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DNA barcoding and taxonomic validation of *Caranx* spp. larvae

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Abstract

Of the five nominal species in the genus *Caranx* Lacepède 1801 distributed throughout the Eastern Central Pacific, *Caranx caballus* and *Caranx sexfasciatus* are the only two that have formal fish larval descriptions based on diagnostic characteristics (morphology, meristics and pigmentation). In this study, the diagnostic characteristics of three *Caranx* species larvae were validated using DNA barcoding analysis cytochrome *c* oxidase subunit I (COI; 651 bp). For the first time, the morphological taxonomic assignation of *C. caballus* fish larvae was confirmed using COI gene partial sequences of adults, with a genetic similarity between 99.8–100%. However, molecular evidence demonstrated that fish larvae previously described as *C. sexfasciatus* had high genetic similarity (99.7–100%) and low genetic distance (<1%) to *Caranx caninus* adults. An undescribed larval morphotype collected in the present study genetically matched (100%) with COI sequences of *C. sexfasciatus* adults. The diagnostic characteristics of this new morphotype were a lack of pigmentation in the supraoccipital crest, over the gut, and at the terminal region of the gut. The combination of diagnostic characteristics and DNA barcoding evidence allowed the discrimination and validation of *C. caballus*, *C. caninus* and *C. sexfasciatus* larvae. The diagnostic characteristics and COI sequences of *Caranx lugubris* and *Caranx melampygus* larvae, which are also distributed in the Eastern Central Pacific, remain to be investigated.

Introduction

An ongoing international research endeavour is to combine larval morphology and DNA barcoding to investigate the diversity of fish species; this combination provides unprecedented precision in larval taxonomy (Pegg *et al.*, 2006; Hui-Ling *et al.*, 2013). With the rise of molecular techniques, it is worth validating published fish larval taxonomic descriptions. This approach can also provide new information to identify larvae that currently lack diagnostic morphological criteria. The proportion of fish with known taxonomic descriptions of their larvae varies geographically, depending on regional species richness and the amount of time and expertise that has been invested in taxonomic research in that region (Fahay, 2007). There has been substantial effort to describe early larval stages of fish in the eastern Pacific. This research has been published in landmark identification guides from the North-east Pacific (Matarese *et al.*, 1989), the California Current System (Moser, 1996) and the Colombian Pacific (Beltrán-León & Ríos-Herrera, 2000). However, these guides are still incomplete when compared with the fish species richness in the Mexican Pacific and Gulf of California, a transitional faunistic region among the California, Panamanian (Panamic) and Cortez biogeographic provinces (Spalding *et al.*, 2007; Briggs & Bowen, 2012).

The species identification of fish larvae using diagnostic characteristics based on morphology, meristics and pigmentation is a difficult task, particularly for rare species or genera that include sibling and cryptic species. The precise identification of fish larvae has been largely solved by comparing diagnostic morphological features with modern molecular methods (Victor *et al.*, 2009; Matarese *et al.*, 2011; Hui-Ling *et al.*, 2013). DNA barcoding provides additional information to discriminate among closely related species that are morphologically similar (Hebert *et al.*, 2003*a*, 2003*b*; Hebert & Gregory, 2005; Ward *et al.*, 2005). Indeed, this method has frequently provided decisive evidence to discriminate problematic taxonomic groups of species (Taylor & Watson, 2004; Watson *et al.*, 2015). DNA barcoding has been widely used in taxonomic, ecological and biogeographic studies to identify fish eggs (Harada *et al.*, 2015; Lewis *et al.*, 2016; Ahern *et al.*, 2018) and larvae (Pegg *et al.*, 2006; Hubert *et al.*, 2012; Hui-Ling *et al.*, 2013; I-Shiung *et al.*, 2013; Thirumaraiselvi *et al.*, 2015; Camacho-Gastélum *et al.*, 2017).

The genus *Caranx* (Carangidae) currently includes 18 extant nominal species, with numerous cases of synonymies and misspelled names (Froese & Pauly, 2021). Only five nominal

