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## Bridging the gaps: New records of *Hazeus ingressus* Engin, Larson & Irmak, 2018 from the Eastern Mediterranean with the first morphological description of larvae

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### Abstract

*Hazeus ingressus* Engin, Larson & Irmak, 2018, a new Indo-Pacific goby, was first discovered and described on Türkiye's Mediterranean coasts before being detected in its native location. It may have entered the Mediterranean through the Suez Canal, as a native population was later found in the northern Red Sea. This study provides further reports of *H. ingressus* from İskenderun and Antalya Bay, indicating that the species is also found in the eastern and central parts of the Levant Basin. Samples were obtained from Antalya Bay on July 26, September 27, and İskenderun Bay on August 26, 2021. These reports are based on larval specimens identified through DNA barcoding, highlighting the efficacy of ichthyoplankton samplings as a valuable tool for monitoring non-indigenous fish species in the Eastern Mediterranean—an area prone to biological invasions. Observing larval stages also suggests local spawning, indicating the presence of reproducing populations. Our study provides the first morphological description of *H. ingressus* larvae, which may serve as a valuable reference for future studies.

**Keywords:** alien species; Antalya Bay; Gobiidae; ichthyoplankton; İskenderun Bay; Lessepsian fish; Levant basin; non-indigenous fish.

### Introduction

The family Gobiidae (Gobiiformes) has the highest number of species among fish families globally. Gobiids, comprising 260 valid genera and around 2000 valid species, are found worldwide in various marine, brackish, and freshwater habitats (Fricke *et al.*, 2024; Froese & Pauly, 2024). Kovačić (2020) listed 62 native and 11 non-native goby species in the Mediterranean region. Three years after Kovačić (2020) published the checklist, three additional gobiid species were discovered, bringing the total number of alien gobies in the Mediterranean to 14 (Kovačić & Sacchetti, 2023). Despite extensive research efforts, there are still gaps in our understanding of the taxonomy, phylogeny, and distribution of gobies (Allen & Erdmann, 2021; Kovačić, 2020; Parenti, 2021). Challenges emerge from these species' diverse and cryptic nature, their small size, limited commercial importance, and their affinity for environments typically neglected in regular monitoring programs (Engin *et al.*,

2018; Kovačić, 2020).

*Hazeus* Jordan & Snyder, 1901 is an Indo-Pacific gobiid fish genus of six known species (Fricke *et al.*, 2024). *Hazeus ingressus* Engin, Larson & Irmak, 2018, a new species originally from the Indo-Pacific region, was first recognized and described on the Mediterranean coasts of Türkiye (Engin *et al.*, 2018) before being discovered in its native area. It is believed to have entered the Mediterranean through the Suez Canal, as a native population was later observed in the northern Red Sea (Bogorodsky & Goren, 2023). Engin *et al.* (2018) reported the existence of hundreds of individuals in Fethiye Bay, the only documented distribution location in the Levantine Sea, indicating a possible established population in the Mediterranean Sea. There is a substantial distance between the Suez Canal, where it was most likely introduced, and the location where it was initially discovered and described. This study reports the occurrence of two postflexion larvae from Antalya Bay and one flexion larva from İskenderun Bay, identified using DNA barcod-

ing. These occurrence reports fill a significant gap in the distribution of *H. ingressus* in other parts of the northern Levant Sea.

Because it is a recently described species, very little is known about the biology and ecology of *H. ingressus*. It is a small goby with a currently known maximum standard length of 26.1 mm. It is primarily distributed in shallow vegetated habitats at depths ranging from 5 to 20 meters (Engin *et al.*, 2018). So far, no information exists on its reproduction, larval morphology, and habitat characteristics. This study also includes the first description of the larval morphology of this species, along with details on the spawning period and the environmental conditions in which its larvae were found.

