

# An integrative study of morphological and molecular characterization of *Diplectanum aequans* (Diplectanidae: Monogenea) infecting European sea bass *Dicentrarchus labrax* (Linnaeus, 1758) from Turkish coasts

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## ABSTRACT

The diplectanid monogenean *Diplectanum aequans* (*D. aequans*) (Wegener, 1857) is one of the most important ectoparasites in the wild and cultured European sea bass *Dicentrarchus labrax* (*Dic. labrax*) (Linnaeus, 1758) worldwide. Presently, there is no information on the molecular data of *D. aequans* from Turkish coasts. In the present study, diplectanid monogeneans were collected from wild and cultured *Dic. labrax* in the Black Sea (FAO area 37.4.2) and Aegean Sea (FAO area 37.1.3) coasts of Türkiye, morphologically identified, and genetically characterized by sequencing of 28S rRNA and 18S rRNA fragments. The collected diplectanids were unambiguously identified as *D. aequans* based on detailed morphological features and sequence characterization of partial 28S rRNA and 18S rRNA genes. The overall prevalence and mean intensity of *D. aequans* were 100% and 15.1, respectively. This study reports first information of molecular (28S and 18S rRNA) evidence of *D. aequans* from *Dic. labrax* in the Turkish coasts. The new 18S and 28S rRNA sequences for *D. aequans* isolated from the Black Sea and Aegean Sea are genetically characterized. The 28S and 18S rRNA sequences of *D. aequans* can be used to resolve the phylogenetic positions of species found in the family Diplectanidae from the Black and Mediterranean Sea.

## Introduction

The European sea bass, *Dicentrarchus labrax*, is the most commercially important fish species widely captured and cultured in the Mediterranean and the Black Sea. The larger producers of European sea bass are Türkiye, Greece, Italy, Spain, and Egypt in the Mediterranean countries, and Türkiye is a leader producer (9, 20). Monogeneans are common ectoparasitic flatworms of marine fish. The taxonomy of the monogenean family Diplectanidae (Platyhelminthes: Monogenea) comprises approximately 20 genera and >200 described species that attach to the gills of a wide diversity of marine fish (40).

Two nominal species of the Diplectanidae Monticelli, 1903 were described from the gills of European sea bass: *Diplectanum aequans* (Wegener, 1857) Diesing, 1858 and *D. laubieri* Lambert and Maillard, 1974 (18, 21), and exhibit strict host specificity (33). These two diplectanid species cause severe pathological damages in the gills and often death of the infected European sea bass (7, 10, 22, 35). Two diplectanids have also been reported in wild and cultured populations of European sea bass from the eastern Atlantic Ocean, English Channel, Aegean, Adriatic, Black, Mediterranean, and Red Sea (22, 35).

The ITS region, 18S, and 28S rRNA have been proven to be particularly useful for molecular characterization and the accurate identification of Diplectanidae species (4, 5, 25, 32). Although *D. aequans* has a wide geographic distribution, there has been limited information on its genetic diversity with only one publication from the Balearic Sea (Western Mediterranean Sea, 80 FAO area 37.1.1) (32). There had been no reports of characterizing the *D. aequans* from *Dic. labrax* using partial 28S and 18S gene fragments in the Black Sea (FAO area 37.4.2) and Aegean Sea (FAO area 37.1.3).

Our study aimed to gain new knowledge about the prevalence, intensity, and genetic diversity of *D. aequans* in wild and cultured European sea bass in the Black Sea and the Aegean Sea coasts of Türkiye using a combination of morphological and molecular analyses.

## Materials and Methods

**Sampling:** A total of 40 wild and cultured European sea bass, *Dic. labrax*, were sampled from the Black Sea and Aegean Sea coasts of Türkiye. All fresh fish samples were purchased from fish markets and immediately transferred to the laboratory for parasitological examinations. Gills were removed and placed in 0.9 % saline solution and examined for diplectanid monogeneans under a stereomicroscope. Monogenean parasites were collected, counted, and preserved in 70 % ethanol. All specimens were individually mounted on slides in glycerol-gelatine and, then identified using morphological characters under a light microscope according to taxonomic keys (10, 18, 21, 26). Morphologically identified *D. aequans* were also measured, photographed using a light microscope with Leica application suite software and Leica MC 190 HD digital camera, and stored in 96 % ethanol until DNA extractions. Five representatives (3 diplectanids; two from cultured and one from wild population from the Black Sea and 2 diplectanids; one from cultured and one from wild population from the Aegean Sea) were subjected to molecular analysis. Prevalence (P) and mean intensity (ml) were calculated according to Rózsa et al. (24) by using Quantitative Parasitology software (23).