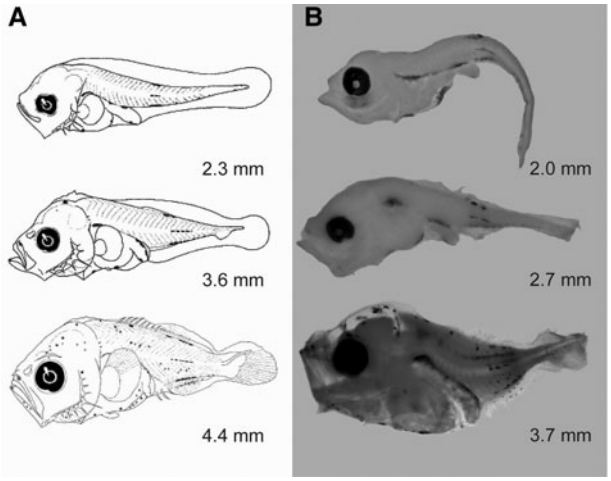

Fig. 1. Illustrations of *Caranx caballus* larvae. (A) Original illustrations according to Sumida *et al.* (1985) and (B) *C. caballus* larvae confirmed using DNA barcoding in the present study. Note the identical pigmentation patterns in larvae shown in A and B.

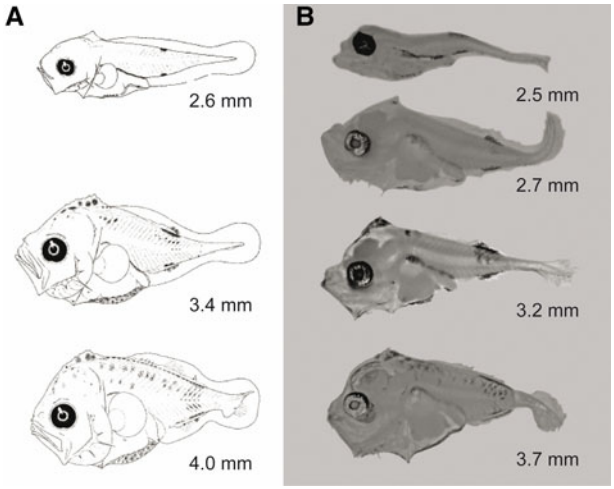

Fig. 2. Illustrations of *Caranx caninus* larvae. (A) Original illustrations of *Caranx sexfasciatus* according to Sumida *et al.* (1985) and (B) *C. caninus* larvae confirmed using DNA barcoding in the present study. Note that these larvae were morphologically identical to those described previously as *C sexfasciatus* by Sumida *et al.* (1985), but they genetically correspond to *C. caninus* larvae, with high mitochondrial cytochrome *c* oxidase subunit I (COI) similarity (99.7–100%).

Caranx species are distributed in the Eastern Central Pacific, namely *Caranx sexfasciatus* Quoy & Gaimard, 1825; *Caranx melampygus* Cuvier, 1833; *Caranx lugubris* Poey, 1860; *Caranx caninus* Günther, 1867; and *Caranx caballus* Günther, 1868 (Froese & Pauly, 2021). The cytochrome *c* oxidase subunit I (COI) gene sequences for each of those species have been deposited in GenBank and/or BOLDSystems. However, there are only diagnostic descriptions of the larval stages of *C. caballus* and *C. sexfasciatus* (Sumida *et al.*, 1985). The larvae of both species are distinguished by a characteristic pigmentation pattern (Sumida *et al.*, 1985). The diagnostic characteristics used to identify *C. caballus* larvae (<4 mm standard length) are the presence of pigments on the dorsal margin of the body (Figure 1A); *C. sexfasciatus* reportedly has a conspicuously pigmented supraoccipital crest (Figure 2A) (Sumida *et al.*, 1985). All other described carangid larvae lack pigmentation on the supraoccipital crest (Sumida *et al.*, 1985). In this study, we combined diagnostic characteristics (morphology, meristics and pigmentation) and DNA barcoding using COI gene partial sequences to clarify and validate the taxonomic species identification of *Caranx* larvae. This integrative analysis supports the separation and identification of *Caranx* species, an approach to identify reproductive and nursery areas useful in management of coastal fisheries in the Eastern Central Pacific (Froese & Pauly, 2021).

Materials and methods

Taxonomic analysis of *Caranx* fish larvae was based on zooplankton samples collected from three regions of the Mexican Pacific, two in Baja California Sur (Cabo Pulmo National Park, sampled weekly between 2016 and 2017, and Ensenada de Muertos, October 2013) and a third in Jalisco (Cabo Corrientes, April 2015) (Table 1). Zooplankton samples were collected using a conical net (60-cm mouth diameter with a 333-µm mesh size) towed near the surface (<5 m depth), following a semicircular path at a mean speed of 1 m s^{-1} for 5 min. All zooplankton samples were collected during daylight hours and preserved in 95% ethanol, which was replaced after 24 h to ensure adequate preservation. A total of 149 *Caranx* fish larvae were identified to the most precise taxonomic level possible using meristic, morphometric and pigmentation criteria (Sumida *et al.*, 1985). The standard length of all larvae was measured with a calibrated micrometer. The 35 best-preserved *Caranx* larvae were selected for molecular analysis; the specimens represented pre-flexion and flexion larval stages between 2.0 and 4.4 mm in length. These specimens provide adequate representation of their morphotypes. Specimens were photographed with a digital camera attached to a stereoscope. Curatorial information was uploaded to BOLDSystems (project: Identifying early life stages of fish from waters of the Mexican Pacific through DNA barcoding).