## Material and Methods

### Sample Collection

The samples were collected from two different ichthyoplankton studies conducted in Antalya Bay and İskenderun Bay. The first sampling was conducted at 26 points (11 x 11 nm regular grid) in Antalya Bay in June 2018 and June 2019, and then at 9 stations from July to October 2019. The research vessel R/V Akdeniz Araştırma - 1 (T.R. Ministry of Agriculture and Forestry, Mediterranean Fisheries Research, Production, and Training Institute, 32 m, 2x 788 HP Engines) was used for sampling. The samples were collected by performing double oblique tows (0-100 m) using a Bongo-60 Net equipped with 200 and 500  $\mu\text{m}$  meshes (KC-Denmark - 23.300), calibrated mechanical flowmeters (KC-Denmark - 23.090), and a depth-time tracker (Star Oddi). Temperature and salinity profiles were recorded at each station using a YSI Exo2 multiparameter sonde. Ichthyoplankton samples were preserved in 10% borax buffered formalin for the 200  $\mu\text{m}$  mesh and 96% ethanol for the 500  $\mu\text{m}$  mesh.

The second survey in İskenderun Bay lasted for 13 months, from December 2020 to December 2021, and was carried out at 13 stations (~1 x 2 miles [lat x lon] regular grids) using a 50 hp boat. Samples were collected with a rectangular frame plankton net (40 x 70 cm) equipped with a 500  $\mu\text{m}$  mesh, a mechanical flow meter (HydroBios, 438-110), and a depth-time tracker (Suunto D4). The net was towed obliquely from the bottom or a depth of 30 m to the surface. Each station's salinity and temperature profiles were recorded using a YSI 6600 v2 multiparameter sonde. The samples were preserved in 96% ethanol. In both studies, ethanol samples were kept at -18 °C pending molecular analysis.

### Morphological Identification

Due to the lack of appropriate literature, larval gobies were identified as morphotypes. Identification at the family level was conducted based on the slender body shape, the presence of a large gas bladder, the number of myomeres (between 25 and 30), and the shape and position

of the anus (Alemany, 1997; Okiyama, 1988; Rodríguez *et al.*, 2017). Subsequently, pigmentation patterns and fin development were used for morphotyping (Maddox, 1992; Ruple, 1984; Victor, 2015). After identification, larvae were photographed, and morphometry was measured using ImageJ V1.53 software (Schindelin *et al.*, 2012).

### DNA Barcoding

The cytochrome oxidase 1 gene sequence of mitochondrial DNA (COI) was barcoded to identify the species. One of two specimens from Antalya Bay (TA\_Ant\_HI\_F1\_4\_9\_2019\_Sn165, hereinafter Sn165, GenBank ID: OK287027) and the only specimen from İskenderun Bay (WWF\_Y\_93\_HI\_2021\_Aug\_St1\_Sn93, hereinafter Sn93, GenBank ID: PP469639) were identified using DNA barcoding. The other specimen from Antalya Bay was stored in the ichthyoplankton collection of the ELS-Fish Laboratory at Çukurova University with the following collection code: TA\_Ant\_HI\_F1\_2\_7\_2019\_Sn205 (herein after Sn205).

For DNA barcoding, the entire flesh was used for DNA extraction employing the phenol-chloroform method, the details of which are given in Mavruk *et al.* (2022). The COI gene region of mtDNA was amplified using the fish-F1 primer (Ward *et al.*, 2005) and DreamTaq DNA polymerase (Thermo Fisher Scientific Baltics, UAB, Vilnius, Lithuania). A 20- $\mu\text{L}$  PCR mixture was prepared with 2  $\mu\text{L}$  isolated genomic DNA (29 ng/ $\mu\text{L}$ ), 5  $\mu\text{L}$  10X buffer containing 25 mM Mg<sup>2+</sup>, 1  $\mu\text{L}$  10 mM dNTP mix, 1.4  $\mu\text{L}$  of primer (10 pmol/ $\mu\text{L}$ ), 0.25  $\mu\text{L}$  Taq DNA polymerase (5 U/ $\mu\text{L}$ ), and 37.95  $\mu\text{L}$  nuclease-free water. Then, using a Veriti® thermal cycler (Applied Biosystems, Foster City, CA, USA), thermal cycling was performed. After gel electrophoresis examination, the PCR product was purified with the NucleoSpin® gel and PCR purification kit (Macherey Nagel, Düren, Germany). Sanger sequence analysis with the fish F1 primer was performed. The obtained sequence was edited with 4Peaks v1.8 software (A. Griekspoor and Tom Groothuis, <https://nucleobytes.com>). Then, using the edited COI sequence, we performed a nucleotide BLAST (<http://www.ncbi.nlm.nih.gov>) and BOLD identification engine search (<https://www.boldsystems.org>) for molecular identification. Finally, specimen Sn165 was registered in GenBank with the following accession code: OK287027 and Sn93 with PP469639.

