1 **Westernmost record of the diamondback puffer, *Lagocephalus guentheri* (Tetraodontiformes:**

2 **Tetraodontidae) in the Mediterranean Sea: First record from Greek waters**

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

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15 **Introduction**

16 The on-going climate change and human pressures in the Mediterranean Sea have led to substantial

17 shifts of the species' communities, and expansion of tropical and subtropical species, particularly

18 along the Eastern Mediterranean continental shelf which is susceptible to intoductions via the Suez

19 Canal (Arndt et al., 2018; Givan et al., 2018). In recent years, an expansion of pufferfish species

20 (family Tetraodontidae) across the Mediterranean has been observed; raising concerns due to the

21 accumulation of a potentially lethal neurotoxin in their tissues, and their potential impacts to the

22 ecosystem, fisheries and economy (Kalogirou, 2013; Giousti et al., 2018).In this respect, carefully

23 identifying the timing of their western advance in the Mediterranean is important, in order to better

24 understand the dynamics of their invasion.

25 *Lagocephalus* is the most speciose genus, with five species recorded so far; namely, the native *L.*

26 *lagocephalus* (Linnaeus, 1758), and four Indo-Pacific Lessepsian immigrants, *L. sceleratus* (Gmelin,

27 1789), *L. spadiceus* (Richardson, 1845), *L. suezensis* Clark & Gohar, 1953, and L. *guentheri* Miranda

28 Ribeiro, 1915. *Lagocephalus* identification to species level is often ambiguous, leading to

29 misidentifications and difficulties in allowing correct distribution assessments of the species in the

30 Mediterranean. A strong example is the taxonomic issue that occurred with the misidentification of

31 *Lagocephalus guentheri* Miranda Ribeiro, 1915 for its congeneric, *L. spadiceus* (Richardson, 1845).

32 Their subtle distinguishing features led to several Mediterranean records of *L. spadiceus* to be

33   questionable (Vella et al., 2017). The misidentification issue has been addressed by Matsuura et al.

34   (2011), who provided a clear description of the species, and identified morphological differences

35   between the two species that are easy to score. Nevertheless, misidentifications may persist. Studies

36   that utilize combined identification tools are likely to improve taxonomic knowledge and enable

37   correct assessment of the species' spread in the basin (e.g. Farrag et al., 2016; Vella et al., 2017). This

38   report presents the first record of *L. guentheri* in Greece, and the western-most record for the species

39   in the Mediterranean basin.

Materials and Methods

41   On 5th November 2017, a recreational fisherman caught a specimen of *L. guentheri* off the coasts of

42   Pachi (Attiki) (37°57'22.7"N 23°21'16.9"E; Figure 1) at a depth of 24 m. The specimen was submitted

43   in a citizen-science program and was collected for macroscopic analysis. Morphometric

44   measurements were obtained and the specimen was identified based on macroscopic examination. In

45   addition, DNA was extracted from the sample and the mitochondrial barcode gene CO1 (Cytochrome

46   oxidase c subunit 1) was amplified following published protocols (Bariche *et al.*, 2015). Briefly, the

47   amplification of COI used fish specific primers VF2T1 and VR1dT1 (Ivanova et al., 2007). PCR

48   amplified fragments were sequenced in both directions using the same primers, and then compared

49   with available sequences in GenBank. Phylogenetic reconstructions were performed based on the

50   Neighbour-Joining method generated in R (R Core Team, 2016) with the use of the ape package

51   (Paradis et al., 2004). Genetic distances were calculated using a Kimura 2-parameter method. The

52   maximum likelihood (ML) method has been also used as a second phylogenetic reconstruction

53   approach, as implemented in GARLI (Zwickl 2006). To estimate support for the nodes, 1000

54   bootstrap replicates were performed and we retained only the values supporting the nodes accounting

55   for more than 50% of the bootstrap replicates.

Results

57   The specimen was identified as *L. guentheri* based on the morphological characteristics described by

58   Matsuura et al. (2011). Specifically, pectoral fins were pale, while the caudal fin was slightly lunate,

59   and an apparent posterior extension medially that made it appear to be doubly emarginated (Figure

60   1b); distinguishing it from its closest congener *L. spadiceus.* In addition, the caudal fin of the

61   specimen was almost entirely dark-brown except of dorsal and ventral white tips, whereas in *L.*

62   *spadiceus,* the ventral one-third of the caudal fin is white. The morphometric measurements collected

63   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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146   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

147

148 **Figure legends**

149 **Figure 1**

150 **A:** The red circle indicates the catch reported in this study, and the blue indicate confirmed sightings

151 of *Lagocephalus guentheri* in the Mediterranean Sea; **B:** Specimen analysed in this study.

152 **Figure 2**

153 Neighbour-Joining (NJ) phylogenetic tree based on K2P distances of mitochondrial cytochrome

154 oxidase c subunit 1 sequences of *Lagocephalus* species (and identical general topology was obtained

155 using Maximum Likelihood, ML). Bootstrap support values higher than 50% are shown next to the

156 corresponding nodes and represent the NJ distance and ML value respectively. The sequence

157 generated in this study (LGU Med) is highlighted in red. All other sequences are from GenBank.

158 Three sequences that are considered as bona fide BOLD specimens are boxed in red. *L. spadiceus*

159 and *L. guentheri* clades are boxed in green and yellow, respectively. Species native to the

160 Mediterranean are boxed in blue and Indo-Pacific immigrants in red.