DNA was extracted with a modified spin-column version of the fibreglass membrane method (Ivanova *et al.*, 2006). A 651-base pair (bp) fragment was amplified from the 5′ region of the mitochondrial COI gene using primers FishF2-t1 (5′-TGTAAAACGACGG CCAGTCGACTAATCATAAAGATATCGGCAC-3′) and FishR2-t1 (5′-CAGGAAACAGCTATGACACTTCAGGGTGACCGAAGAAT CAGAA-3′) (Ward *et al.*, 2005; Ivanova *et al.*, 2007). PCR amplifications were performed in 18-µl including 30 ng DNA template, 5× MyTaq Buffer (Bioline®), 10 µM of each primer, and 1 U of MyTaq DNA polymerase. PCR was performed in an Eppendorf Mastercycler Pro thermocycler, with the following thermal cycling conditions: 3 min at 96°C; 35 cycles of 30 s at 94°C, 40 s at 52°C, 1 min at 72°C; and a final extension of 5 min at 72°C. PCR products were visualized by electrophoresis in 1.0% agarose gels stained with ethidium bromide. PCR products were purified and sequenced in forward and reverse directions at the Instituto de Biología, Universidad Nacional Autónoma de México (IB-UNAM, Mexico City).

All COI sequences were manually edited and aligned using GENEIOUS® Prime 2020 software (https://www.geneious.com; Kearse *et al.*, 2012). We used the basic local alignment search tool (BLAST) included in GENEIOUS® and the Identification System of Barcode of Life Data Systems (BOLDSystems; http://www.boldsystems.org) to determine homology between the COI sequences from our study and previously deposited sequences. Each sequence was assigned a barcode index number (BIN) in BOLDSystems. The BIN was used for the interpretation of species boundaries based on the analysis of nucleotide variation patterns in the barcode region (Ratnasingham & Hebert, 2013). We used DnaSP software to obtain the number of haplotypes for each morphotype observed in the collected *Caranx* larvae and to remove redundancy in sequence data sets (Rozas *et al.*, 2003). The COI sequences of the five nominal *Caranx* species distributed in the Eastern Central Pacific were searched in the National Center for Biotechnology Information (NCBI) and BOLDSystems databases.

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Table 1. Taxonomic identification of *Caranx* spp. fish larvae collected at three regions of the Mexican Pacific comparing morphological and molecular criteria