**PCR assays, DNA sequencing and phylogenetic analyses:** Total genomic DNA (gDNA) was extracted from individual diplectanids using a commercial genomic DNA extraction kit (GeneJET Genomic DNA Purification Kit, Thermo Scientific, Lithuania EU) following the manufacturer's instructions. Concentration of extracted gDNA was measured using a spectrophotometer (Thermo Scientific, USA) at 260 nm, diluted to 10–50 ng/μl in TE buffer, and stored at –20 °C. The partial large subunit ribosomal RNA (28S rRNA) gene was amplified using the primers C1 (5'–ACCCGCTGAATTTAAGCAT–3') and D2 (5'–TGGTCCGTGTTTCAAGAC–3') (12). PCR reaction was performed in 50 μl volumes that contained

10–50 ng gDNA, 1X PCR Buffer with KCl (Thermo Scientific), 1.5 mM of MgCl<sub>2</sub> (Thermo Scientific), 200 μM of each dNTP (Thermo Scientific), 0.5 μM each of primers, 1.5 U of Taq DNA polymerase (Thermo Scientific), and nuclease-free water (Thermo Scientific). Cycle conditions were initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 60 s, 56 °C for 60 s, 72 °C for 60 s, and a final extension at 72 °C for 10 min (12). The partial small subunit ribosomal RNA (18S rRNA) with internal transcribed spacer 1 (ITS1) region was amplified using L7 (5'–TGATTTGTCTGGTTT ATTCCGAT–3') and IR8 (5'–GCTAGCTGCGTTCTT CATCGA–3') primer pairs (27, 31). PCR reaction was performed in a final volume of 50 μl containing 10–50 ng gDNA, 1X PCR Buffer with KCl (Thermo Scientific), 1.5 mM of MgCl<sub>2</sub> (Thermo Scientific), 200 μM of each dNTP (Thermo Scientific), 0.5 μM each of primers, 1.5 U of Taq DNA polymerase (Thermo Scientific), and nuclease-free water (Thermo Scientific). Reaction conditions were as follows: 4 min at 95 °C, then 35 cycles of 60 s at 92 °C, 60 s at 53 °C and 90 s at 72 °C followed by a final elongation of 10 min at 72 °C (27, 31). PCR products were visualized using a 1.5% agarose gel, and purified using a commercial kit (GeneJET PCR Purification Kit, Thermo Scientific). Purified products were bidirectionally sequenced with the same primers using an ABI PRISM 3130xl by Macrogen Company.

The quality of the sequences was checked with Phred scores (Q ≥ 20), and sequences were assembled using Geneious R11 (16). The consensus sequences were blasted in GenBank for species identification (1). Subsequently, previously published sequences for species belonging to the Diplectanidae were selected from GenBank. Consensus sequences were aligned with those of Diplectanidae sequences using ClustalW (29) within MEGA X (17). Alignments were cleaned from ambiguous positions using Gblocks Version 0.91b (3). After editing the partial 28S and 18S rRNA with ITS1 region sequence alignments using Gblocks comprised 880 bases (90 % of the original 973 bases) and 402 bases (87 % of the original 457 bases), respectively. Pairwise estimates of evolutionary divergence (p–distance) between trimmed, aligned sequences were calculated as the percentage using the Kimura two-parameter model using uniform rates and a partial deletion of 95 % in MEGA X (17). All positions with less than 95 % site coverage were eliminated. That is, fewer than 5 % alignment gaps, missing data, and ambiguous bases were allowed at any position.

