ARTICLE

Molecular identification of *Auxis* spp. larvae (Pisces: Scombridae) from the Gulf of California: Solving morphological identification limits

Identificación molecular de larvas de *Auxis* spp. (Pisces: Scombridae) del Golfo de California: Resolviendo límites en la identificación morfológica

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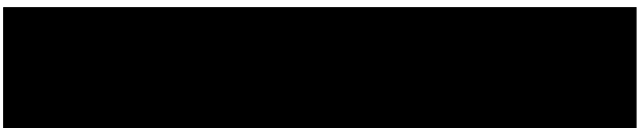
Resumen.- Las larvas del género *Auxis* son abundantes en el Golfo de California durante verano, sin embargo su identificación a nivel de especie mediante métodos morfológicos es un desafío. El objetivo de este estudio fue identificar larvas de *A. thazard* y *A. rochei* por primera vez a través de marcadores moleculares, usando secuencias de COI de ADNmt y buscar características morfológicas distintivas entre especies, principalmente en los patrones de pigmentación. Se obtuvieron larvas mediante arrastres de zooplancton en tres cruceros oceanográficos en el sur del Golfo de California y aguas adyacentes. Se confirmó genéticamente la presencia de larvas de *A. thazard* y de *A. rochei*. Las secuencias de 7 larvas mostraron divergencias genéticas menores al 1% al ser comparadas con secuencias de adultos de *A. thazard*, mientras que 15 larvas mostraron divergencias genéticas menores al 2% al ser comparadas con secuencias de adultos de *A. rochei*. Las divergencias genéticas entre ambas especies de *Auxis* fueron mayores al 2%. Estos resultados sugieren el desove de ambas especies en el Golfo de California. Por otro lado, los patrones de pigmentación y características morfométricas en todos los estadios larvarios no permitieron la identificación segura entre especies. Por lo tanto, se recomienda el uso de la identificación molecular mediante COI para identificar las larvas de *Auxis* a nivel de especie, así como en otras larvas de peces marinos recolectados en otras regiones del mundo, que tienen problemas de identificación.

Palabras clave: Identificación molecular, COI, larvas, *Auxis rochei, Auxis thazard*

Abstract.- The larvae of the *Auxis* genus are abundant in the Gulf of California during summer; however, their identification to the species level by morphological methods is a challenge. The goal of this study was to identify *A. thazard* and *A. rochei* larvae for first time, through molecular markers using COI sequences of mtDNA, and look for distinctive morphological characteristics between species, mainly in pigmentation patterns. Larvae were obtained by zooplankton tows in 3 oceanographic cruises in the southern Gulf of California and adjacent waters. The presence of *A. thazard* and *A. rochei* larvae was genetically confirmed. The sequences of 7 larvae showed genetic divergences lower than 1% when were compared to sequences of *A. thazard* adults, while 15 larvae showed genetic divergences lower than 2% when where compared to sequences of *A. rochei* adults. Genetic divergences between both *Auxis* species were higher than 2%. These results suggest the spawning of both species in the Gulf of California. On the other hand, pigmentation patterns and morphometric characteristics, in all larval stages, did not permit the secure differentiation between species. Thus, the use of molecular identification by COI is recommended to identify *Auxis* larvae to the species level, as well as in other marine fish larvae collected in other regions of the world, that have identification troubles.

Key words: Molecular identification, COI, larvae, *Auxis rochei, Auxis thazard*

Introduction

Scombridae, which includes tunas, bonitos, and mackerels, is one of the pelagic fish families highly important in sport and commercial fisheries around the world (Richards 2006). Within this family is the genus *Auxis* Cuvier, 1829, which is composed of small tunas (40 cm of fork length approx.) of 2 species: Frigate tuna *Auxis thazard* Dresslar & Fesler, 1889 and Bullet tuna *A. rochei* Risso, 1810. Their distribution has been registered from temperate, subtropical, and tropical waters along the equatorial belt, inhabiting near-shore waters during the reproductive season, after which they migrate to the open ocean to feed and continue with their life cycle (Majkowski 2007). The larvae of the *Auxis* genus have been reported as one of the most abundant taxa in the Gulf of California during summer (Sánchez-Velasco *et al.* 2013, Apango-Figueroa *et al.* 2015).