Specimen ID	Regions	Fish larval stages	Identification according with Sumida *et al.* (1985) criteria	*COI* Similarity (%)	Species name at GenBank and BOLDSystems	GenBank accession number	BIN
ILC049	CP	preflexion	*C. caballus*	99.8	*C. caballus*	MK670988	BOLD: AAC4853
ILC069	CP	preflexion	*C. caballus*	100	*C. caballus*	MK670991	
ILC225	CP	preflexion	*C. caballus*	100	*C. caballus*	MK671005	
ILC233	CP	preflexion	*C. caballus*	100	*C. caballus*	MT641332	
ILC247	CP	preflexion	*C. caballus*	100	*C. caballus*	MT641333	
ILC257	CP	preflexion	*C. caballus*	100	*C. caballus*	MT641334	
ILC263	CP	preflexion	*C. caballus*	99.8	*C. caballus*	MT641335	
ILC266	CP	flexion	*C. caballus*	100	*C. caballus*	MT641336	
IPM115	EM	flexion	*C. caballus*	100	*C. caballus*	MK670995	
IPM116	EM	flexion	*C. caballus*	100	*C. caballus*	MK670996	
ILC226	CP	preflexion	*C. caballus*	100	*C. sexfasciatus*	MT641337	BOLD: AAB0584
ILC250	CP	preflexion	*C. caballus*	99.8	*C. sexfasciatus*	MT641338	
ILC251	CP	preflexion	*C. caballus*	100	*C. sexfasciatus*	MT641339	
ILC253	CP	preflexion	*C. caballus*	99.8	*C. sexfasciatus*	MT641340	
ILC051	CP	preflexion	*C. sexfasciatus*	99.8	*C. caninus*	MK670989	BOLD: AAE2948
ILC053	CP	flexion	*C. sexfasciatus*	100	*C. caninus*	MK670990	
ILC111	CP	preflexion	*C. sexfasciatus*	100	*C. caninus*	MK670997	
ILC114	CP	flexion	*C. sexfasciatus*	99.8	*C. caninus*	MK670998	
ILC115	CP	flexion	*C. sexfasciatus*	100	*C. caninus*	MK670999	
ILC146	CP	preflexion	*C. sexfasciatus*	100	*C. caninus*	MK671000	
ILC219	CP	preflexion	*C. sexfasciatus*	100	*C. caninus*	MK671001	
ILC220	CP	preflexion	*C. sexfasciatus*	100	*C. caninus*	MK671002	
ILC222	CP	preflexion	*C. sexfasciatus*	99.7	*C. caninus*	MK671003	
ILC223	CP	flexion	*C. sexfasciatus*	100	*C. caninus*	MK671004	
ILC237	CP	preflexion	*C. sexfasciatus*	100	*C. caninus*	MT641341	
ILC238	CP	preflexion	*C. sexfasciatus*	99.8	*C. caninus*	MT641342	
ILC240	CP	preflexion	*C. sexfasciatus*	100	*C. caninus*	MT641343	
ILC241	CP	preflexion	*C. sexfasciatus*	100	*C. caninus*	MT641344	
ILC242	CP	preflexion	*C. sexfasciatus*	100	*C. caninus*	MT641345	
ILC249	CP	preflexion	*C. sexfasciatus*	100	*C. caninus*	MT641346	
ILC254	CP	preflexion	*C. sexfasciatus*	99.8	*C. caninus*	MT641347	
ILC262	CP	preflexion	*C. sexfasciatus*	99.8	*C. caninus*	MT641348	
IPM110	CC	preflexion	*C. sexfasciatus*	99.8	*C. caninus*	MK670992	
IPM111	CC	preflexion	*C. sexfasciatus*	100	*C. caninus*	MK670993	
IPM112	CC	flexion	*C. sexfasciatus*	99.8	*C. caninus*	MK670994	

CP, Cabo Pulmo National Park (23°27′57.99″N 109°24′40.99″W); EM, Ensenada de Muertos (23°59′22.45″N 109°49′41.73″W), Baja California Sur; CC, Cabo Corrientes (20°17′ 50.31″N 105° 53′40.88″W), Jalisco, Mexico.
Similarity of COI barcoding of fish larvae collected north-west of Mexico compared with known DNA sequences typically from adults obtained from GenBank (GB) and BOLDSystems. Barcode Index Number assignation (BIN); clustered barcode sequences that create OTUs (operational taxonomic units) closely reflect species groupings.

Sequences of adult specimens with the same length (651 bp) were downloaded independently to the collection site. All sequences corresponded to the BINs of the five *Caranx* species: *C. sexfasciatus* (BOLD:AAB0584), *C. melampygus* (BOLD:AAB0585), *C. lugubris* (BOLD:AAI6630), *C. caninus* (BOLD:AAE2948) and *C. caballus* (BOLD:AAC4853). The haplotypes that characterized each adult *Caranx* species were also obtained from GenBank and BOLDSystems for comparison with the larval haplotypes obtained in the present study. All haplotypes were aligned using MEGA 10.0.5 software to calculate the intra- and inter-specific genetic distances (Kimura 2-parameter (K2P) method) and neighbour-joining (NJ) tree reconstruction with 10,000 bootstraps (Kumar *et al.*, 2016). The mackerel scad, *Decapterus macarellus* (Cuvier, 1833), was used as an outgroup because the genus *Decapterus*

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has been placed in a sister clade of *Caranx* according to phylogenetic analyses of species of the family Carangidae (Reed *et al.*, 2002). The complete *D. macarellus* COI genome sequence was downloaded from GenBank (accession number KM986880) (Zou *et al.*, 2016).

Results

A total of 149 *Caranx* fish larvae were analysed. The 35 best-preserved specimens were used to compare morphological and DNA barcoding information. Fourteen larvae were identified as *C. caballus* and 21 as *C. sexfasciatus* according to the diagnostic characteristics reported by Sumida *et al.* (1985). The 14 larvae identified as *C. caballus* were based on the presence of sparse pigmentation in the larval stages; opposing dorsal, lateral, and ventral streaks on the body; and melanophores on the top of the head and over and along the abdominal region (Table 1, Figure 1). The remaining 21 larvae were identified as *C. sexfasciatus* based on the presence of a characteristic pigment in the supraoccipital crest, which is absent in other Carangidae species. The smallest larvae (2.4–2.6 mm) had no pigmentation on the crest (Table 1, Figure 2).

