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First record of darter goby, *Ctenogobius boleosoma* (Jordan & Gilbert, 1882) (Gobiiformes: Gobiidae), in the Mediterranean with notes on larval morphology and cryptic diversity

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Abstract

Darter goby, *Ctenogobius boleosoma* (Jordan & Gilbert, 1882), is a small euryhaline fish of the Gobiidae family (Gobiiformes). It is naturally found in the shallow marine and brackish waters of the Western Central Atlantic. This study reports the first record of *C. boleosoma* in the Mediterranean Sea. In August and October 2019, two postflexion stage larvae were sampled during ichthyoplankton surveys in Antalya Bay in the Northeastern Mediterranean. The larvae were identified using the barcoding of cytochrome oxidase 1 gene sequence of mitochondrial DNA (COI). The morphology of the pre-transitional stage larvae was described and compared to previously published data. A phylogenetic analysis revealed that two distinct clades of *C. boleosoma* exist in its distribution range. The COI records belonging to one of the clades were generally collected within the Caribbean Sea, while the others were collected primarily in North American Western Atlantic waters from Florida to North Carolina. The average divergence between the clades was calculated to be 9.36 ± 1.34 (±95% confidence interval), which likely indicates the existence of undescribed species. The specimen from the Mediterranean was identified as most closely related to the North American clade. This study shows how the integrative taxonomy of ichthyoplankton can improve knowledge about non-indigenous fish in the Mediterranean.

Keywords: alien species; DNA barcoding; integrative taxonomy; larvalfish; non-indigenous species; phylogeny.

Introduction

Gobiidae (Gobiiformes) is currently the richest fish family, consisting of 258 valid genera and 1957 species (Eschmeyer *et al*., 2021). Gobies inhabit various marine, brackish and freshwater habitats around the world, mostly in tropical and warm-temperate waters (Froese & Pauly, 2020). Gobiidae is also the most diversified fish family in the Mediterranean, where Kovačić (2020) listed 62 native and 11 alien Mediterranean gobies. Although extensive research has been carried out on Mediterranean gobies, knowledge of their taxonomy, phylogeny and distribution is still insufficient (Kovačić, 2020; Kovačić *et al*., 2021 and references therein) due to their high cryptic diversity, the small size of many species, lack of commercial or recreational importance, and also because many Mediterranean gobies inhabit cryptic habitats that are largely ignored in routine monitoring activities (Engin & Seyhan, 2017; Iglésias *et al*., 2021; Kovačić *et al*., 2021; Murdy & Hoese, 2002).

The darter goby, *Ctenogobius boleosoma* (Jordan & Gilbert, 1882) is a small sized euryhaline fish inhabiting shallow, sandy-muddy marine and brackish water habitats (Hildebrand & Cable, 1938) in the tropical and subtropical areas of the Western Central Atlantic from Brazil to North Carolina (Aquamaps, 2019) (Fig. 1). The species spawns in marine waters during summer and autumn months (Wyanski & Targett, 2000). Larval darter gobies are amongst the most dominant species of estuarine and coastal ichthyoplankton in their native range (Castro & Bonecker, 2017; De Macedo-Soares *et al*., 2014; Gomes *et al*., 2014).

So far, no previous record of *C. boleosoma* exists either in the Eastern Atlantic or in the Mediterranean. This study recorded the presence of *C. boleosoma* in the Mediterranean based on two postflexion-stage larvae collected