For the phylogenetic analyses, publicly available data of *H. ingressus* (Engin *et al.*, 2018) and an outgroup [Hazeus sp.; GenBank ID: OL512817.1; (Huang *et al.*, 2022)] were downloaded from GenBank and aligned using the Clustal algorithm. Then, the nearest neighbor trees were constructed with 500 bootstrap resamplings (Felsenstein, 1985) using MEGA X v11 software (Kumar *et al.*, 2018).

## Results

### Occurrence Records

During the sampling in Antalya Bay, two gobiid specimens at the postflexion stage were identified as a morphotype named Gobiidae sp. L3 due to their identical morphology, pigmentation patterns, and early emergence of the pelvic fins. Subsequent DNA barcoding analysis revealed that the morphotype was *H. ingressus*. The specimens were collected on July 26, 2019, and September 27, 2019 (Fig. 1, Table 1). Another *H. ingressus* larva was sampled in İskenderun Bay, near Yumurtalık Bight, on August 26, 2021, and was likewise identified by DNA

barcoding. The İskenderun Bay sample was at the flexion stage. Therefore, larval specimens were collected from July to September, with sea surface temperatures varying from 27.9 to 29.4 °C at the locations where *H. ingressus* larvae were detected (Fig. 1, Table 1).

### Phylogeny

The phylogenetic analysis showed that all *H. ingressus* samples were grouped, with an average within-group divergence value of  $0.0025 \pm 0.0015$  ( $\pm$  bootstrapped confidence intervals). *H. ingressus* samples were separated from the outgroup, leaving a distinct barcode gap. The



**Fig. 1:** Known and updated distribution of *Hazeus ingressus* in the Eastern Mediterranean. The red rectangle shows Fethiye Bay (FB), where Engin *et al.* (2018) described the species for the first time. The samples of the present study are shown with green spots in Antalya Bay (AB) and İskenderun Bay (IB). The most likely scenario on the introduction vector through Suez Canal and on the Mediterranean dispersal route is shown with red arrows.

**Table 1.** GenBank accession codes, sampling dates and locations, and the environmental conditions where *Hazeus ingressus* larvae were observed. Temperature and salinity values represent the average of the surface mixed layer.