Each COI sequence obtained from the 35 *Caranx* larvae was 651 bp, without evidence of stop codons, insertions or deletions in the reading frame. Hence, these sequences represent functional COI sequences. All sequences were deposited in GenBank; the accession numbers are shown in Table 1. BLAST analysis showed that 10 of 14 larvae identified with diagnostics characters as *C. caballus* matched with *C. caballus* sequences from GenBank and BOLDsystems (99.8–100% similarity). Indeed, these 10 larvae were assigned a BIN (BOLD:AAC4853) that contained only *C. caballus* sequences. Thus, the diagnostic description of *C. caballus* larvae was genetically corroborated. However, the remaining four larval specimens identified as *C. caballus* matched *C. sexfasciatus* COI sequences (99.8–100% similarity); they were assigned a BIN (BOLD:AAB0584) that included only *C. sexfasciatus* sequences. The main diagnostic characteristics of these four fish larvae (2.7–4.0 mm in length) were the lack of pigmentation in the supraoccipital crest, over the gut, and in the terminal region of the gut (Figure 3). The 21 fish larvae previously identified as *C. sexfasciatus* based on the published diagnostic characteristics (Sumida *et al.*, 1985) were genetically similar to *C. caninus*. Indeed, these specimens showed 99.7–100% similarity to *C. caninus* COI sequences and were assigned a BIN (BOLD:AAE2948) that exclusively included *C. caninus* sequences (Table 1).

COI sequences of the 10 *C. caballus* larvae showed four distinct haplotypes (Hd = 0.73; $\pi = 0.0014$; three polymorphic sites) (Table 2). The COI sequences of the four *C. sexfasciatus* larvae included three distinct haplotypes (Hd = 0.83; $\pi = 0.0017$; two polymorphic sites). The COI sequences of the 21 *C. caninus* larvae included seven distinct haplotypes (Hd = 0.69; $\pi = 0.0014$; six polymorphic sites) (Table 2). Of the total of 91 *Caranx* COI sequences distributed in different regions of the world (downloaded from GenBank and/or BOLDSystems), four haplotypes belonged to *C. caballus*, 11 to *C. sexfasciatus*, three to *C. caninus*, seven to *C. melampygus* and two to *C. lugubris* (Table 3).

The intraspecific genetic distances among the haplotypes of the five *Caranx* species were between 0.15–0.42%; and the interspecific between 6.16–14.34%. The low intraspecific genetic distance among haplotypes of *Caranx* larvae confirms the precise species identification of the larval morphotypes of the *C. caballus* (0.25%), *C. caninus* (0.27%) and *C. sexfasciatus* (0.26%) morphotypes collected in the present study (Table 4). The NJ tree of *COI* sequences shows five distinct clades that match the five nominal *Caranx* species; the haplotypes of the fish larvae sequenced in the present study were placed in each corresponding clade (Figure 4).

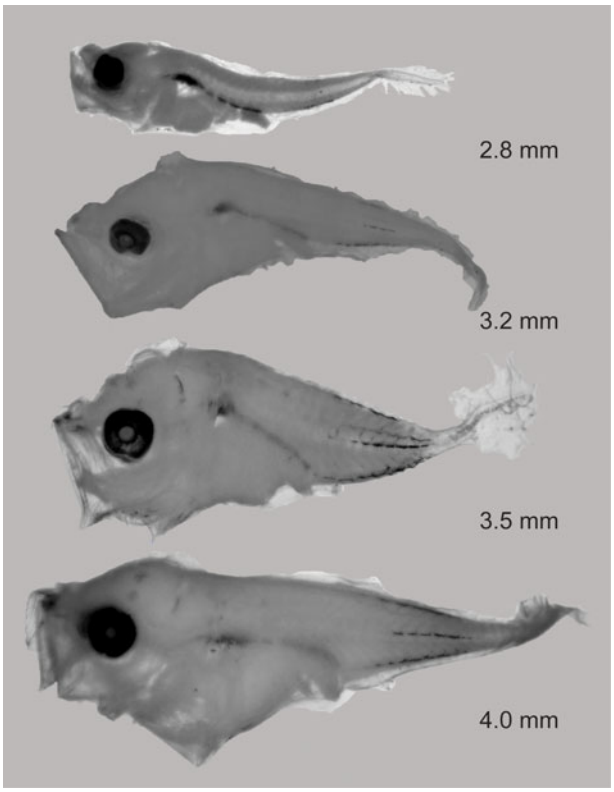

Fig. 3. Illustrations of *Caranx sexfasciatus* larvae. The unidentified *Caranx* morphotype was later identified as *C. sexfasciatus* based on DNA barcoding evidence from larvae collected in the present study.