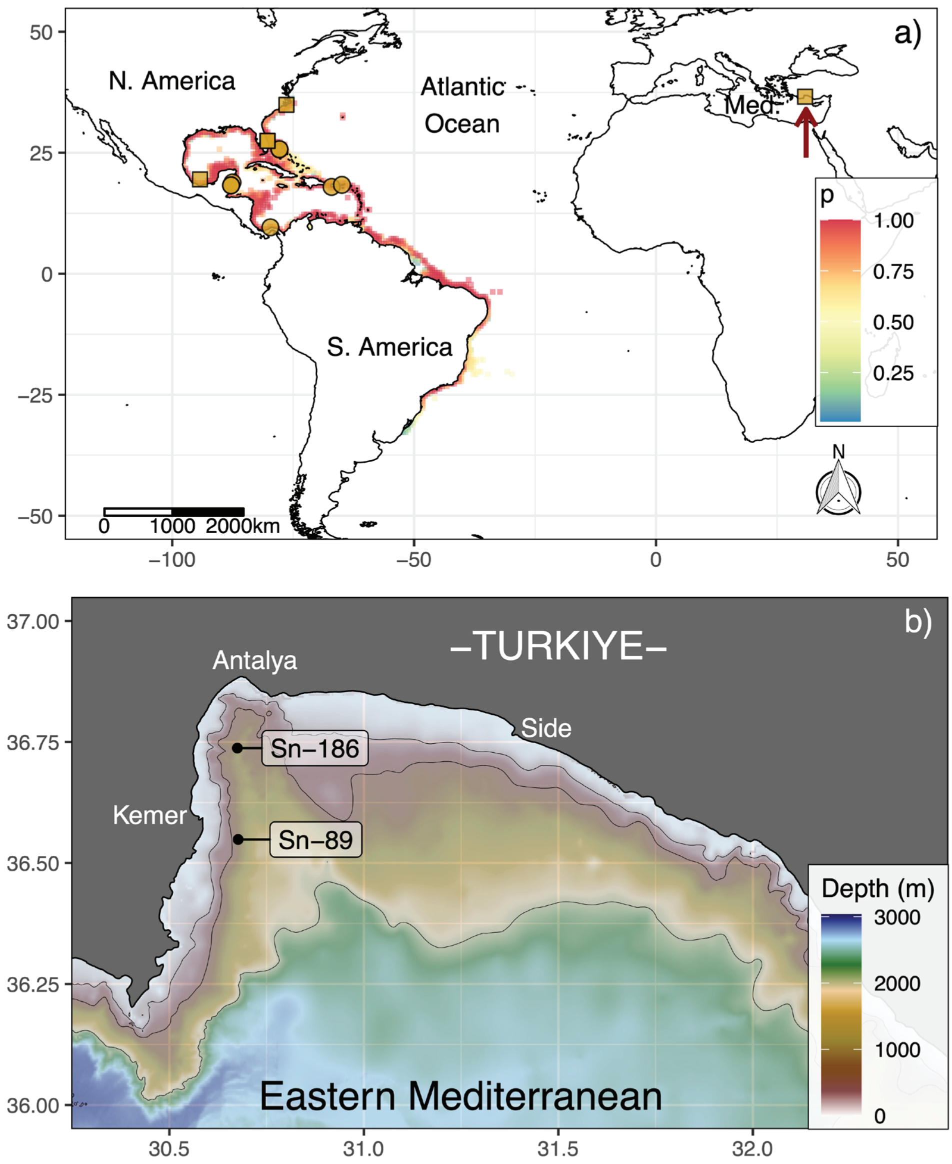

Fig. 1: a) The global distribution area of *Ctenogobius boleosoma* and b) ichthyoplankton sampling stations where larval specimens were collected. a) Tiles are locations where *C. boleosoma* has so far been recorded in Aquamaps (2019). Color of tiles represents probability of occurrence values from Aquamaps (2019). Large squares and circles represent sampling locations of species with COI sequence publicly available in GenBank or in BOLD. Squares belong to the northern clades, and circles belong to the southern clades in the phylogenetic analysis (see Fig. 2). Red arrow shows the sampling location of specimens reported in this study. b) Sampling locations. Sn-89 was registered to GenBank with accession code: OK284603.

during an ichthyoplankton survey in Antalya Bay, Turkiye. One individual was identified using DNA barcoding of the cytochrome oxidase gene region of mitochondrial DNA (COI). The phylogeny of the species was analyzed and the existence of a cryptic species within the distribution range of *C. boleosoma* is discussed. Finally, the morphology of *C. boleosoma* larvae is described and compared to published data.