The identification of *Auxis* species in adult specimens is largely based on some particular characteristics: teeth of the jaws are thin and conical, corselet well developed, first and second dorsal fins are widely separated, the position of the tip of the pectoral fin in reference to the naked dorsal area and the number of gill rakers (Collette 2001, Joshi *et al.* 2012). However, as such morphological characteristics are not developed at larval stages, it is not possible to differentiate between *Auxis* species at those stages. In general, larvae strongly differ in morphology from the adults, and identifying them to the species level remains challenging (Ko *et al.* 2013; Leis 2015; Collet *et al.* 2017). Traditionally, larval identification has always used morphological characters, such as body shape, pigmentation, meristic count and measurements (Azmir *et al.* 2017). However, identification based on morphology is not always effective when many pre-bend larval species share meristic and morphometric characters making their recognition very complex or even ambiguous (Pappalardo *et al.* 2015). In addition, larval fish are so delicate that they are often badly damaged during collection and fixation that caused loss of pigmentation pattern which greatly help during identification process (Valdez-Moreno *et al.* 2010).

Studies about larvae distribution in the Mediterranean Sea and the Pacific Ocean, refer imprecisely to both species as *Auxis* spp., despite the presence of adults of both species in their respective areas (Ayala 1998, Rebollar-Prudente 2000, García *et al.* 2002, Avendaño-Ibarra *et al.* 2013). The available bibliography of fish larvae taxonomy in the north-eastern Pacific (Moser 1996) does not show clear differences in morphometric, meristic, and pigmentation patterns that support differentiation between *A. thazard* and *A. rochei* larvae. Even though a previous morphological analysis differentiated between two *Auxis* larval types as type I and type II, it was not clear how the type I and type II were identified as *A. rochei* and *A. thazard*, respectively, based only in their ossification degrees (Rebollar-Prudente 2000). However, the traditional taxonomy of larvae has implemented molecular techniques that permit species identification (Ko *et al.* 2013).

In the last decade, genetic techniques have been applied to clarify the presence of one or more scombrid species of fish in different regions of the world. Chow *et al.* (2003) used morphology and 3 molecular markers of mitochondrial DNA (mtDNA): restriction fragment length polymorphism (RFLP), cytochrome *b* (cyt *b*), and a flanking region between ATPase and cytochrome oxidase subunit III, to distinguish larvae and juveniles of different tuna fish in the tropical and subtropical West Pacific. Species of *A. thazard* and *A. rochei* were genetically identified; however, morphologic characters alone did not permit their classification in specimens smaller than 6 mm of length. Paine *et al.* (2007, 2008), using sequences of cytochrome oxidase *c*, subunit I (COI) of mtDNA, showed genetic differentiation between *A. thazard* and *A. rochei* in the western Atlantic Ocean and Hawaii. Robertson *et al.* (2007), using cytochrome *b* (cyt *b*) sequences, differentiated 9 species of Scombridae, with similar morphologies in postlarval and juvenile stages in north-western Australia. Mudumala *et al.* (2011), using COI sequences, showed low intra-specific (0.002-0.003) and high inter-specific (0.028) divergences between *A. thazard* and *A. rochei*. Kumar *et al.* (2013), with COI, found evolutionary differences between *A. thazard* and *A. rochei*, concluding that *A. rochei* is the more ancestral species. Puncher *et al.* (2015), found misidentification of tuna larvae following morphology-based methods for the Mediterranean Sea and concluded that genetic barcoding is a legitimate technique that can support species identification and play a crucial role in fisheries management efforts.

The objective of this study was to test the current taxonomic hypothesis of morphological differentiation of *Auxis* spp. larvae, coupled with the COI based identification system. Once identified, was looked for morphological characteristics that could possibly help in larval identification to the species level.

MATERIALS AND METHODS

SAMPLING

Larvae of *Auxis* spp. were collected during 3 oceanographic cruises at the entrance and southern Gulf of California (Fig. 1). The first (GOLCA1107) was carried out from the southern Gulf of California to the Midriff Archipelago region (25°-29°N and 109°-113°W), aboard the R/V El Puma, from 26th July to 6th August 2011. The second (GOLCA1204) was performed from the southern Gulf of California to Cabo Corrientes (21°-25°N and 106°-110°W), aboard the R/V Francisco de Ulloa, from 26th April to 4th May 2012. The third was done to the west of San Lorenzo channel in Bahía de La Paz (24.3°-24.6°N and 110°W) aboard a boat with outboard motor (CICIMAR1), on 28th June 2014.

