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ORIGINAL PAPER

Engraulidae (Teleostei, Clupeiformes) in tropical estuarine ecosystems: identification of fish larvae based on morphological analysis and molecular evidence

Mariana Muguet Julio[1] · Ana Cristina Teixeira Bonecker[1] · Cristina de Oliveira Dias[1] · Rosane Silva[2] · Sérgio Luiz Costa Bonecker[1]

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Abstract

Fish larvae identification in estuarine environments is of major importance for fishing resources verifications, environmental monitoring, and establishment of protection areas. Species of the Engraulidae family are morphologically very similar; therefore, taxonomic characterization can be insufficient to reach a precise identification. Molecular-level analysis has become an important tool to assist on taxonomy, as it does not depend on morphological expressions that only occur in juvenile or adult stages. Fish larvae were sampled in tropical estuarine ecosystems, in a single water body, with salinities ranging between 15 and 25, using a bongo net with a 500-μm mesh, towed obliquely from the surface. Around 73% of identified individuals are from the Engraulidae family, and are classified into nine morphotypes according to morphological characteristics. DNA analyses were performed, with the sequencing of the mitochondrial gene Cytochrome C Oxidase I. These analyses suggest the occurrence of two distinct genera: *Anchoa* and *Lycengraulis*, among the investigated specimens. The present study contributes to enriching knowledge on the morphology of larvae of the Engraulidae family, and highlights the potential of molecular analysis techniques to elucidate taxonomic issues.

Keywords Morphology · Estuary · COI · Ichthyoplankton

Introduction

The Engraulidae family, known popularly as anchovy, belongs to Clupeiformes. This order includes seven families and around 419 species (Fricke et al. 2020). Examples of Engraulidae can be found among pelagic and coastal fishes (Inoue et al. 2001), and in saltwater and even freshwater environments (Malabarba et al. 2013). They occur in the Atlantic, Indian, and Pacific Oceans (Bonecker et al. 2014), and present migratory behavior (Fuster de Plaza and Boschi, 1961; Loeb 2009). It is a family of small fishes, with distinguished elongated bodies, wide mouths, long intestines reaching about 75% of the body length, round eyes located closer to the end of the snout than to the operculum extremity, and a prominent swim bladder. Specimens of this family usually present a longitudinal silver band in each side of the body, extending from the posterior end of the head until the caudal peduncle (Figueiredo and Menezes 1978; Bonecker et al. 2014).

Sixteen species of the Engraulidae family are known to occur in the Brazilian southeastern coast. Genus *Anchoa* is the most typical, comprehending nine species: *Anchoa cubana* (Poey, 1868), *Anchoa filifera* (Fowler, 1915), *Anchoa hepsetus* (Linnaeus, 1758), *Anchoa januaria* (Steindachner, 1879), *Anchoa lyolepis* (Evermann & Marsh, 1900), *Anchoa marinii* Hildebrand, 1943, *Anchoa pectoralis* Hildebrand, 1943, *Anchoa spinifer* (Valenciennes, 1848), and *Anchoa tricolor* (Spix & Agassiz, 1829). There are also records of the species *Anchovia clupeoides* (Swainson, 1839);

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✉ Mariana Muguet Julio
marianamuguet@gmail.com

[1] Instituto de Biologia, Departamento de Zoologia, Universidade Federal do Rio de Janeiro, Av. Carlos Chagas Filho, 373, CCS, Bloco A, Sala A0-084, Ilha do Fundão, Rio de Janeiro, RJ 21941-902, Brazil

[2] Instituto de Biofísica Carlos Chagas Filho, Departamento de Biofísica, Universidade Federal do Rio de Janeiro, Av. Carlos Chagas Filho, 373, CCS, Bloco G, Sala G1-019, Ilha do Fundão, Rio de Janeiro, RJ 21941-902, Brazil

of three species of the genus *Anchoviella*: *Anchoviella brevirostris* (Günther, 1868), *Anchoviella lepidentostole* (Fowler, 1911), and *Anchoviella cayennensis* (Puyo, 1946); and also of the species *Cetengraulis edentulus* (Cuvier, 1829), *Engraulis anchoita* Hubbs & Marini, 1935, and *Lycengraulis grossidens* (Spix & Agassiz, 1829) (Whitehead et al. 1988).

Species-level identification of fish larvae is of major importance for fishing resources verifications (Bonecker and Castro 2006), environmental monitoring and establishment of protection areas, for clearing doubts regarding group taxonomy, for the field of conservation biology (Metcalf et al. 2007), and also for economic matters (Smith et al. 2008; Carvalho et al. 2014). During identification, morphological and meristic characters are taken into consideration; however, they are insufficient for the identification of all species (Ko et al. 2013) due to the resemblance among individuals (Weiss and Souza 1977). Larvae of the Engraulidae family are morphologically similar to those from the Clupeidae, Argentinidae, Bathylagidae, and Phosichthyidae families (Bonecker et al. 2014). Carvalho (1950) already mentioned the difficulty in the morphological identification of the engraulids in his work. The biggest hindrance in Clupeiformes identification is usually the external morphological similarity among larvae (Silva et al. 2010).

Discrepancy regarding morphological identification of larval stages represents a major challenge, stumbling upon limitations and requiring a new approach (Hebert et al. 2003a; Packer et al. 2009; Wibowo et al. 2016). The study of mitochondrial DNA is a widely used taxonomic tool, and efficient as a molecular marker in identification systems and phylogenetic studies, even among cryptic species (Hebert et al. 2003b; Hebert et al. 2004). In these cases, molecular techniques are necessary for a more consistent identification (Chairi and Rebordinos 2014) to assist on species classification, being an important option to confirm the systematic (Oliveira et al. 2013; Tresbach et al. 2015) and add to the phylogeny of varied groups (Silva et al. 2012). A noted advantage of this method is the use of a small fragment of tissue for mitochondrial DNA extraction, with high material recovery rates (Bolzan 2011), a technique adopted in several Engraulidae studies (Ribeiro et al. 2012; Weigt et al. 2012; Elías-Gutiérrez et al. 2017; Bingpeng et al. 2018).

The Cytochrome C Oxidase I gene is used in molecular techniques as a complement to morphological identification. There are some examples of the use of this gene for species of the Engraulidae family, such as the works of Afrand et al. (2020), Díaz-Viloria et al. (2015), Yang et al. (2016), Rosas et al. (2018), Bingpeng et al. (2018), among others. Afrand et al. (2020) report the use of morphological data along with molecular information for Engraulidae identification, since the identification based only on morphology is hindered by the shortage of taxonomic studies, the family's multiplicity, and the resemblance among some of the species.

In an attempt to lead to more reliable identifications, this study's aim is to propose a methodology based on the integrated analysis of morphological and molecular data for the species-level identification of fish larvae of the Engraulidae family present in the estuaries of the Macaé, São João, Bracuí, and Perequê-Açu Rivers, in the state of Rio de Janeiro.