Material and Methods

Ichthyoplankton samplings were carried out at 26 stations in June 2018 and June 2019, and at nine stations in July, August, September and October in 2019 in Antalya Bay in the Eastern Mediterranean (Fig. 1). Samples were collected with double oblique tows of a Bongo-60 net equipped with 200 and 500 µm meshes (KC-Denmark - 23.300). The temperature and salinity profiles were recorded at each station using a YSI Exo2 multiparameter sonde. The larvae were sorted onboard and fixed in 99.5% ethanol.

On August 23rd and October 22nd of 2019, two postflexion stage larvae of *C. boleosoma* –recorded as Sn89 and Sn186 – were sampled in Antalya Bay at the following coordinates, 36.6°N/30.8°E and 36.8°N/30.7°E, respectively. The water depths were 1150 m and 373 m at the sampling locations, respectively (Fig. 1). The distance of each station from the nearest shore was 9.1 and 2.3 nautical miles and the sea surface temperature was recorded at 29.0 and 25.3°C, respectively.

While one specimen was preserved in the Çukurova

University ichthyoplankton collection (TA_Ant_CB_F1_1_10_2019_Sn186, hereinafter Sn186), the identification of the other (TA_Ant_CB_F1_5_8_2019_Sn89, hereinafter Sn89) was performed using DNA barcoding. To do this, DNA was extracted from the whole flesh employing the phenol–chloroform method. Then the COI gene region of mitochondrial DNA (mtDNA) was amplified using fish-F1 primer (Ward *et al.*, 2005) and DreamTaq DNA polymerase (Thermo Fisher Scientific Baltics, UAB, Vilnius, Lithuania). A 20 µL polymerase chain reaction (PCR) mix was prepared with 2 µL of isolated genomic DNA (29 ng/µL), 5 µL of 10×buffer containing 25 mM of Mg^{2+}, 1 µL of 10 mM deoxynucleotide (dNTP) mix, 1.4 µL of primer (10 pmol/µL), 0.25 µL of Taq DNA polymerase (5 U/µL) and 37.95 µL of nuclease-free water. Thermal cycling was then performed using a Veriti® thermal cycler (Applied Biosystems, Foster City, CA, USA). After a gel electrophoresis investigation, the PCR product was purified using NucleoSpin® gel and a PCR clean-up kit (Macherey Nagel, Düren, Germany). Finally, sequence analysis was performed using the same primer, and the sequence obtained was edited with geneious prime 2021.0.3 software (https://www.geneious.com). Then, this information was uploaded to the GenBank (http://www.ncbi.nlm.nih.gov) and BOLD Identification Engine (https://www.boldsystems.org/index.php) to perform molecular identification. Finally, the specimen was registered to GenBank with the following accession code: OK284603.

For the phylogenetic analyses, publicly available data belonging to the same species and to one out group (*C. stigmaticus*, BOLD ID: LIDM1315-08) were accessed and downloaded from the databases mentioned and aligned with the Clustal Omega program (http://www.clustal.org/omega/). Before the phylogenetic analysis, the best substitution models (HKY+G+T) were determined using the Qiagen CLC Main Workbench 21.0.5 software model testing tool. A PhyML algorithm was used for phylogenetic estimation by using the maximum likelihood method (Guindon & Gascuel, 2003). Trees were constructed using Qiagen CLC Main Workbench 21.0.5 software. Bootstrapping with 1000 replications was performed to assess the reliability of the topology of the phylogenetic tree (Felsenstein, 1985). Clades were determined according to the bootstrap agreement values higher than 90%. Then, within and between groups average similarity values and their bootstrap confidence intervals (500 replications) were calculated using Mega X software (Kumar *et al.,* 2018).

The morphology of the larvae was described following the earlier descriptions of darter goby larvae by Hildebrand & Cable (1938), Wyanski & Targett (2000) and Victor (2015). The larvae were photographed and morphometric measurements were taken using ImageJ V1.53 open source software (Schindelin *et al.,* 2012).