The zooplankton samples were collected with a conical net (60 cm mouth diameter, 250 cm long, 0.505 mm mesh) towed in an oblique trajectory at depths from 50 m to the surface in a circular path at 2.5 knots for 5 min during the day or night (Smith & Richardson 1979). Complete samples of zooplankton were preserved in 80% ethanol. Tissue samples of *A. thazard* and *A. rochei* adults were obtained from sport fishing site in San Jose del Cabo, Baja California Sur on 5th July 2014. Adult specimens were identified with the taxonomic keys of Scombridae (Collette 2001). Such tissue samples were used to obtain reference sequences of *Auxis* spp.

Larvae were sorted from zooplankton samples, identified to the *Auxis* genus level (Moser 1996), and divided into 3 developmental stages: preflexion, flexion and postflexion, based on the degree of flexion of the terminal section of the notochord during formation of the caudal fin (Kendall *et al*. 1984).

MORPHOLOGICAL REVISION

A total of 23 larvae were used in the revision of pigmentation patterns (number and position of pigment spots). Five measurements used by Rebollar-Prudente (2000) were recorded: notochord or standard length (NL), head length (HL), snout-to-anus length (PAL), horizontal eye diameter (ED), and upper jaw length (UJL) (Fig. 2), were measured for each larva in a stereomicroscope (Stemi SV6, Carl Zeiss®, Oberkochen, Germany) with a calibrated micrometer.
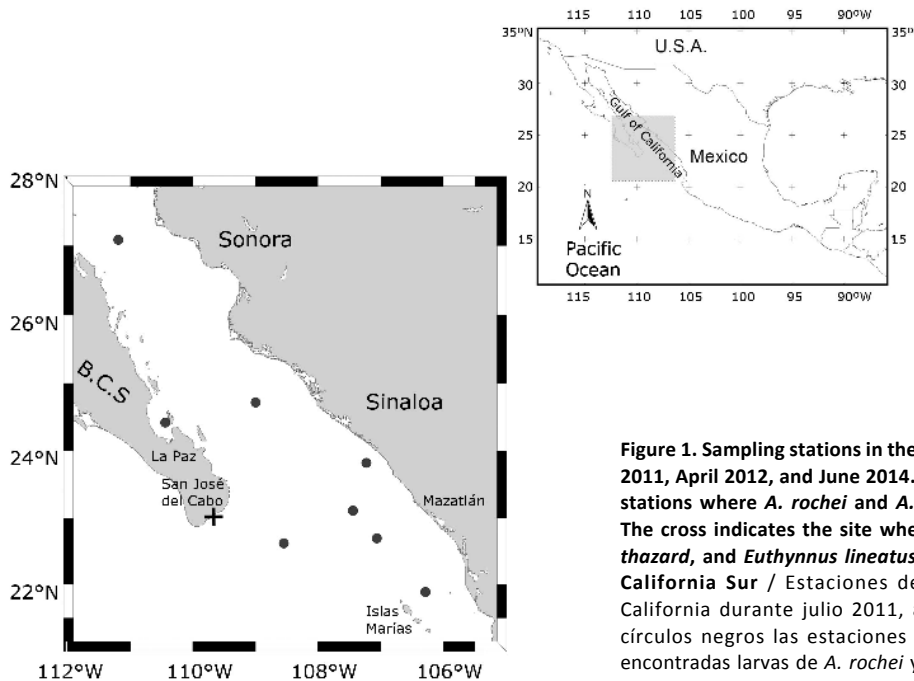
Figure 1. Sampling stations in the Gulf of California during July 2011, April 2012, and June 2014. In black circles the sampling stations where *A. rochei* and *A. thazard* larvae were found. The cross indicates the site where adults of *Auxis rochei*, *A. thazard*, and *Euthynnus lineatus* were collected. B.C.S.: Baja California Sur / Estaciones de muestreo en el Golfo de California durante julio 2011, abril 2012 y junio 2014. En círculos negros las estaciones de muestreo donde fueron encontradas larvas de *A. rochei* y *A. thazard*. La cruz indica el sitio donde fueron recolectados los adultos de *Auxis rochei*, *A. thazard* y *Euthynnus lineatus*. B.C.S.: Baja California Sur
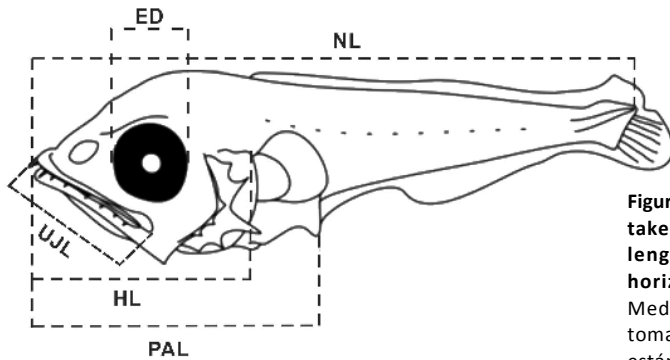
Figure 2. Morphometric measurements applied in *Auxis* spp. larvae, taken from Rebollar-Prudente (2000). Notochord or standard length (NL), head length (HL), snout-to-anus length (PAL), horizontal eye diameter (ED), and upper jaw length (UJL) / Mediciones morfométricas aplicadas en larvas de *Auxis* spp., tomado de Rebollar-Prudente (2000). Longitud de notocorda o estándar (NL), longitud cefálica (HL), longitud hocico-ano (PAL), diámetro horizontal del ojo (ED) y longitud de la mandíbula superior (UJL)