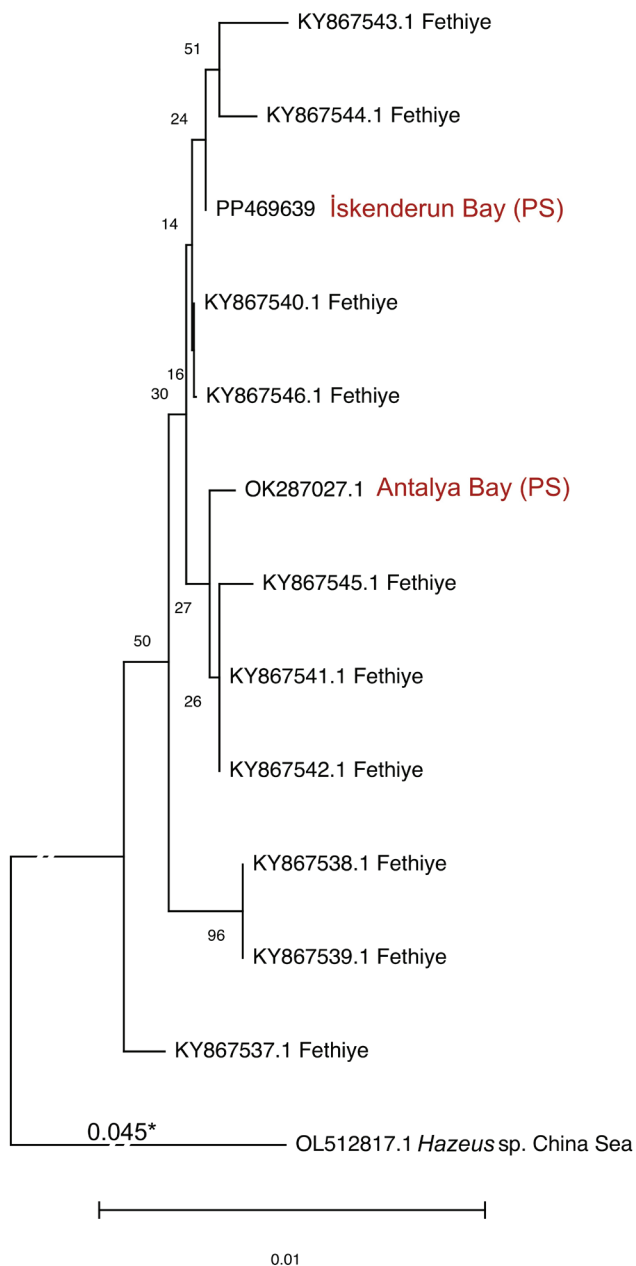
Specimen	Sn205	Sn165	Sn93
GenBank ID	-	OK287027	PP469639
Sampling Date	26.07.2019	27.09.2019	26.08.2021
Sampling Location	36.743° N, 30.773° E	36.823° N, 30.757° E	36.744° N, 35.790° E
Temperature (°C)	28.69	27.87	29.39
Salinity (psu)	39.41	40.16	-
Bottom Depth (m)	306	424	25



average divergence between *H. ingressus* samples, and the outgroup was determined to be  $0.0893 \pm 0.0356$ . The Fethiye, Antalya, and İskenderun Bay samples did not show any significant differences, and the bootstrap agreement values were mainly below 50%, indicating that the branches were randomly formed within the *H. ingressus* samples available (Fig. 2).

### Description of Larvae

The larva collected from İskenderun Bay (Sn93) was at flexion stage, whereas two specimens from Antalya Bay were at postflexion stage (Sn205 and Sn165). All



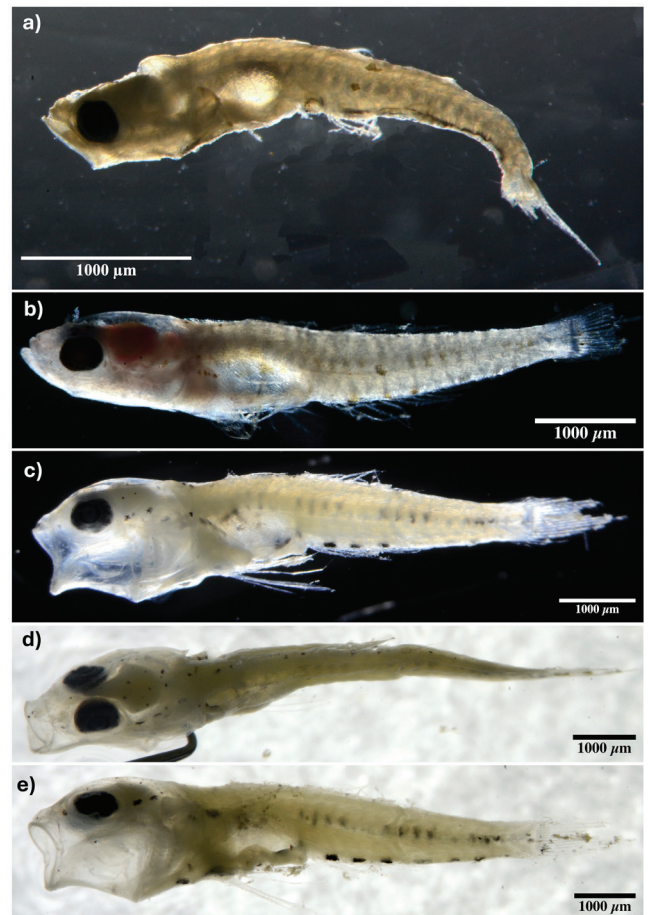
**Fig. 2:** Phylogenetic tree of *Hazeus ingressus* based on nearest neighbor method (The number above nodes are bootstrap agreement percentages. (PS: Samples of the present study. *Hazeus* sp. (OL512817.1) specimen from the south China Sea was used as an outgroup, \*: The original length of the branch is 0.045, but it was reduced to fit within the plot.))