Materials and methods

Four estuaries were selected for the study, all in the state of Rio de Janeiro: (1) the Macaé river estuary (22°22′28″S – 41°46′30″W) and (2) the São João river estuary (22°35′54″ S – 41°59′32″W), both located in the northern region of the state and flowing into the open sea; (3) the Bracuí river estuary (22°57′12″S – 44°24′05″W); and (4) the Perequê-Açu river estuary (23°13′01 ″S and 44°42′40″W), located in the southern region and flowing into the Ilha Grande bay (Figure 1). These four estuaries have been studied by the Integrated Zooplankton and Ichthyoplankton Laboratory, for identification and dynamics of planktonic organisms (Carvalho et al. 2016; Araújo et al. 2017a, b; Santos et al. 2017a, b).

The Macaé and São João Rivers are big systems, with 130 and 120 km of extension, respectively, when compared to the Bracuí and Perequê-Açu Rivers, which are considered smaller systems, comprising about 32 and 22 km respectively (Santos et al. 2017a). The four estuaries selected for this study present partially mixed salinity profile and shallow waters, with depths ranging from 1 to 5 m.

Fish larvae sampling

Sampling campaigns in each estuary were performed every 2 months from March 2013 to March 2015, adding up to a total of 12 campaigns, all held during nocturnal syzygy ebb tides to guarantee higher continental influence and organism density. The exception was the Perequê-Açu estuary, in which samplings were carried out during quadrature tides due to the shallow depths. In August 2013, due to logistic issues, sampling in the Perequê-Açu river estuary was not possible; therefore, it was carried out in November 2014. Samplings were performed in a fixed point, determined according to salinity — which was standardized between 15 and 25—and measured through a HQ40D portable multimeter (Hach Company, Loveland, USA). Ichthyoplankton sampling was carried out by using bongo nets with 500-μm mesh size, towed obliquely for 10 min, from the bottom to the surface. Immediately after sampling, specimens were fixed in anhydrous ethanol 99.5%. The medium was renewed 24 h after collection to maintain DNA integrity for molecular analysis.

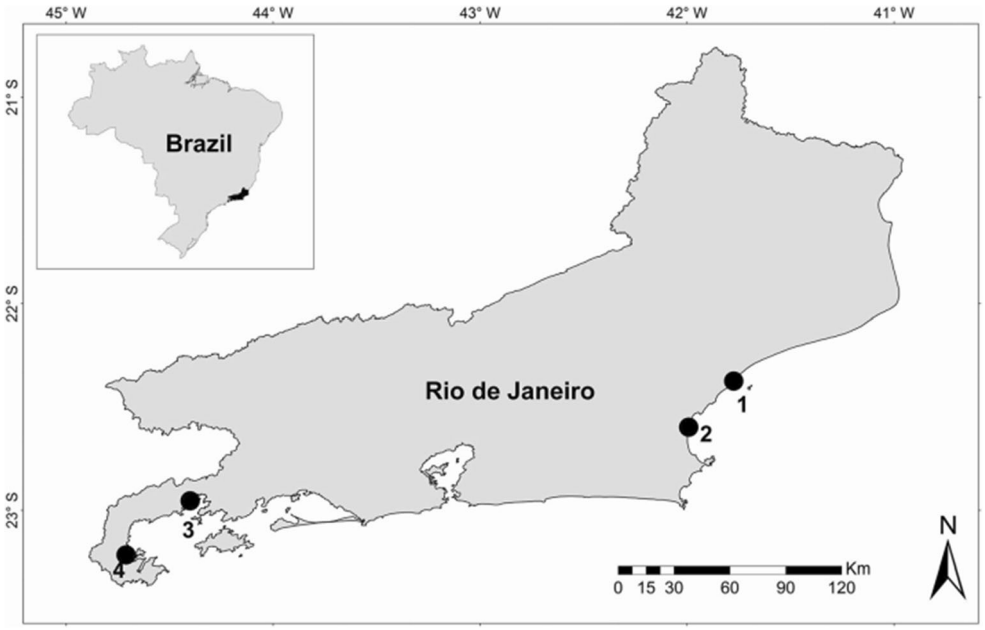


Fig. 1 Map of the sampled estuaries in Rio de Janeiro, Brazil. 1, Macaé river estuary; 2, São João river estuary; 3, Bracuí river estuary; 4, Perequê-Açu river estuary. Map made in ArcGIS software.

Morphological identification

Fish larvae were sorted and identified to the lowest possible taxonomic level with the aid of stereo microscopes (Olympus SZ and Zeiss Stemi SV6). Larvae identification was based on morphological (shape of the body, head and fins; position of the anus and of caudal and dorsal fins) and meristic characters (number of fin rays, pigmentation pattern, number of lower gills rakers on the first branchial arch) according to Whitehead et al. (1988) and Richards (2005), and based on data from the zoological collection of the Integrated Zooplankton and Ichthyoplankton Laboratory (LIZI-UFRJ). Figure 2 shows the main morphological structures observed during the present study to identify larvae of the Engraulidae family. Fish larvae classification was based on Fricke et al. (2020). Specimens were classified to family level and later into morphotypes.

Fish larvae were photographed with an Olympus digital camera attached to an Olympus SZX12 stereo microscope. Measurements were performed afterwards (Richards 2005) using the Image Pro-Plus 6.1. software to assist in the identification of species and in the characterization of the stage of development. To illustrate and show the different types of lower gill rakers on the first branchial arch identified in the specimens throughout this investigation, these structures were photographed in a differential interference contrast (DIC) microscope (Zeiss Imager.A2 Axio, Carl Zeiss, Oberkochen, Germany).

DNA extraction, PCR, and sequencing

Larval tissue samples from specimens of the Engraulidae family (DZUFRJ60211; 60220; 60222; 60229) were used for DNA extraction, except in the case of specimens in preflexion stage, in which the whole individual was used. Tissues were washed and hydrated with TE 1× (Tris-HCl 10 mM; EDTA 1 mM; pH 8.0) and individually placed in 1.5-mL microtubes. Genomic DNA extraction followed two protocols: phenol-chloroform (Green and Sambrook 2012) and the DNEasy Blood & Tissue kit (Qiagen), with the aim to verify which method is the most effective in this case.

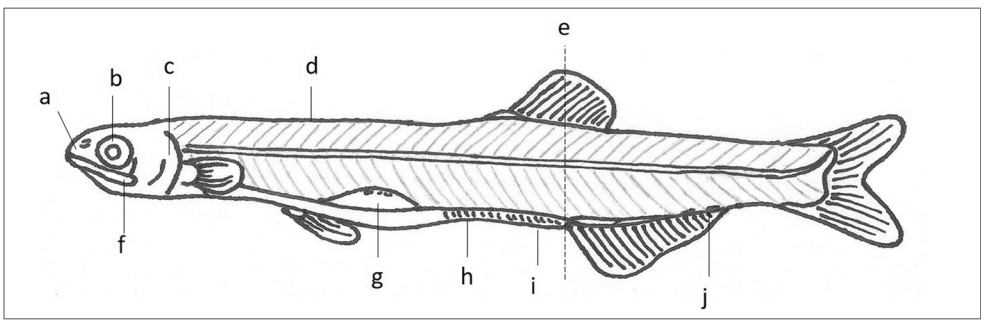


Fig. 2 Main morphological characters analyzed to identify larvae of the Engraulidae family in the present study. **a** Snout; **b** round eyes; **c** operculum (gill arches); **d** elongated body; **e** dorsal and anal fins overlap; **f** upper jaw; **g** prominent gas vesicle; **h** striated intestine; **i** elongated intestine; **j** melanophores.