MOLECULAR IDENTIFICATION OF *AUXIS* SPECIES

Genomic DNA from adult tissue samples and larvae was extracted following a modified salt extraction technique of Lopera-Barrero *et al*. (2008) and a Chelex extraction technique (Hyde *et al*. 2005), respectively. DNA extractions were done from isolated tissues of 7 scombrid adults (*Auxis rochei*, n = 4; *A. thazard,* n = 2 and *Euthynnus lineatus*, n = 1), and 23 putative larvae of *Auxis* spp. (5 in preflexion, 6 in flexion and 12 in postflexion). A 700 bp fragment of the COI gene of mtDNA was amplified with primers VF1 (52-TCTCAACCAACCACAAAGACATTGG-32) (Ivanova *et al*. 2006) and FishR1 (52-TAGACTTCTGGGTGGCCAATCA-32) (Ward *et al*. 2005). DNA amplifications were performed by PCR (Bibby Scientific Ltd, Staffordshire, UK) under the following conditions: 2 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 50°C, 1 min at 72°C and a final extension of 4 min at 72°C. PCR products (20 µl, 100 ng µl^{-1}) were purified and sequenced in sense and antisense directions in an automatic sequencer (ABI Prism 3730XL, Applied Biosystems, Carlsbad, CA) at Macrogen (Seoul, South Korea). Sense and antisense sequences were aligned using Clustal W, implemented in MEGA version 6.0 software (Tamura *et al*. 2013). The best sequences were deposited in the GenBank (Accession numbers: KX419574 - KX419603). Five COI sequences of 4 Scombridae species from the GenBank were used as references (Accession numbers between parentheses): *A. rochei* (LN908924), *A. thazard* (LN908925), *E. lineatus* (GU440322 and LN908928), and *Scomber japonicus* (GU440513).

Genetic distances of Kimura's two-parameter model (Kimura 1980) among adults, larvae at different developmental stages, and reference sequences of COI were used to display phylogenetic relationships by Neighbor Joining (NJ) tree (5000 bootstraps) with MEGA version 6.0 software, using the sequence of *S. japonicus* as outgroup. The interspecific and intraspecific cutoff criterion were established in agreement to those proposed by Avise (2000) and Hebert *et al*. (2003), who suggested that interspecific genetic variation is higher than 2% and intraspecific is rarely greater than 2%, most being less than 1%.