Table 2. Haplotypes of mitochondrial gene cytochrome *c* oxidase subunit I (COI, 651 bp) of larvae of the three *Caranx* species collected at the Mexican Pacific

Haplotypes	Sequence number	Specimen ID
Caranx caballus		
C. cab H1	1	ILC049
C. cab H2	4	ILC069, ILC247, IPM115, IPM116
C. cab H3	4	ILC225, ILC233, ILC257, ILC266
C. cab H4	1	ILC263
Caranx sexfasciatus		
C. sex H1	1	ILC226
C. sex H2	2	ILC250, ILC253
C. sex H3	1	ILC251
Caranx caninus		
C. can H1	5	ILC051, ILC114, ILC220, ILC240, IPM112
C. can H2	11	ILC053, ILC111, ILC115, ILC146, ILC219, ILC223, ILC237, ILC241, ILC242, ILC249, IPM111
C. can H3	1	ILC222
C. can H4	1	ILC238
C. can H5	1	ILC254
C. can H6	1	ILC262
C. can H7	1	IPM110

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Table 3. Haplotypes of mitochondrial gene cytochrome *c* oxidase subunit I (COI, 651 bp) of *Caranx* spp. sequenced from different regions of the world downloaded from GenBank and BOLDSystems

Haplotype	Sequence number	GenBank accession number or sequence ID	BIN ID and DOI
Caranx caballus			
C. cab H1	4	RDFCA268-05[1], RDFCA270-05[1], RDFCA272-05[1], RDFCA384-05[1]	BOLD:AAC4853 dx.doi.org/10.5883/BOLD:AAC4853
C. cab H2	1	RDFCA269-05[1]	
C. cab H3	1	RDFCA271-05[1]	
C. cab H4	1	RDFCA393-05[1]	
Caranx sexfasciatus			
C. sex H1	8	HQ560961.1[12], F952695.1[13], JN312937.1[1], JQ431547.1[3], JX261414.1[12], JX261569.1[12], KF378587.1[15], KU692408.1[16]	BOLD:AAB0584 dx.doi.org/10.5883/BOLD:AAB0584
C. sex H2	17	EF609305.1[11], HQ560947.1[12], JN312936.1[1], JQ431548.1[3], JX261259.1[12], JX261315.1[12], KF378586.1[15], KF714907.1[18], KJ202139.1[18], KJ202140.1[18], KJ202142.1[18], KU535573.1[18], KU692409.1[16], KX064466.1[18], KX064467.1[18], KX064468.1[18], MH638724.1[17]	
C. sex H3	1	JF952696.1[13]	
C. sex H4	4	JF493042.1[4], JF493044.1[4], KF009576.1[18], KU176334.1[4]	
C. sex H5	2	HQ560966.1[12], KU176404.1[4]	
C. sex H6	1	KJ202141.1[18]	
C. sex H7	1	KJ013038.1[18]	
C. sex H8	1	KC970458.1[14]	
C. sex H9	1	JX261464.1[12]	
C. sex H10	1	JQ431549.1[3]	
C. sex H11	1	JQ431546.1[3]	
Caranx caninus			
C. can H1	3	JN313923.1[1], RDFCA231-05[1], RDFCA385-05[1]	BOLD:AAE2948 dx.doi.org/10.5883/BOLD:AAE2948
C. can H2	5	HQ974525.1[1], HQ974567.1[1], EU752066.1[2], RDFCA324-05[1], RDFCA392-05[1]	
C. can H3	1	EU752067.1[2]	
Caranx melampygus			
C. mel H1	24	DQ427063.1[9], DQ427064.1[9], FOAJ803-09[1], FOAJ890-09[1], FOAJ892-09[1], HQ564390.1[1], JF493040.1[4], JQ431544.1[3], KC970375.1[18], KF649843.1[10], KF929686.1[18], KU943758.1[4], KU943761.1[4], KU943804.1[8], KY371306.1[7], KY371307.1[7], KY371308.1[7], KY371309.1[7], KY371310.1[7], MG816665.1[18], MK566836.1[5], RDFCA388-05[1], SAIAB247-06[1], SAIAB414-06[1]	BOLD:AAB0585 dx.doi.org/10.5883/BOLD:AAB0585
C. mel H2	1	KP194436.1[6]	
C. mel H3	1	JF493039.1[4]	
C. mel H4	2	DQ427059.1[9], DQ427062.1[9]	
C. mel H5	1	FOAJ891[1]	
C. mel H6	1	FOAN703[1]	
C. mel H7	1	FTWS948[1]	
C. lugubris			
C. lug H1	5	FOAC434-05[1], JQ431541.1[3], JQ431542.1[3], MK566835.1[5], MK657661.1[5]	BOLD:AAI6630 dx.doi.org/10.5883/BOLD:AAI6630
C. lug H2	1	KU176344.1[4]	