DNA quantification was performed by a NanoDrop 2000c spectrophotometer (Thermo Scientific). A ~500-pb fragment of the COI gene was amplified through PCR using specific forward and reverse primers (Invitrogen): COI_ENG_F (TCAAATTTATAACGTAATCG) and COI_ENG_R (GCTGGGTCGAAGAAAGTAGT), respectively, developed in the Geneious 8.1.3 software and based on the corresponding sequence alignments in species of the Engraulidae family registered in the GenBank (JX983289; KC208636; KF489773; KF614701; KF614703; KF929839; KJ128482; KJ204858; KJ709524). PCR reactions were performed in a final volume of 25 μL, containing: 2.5 μL of 10× reaction buffer (50 mM KCl; 75 mM Tris – HCl; pH 9.0; 20 mM of $(NH4)_2(SO4)$ (Invitrogen), 1.5 mM of $MgCl_2$ (Invitrogen), 0.3 μM of each primer, 0.2 mM of dNTP (dATP, dTTP, dCTP, and dGTP) (Invitrogen), 0.05 U/μL of recombinant Taq DNA polymerase (Invitrogen), and 1–2 μL of the DNA sample with 25 ng/μL of Milli-Q water (Merck Millipore Burlington, USA), enough to obtain the final volume. The program of the Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, USA) consisted in: denaturation at 95° for 10 min; 40 cycles of denaturation at 95° for 30 s, annealing at a temperature of 42° for 30 s, extension at 72° for 1 min, and a final extension step at 72° for 4 min. PCR products were analyzed by agarose gel electrophoresis 1.5% (Invitrogen), and next prepared for sequencing using the BigDye Terminator v. 3.1 Cycle Sequencing Kit protocol (Applied Biosystems). Samples were then placed in an ABI 3500 Genetic Analyzer (Applied Biosystems). Some samples were also sequenced by Macrogen Inc., in Seoul, South Korea.

Sequence editing and analysis

After sequencing, the results were imported and sequence quality analysis was performed using electropherograms. Consensus sequences of the COI gene were edited and aligned using the Geneious Prime 2019.1 software (Biomatters Ltd, Auckland, New Zealand; Larkin et al. 2007). A Basic Local Alignment Search Tool (BLAST; Altschul et al., 1990) was used along with a network service (http://www.ncbi.nlm.nih.gov/) to search for homologous sequences in the GenBank nucleotide database. The sequences were deposited in GenBank with accession numbers MZ209216 to MZ209224.

A phylogenetic tree was established with the aim to interpret the relationships between data according to the Maximum Likelihood method (ML) and built with ClustalW v. 2.0 (Kumar et al. 2016) using the MEGA7.0 program (Kumar et al. 2016) and nucleotide substitution model HKY+I+G (Hasegawa et al. 1985). Cladistic support for the analyses was indicated in the tree's knots, omitting bootstrap values under 70%. A consensus tree was developed and exhibited in the Fig Tree v.1.4.2. software. Pairwise genetic distances (matrix in supplemental material 1) were estimated in MEGA7.0.

The phylogenetic tree was built based on the sequences obtained in this study and the sequences available in the GenBank Database for six genera of the Engraulidae family (*Anchoviella*, *Anchoa*, *Engraulis*, *Lycengraulis*, *Cetengraulis*, and *Coilia*). The external group was formed by three sequences of the species *Harengula jaguana* (Poey, 1865), from the Clupeidae family (order Clupeiformes). The codes of the species used to build the consensus tree are listed in table 2 in the supplemental material 2.

Results

Morphological study

In the four studied estuaries, 73% of the morphologically identified specimens were classified as belonging to the Engraulidae family. Standard length ranged from 4.43 to 50.5 mm (SL, length measured from the tip of the snout to the caudal peduncle) in the investigated estuaries. A total of nine morphotypes were morphologically identified among the individuals of the Engraulidae family collected: morphotype 1 (Figure 3), morphotype 2 (Figure 4), morphotype 3 (Figure 5), morphotype 4 (Figure 6), morphotype 5 (Figure 7), morphotype 6 (Figure 8), morphotype 7 (Figure 9), morphotype 8 (Figure 10), and morphotype 9 (Figure 11). The diagnostic characteristics of the morphotypes identified in this work are listed in Table 1 below.

Molecular study

In total, DNA extractions were performed in 71 individuals, resulting in nine consensus sequences of the COI gene, which were submitted to the GenBank with accessions MZ209216 to MZ209224. Successive DNA sequencing and protocol adjustments were performed. However, no positive results were obtained in most of the analyses. The topology resulting from the analyses (Figure 12) shows three distinct clades among the available sequences of the COI gene. In the blue clade, it is possible to see that the sequences of morphotypes 2 and 8 are conspecific (interspecific *p* distance 0.009), grouped to the sequences of the species *Anchoa hepsetus* (interspecific *p* distance 0.053–0.058). In the green clade we can see the sequences of Morphotype 3, 3a, 3b, 3c, and 3d are conspecific (interspecific *p* distance 0.000–0.002). These four sequences are shown grouped to the species *Anchoa* sp. (interspecific *p* distance 0.021–0.023), and in the same clade as the species *Anchoa lyolepis* (interspecific *p* distance 0.074–0.076), and as the *Anchoa mitchilli* (interspecific *p* distance 0.081–0.083). The red clade wraps the sequences of Morphotype 9, 9a, 9b,

Table 1 Diagnostic characteristics used to identify the morphotypes of the Engraulidae family present in the estuaries sampled in this work

Morphotype	Teeth	Number of rays in the anal fin	Anal fin position	Lower gill rakers (1st arch)	Pseudo-gills	Lower jaw margin	Upper jaw
1	Small, very visible (postflexion and juvenile)	19–22	Starting on the posterior half of the dorsal fin	Long and thin, 20–25	Short	Between the tip of the snout and the anterior margin of the eye socket	Short, rounded border, close to the preoperculum
2	Not visible	17–18	Starting on the posterior half of the dorsal fin	Long, 14–15	Not visible	Extends to the tip of the snout	Short border, does not reach the preoperculum
3	Small	20–22	Starting on the posterior half of the dorsal fin	Medium-long, 19-20	Not visible	Extends to the tip of the snout	Short, rounded border, does not reach the preoperculum
4	Very small, barely visible	22–24	Starting on the posterior half of the dorsal fin	Long, 28	Short	Between the tip of the snout and the anterior margin of the eye socket	Pointed border, reaches or surpasses the preoperculum
5	Not visible	19–22	Starting on the posterior half of the dorsal fin	Long and thin, 20–25	Short	Between the tip of the snout and the anterior margin of the eye socket	Long, rounded border, reaches the preoperculum
6	Not visible	22–26	Originates towards the last rays of the dorsal fin	Medium–long, 14–15	Not visible	Extends to the tip of the snout, or very close to the tip	Very short, rounded border, does not reach the preoperculum
7	Not visible	20–22	Starting on the posterior half of the dorsal fin	Short, 14–15	Not visible	Extends to the tip of the snout	Short, rounded border, does not reach the preoperculum
8	Not visible	17–18	Starting on the posterior half of the dorsal fin	Short, 14–15	Not visible	Extends to the tip of the snout	Short border, does not reach the preoperculum
9	Not visible	22–26	Originates towards the last rays of the dorsal fin	Short, 9–10	Not visible	Extends to the tip of the snout, or very close to the tip	Very short, rounded border, does not reach the preoperculum