RESULTS

MOLECULAR IDENTIFICATION (COI)

The intra-specific genetic divergences in adults of *Auxis rochei*, *A. thazard*, and *Euthynnus lineatus* ranged from 0-0.8% (Table 1). The inter-specific genetic divergences among adults of *A. rochei*, *A. thazard* and *E. lineatus* ranged from 2.7-11.2%. The sequences of 15 larvae (4 in preflexion, 3 in flexion, and 8 in postflexion), had genetic divergences ranging from 0-1.2% when were compared to sequences of *Auxis rochei* adults and higher than 3% when were compared to sequences of *A. thazard* (3.1-3.7%) and *E. lineatus* adults (10.7-11.9%). The sequences of 7 larvae (1 in preflexion, 3 in flexion, and 3 in postflexion) had genetic divergences ranging from 0-0.4% when they were compared to sequences of *A. thazard* adults and higher than 2% when were compared to sequences of *A. rochei* (2.7-4.0%) and *E. lineatus* adults (9.7-10.5%). The sequence of 1 larva in postflexion had genetic divergences from 0-0.2% when it was compared to sequences of *E. lineatus* adults and higher than 9% when it was compared to sequences of *A. thazard* (9.7%) and *A. rochei* adults (10.2-10.9%).

Table 1. Minimum, maximum (between parentheses), and mean percentages values of genetic divergences of COI mtDNA fraction obtained with Kimura's two-parameter model, among _Auxis_ species (_A. rochei_ and _A. thazard_), _Euthynnus lineatus_, and _Scomber japonicus_. N: number of individuals / Porcentajes promedio, mínimo y máximo (entre paréntesis) de divergencia genética de la fracción COI de ADNmt, obtenidos con el modelo parámetro dos de Kimura, entre especies de _Auxis_ (_A. rochei_ y _A. thazard_), _Euthynnus lineatus_ y _Scomber japonicus_. N: número de individuos

	Adults of _Auxis rochei_	Adults of _Auxis thazard_	Adults of _Euthynnus lineatus_	Putative larvae of _Auxis rochei_	Putative larvae of _Auxis thazard_	Putative larva of _Euthynnus lineatus_	Adult of _Scomber japonicus_
	N= 5	N= 3	N= 3	N= 15	N= 7	N= 1	N= 1
Adults of _Auxis rochei_	(0-0.8) 0.5	(2.7-3.5) 3.2	(10.2-11.2) 10.8	(0-1.2) 0.5	(2.7-4.0) 3.2	(10.2-10.9) 10.7	(15.4-16.2) 15.9
Adults of _Auxis thazard_		0	(9.7-10.0) 9.9	(3.1-3.7) 3.5	(0-0.4) 0.2	(9.7-9.7) 9.7	(15.7-15.7) 15.7
Adults of _Euthynnus lineatus_			(0.2-0.4) 0.3	(10.7-11.9) 11.2	(9.7-10.5) 10.0	(0-0.2) 0.1	(18.1-18.7) 18.4
Putative larvae of _Auxis rochei_				(0-1.2) 0.5	(3.1-4.2) 3.5	(10.7-11.7) 11.1	(15.7-16.5) 16.2
Putative larvae of _Auxis thazard_					(0-0.8) 0.3	(9.7-10.2) 9.9	(15.7-15.9) 15.8
Putative larva of _Euthynnus lineatus_						--	18.4
Adult of _Scomber japonicus_							--

--, No data because there was only one individual of that species
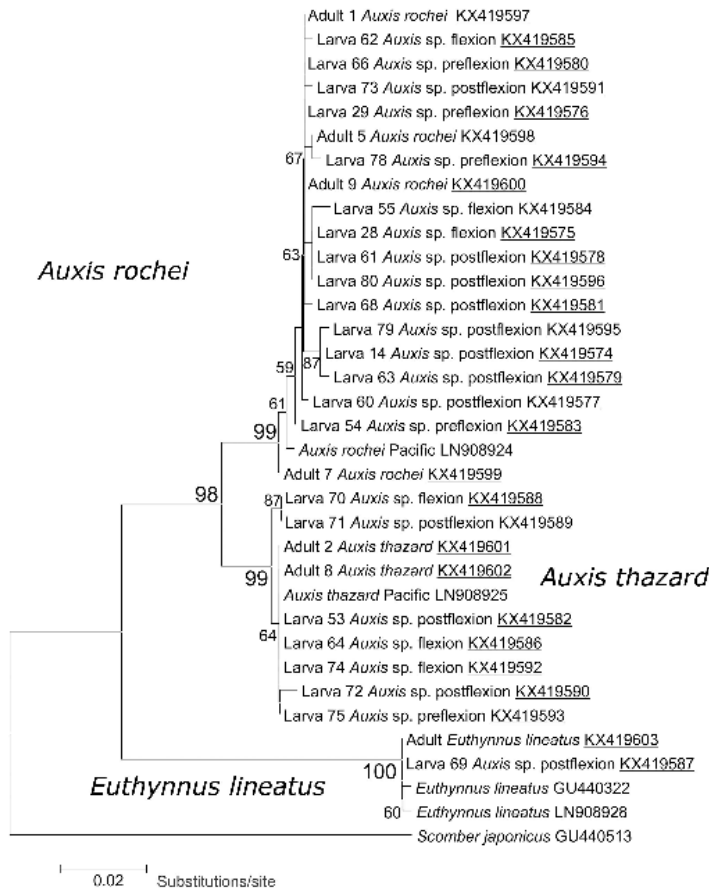
Figure 3. Neighbor-joining trees based on the partial sequences of COI (504 bp) gene of the mitochondrial DNA. Numbers beside the branches indicate bootstrap values (>50%), based on 5,000 replicates. Accession numbers of new sequences (this study) are underlined. Scale bar represents the genetic distance of Kimura's two-parameter model / Árbol filogenético de unión del vecino más próximo basado en las secuencias parciales del gen COI (504 pb) del ADN mitocondrial. Los números a los lados de las ramas indican los valores de remuestreo (>50%), basados en 5.000 réplicas. Los números de acceso de nuevas secuencias (este estudio) están subrayados. La barra de la escala representa la distancia genética del modelo parámetro dos de Kimura