Results

Molecular identification

A total of 45 records of *C. boleosoma* formed a branch above the 90.90% similarity level in BOLD. Two clades were obtained with more than 90% bootstrap agreement in the phylogenetic tree (Fig. 2). The first clade, which had a 99% bootstrap agreement, was formed by specimens distributed mainly in the Caribbean Sea (southern clade); the northernmost records of this group were from the Bahamas. A second clade with a bootstrap agreement value of 96%, consisted of records from Florida to North

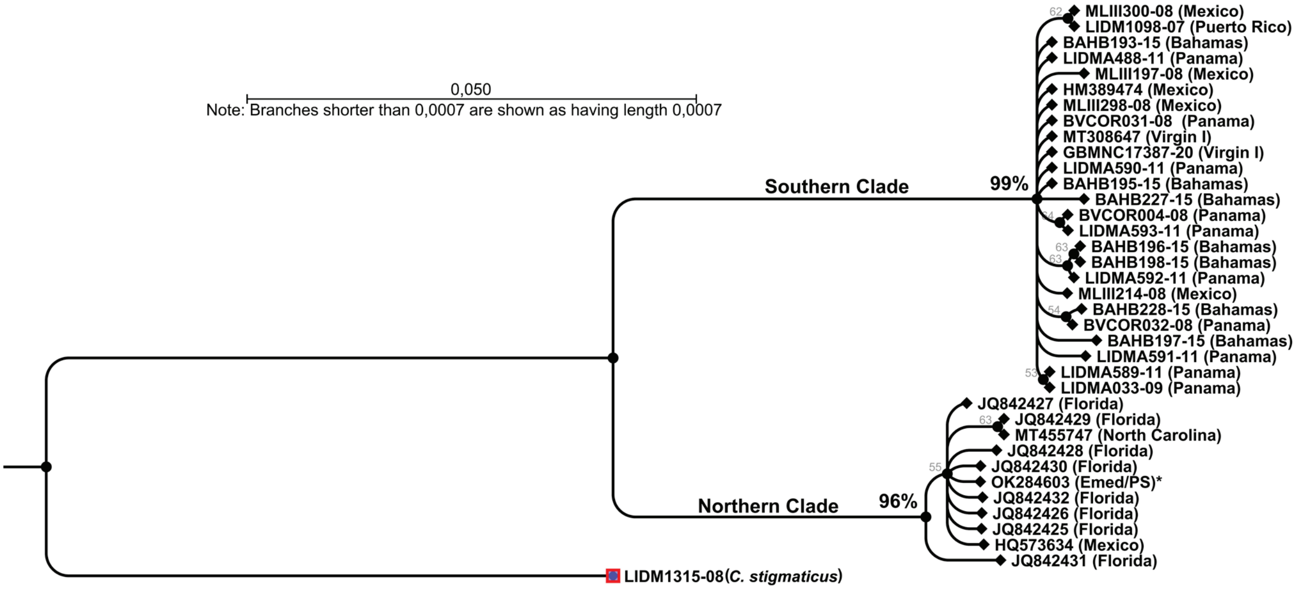

Fig. 2: Phylogenetic tree of *Ctenogobius boleosoma*. The numbers above nodes are bootstrap agreement percentages. * Specimen Sn89 (OK284603), present study. *Ctenogobius stigmaticus* was used as an outgroup.

Carolina (northern clade). Only one specimen of this clade was recorded within the Gulf of Mexico (HQ573634), along the mainland coast distinctly from the southern clade localities. The Mediterranean specimen of this current study (Sn89; OK284603) was also included in the northern clade (Fig. 2), indicating a North American origin. Within-group average divergence values (WD) were calculated to be 0.34 ± 0.09% and 0.35 ± 0.12% for the southern and northern clades, respectively. A deep divergence was detected between clades with an average value of 9.36 ± 1.34%.