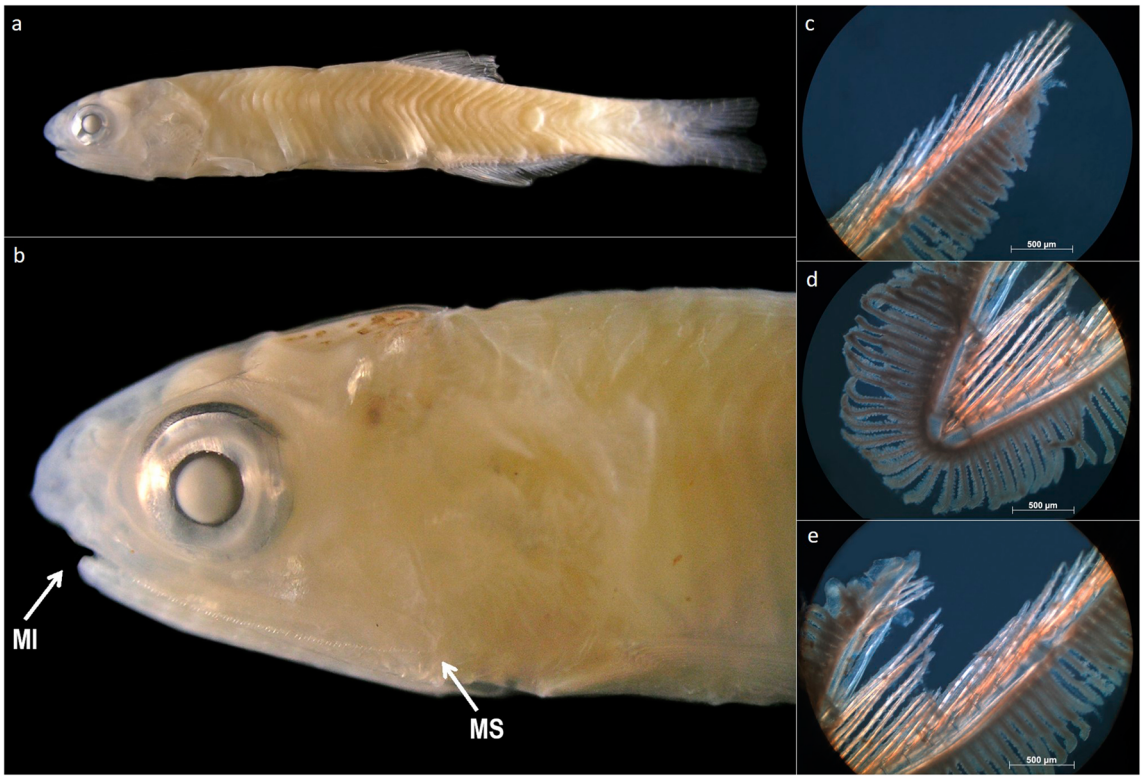


Fig. 3 Engraulidae— Morphotype 1. **a** Standard length=35.6mm; **b** details of the upper (MS) and lower (MI) jaws, head length =9.3mm; gill rakers in the first branchial arch; **c** initial portion; **d** final portion; **e** middle portion (scale = 500µm).

and 9c, conspecific (interspecific *p* distance 0.000–0.004), grouped with the species *Lycengraulis grossidens* (interspecific *p* distance 0.032–0.038). The genetic distance between the morphotypes that were grouped into different clades was not significant. It is possible to suggest that they are of the same species.

Discussion

The present study highlights the importance of morphological studies allied with molecular techniques to solve issues on fish larvae identification. A recent study with the same purpose uses the conjunction of these data for larvae of the

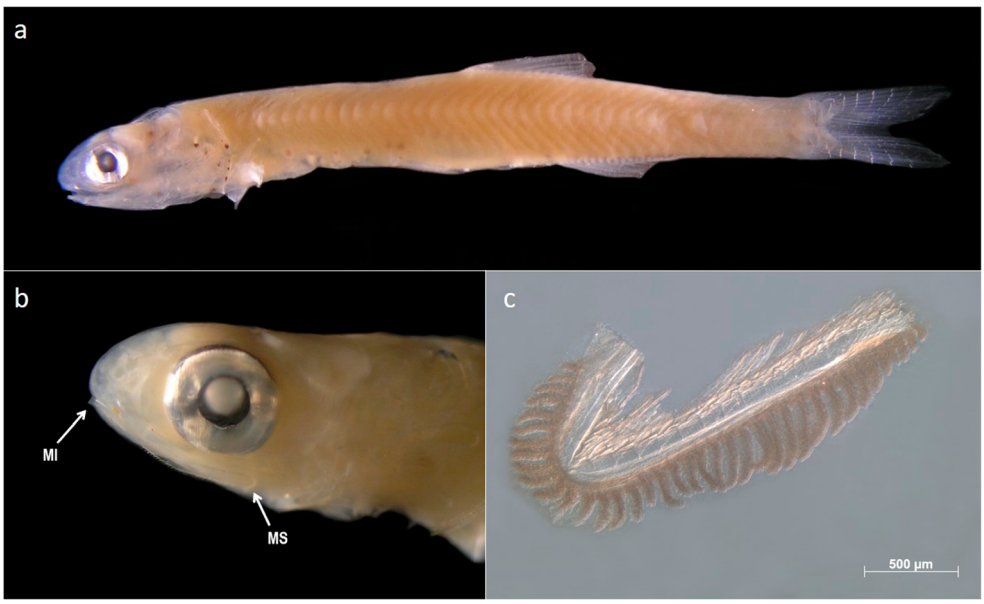


Fig. 4 Engraulidae — Morphotype 2. **a** Standard length=40.7mm; **b** details of the upper (MS) and lower (MI) jaws, head length=9.02mm; **c** gill rakers in the first branchial arch (scale=500µm).

Fig. 5 Engraulidae — Morphotype 3. **a** Standard length=32.1mm; **b** details of the upper (MS) and lower (MI) jaws, head length=7.3mm; **c** gill rakers in the first branchial arch (scale=500µm).