The NJ tree showed that sequences from each species were confined to a single clade, supported by bootstrap confidence levels of 99-100% (Fig. 3). Fifteen larvae were contained within *A. rochei*, 7 within *A. thazard*, and 1 within *E. lineatus* clades. These results statistically corroborate the identity of the 23 larvae of Scombridae collected at the entrance and southern Gulf of California.

MORPHOLOGICAL DESCRIPTION

The morphological characteristics for *A. rochei* and *A. thazard* larvae (15 and 7, respectively) were described based on the genetic identity of each species as defined above.

Pigmentation (Table 2, Figs. 4-7): from 1 to 4 pigment spots were observed on the tip of the lower jaw (TLJ) in both species; however, 2 larvae of *A. rochei* (preflexion and flexion stages) did not show this pigmentation. Several pigments spots were observed at the posterior cephalic dorsal region (PCDR) in almost all *A. rochei* and

A. thazard larvae, with 2 larvae of *A. rochei* (in preflexion and flexion) not showing such pigmentation. No pigment spots were observed in the anterior cephalic dorsal region (ACDR) of preflexion larvae and most of the flexion and postflexion larvae. From one to several pigment spots were observed in the ACDR of some flexion and postflexion larvae of both species. The biggest *A. thazard* (5.04 mm) and *A. rochei* (10.58 mm) larvae showed several pigment spots at ACDR. A pigment spot was observed at cleithral symphysis (CS) of all larvae of *A. thazard* and *A. rochei*, at all developmental stages, except in 2 larvae of *A. rochei* at preflexion.

Several pigment spots were observed dorsally on gut (DG) of both species at all developmental stages except in 2 larvae of *A. rochei* (preflexion and flexion). One pigment spot anterior to anus (AA) was observed in larvae of both species, at all developmental stages, except in 1 larva of *A. thazard* in postflexion. One larva of *A. rochei* in postflexion showed as much as 11 pigment spots AA.

Table 2. Pigment spot patterns of putative species of *Auxis* and *Euthynnus* larvae, after molecular identification with COI. Standard length (NL) in mm, tip of lower jaw (TLJ), posterior cephalic dorsal (PCDR), and anterior cephalic dorsal (ACDR) regions, cleithral symphysis (CS), dorsal gut (DG), anterior to anus (AA), postanal ventral midline (PVM), dorsal caudal (DC), No data (--) / Patrones de pigmentación de especies putatitvas de larvas de *Auxis* y *Euthynnus*, después de la identificación molecular con COI. Longitud estándar (NL) en mm, punta de la mandíbula inferior (TLJ), regiones cefálicas posteriores dorsales (PCDR) y cefálicas anteriores dorsales (ACDR), sínfisis cleitral (CS), dorsal del estómago (DG), anterior al ano (AA), línea media ventral postanal (PVM), dorsal caudal (DC), Sin dato (--)