Morphological details

The standard lengths (SL) of the two postflexion stage larvae were 4.25 mm for Sn186 and 4.40 mm for Sn89. Both larvae had a prominent gas bladder located anterior to the anus, which is characteristic of gobiids (Ruple, 1984). The body was elongated, with a maximum depth of 15 to 17% of the SL. The anus was slightly posterior to the midbody. The head was moderate, with a depth (HD) of about 48% of head length. The eyes were large and round and cover about 55% of the HD. The mouth was

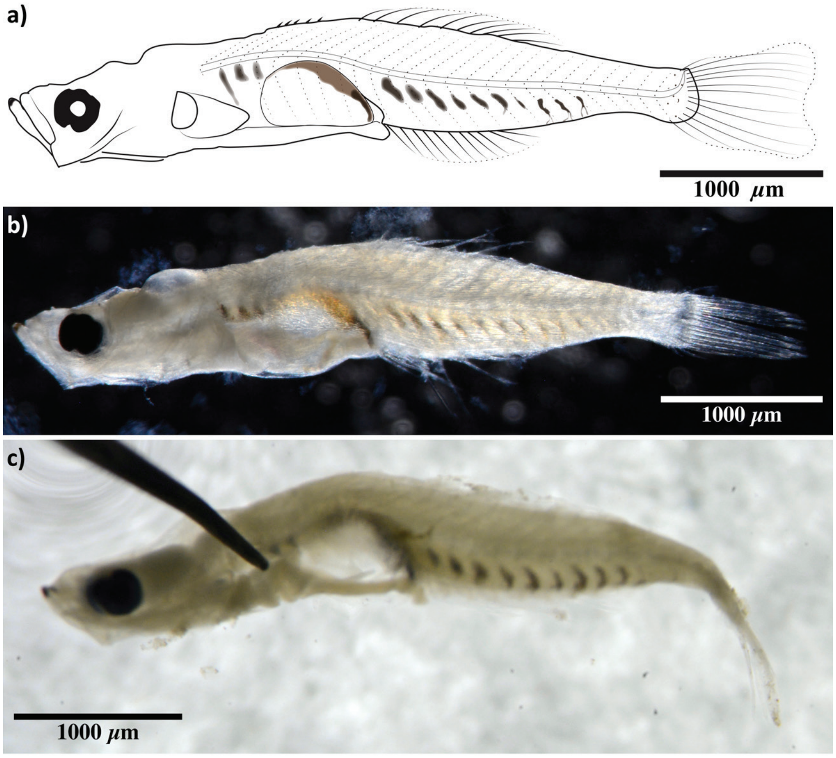

Fig. 3: Schematic drawing of *Ctenogobius boleosoma* (a) and specimens sampled in Antalya Bay (b: Sn186, 4.3 mm SL; c: Sn89, 4.4 mm SL, OK284603; both samples were fixed in 99.5% ethanol).

Table 1. Morphometrics of larval *Ctenogobius boleosoma* (Sn186). The specimen was sampled on 22 October 2019 at 36.8°N, 30.7°E.

	Measurement (mm)	**% of SL**
Standard length	4.25	
Head length	0.92	22
Head width	0.44	10
Head depth	0.53	12
Eye diameter	0.25	6
Predorsal fin length (second dorsal)	2.29	54
Preanal length	2.25	53
Body depth at first dorsal fin spine	0.71	17
Body depth at anus	0.67	16
Body depth at last anal fin ray	0.43	10
Caudal peduncle min depth	0.27	6
Length of anal fin base	1.12	26
Length of second dorsal fin base	1.09	26

oblique, and the maxillary reached the anterior margin of the eye (Fig. 3, Table 1). There were nine preanal and 16 to 17 postanal myomeres (a total of 25 to 26 myomeres). The first dorsal fin and pelvic fin were barely visible in either specimen, while the second dorsal, anal, pectoral and caudal fins were well developed. The formation of the total elements in the unpaired fins were D: V + 10, A: 11, C 7+6 (the upper and lower hypural bones).

Both specimens showed similar pigmentation: the dorsal and posterior surfaces of the gas bladder was covered with crescent-shaped pigmentation, a prominent melanophore on the lower jaw tip, three internal melanophore stripes located anterior to the gas bladder following the myosepta, and a series of nine to 10 internal stripes following the ventrolateral sections of the myosepta in the postanal area, posterior to the gas bladder. The latter pigment row began at the 2nd or 3rd postanal myomeres and ended at the 5th or 6th myomeres before the caudal fin. The last three of these pigments were in a pyramid shape reaching to the ventral body. There was an indistinct pigment stripe posterior to the cleithrum in the abdominal ventral side of one specimen; however, this pigment was not visible in the other specimen. A few small pointed melanophores were irregularly scattered on the caudal peduncle (Fig. 3). Based on the lack of morphological or coloration differences between the two specimens, it was concluded that the two specimens were conspecific.