Engraulidae family (Afrand et al. 2020). Barcode identification techniques can differentiate species that are physically indistinguishable, although they cannot replace morphology, being considered by some authors as a competitor for taxonomy (Will and Rubinoff 2004; Ebach and Holdrege 2005). In this work, we support the use of the two techniques together to assist in these issues of distinguishing similar species, as they are complementary. According to Afrand et al. (2020), reconciling morphological taxonomy and molecular analysis is laborious; however, it is essential that such approaches complement each other. This combination, called an integrated approach, is defended in several works (Lefébure et al. 2006; Padial and De La Riva 2007; Ward et al. 2009; Pires and Marinoni 2010; Rajpoot et al. 2016).

During the development of this work, several difficulties arose regarding the precise morphological identification of the material at species level, as a result of the lack of specific bibliography for this group in the larval stage. The Engraulidae family has been periodically reviewed, in order to solve the existing taxonomic problems in the identification

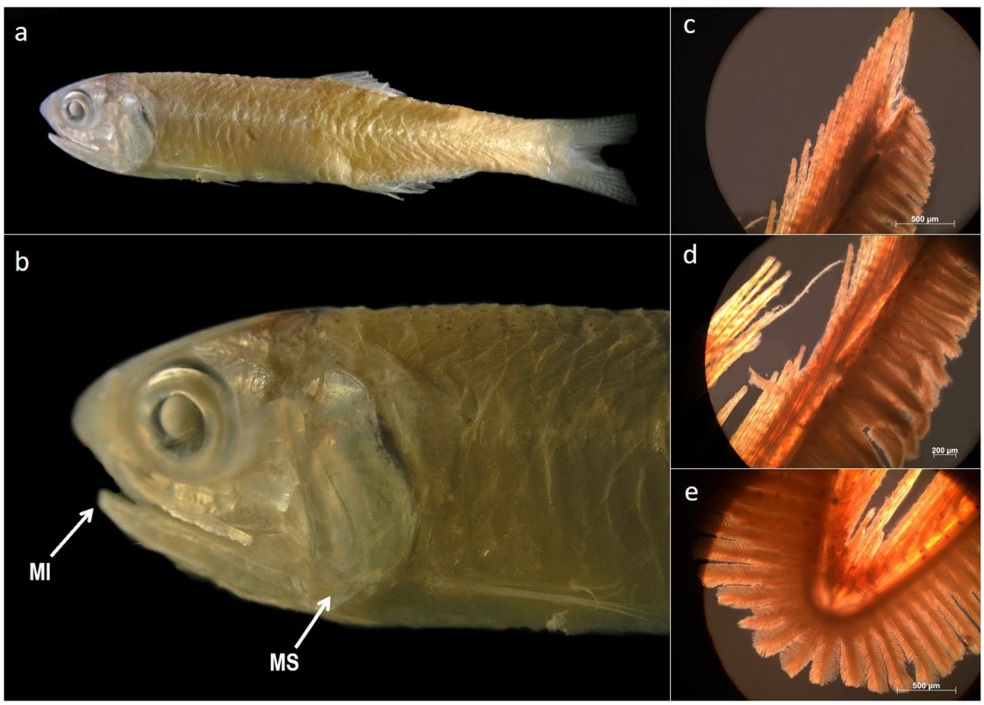


Fig. 6 Engraulidae — Morphotype 4. **a** Standard length=60.4mm; **b** details of the upper (MS) and lower (MI) jaws in Morphotype 4, head length=13.6mm; gill rakers in the first branchial arch; **c** initial portion (scale=500µm); **d** middle portion (scale=200µm); **e** final portion (scale=500µm).

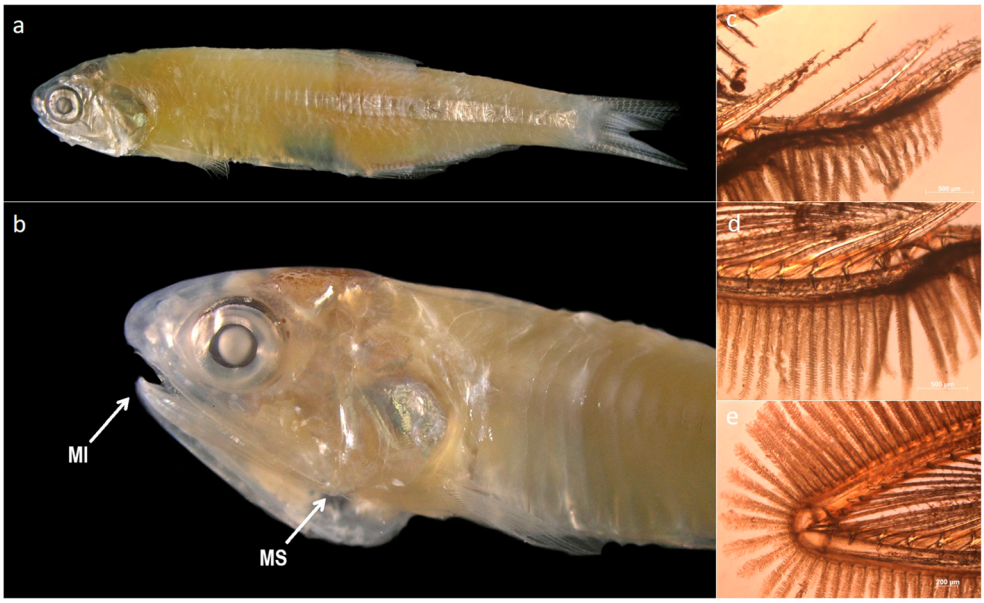


Fig. 7 Engraulidae—Morphotype 5. **a** Standard length=75.8mm; **b** details of the upper (MS) and lower (MI) jaws, head length=16.0mm; gill rakers in the first branchial arch: **c** initial portion (scale=500μm); **d** middle portion (scale=500μm); **e** final portion (scale=200μm).

of larval, juvenile, and even adult stages. In the works of Silva et al. (2010) and Afrand et al. (2020) with Engraulidae, the authors report the main difficulties for research, due to incomplete descriptions and the scarcity of taxonomic studies that help morphological identification. Morphotyping was an alternative used in the present study to classify specimens with taxonomic difficulties.

First, based on the morphological identification of the morphotyped specimens, it is possible to suggest some relationships based on Whitehead et al. (1988) and Richards (2005). Morphotypes 1, 4, and 5 resemble the species *A. lepidentostole* because they share diagnostic features—such as position and number of rays in the anal fin, shape, and number of the lower gill rakers in the first branchial arch, pseudo-gills, and extension of the lower jaw. However, they differ in the characteristics of the upper jaw, which in *A. lepidentostole* is rounded, reaching the preoperculum. The relationship between morphotypes 2 and 8 will be discussed later, integrating the morphological and molecular data. The morphological characteristics of morphotype 3 converge with morphotype 7, differing only in the number of lower gill rakers in the first branchial arch. Morphotype 6 is morphologically similar to morphotype 9, which, however, presents shorter lower gill rakers in the first branchial arch. It is possible to suggest that these small morphological differences occur due to the larval development stage, and may not be