As there are more 28S rRNA sequences for *Diplectanum* specimens in the GenBank, we used only partial 28S rRNA sequences for the phylogenetic tree. There were a total of 804 positions in the final sequence dataset. Maximum-likelihood (ML) phylogenetic analysis was performed with PhyML (11) with automatic model selection using the AIC criterion (19) with topology

assessed by bootstrapping with 100 replicates. We used a GTR + I + G model and *Tetrancistrum* sp. and *Cichlidogyrus* sp. as outgroups to do the ML analysis. Only bootstrap values above 70 were considered well supported (13).

## Result

The P (%), mI and number of *Diplectanum* spp. infecting cultured *Dic. labrax* from the Black and Aegean Sea were 100 %, 26.40 and 264, and 100 %, 11.1 and 110, respectively. While the P (%), mI and number of *Diplectanum* spp. infecting wild *Dic. labrax* from the Black and Aegean Sea were 100 %, 9.90 and 99, and 100 %, 13.10 and 131, respectively. A total of 604 diplectanids were collected from gills of wild and cultured European sea bass in the Black and Aegean Sea coasts of Türkiye. The overall P and mI of diplectanids from the Black and Aegean Sea were 100 % and 15.1, respectively (Table 1).

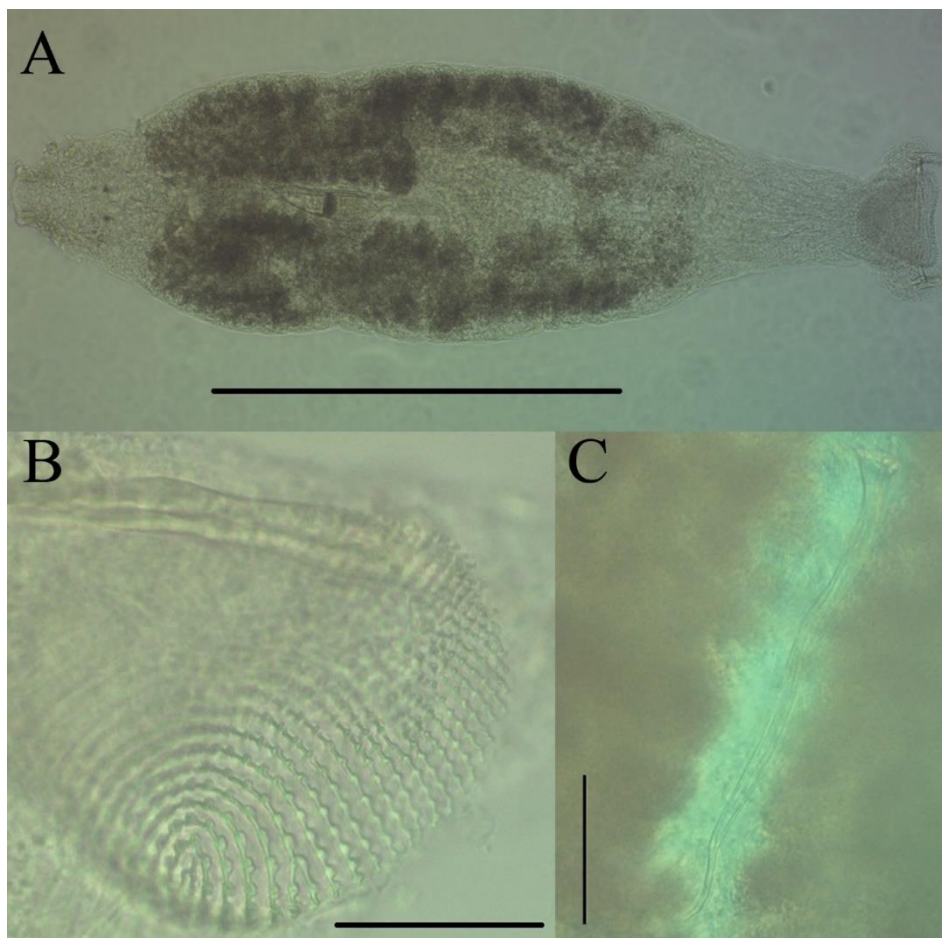
All diplectanids were morphologically identified as *Diplectanum aequans* according to the identification keys. Ethanol-preserved our specimens ( $n = 10$ ) were measured about 13.8 (11.3–17.8) mm in length and 0.32 (0.2–0.38) mm in width. Four eyespots were clearly visible in the

cephalic part. There was a straight cirrus measured 170 (156–188)  $\mu$  in length. The end of cirrus characteristically tapered and slightly ended as curved. We observed two squamodiscs (one dorsal and one ventral). A squamodisc was measured 131 (110–145)  $\mu$  in diameter and typically exhibited 24–25 rows of sclerotized pieces (Figure 1).