specimens have a large gas bladder in the dorsal portion of the abdominal cavity (Fig. 3).

Description of Sn93 at the flexion stage: body elongated, maximum depth 17% of standard length (SL) (3.2 mm); anus mid-body; head moderate, head length (HL) 26% of SL; eyes round, equal vertical and horizontal diameters, 30% of HL (Table 2); 26 myomeres; anal and dorsal fin knobs visible; pelvic cup absent.

Pigmentation of Sn93: no head, lateral, or dorsal body pigmentation; no pigmentation on caudal peduncle or hypural area; small punctate and stellate melanophores on cleithrum, anterior ventral abdomen, and dorsal gas bladder surface; large branched melanophore on anus; paired dashed row of melanophores along anal fin knob; single row of extensively branched melanophores along medioventral tail line after anal fin, with first anterior melanophore larger than subsequent ones (Fig. 3a).

Description of Sn205 and Sn165: postflexion stage, SL 5.5 mm, and 6.5 mm, respectively (Table 2). Anus medioventral in Sn205 (Fig. 3b), slightly shifted backward in Sn165 (Fig. 3c). Body elongated, moderate head; eyes slightly compressed transversally; myomeres: 10 preanal + 16 caudal. Caudal fin segmented ray formation is complete in both specimens. Fin meristics for Sn205 are as follows: D1: VI, D2: I+8, A: I+9, P: 17, C: 8+8 (upper



**Fig. 3:** Larval *Hazeus ingressus* from İskenderun Bay (a: Sn93, 3.2 mm SL) and Antalya Bay (b: Sn205, 5.5 mm; c, d, and e: Sn165, 6.5 mm). Panels d and e show the dorsal and ventral perspectives of Sn165. Photo credits: Sinan Mavruk.

**Table 2.** Metric characteristics of postlarval *Hazeus ingressus* specimens from İskenderun Bay (Sn93) and Antalya Bay (Sn205, Sn165).