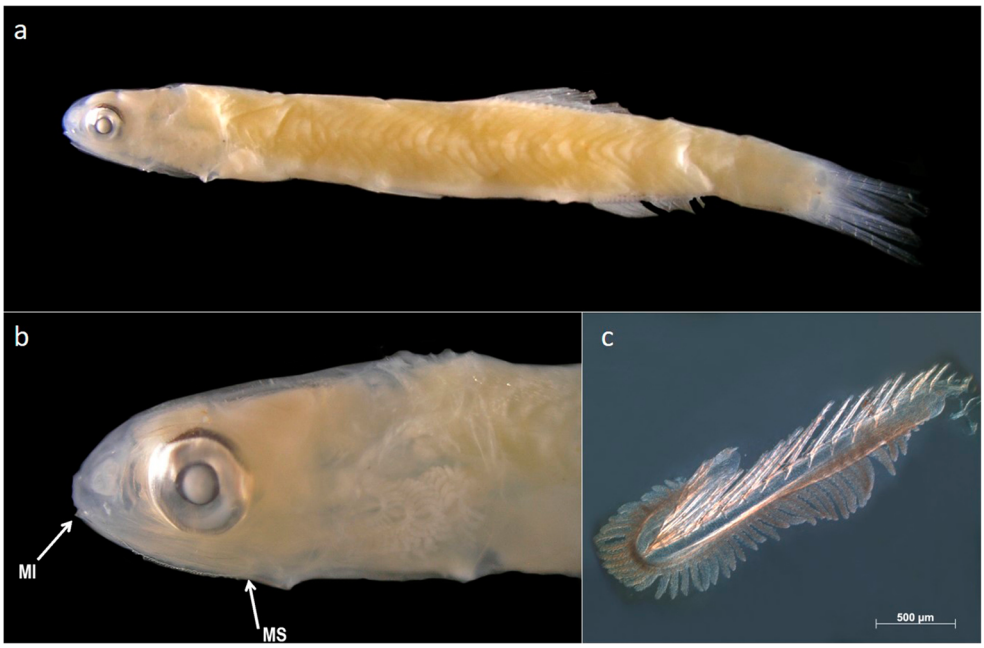


Fig. 8 Engraulidae—Morphotype 6. **a** Standard length=36.9mm; **b** details of the upper (MS) and lower (MI) jaws, head length=8.01mm; **c** gill rakers in the first branchial arch (scale=500μm).

Fig. 9 Engraulidae — Morphotype 7. **a** Standard length=36.0mm; **b** details of the upper (MS) and lower (MI) jaws, head length=7.1mm; **c** gill rakers in the first branchial arch (scale=500µm).

significant to separate them into different species. The relationships between these morphotypes and described species of the Engraulidae family can be explained by the molecular results obtained in the present study.

Regarding molecular analysis, we used two different methods of DNA extraction, in order to obtain greater yield in the samples of species of the Engraulidae family. Total DNA extraction protocols were applied: organic phenol-chloroform extraction and the DNEasy Blood and Tissue kit. The use of a DNA extraction kit is a relatively quick method and is mentioned and used in the works of several authors (Ribeiro et al. 2012; Keskín and Atar 2013; Díaz-Viloria et al. 2015; Azmir et al. 2017; Isari et al. 2017; Rosas et al. 2018). Hajibabaei et al. (2005) report the extraction of DNA by phenol-chloroform as a less attractive method, as it demands a lot of time. However, it is widely used in this type of analysis (Rüber et al. 2003; Yu et al. 2005; Paine et al. 2007; Jerôme et al. 2008; Santos et al. 2013; Ma et al. 2015). Both the mentioned methods are efficient, but the phenol-chloroform protocol showed better recovery in DNA quality, in the case of the present study.

Chairi and Rebordinos (2014) mention in their work the difficulty in the conclusive differentiation of species of Engraulidae through molecular techniques, occasioning confusing results, and suggest an adjustment in the method, with the use of specific primers to provide reliable results. In the present work, specific primers for the Engraulidae family were used, built from the alignment of sequences registered in the GenBank for this group. In other works, universal primers are suggested for fish species, and are used effectively (Ward et al. 2005; Ivanova et al. 2007; Zhang and Hanner 2012). The use of universal primers is a valid option to be applied in future works, seeking an improvement in the amplification of the samples.

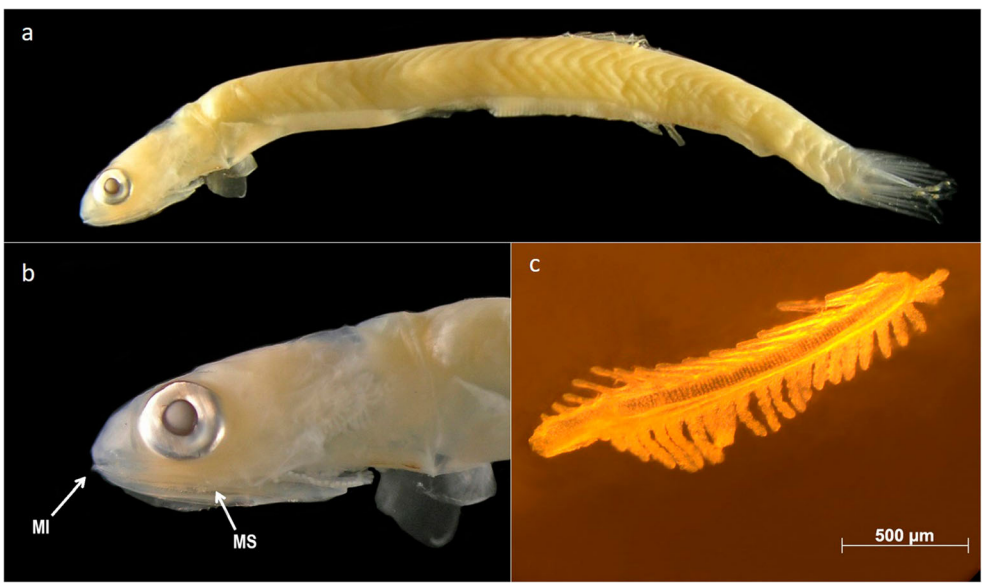


Fig. 10 Engraulidae— Morphotype 8. **a** Standard length=34.4mm; **b** details of the upper (MS) and lower (MI) jaws, head length =6.6mm; **c** gill rakers in the first branchial arch (lower section) (scale=500µm).