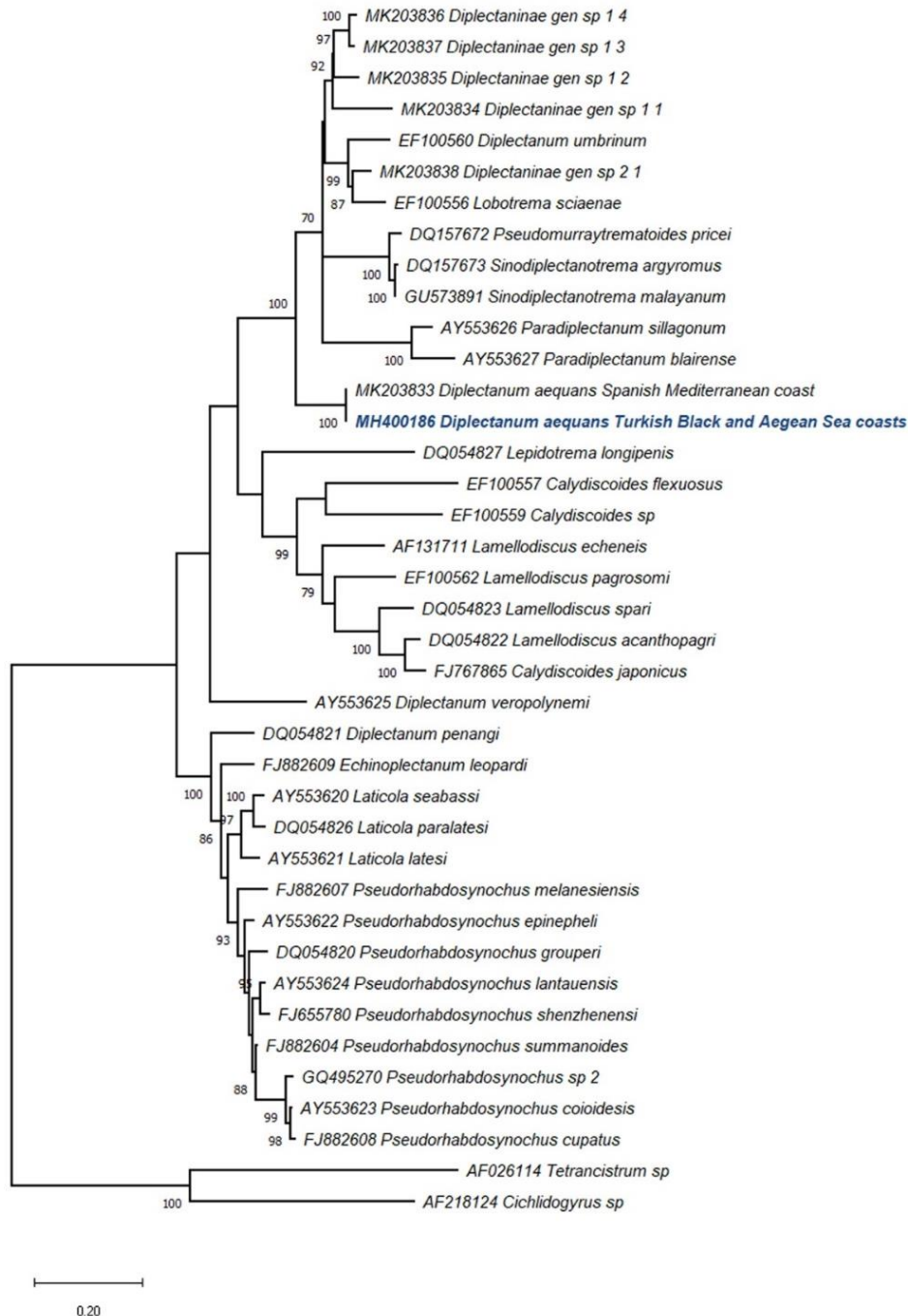
**Table 1.** Epidemiological parameters of *Diplectanum* spp. in *Dic. labrax* from Turkish water.