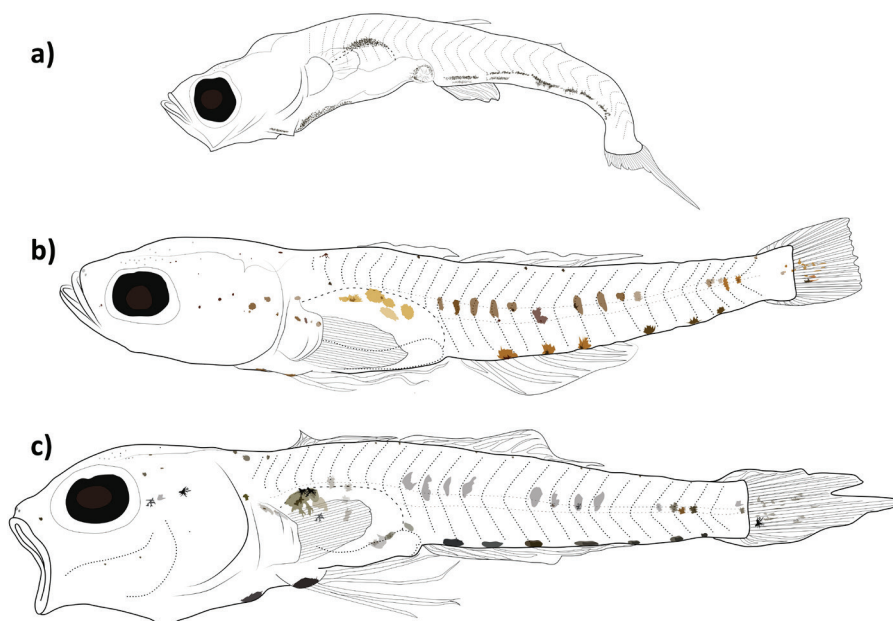
		Sn93	Sn205	Sn165
<b>Standard Length (mm)</b>		3.2	5.5	6.5
<b>Head Length</b>	% of SL	26	29	32
<b>Head Width</b>	% of SL	-	15	24
<b>Head Depth</b>	% of SL	16	17	-
<b>Vertical Eye Diameter</b>	% of HL	29	19	19
<b>Horizontal Eye Diameter</b>	% of HL	30	28	26
<b>Predorsal (second) Fin Length</b>	% of SL	-	53	55
<b>Preanal Length</b>	% of SL	52	50	55
<b>Body Depth at First Dorsal Fin Spine</b>	% of SL	17*	17	20
<b>Body Depth at Last Anal Fin Ray</b>	% of SL	-	10	11
<b>Caudal Peduncle Minimum Depth</b>	% of SL	-	7	8
<b>Anal Fin Base Length</b>	% of SL	-	22	23
<b>Second Dorsal Fin Base Length</b>	% of SL	-	23	23

\*: The maximum body depth was measured because the formation of the dorsal fin was not yet complete. Vertical and horizontal eye diameters represent the longest and shortest axes of the eye in postflexion larvae.

and lower hypural bones' segmented fin rays). Sn205 has 6 superior and 6 inferior procurrent rays, while Sn165 has 8 superior and 6 inferior procurrent rays. Pelvic cup well-developed, extending beyond anus, although marginal shape unclear due to sampling or fixation damage.

Pigmentation of Sn205 and 165: moderate on dorsal, lateral, and ventral sides (Fig. 4). Small spots randomly scattered on occipital; one (Sn165) or two (Sn205) punctate (Sn205) or satellite (Sn165) melanophores on antorbital; three punctate (Sn205) or satellite (Sn165) melanophores in a transverse series on postorbital; trian-

gular group of melanophores dorsally on snout; two large melanophores anterior and posterior to cleithrum (barely visible on Sn205, more prominent on Sn165); 6 (Sn205) to 9 (Sn165) punctate melanophores irregularly spaced on dorsal body surface from just behind head to caudal peduncle; four large stellate melanophores on dorsal surface of pectoral fin base; group of melanophores on anterior surface of gas bladder (Fig. 4). Large prominent melanophore mediolaterally on second preanal myomere of Sn205, absent in Sn165. Notochordal melanophores form three prominent pigment groups on lateral surface



**Fig. 4:** Schematic drawings of larval *Hazeus ingressus* from İskenderun Bay (a: Sn93, 3.2 mm SL) and Antalya Bay (b: Sn205, 5.5 mm; c: Sn165, 6.5 mm). Drawings by Tahani Chargui.

of postanal body in both specimens, with additional smaller internal melanophores between these groups. Two (Sn205) or three (Sn165) mediolateral satellite melanophores on caudal peduncle and caudal fin; one large satellite melanophore ventrolaterally on caudal fin. Six melanophores in a row ventrally on postanal body surface; first three proximal ones form a double row on both sides of anal fin base in Sn205. These melanophores are more prominent and united, forming a single row, with an additional punctate melanophore on the caudal peduncle in Sn165 (Fig. 3a, 4a).

