, 1845). Their subtle distinguishing features led to several Mediterranean records of *L. spadiceus* to be questionable (Vella et al., 2017). The misidentification issue has been addressed by Matsuura et al. (2011), who provided a clear description of the species, and identified morphological differences between the two species that are easy to score. Nevertheless, misidentifications may persist. Studies that utilize combined identification tools are likely to improve taxonomic knowledge and enable correct assessment of the species' spread in the basin (e.g. Farrag et al., 2016; Vella et al., 2017). This report presents the first record of *L. guentheri* in Greece, and the western-most record for the species in the Mediterranean basin.

Materials and Methods

On 5th November 2017, a recreational fisherman caught a specimen of *L. guentheri* off the coasts of Pachi (Attiki) (37°57'22.7''N 23°21'16.9''E; Figure 1) at a depth of 24 m. The specimen was submitted in a citizen-science program and was collected for macroscopic analysis. Morphometric measurements were obtained and the specimen was identified based on macroscopic examination. In addition, DNA was extracted from the sample and the mitochondrial barcode gene CO1 (Cytochrome oxidase c subunit 1) was amplified following published protocols (Bariche *et al.*, 2015). Briefly, the amplification of COI used fish specific primers VF2T1 and VR1dT1 (Ivanova et al., 2007). PCR amplified fragments were sequenced in both directions using the same primers, and then compared with available sequences in GenBank. Phylogenetic reconstructions were performed based on the Neighbour-Joining method generated in R (R Core Team, 2016) with the use of the ape package (Paradis et al., 2004). Genetic distances were calculated using a Kimura 2-parameter method. The maximum likelihood (ML) method has been also used as a second phylogenetic reconstruction approach, as implemented in GARLI (Zwickl 2006). To estimate support for the nodes, 1000 bootstrap replicates were performed and we retained only the values supporting the nodes accounting for more than 50% of the bootstrap replicates.

Results

The specimen was identified as *L. guentheri* based on the morphological characteristics described by Matsuura et al. (2011). Specifically, pectoral fins were pale, while the caudal fin was slightly lunate, and an apparent posterior extension medially that made it appear to be doubly emarginated (Figure 1b); distinguishing it from its closest congener *L. spadiceus.* In addition, the caudal fin of the specimen was almost entirely dark-brown except of dorsal and ventral white tips, whereas in *L. spadiceus*, the ventral one-third of the caudal fin is white. The morphometric measurements collected are presented in Table 1.

The PCR amplification and sequencing of the cytochrome oxydase 1 resulted in a 652bp fragment (GenBank accession number MH277032). A BLAST comparison of this sequence with available sequences in GenBank resulted in a 100% match with both *Lagocephalus spadiceus* and *L. guentheri.* In contrast, a query in BOLD resulted in a 100% positive identification to *L. guentheri.*

Phylogenetic analysis was performed by comparing our sequence to 52 sequences extracted from GenBank (Figure 2). Misidentification of *Lagocephalus* individuals resulting in wrong taxonomic assignments of sequences is rampant. Vella et al. (2017) addressed this question and present convincing evidence as to the proper matches between good taxonomic individuals and their sequences. We therefore also included sequences that are considered as representative of *bona fide* species according to Vella et al. (2017), for *L. lagocephalus*, *L. spadiceus*, and *L. guentheri* from BOLD (boxed in red in Figure 2). Our sequence clustered with 30 GenBank sequence assigned to *L. spadiceus* (18 sequences), *L. guentheri* (10 sequences), and *Dactyloptena orientalis* (2 sequences) (Figure 2). The *bona fide L. guentheri* species belonged to that cluster (although originally labeled *L. spadiceus*).

Discussion

Continuous, directed monitoring and management plan for the detection and abundance monitoring of alien species is imperative as the biodiversity of the Mediterranean basin is changing (Farrag et al., 2016). Inevitably, the addition of new tools such as genetics, enable the accurate taxonomic identification and taxonomic revision of the marine alien species in the Mediterranean as distributions, identifications and nomenclatures are updated. (Zenetos et al., 2017).

Vella et al. (2017) have pointed out that BOLD accession numbers AAD4510 and ADG5739 correspond to *L. spadiceus* and *L. guentheri*, respectively. The morphological analysis of our specimen is consistent with the characteristics of *L. guentheri,* and its COI sequence does cluster with the ADG5739 sequence showing concordance between morphological and genetic assignment of our specimen.

To date, the presence of *L. guentheri* (or individuals previously identified as *L. spadiceus*) remained confined to the Eastern Mediterranean and had not shown signs of westward expansion (Azzurro et al., 2016). Driven by the climate change and the synergistic effects of human pressures, it is possible that the species' dispersal, niche availability and establishment across the Mediterranean will be expanded on the following years. This study reports the westernmost record of the species in the Mediterranean basin. The improvement of taxonomic knowledge and utilization of identification tools that will incorporate morphological and molecular data should be facilitated to avoid common taxonomic issues of the past and monitor species' expansion to forestall any possible latent events.

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Table 1. Morphometric measurements of the *Lagocephalus guentheri* analysed in the present study.

Total Length	23.9 cm
Fork Length	23.3 cm
Standard Length	20.6 cm
Head Length	6.4 cm
Pre-anal length	11.5 cm
Pre-pectoral length	5.7 cm
Body Depth	5.3 cm

Figure legends

Figure 1

A: The red circle indicates the catch reported in this study, and the blue indicate confirmed sightings of *Lagocephalus guentheri* in the Mediterranean Sea; **B:** Specimen analysed in this study.

Figure 2

Neighbour-Joining (NJ) phylogenetic tree based on K2P distances of mitochondrial cytochrome oxidase c subunit 1 sequences of *Lagocephalus* species (and identical general topology was obtained using Maximum Likelihood, ML). Bootstrap support values higher than 50% are shown next to the corresponding nodes and represent the NJ distance and ML value respectively. The sequence generated in this study (LGU Med) is highlighted in red. All other sequences are from GenBank. Three sequences that are considered as bona fide BOLD specimens are boxed in red. *L. spadiceus* and *L. guentheri* clades are boxed in green and yellow, respectively. Species native to the Mediterranean are boxed in blue and Indo-Pacific immigrants in red.