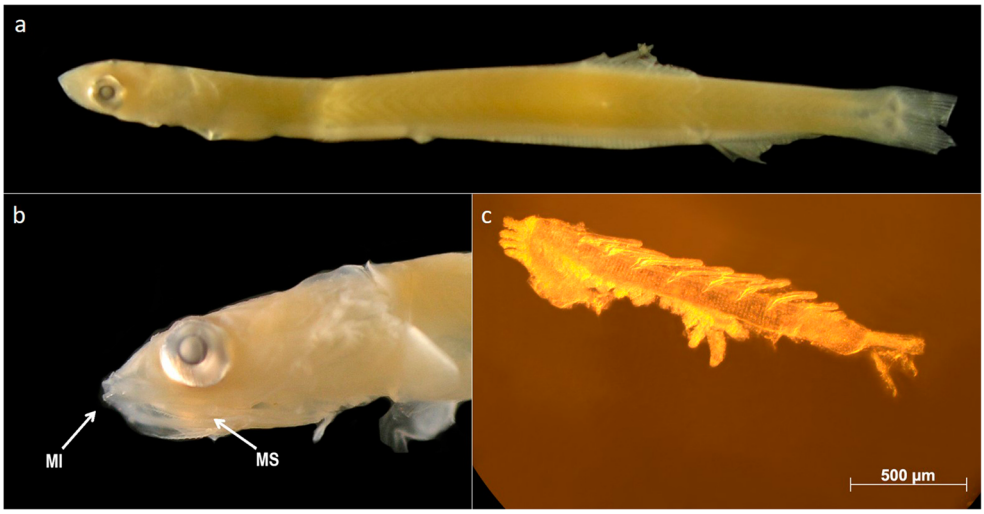


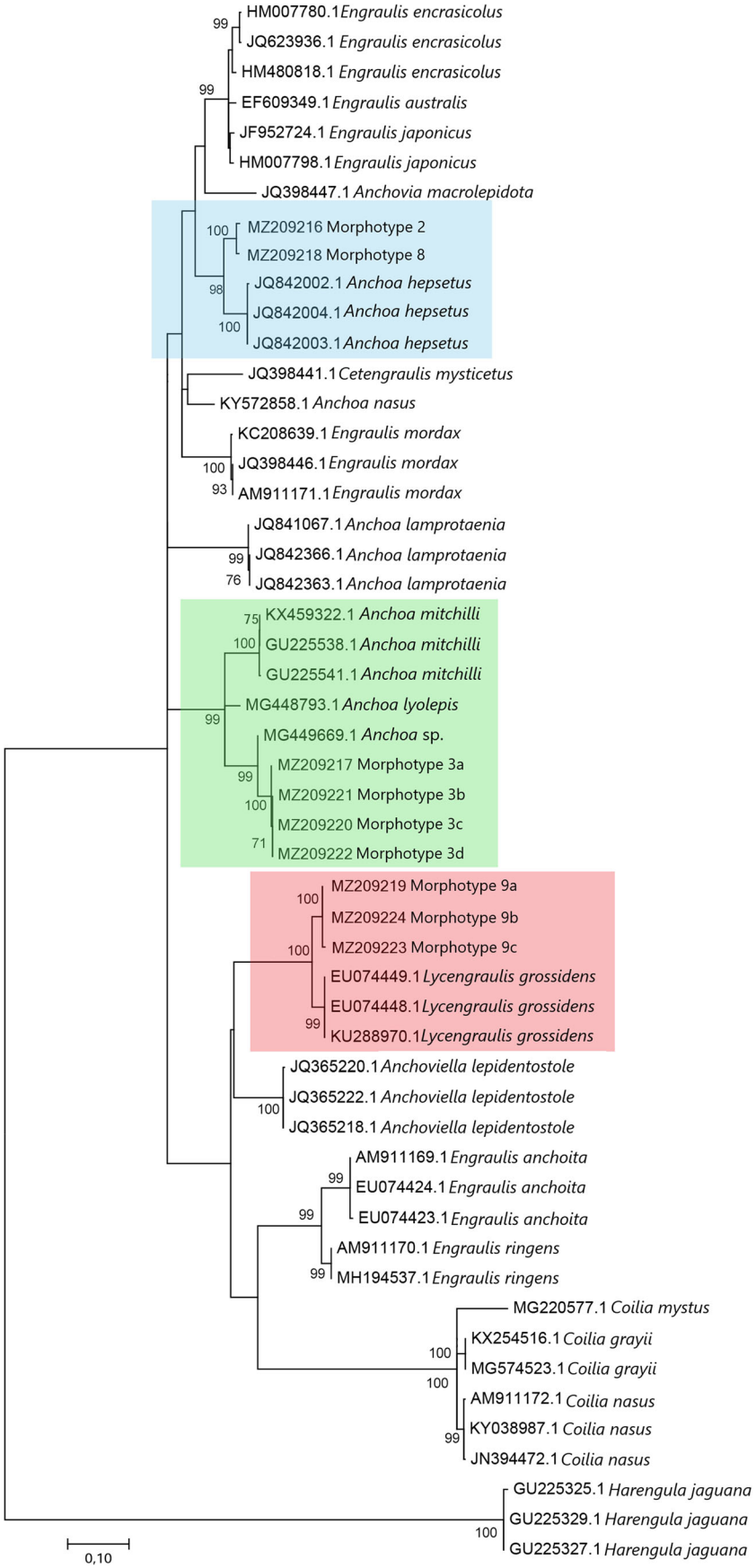
Fig. 11 Engraulidae—Morphotype 9. **a** Standard length=38.9mm; **b** details of the upper (MS) and lower (MI) jaws, head length=7.5mm; **c** gill rakers in the first branchial arch (lower section) (scale=500µm).

According to the result of the phylogenetic analysis, we suggest some relationships. It is possible to point out two distinct genera in the investigated estuarine ecosystems: genus *Anchoa* and genus *Lycengraulis*. In the clade highlighted in blue, we can indicate that the sequences of Morphotypes 2 and 8 are conspecific and it is possible to suggest that they belong to the genus *Anchoa*, but not to the species *A. hepsetus*, since they are separated, even with a high bootstrap value and the same number of rays in the anal fin. These morphotypes were related in the tree to the species *A. hepsetus*, with the sequences from a study carried out in the Caribbean region (Weigt et al. 2012). Analyzing the morphological characteristics of Morphotypes 2 and 8, in parallel with the phylogenetic analysis, it is possible to suggest that they belong to the same species, since the morphological differences are not significant, except for having shorter, 14–15 lower gill rakers the first branchial arch, which can be explained by a difference in the stage of development in the analyzed specimens. Observing the diagnostic characters described in the present study, these morphotypes would be more related to the species *Anchoviella brevirostris*, according to bibliography (Whitehead et al. 1988), due to the similar number of rays in the anal fin, similar position of the anal fin in relation to the dorsal, extension from the lower jaw to the tip of the snout and short upper jaw. It is not possible to establish this relationship in a phylogenetic tree as there is no record of the COI gene for *A. brevirostris* in the GenBank until the present moment. Anyway, an approximation to the clade of the other species of *Anchoviella* would be interesting, which did not occur in the present analysis.

In the second clade, indicated in green, it is possible to observe the conspecific Morphotype 3 sequences (3a, 3b, 3c, and 3d) grouped to the sequence referring to the species *Anchoa* sp. from a study carried out in Mexico (Elías-Gutiérrez et al. 2017), suggesting that these samples belong to the genus *Anchoa*. This clade, in its turn, is related to two other species of the genus *Anchoa*: *Anchoa mitchilli*, with the sequences from studies carried out in the Chesapeake Bay, USA (KX459322.1) (Aguilar et al. 2016), and in the Yucatán Peninsula, Mexico (GU225538.1; GU225541.1) (Valdez-Moreno et al. 2010); and *Anchoa lyolepis*, with sequences from a study carried out in Mexico (Elías-Gutiérrez et al. 2017) (MG448793.1), reinforcing relations with the genus, which is the most representative of the Engraulidae family on the southeast coast of Brazil. However, it was not possible to establish further relationships among the sequences in the present study and all occurring species in the estuarine regions studied, as they do not have registered sequences for the COI gene in the GenBank. These information, integrated with the morphology, suggest that morphotype 7 also belongs to the genus *Anchoa*.