## Discussion

*Hazeus ingressus* is an Indo-Pacific fish species that most likely entered the Mediterranean Sea via the Suez Canal (Engin *et al.*, 2018). The species was first described in Fethiye Bay on the northwestern Levantine coast of the Mediterranean by Engin *et al.* (2018) and then confirmed to be present in the northern Red Sea by Bogorodsky & Goren (2023). Before this study, it was only documented in these two locations, leaving a significant geographical gap. Here, we document the presence of *H. ingressus* in Antalya and İskenderun Bay in the central and eastern parts of the northern Levant Basin, addressing an apparent lack of information regarding the distribution of this species.

According to Golani *et al.* (2017), a non-indigenous fish population can be considered established if there are at least three independent records from different locations or periods. In this study, we report the presence of *H. ingressus* from two distinct surveys conducted in different areas and years. Previously, Engin *et al.* (2018) documented hundreds of individuals in Fethiye Bay, indicating an abundant population in that area. Although the number of individuals in our study was limited, the discovery of larval specimens suggests spawning activity near the sampling sites (Clavel-Henry *et al.*, 2021), which implies local reproduction in Antalya and İskenderun Bay. These findings collectively suggest that the species has established populations in the Eastern Mediterranean. Finding reproducing populations of this species along the northern Levant coasts is not unexpected. Because there is substantial evidence that after entering the Mediterranean Sea via the Suez Canal, the principal path taken by many Lessepsian species usually follows the eastern and northern coastlines of the Levant Basin (Azzurro *et al.*, 2022; Mavruk & Avsar, 2008).

*H. ingressus*, a small coastal fish like many gobies, has lack of commercial or recreational value (Froese & Pauly, 2024). Such species are quite likely to be overlooked in conventional biodiversity studies that focus on adult fish (Kovačić, 2020). However, despite laying demersal eggs, gobies have planktonic larval stages (Richards, 2006) that are more likely to be detected during ichthyoplankton samplings in pelagic habitats. But, identifying larvae is difficult due to morphological similarities and scarce literature, especially in highly diverse families such as gobies (Ruple, 1984). In this study,

this challenge was tackled by utilizing DNA barcoding (Hebert *et al.*, 2003). Previously, Mavruk *et al.* (2022) demonstrated the usefulness of this method in detecting alien gobies in the Mediterranean by presenting the first report of *Ctenogobius boleosoma* based on larval specimens. The existence of this species which is native to the West Atlantic, has later been confirmed in the Ionian Sea (Kovačić & Sacchetti, 2023). Similarly, Dulčić *et al.* (2022) reported the presence of *Buenia massutii*, a small goby, for the first time in the Adriatic Sea by identifying larval specimens with DNA barcoding. This study, along with earlier ones, obviously shows that, ichthyoplankton surveys are highly valuable for detecting and monitoring cryptic non-indigenous fish species in the Mediterranean, known as the most invaded marine ecosystem worldwide (Edelist *et al.*, 2013).