[1]International Barcode of Life (IBOL/BOLSystems); [2] Yancy *et al.* (2008); [3]Hubert *et al.* (2012); [4]Steinke *et al.* (2016); [5] Delrieu-Trottin *et al.* (2019); [6] Steinke *et al.* (2017); [7] Hou *et al.* (2018); [8] Chang *et al.* (2017); [9] Murakami *et al.* (2007), [10] Santos *et al.* (2011); [11] Ward & Holmes (2007); [12] Jaafar *et al.* (2012); [13] Zhang & Hanner (2011); [14] Templonuevo *et al.* (2018); [15] Justine *et al.* (2013); [16] Dahruddin *et al.* (2017); [17] Xu *et al.* (2019); and [18] Unpublished.
Barcode Index Number (BIN); clustered barcode sequences that create OTUs (Operational Taxonomic Units) closely reflect species groupings.

The percentage of genetic similarity, BIN assignments, genetic distances and reconstruction of the NJ tree strongly confirm the taxonomic identity of early larval stages of *C. caballus*. However, larvae identified as *C. sexfasciatus* according with diagnostic characteristics from Sumida *et al.* (1985), matched with adult *C. caninus* sequences. This finding indicates the diagnostic characteristics used to identify *C. sexfasciatus* must be reassigned to describe *C. caninus*. Finally, COI sequence indicated

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Table 4. Kimura two-parameters model of genetic distance within *Caranx* species (intraspecific variability, bold font) and among *Caranx* species (interspecific variability) for haplotypes of mitochondrial gene cytochrome *c* oxidase subunit I (COI, 651 bp) of adults obtained from GenBank/BOLDSystems and fish larvae collected in the present study. *Decapterus macarellus* (Cuvier, 1833) was used as an outgroup

	COI Haplotype	1	2	3	4	5	6	7	8	9
GenBank BoldSystems Juveniles and adults	**1** Outgroup	0								
	2 *C. melampygus*	18.57	**(0.42)**							
	3 *C. lugubris*	16.88	8.90	**(0.15)**						
	4 *C. caballus*	15.78	13.44	14.34	**(0.23)**					
	5 *C. caninus*	18.04	8.25	9.71	11.83	**(0.31)**				
	6 *C. sexfasciatus*	17.69	6.16	9.89	11.57	8.50	**(0.33)**			
Fish larvae of the present study	**7** *C. caballus*	15.68	13.52	14.49	**0.25**	11.92	11.76	**(0.23)**		
	8 *C. caninus*	17.95	8.24	9.73	11.73	**0.27**	8.49	11.83	**(0.31)**	
	9 *C. sexfasciatus*	17.82	6.10	9.95	11.57	8.47	**0.26**	11.76	8.45	**(0.21)**

Fig. 4. Neighbour-joining tree using 19 haplotypes of cytochrome *c* oxidase subunit I (COI, 651 bp) of different *Caranx* species obtained from GenBank and/or BOLDSystems compared with 14 haplotypes of *Caranx* larval sequences obtained in the present study (haplotypes: ▲ = *Caranx caninus*; ■ = *Caranx caballus*; ◆ = *Caranx sexfasciatus*). Numbers shown on the tree branches indicate bootstrap values (>70%) based on 10,000 replicates. Numbers between parentheses are the sequences per haplotypes. The scale bar represents the genetic distance of the Kimura two-parameter model. The accession numbers of *Caranx* species sequences downloaded from GenBank and/or BOLDSystems are shown in Table 3.

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that *C. sexfasciatus* larvae correspond to our previously undescribed morphotype (without pigmentation in the supraoccipital crest and over and along the terminal region of the gut; Figure 3). This morphotype was morphologically similar to *C. caballus* collected in the present study (Figure 1). Therefore, early larval stages of *C. sexfasciatus* and *C. caballus* identification is a taxonomic challenge using only diagnostic criteria.