Locality	n	P (%)	mI	Number of collected parasite
<b>Cultured</b>				
Black Sea (FAO area 37.4.2)	10	100	26.40	264
	10	100	11.10	110
<b>Wild</b>				
Aegean Sea (FAO area 37.1.3)	10	100	9.90	99
	10	100	13.10	131
Overall	40	100	15.10	604

n: examined fish, P: prevalence, mI: mean intensity



**Figure 1.** (A–C): Microphotographs showing main morphological features of *D. aequans* collected from *Dic. labrax* in the Turkish coasts: (A) whole body (scale bar = 500  $\mu$ m), (B) detail of 24–25 rows of sclerotized pieces in a squamodisc (scale bar = 50  $\mu$ m), (C) detail of cirrus (scale bar = 50  $\mu$ m).



**Figure 2.** Phylogenetic tree generated by maximum likelihood analysis of the 28S rRNA sequences of Diplectanidae species. Only one representative sequence (coloured with blue and highlighted in bold) was used as all the five sequences were identical. Numbers above/below the branches are bootstrap confidence levels based on 100 replicates. Bootstrap values <70 are not shown. GenBank accession numbers are given beside species name. The scale shows genetic distance.

Five representatives were subjected to molecular analysis. The sequencing of partial large subunit ribosomal RNA gene and partial small subunit ribosomal RNA gene with ITS1 region of *D. aequans* produced two fragments of approximately 933 and 960 base pairs, respectively. Among representatives were not observed intraspecific nucleotide differences for two fragments. The sequences of 28S rRNA and 18S rRNA with ITS1

region of *D. aequans* from the Black Sea were deposited to GenBank with accession numbers MH400186 and MH400167.

## Discussion and Conclusion

The diplectanids collected from wild and cultured *Dic. labrax* in the Turkish coasts were unambiguously identified as *D. aequans* based on detailed morphological



features previously reported (18, 21, 26), and sequence characterization of partial 28S rRNA and 18S rRNA data. The characteristic shape of the cirrus and the rows of sclerotized pieces in a squamodisc are the most diagnostic morphological structures to discriminate among valid two *Diplectanum* species in the European sea bass (10, 18, 21, 26). While *D. aequans* has the end of cirrus characteristically tapered and slightly ended as curved and arranged 18–31 rows of sclerotized pieces in a squamodisc, *D. laubieri* has the end of cirrus characteristically hook-shaped and made up 11–16 rows of sclerotized pieces in a squamodisc (10, 18, 21). Because the end of cirrus of our diplectanids tapering characteristically slightly end as the curve and typically comprise 24–25 rows of sclerotized pieces in a squamodisc, we did not hesitate to identify it as *D. aequans* in the present study (Figure 1). Most morphometric measurements of the same diplectanids which were preserved using the different fixation techniques may be insufficient for identifying the same species because different measurements could be obtained (5, 14). However, the shapes of sclerotized parts (cirrus and squamodisc) in diplectanids are preserved relatively well despite different fixation techniques and can still confidently be used for species morphological identifications (14). Therefore, we considered especially the shapes of the sclerotized parts rather than morphometric measurements in the morphological identification of diplectanids.

Molecular data provide us to understand the taxonomy, systematics, and phylogeny of Trematoda taxa. At least one conserved region (18S rRNA or 28S rRNA) and one spacer (ITS1, ITS2, or the entire ITS1–5.8S–ITS2) region of ribosomal DNA should be targeted for trematode taxonomy and systematics in the genetic analyses (2). The molecular methods combined with morphological identifications have been commonly used to identify species of diplectanids and estimate their phylogenetic relationships (4, 5, 25, 32). The 28S rRNA sequence of *D. aequans* in the present study matched 100 % with the 28S rRNA sequence of *D. aequans* (accession number MK203833) from the Spanish Mediterranean coast available in GenBank (32). Our diplectanid species had 85.38–87.04 % similarity with 28S rRNA sequences of Diplectaninae gen. spp. (accession numbers MK203834–MK203838) from the Spanish Mediterranean coast (32) (p-distance = 12.98–16.57 %), 83.73% with *D. umbrinum* larvae (accession number EF100560, unpub. data) (p-distance = 18.02%), 86.77 % with *Paradiplectanum (Diplectanum) sillagonum* (accession number AY553626) from the South China Sea (p-distance = 19.88 %) (34), 85.33 % with *P. (D.) blaiense* (accession number AY553627) from the South China Sea (p-distance = 21.38 %) (34), 85.71 % with *D. penangi* (accession