One important obstacle in using ichthyoplankton data for monitoring marine fish biodiversity is the potential advection of the planktonic stage (Mavruk *et al.*, 2023). Pelagic propagules can drift away from the places where the reproducing population resides, and the drift distance can be up to hundreds of kilometers under some circumstances (Kinlan & Gaines, 2003). However, there are several factors that limit the maximum potential advection distance of the larvae. First, gobies produce precocial demersal eggs in which only the postlarval period is planktonic (Ruple, 1984). Additionally, *H. ingressus* larvae were observed during the warmest season and it is well documented that high temperatures reduce drift distance by accelerating growth and reducing the duration of the larval stage in fish (O'Connor *et al.*, 2007). Furthermore, both samples were collected from bays that can locally retain ichthyoplankton (Houde, 2002). Previously, Mavruk *et al.* (2018) demonstrated that the sea breeze in İskenderun Bay forms a closed circulation system consisting of two mesoscale eddies. The propagules of coastal fish in İskenderun Bay are retained by this system, which influences the composition of larval fish assemblages. Similarly, Mavruk *et al.* (2023) used particle backtracking simulations to analyze the local retention capabilities in Antalya Bay. They found that the average linear drift distance is 48 km in 30 days due to the presence of mesoscale eddies in the local circulation system. Therefore, while the larvae sampling sites may not precisely indicate the spawning location and distribution of adult fish, ichthyoplankton samples can offer a reliable estimate of this, especially in enclosed and semi-enclosed coastal areas.

Understanding reproduction is important for managing the spread and potential invasion of an alien species (Sakai *et al.*, 2001). Ichthyoplankton data can offer vital insights on fish reproduction. In our study, *H. ingressus* larvae were collected in July and September during the June to October sampling period, as well as in August during the year-round sampling period, implying that the species reproduces during the summer, the warmest time of year. However, we could not ascertain the timing and duration of the peak spawning season.

In this study, we adhered to the recommended best practices with five specified criteria for reporting new fish



species records (Bello *et al.*, 2014), which were adapted for DNA barcoding identification. A comprehensive morphological description of the material was provided, along with images of each individual. The remaining specimen was then catalogued and stored in the collection. However, due to a lack of comparable data for most Mediterranean and Red Sea gobiid larvae, species-level morphological identification or diagnosis was not possible. In general, information regarding the morphology of larval gobies is scarce because the early life stages of many remain unidentified (Maddox, 1992). The lack of data is evident also in the Mediterranean region, where the gobiid fish species are continuously increasing due to newly identified species and new occurrences of non-native fishes (Kovačić, 2020). In such groups with limited knowledge, DNA barcoding can be an efficient tool for describing the morphological characteristics of the larval stage (Mavruk *et al.*, 2022).

Using DNA barcoding, this study provides the first larval description for *H. ingressus*. During the flexion stage, our specimen's morphology is similar to that of numerous gobies (Ruple, 1984), with a large gas bladder and a large expanded melanophore present on the ventral side of the tail. Thus, using morphological characteristics to distinguish *H. ingressus* is inappropriate for flexion larvae. During the postflexion stage, the morphology becomes more specific, suggesting that the postflexion stages of this species may be distinguished from other gobies, especially by pigmentation patterns and the early emergence of the pelvic cup. However, caution should be applied when using pigmentation patterns for identification, as we observed differences in the shape (punctate or stellate) and the number of melanophores within the postflexion stage larvae. These differences may be attributable to the varied sizes of the larvae (Borges *et al.*, 2003) or could reflect within-species variability (Gray *et al.*, 2006). So far, only a limited number of gobies have been described during their larval stage (Alemany, 1997; Russell, 1976). Currently, attaining precise morphological identification by describing all unique characteristics may be challenging. However, it offers a potential direction for future studies focused on developing an identification guide for larval gobies in the Mediterranean.

In conclusion, cryptic non-indigenous species may quietly establish themselves in new habitats, evading immediate detection due to their inconspicuous appearance or subtle ecological impacts. Unlike high-profile invasive species that attract attention, such species may operate under the radar, influencing the community structure and ecosystem resilience. Recent studies have highlighted the prevalence of cryptic diversity, suggesting that many species remain undiscovered despite prominent efforts (Katsanevakis *et al.*, 2020). The discovery of cryptic non-natives, such as the recent identification of *H. ingressus* in Antalya and İskenderun Bay, underscores the need for continued exploration and monitoring to unravel the existence of such species. Monitoring ichthyoplankton in semi-enclosed environments like bays and gulfs can improve biodiversity assessments by revealing such overlooked species (Dulčić *et al.*, 2022; Mavruk *et al.*, 2022, 2023).

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