Discussion

Diagnostic characteristics (morphology, meristics and pigmentation) are useful to taxonomists and ecologists because they allow fast identification of fish larval specimens collected in the field. However, the identification of fish eggs and larval stages is considerably more complex than juveniles and adults when discriminating among rare species or sibling and cryptic species (Ahern *et al.*, 2018). The combination of diagnostic characteristics and DNA barcoding allows for greater precision in species identification (Hui-Ling *et al.*, 2013). Both methodological approaches are required to evaluate closely related species that show overlap in meristic and/or morphological diagnostic characteristics (Victor *et al.*, 2009; Matarese *et al.*, 2011), which are used for precise identification of target species in ecological studies. Species identification using molecular confirmation (e.g. DNA barcoding) allow the distinction among nominal species in regions with great fish diversity, such as the Mexican Pacific and the Gulf of California (Thomson *et al.*, 1979; Allen & Robertson, 1994; Fischer *et al.*, 1995; Camacho-Gastélum *et al.*, 2017).

Although there has been significant progress in describing the larvae of fish distributed in the North-east Pacific, the California Current System and the Colombian Pacific (Matarese *et al.*, 1989; Moser, 1996; Beltrán-León & Ríos-Herrera, 2000), only two of the five nominal *Caranx* species (*C. caballus* and *C. sexfasciatus*) distributed in the Eastern Central Pacific are currently known by their larval morphological, meristic and pigmentation descriptions (Sumida *et al.*, 1985). Although *C. caninus*, *C. lugubris* and *C. melampygus* adults have also been recorded in the Eastern Central Pacific (Froese & Pauly, 2021), their larval morphology is unknown. Kim *et al.* (2001) analysed the digestive enzymes of early larvae from *C. melampygus* females that spawned under laboratory conditions; however, they did not provide morphological descriptions or photographs of those larvae. Avendaño-Ibarra *et al.* (2014) reported *C. caballus*, *C. sexfasciatus* and other morphotypes identified as *Caranx* spp. in an updated taxonomic list of marine fish larvae from the region between the Gulf of California and Colima (19–30°N, Mexico). We initially identified *C. caballus* larvae based on the diagnostic characteristics described by Sumida *et al.* (1985) and then confirmed their taxonomic identity using COI sequences from those larvae. However, larvae identified as *C. sexfasciatus* – according to the main diagnostic characteristic of a conspicuously pigmented supraoccipital crest (Sumida *et al.*, 1985; Moser, 1996) – were actually *C. caninus* confirmed by: high genetic similarity (99–100%); clustered into a particular BIN; and low genetic distance among *C. caninus* haplotypes (0.27%). The larval morphotype genetically identified as *C. sexfasciatus* has no pigment in the supraoccipital crest and lacks pigments over the gut and along the terminal region of the gut. Thus, the genetic evidence indicates that the previous morphological and pigmentation description of *C. sexfasciatus* reported by Sumida *et al.* (1985) must be considered diagnostic characteristics of *C. caninus* larvae.

Although there is a lack of diagnostic descriptions of the larval stage of *C. lugubris* and *C. melampygus*, both of which inhabit the Eastern Central Pacific, it is possible to identify the species within the genus *Caranx* using genetic divergence. In this sense, DNA barcoding is an effective tool to identify Carangidae species, based on their average genetic distance (K2P) among individuals (0.37%), species within genera (10.53%) and genera within the Carangidae family (16.56%) (Jaafar *et al.*, 2012). The genetic distances obtained in the present study (intraspecific, 0.15–0.42%; interspecific, 6.16–14.34%) were similar to those reported by Jaafar *et al.* (2012); these genetic distances increase with the change in the taxonomic level. This information can be used to identify and distinguish among highly related species (Ward *et al.*, 2005; Jaafar *et al.*, 2012).

In summary, we have shown that the larval morphology of three of five *Caranx* species distributed in the Eastern Central Pacific is supported by their diagnostic characteristics and DNA barcoding evidence. Diagnostic and genetic characteristics of different ontogenetic larval stages of *C. lugubris* and *C. melampygus* must be investigated in the future. The present study is part of a continuous research effort to identify taxonomically larvae of poorly known species, genera or families; to collect their morphological descriptions; and to expand international COI sequence databases (GenBank, BOLDSystem). This taxonomic information will increase the precision of species identification in ecological, systematics, evolution and fishery management studies of marine fish resources.

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