Discussion

Larval gobies can be distinguished from the larvae of other families by having a slender body, a large and laterally visible gas bladder, a gut that reaches or slightly exceeds the midbody (Ruple, 1984) and the number of myomeres usually ranges from 25 to 30 (Okiyama, 1988). Although the available identification keys for larval gobies are far from being conclusive, some prominent pigmentation patterns appear to be useful for identification at the species level (Maddox, 1992; Okiyama, 1988; Ruple, 1984; Victor, 2015).

Detailed pigmentation patterns of pre-transitional *C. boleosoma* have only been provided by Hildebrand & Cable (1938). Distinguishing characteristics of larvae of the species include the presence of crescent-shaped pigmentation on the dorsal and posterior surfaces of the gas bladder (Hildebrand & Cable, 1938; Ruple, 1984; Wyanski & Targett, 2000), a prominent melanophore at the lower jaw tip (Victor, 2015) and one, or rarely two pyramid shaped internal melanophores anterior to the caudal peduncle (Maddox, 1992; Victor, 2015). The combination of these characteristics was also observed in the specimens in this current study.

The specimens from the Mediterranean had a strong lateral pigmentation, consisting of internal melanophore stripes, which had not been mentioned or illustrated in earlier descriptions by Hildebrand & Cable (1938). This pigmentation pattern can also be seen in several other photos available in the identification keys or databases. For example, Victor (2015) provided photos of a 6.4 mm larva that was identified as *Ctenogobius* sp. that has similar internal melanophore stripes as the specimens in this current study. In addition, photos of two specimens available in BOLD (BOLD id: MFVI507-10; GenBank id: HQ573634 and BOLD id: MFLIV299-09; GenBank id: HM389474) also show stripes on the myosepta. The variation in pigmentation pattern does not appear to be phylogenetically informative, because the specimens under study here phylogenetically belonged to the northern clade along with previously recorded North Carolina specimens. Hildebrand & Cable (1938) also collected their samples from North Carolina, and these samples showed no lateral pigmentation. In addition, one larval specimen in BOLD that had identical pigmentation patterns to the specimens of this current study was assigned to the southern clade (HM389474), while another was assigned to the northern clade (HQ573634). Because the phylogeny does not match, the differences in pigmentation patterns may be caused by ontogenetic factors, environmental conditions, and/or differences in fixation procedures (Wyanski & Targett, 2000). This inconsistency also indicates incomplete knowledge on lateral pigmentation variability in larval descriptions of *C. boleosoma* (Hildebrand & Cable, 1938).

The average divergence between the northern and southern clades was significantly higher than 10-fold within group divergence ($p<0.05$), suggesting that these two clades may be separate species based on the species screening threshold approach of Witt *et al.* (2006). Cryptic diversity is very high among gobies inhabiting the Western Atlantic, where Victor (2014) described three new previously overlooked species using DNA barcoding combined with morphological assessment. Obviously, further investigations of genetic and morphological characteristics of *C. boleosoma* are needed to understand if the nominal species is actually a species complex.

Kovačić (2020) listed the existence of 11 alien gobiid fish species in the Mediterranean Sea. After Kovačić's (2020) checklist has been published, Goren & Stern (2021) described Indo-Pacific *Cryptocentrus steinhardti* as a new alien goby off the Mediterranean coasts of Israel. Recently Özden *et al.* (2022) reported the occurrence of five specimens of the Indo-Pacific *Oxyurichthys keiensis* (Smith, 1938) in Fethiye Bay on the Southern Aegean coasts of Turkiye. With *C. boleosoma*, the number of alien gobies in the Mediterranean increased to 14 species.