number DQ054821) from the South China Sea (p-distance = 25.78 %) (37), and 84.05 % with *D. veropolynemi* (accession number AY553625) from South China Sea (p-distance = 25.78 %) (34).

In the present study, the length of the partial 18S rRNA and ITS1 fragments of *D. aequans* were comprised 457 and 503 bp, respectively. The partial 18S rRNA fragments of *D. aequans* reported herein from Turkish coasts showed 100% identity with the 18S rRNA of *D. aequans* (AJ276439 and AM943816) from the French Atlantic coast (6) and the Italian Mediterranean coast (28), respectively. The 18S rRNA sequence of our specimen was 96.96% identical to 18 rRNA of *P. (D.) sillagonum* (accession number AY553617) from the South China Sea (p-distance = 3.38 %) (34) and 95.78 % with *P. (D.) blaiense* (accession number DQ537356) (p-distance = 4.45 %). Comparison of partial 28S and 18S rRNA sequences confirmed that our diplectanid species in the Turkish coast are the same taxon as reported from the French Atlantic coast, the Italian Mediterranean coast, and the Spanish Mediterranean coast (6, 28, 32). This study also provides the first molecular confirmation of *D. aequans* sampled from cultured and wild *Dic. labrax* in the Black and Aegean Sea coasts of Türkiye.

Recently, it has been understood that *Diplectanum* species from the Spanish Mediterranean coast do not form a monophyletic group with previously sequenced diplectanids in the phylogenetic analyses using the 28S rRNA gene and therefore do not belong to a single genus, and *Diplectanum* species may represent three different genera as *Diplectanum* sensu stricto, Diplectaninae gen. clade B1 and Diplectaninae gen. clade B2 (32). The cladistic methods using comparative morphological characters were already supported the paraphyletic of *Diplectanum* genera (8). The phylogenetic results of 28S rRNA in congruence with those stated by Villar-Torres et al. (32), and our isolate was placed at the base of clade B included Diplectaninae gen. spp. from sciaenids (32) (Figure 2). Moreover, the polyphyletic status of the subfamily Diplectaninae has been reflected in the position of *Lobotrema* and *Murraytrema (Pseudomurraytremaoides)* genera as a sister group of *D. aequans* in clade “P” in the cladistic analysis (8). In the present study, our *D. aequans* phylogenetically represents a sister group of *Lobotrema* and *Murraytrema (Pseudomurraytremaoides)* genera and also supports the view of Domingues and Boeger (8) (Figure 2). Additionally, there are only available two 18S sequences from the French Atlantic coast (6) and the Italian Mediterranean coast (28), and one 28S rRNA and one entire ITS sequences from the Spanish Mediterranean coast (35) in the GenBank for *D. aequans*. This study is also the new record of 18S and 28S rRNA sequences for *D. aequans* sampled from the Black Sea in GenBank.

In conclusion, the current study reports molecular (18S rRNA and 28S rRNA) evidence of *D. aequans* from *Dic. labrax* in the Turkish coast for the first time. These molecular data can be used to resolve the phylogenetic position of Diplectanidae. Molecular data for species representing Diplectanidae are still currently lacking from Turkish waters. Further combining morphological and molecular studies are needed for resolving the phylogenetic relationship, taxonomy, and classification of the Diplectanidae in the coasts of Türkiye.

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### Conflict of interest

The authors declare that they have no competing interests.

### Author Contributions

CA and GZP conceived and planned the experiments. CA and GZP carried out the experiments. CA and GZP planned and carried out the simulations. CA and GZP contributed to sample preparation. CA and GZP contributed to the interpretation of the results. GZP took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

### Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

### Ethical Statement

For this study, ethics committee approval was not needed because no handling of live marine teleost specimens was involved.

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