The clade in red groups the conspecific sequences of Morphotype 9 (9a, 9b, and 9c) with the species *Lycengraulis grossidens*. The sequences of this species come from a study along the coastal region of Argentina (EU074448.1; EU074449.1) (Mabragaña et al. 2011), and from a work carried out on the Paraná River, near the city of Rosario in Argentina (KU288970.1) (Díaz et al. 2016). In this case, it is possible to suggest that these sequences belong to the species *L. grossidens*, as it is the only species of the genus occurring in the estuarine regions studied (Whitehead et al. 1988). These information, along with the morphology, may indicate that morphotype 6 also belongs to that species. *L. grossidens* differs from the other species in the family by its canine teeth, visible from juvenile stage (Whitehead et al. 1988). Such characteristic was not observed in the specimens analyzed in the present study, possibly due to the stage of development. In a study carried out with the species *L. grossidens* along the

Fig. 12 Phylogenetic consensus tree, constructed using the maximum ▶ likelihood estimation method (ML). Bootstrap support: ML>70%.


HM007780.1 Engraulis encrasicolus
JQ623936.1 Engraulis encrasicolus
HM480818.1 Engraulis encrasicolus
EF609349.1 Engraulis australis
JF952724.1 Engraulis japonicus
HM007798.1 Engraulis japonicus
JQ398447.1 Anchovia macrolepidota
MZ209216 Morphotype 2
MZ209218 Morphotype 8
JQ842002.1 Anchoa hepsetus
JQ842004.1 Anchoa hepsetus
JQ842003.1 Anchoa hepsetus
JQ398441.1 Cetengraulis mysticetus
KY572858.1 Anchoa nasus
KC208639.1 Engraulis mordax
JQ398446.1 Engraulis mordax
AM911171.1 Engraulis mordax
JQ841067.1 Anchoa lamprotaenia
JQ842366.1 Anchoa lamprotaenia
JQ842363.1 Anchoa lamprotaenia
KX459322.1 Anchoa mitchilli
GU225538.1 Anchoa mitchilli
GU225541.1 Anchoa mitchilli
MG448793.1 Anchoa lyolepis
MG449669.1 Anchoa sp.
MZ209217 Morphotype 3a
MZ209221 Morphotype 3b
MZ209220 Morphotype 3c
MZ209222 Morphotype 3d
MZ209219 Morphotype 9a
MZ209224 Morphotype 9b
MZ209223 Morphotype 9c
EU074449.1 Lycengraulis grossidens
EU074448.1 Lycengraulis grossidens
KU288970.1 Lycengraulis grossidens
JQ365220.1 Anchoviella lepidentostole
JQ365222.1 Anchoviella lepidentostole
JQ365218.1 Anchoviella lepidentostole
AM911169.1 Engraulis anchoita
EU074424.1 Engraulis anchoita
EU074423.1 Engraulis anchoita
AM911170.1 Engraulis ringens
MH194537.1 Engraulis ringens
MG220577.1 Coilia mystus
KX254516.1 Coilia grayii
MG574523.1 Coilia grayii
AM911172.1 Coilia nasus
KY038987.1 Coilia nasus
JN394472.1 Coilia nasus
GU225325.1 Harengula jaguana
GU225329.1 Harengula jaguana
GU225327.1 Harengula jaguana
0,10

Brazilian coast, Silva (2006) evidenced the difference in the gill rakers among individuals of this species, which were indicated in the present work as an important character in the distinction of morphotypes. The author also reports that it was not possible to establish a variation pattern for the number of rays in the fins, but we cannot disregard this characteristic, which is of great importance in the distinction of other species in the family.

Therefore, it is possible to suggest that among the specimens of Engraulidae morphologically identified in this study, there are distinct genera that could be obtained through molecular analysis. These relationships could be established more precisely with a fragment of the COI gene with a greater number of base pairs, or by analyzing another molecular markers for this purpose; for example, Azevedo et al. (2008) used the mitochondrial genes 12S and 16S; and Kochzius et al. (2010), in addition to using the COI gene, also evaluated the applicability of the 16S mitochondrial and cytochrome *b* (cyt *b*) genes.

Observing the genus *Engraulis* in the tree topology presented in this study, it is possible to suggest that it is not monophyletic, as it appears in distinct and distant clades in the analysis. Similar results were found by Bloom and Lovejoy (2012) and Loeb (2015). The Engraulidae family is well supported as a monophyletic group by several authors (Li and Ortí 2007; Lavoué et al. 2010; Bloom and Lovejoy 2012; Lavoué et al. 2013), who analyzed, based on molecular data, the phylogenetic relationships of Clupeiformes. The present work indicates Engraulidae as a monophyletic group.

With the present work, it is possible to observe that the techniques used here produce results to assist in the resolution of taxonomic issues, and suggest the morphotyping of the material as a first step. It is well known that when using genetic data, we corroborate the need to use this tool and the importance of a more in-depth morphological identification to reach a conclusion. However, to perform the morphotyping of the samples is suggested as a first step for the identification of the specimens.

Conclusion

With the studies carried out in the present work in tropical estuarine ecosystems, it was possible to morphologically distinguish fish larvae of the Engraulidae family into morphotypes, and from the molecular analysis of the COI gene it was possible to establish some relationships between these data.

It is concluded that morphotypes 6 and 9 can be grouped into a single morphotype, due to their similar morphological characters, and the differences between the two are considered not significant enough to separate them. Therefore, according to molecular results, they can be associated with the genus *Lycengraulis*. Morphotypes 3 and 7 can also be grouped into a single morphotype, and according to the molecular results, it is possible to associate them with the genus *Anchoa*. Morphotypes 1, 4, and 5, morphologically, are closer to the species *Anchoviella lepidentostole*; however, we do not have molecular data to confirm this relationship. Aside from being morphologically very similar, the molecular results for Morphotypes 2 and 8 indicate that they can be grouped into a single morphotype, and associated with the genus *Anchoa*.

The protocols followed were considered effective to establish an integrated identification analysis, confirming that the morphological identification methods combined with molecular analysis are fundamental and complementary tools. It was possible to contribute effectively to the knowledge of the composition of fish larvae in Brazilian estuarine ecosystems, as well as assist in the elucidation of taxonomic issues in the Engraulidae family. However, further research, complementary to the approach presented here, is needed to enrich the information on this group.

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Declarations

Conflict of Interest The authors declare no competing interests.

Ethical approval This article does not contain any studies with animals performed by any of the authors.

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Data availability All data generated and/or analyzed during the current study are included in this published article and its supplementary information files.

Author contribution MMJ participated in the fieldwork study, analyzed the molecular data, and wrote the paper. COD and ACTB analyzed the data and revised the paper. RS designed the molecular analysis and revised the paper. SLCB designed and participated in the fieldwork study, and revised the paper. All the authors edited the manuscript.

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