The current study could not identify the exact mode of introduction of *C. boleosoma*; however, several potential mechanisms can be suggested. Water gateways, biofouling on ships, ballast transport, sea chests (in ships), aquaculture and the aquarium trade are known as the main pathways for introduction of alien species into the Mediterranean (Katsanevakis *et al*., 2013; Ulman *et al*., 2019).

There is intense maritime traffic in Antalya Bay, and ballast transport therefore seems to be the most likely mode of introduction since darter gobies undergo planktonic larval stages (Hildebrand & Cable, 1938). The aquarium fish trade is another possible route, albeit less likely than ship transport. Despite that, *C. boleosoma* is not an appropriate aquarium fish species (Froese & Pauly, 2020), Darcy (1980) points out that many gobiid species normally appear for sale in ornamental fish markets without known identities.

In this study, the darter goby samples were collected approximately 5000 nautical miles away from the nearest known distribution area of the species (Aquamaps, 2019), the Western Atlantic Ocean. To date, all alien gobies reported in the Mediterranean have been of Indo-Pacific origin (Goren & Stern, 2021; Kovačić, 2020; Özden *et al.*, 2022). Therefore, *C. boleosoma* is the first alien goby of Atlantic origin in the Mediterranean.

Assuming an average water temperature of 25°C, the age of the larger of the two larvae (Fig. 3; 4.40 mm) is about five to six days (Lett *et al*., 2008), and therefore the period is too short for either advection or for ballast water transport into the Mediterranean Sea. Although the population status of darter goby in the Mediterranean is unclear, the presence of two larvae in different months and in different locations strongly suggests the existence of an established and reproducing population. The biology, distribution and status of the darter goby population, as well as its interactions between the indigenous and previously established non-indigenous elements of the Mediterranean biota should be investigated in further studies.

Although the sampling period for Mediterranean specimens was in accordance with the spawning season of the species (Gomes *et al*., 2014; Hildebrand & Cable, 1938), the sampling area could be considered unexpected. *C. boleosoma* is a nearshore species (Hildebrand & Cable, 1938), and its larvae dominate ichthyoplankton assemblages in estuarine and coastal areas of the Western Atlantic Ocean (De Macedo-Soares *et al*., 2014; Gomes & Bonecker, 2014). However, this study encountered the larvae in the offshore waters of Antalya Bay. Hildebrand & Cable (1938) also indicated that larvae of *C. boleosoma* can be carried by currents into offshore waters. Larvae of many coastal species such as *Siganus* spp. and *Nemipterus randalli* have been observed at the same stations (unpublished data) showing that ichthyoplankton dispersal was driven by horizontal advection in the area.

Alien species are increasingly being reported in the Eastern Mediterranean, with approximately one new species detected every 2 to 12 weeks in Turkiye within the last decade (Çinar *et al*., 2021). This frequency increases as research efforts increase and citizen scientists become involved in biodiversity monitoring activities (Zenetos & Galanidi, 2020). However, small-sized species lacking commercial or recreational importance, such as the darter gobies, are generally ignored or overlooked. Therefore, ichthyoplankton studies offer a high potential to uncover the occurrence and distribution of non-indigenous teleost fish species, as most teleost fishes undergo planktonic early life stages (Richards, 2006). On the other hand, identifying early life stages of highly diversified families such as gobies is challenging due to similarities amongst species, and a lack of sufficient information on the morphology and coloration of the species (Ko *et al*., 2013; Ruple, 1984). In this study, this challenge was overcame by using integrative taxonomy that combines morphological data with DNA barcoding (Hebert *et al*., 2003). Previously, Collet *et al.* (2018) showed how DNA barcoding of fish larvae contributes to local biodiversity inventories by revealing the occurrence of nine cryptic species in La Reunion in the Southwest Indian Ocean. Similarly, this study provides another example of how DNA barcoding of larval fish revealed a new record for a non-indigenous species in the tropicalizing Mediterranean. However, it should also be noted that DNA barcoding alone cannot be the answer to the challenge of identifying fish larvae as it also has its own shortcomings, such as discordant molecular assignments (Hubert *et al.,* 2015).

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