| Putative species	Larval stage	NL	TLJ	PCDR	ACDR	CS	DG	AA	PVM	DC
Auxis rochei	Preflexion	3.50	0	No	No	No	Yes	Yes	15	1
	Preflexion	3.57	1	Yes	No	No	No	Yes	14	1
	Preflexion	3.58	2	Yes	No	Yes	Yes	Yes	10	2
	Preflexion	--	1	Yes	No	--	Yes	--	11	0
	Flexion	3.61	1	Yes	No	Yes	Yes	--	9	0
	Flexion	4.40	0	No	No	Yes	Yes	--	9	--
	Flexion	--	--	Yes	No	--	No	--	--	0
	Postflexion	4.82	3	Yes	No	Yes	Yes	--	10	0
	Postflexion	5.00	1	Yes	No	Yes	Yes	--	10	0
	Postflexion	5.05	3	Yes	1	Yes	Yes	Yes	11	0
	Postflexion	5.11	4	Yes	No	Yes	Yes	Yes	11	0
	Postflexion	5.50	1	Yes	1	Yes	Yes	--	9	2
	Postflexion	5.60	1	Yes	No	Yes	Yes	--	9	0
	Postflexion	5.73	2	Yes	No	Yes	Yes	Yes	17	1
	Postflexion	10.58	2	Yes	Yes	Yes	Yes	--	--	--
Auxis thazard	Preflexion	3.20	1	Yes	No	Yes	Yes	--	7	0
	Flexion	3.60	1	Yes	1	Yes	Yes	--	10	0
	Flexion	4.00	1	Yes	No	Yes	Yes	--	11	0
	Flexion	--	2	Yes	No	Yes	Yes	--	7	0
	Postflexion	4.50	2	Yes	No	Yes	Yes	No	11	2
	Postflexion	4.70	--	Yes	1	Yes	Yes	--	10	2
	Postflexion	5.04	--	Yes	Yes	Yes	Yes	Yes	14	--
Euthynnus lineatus	Postflexion	6.00	0	Yes	Yes	Yes	Yes	No	9	0
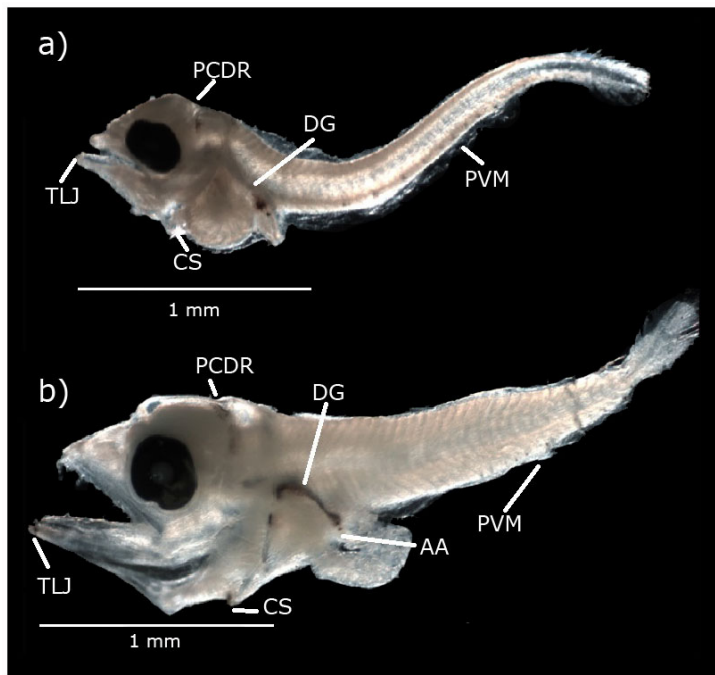
Figure 4. Some of the pigment spot patterns shared by *Auxis thazard* (a) and *A. rochei* (b) larvae in preflexion stage, at 3.2 mm and 3.58 mm NL, respectively. TLJ: tip of lower jaw, PCDR: posterior cephalic dorsal region, CS: cleithral symphysis, DG: dorsal gut, AA: anterior to anus, PVM: postanal ventral midline / Algunos de los patrones de pigmentación compartidos por larvas de *Auxis thazard* (a) y *A. rochei* (b) en estadio de preflexion, a los 3,2 mm y 3,58 mm NL respectivamente. TLJ: punta de la mandíbula inferior, PCDR: región cefálica posterior dorsal, CS: sínfisis cleitral, DG: dorsal del estómago, AA: anterior al ano, PVM: línea media ventral postanal
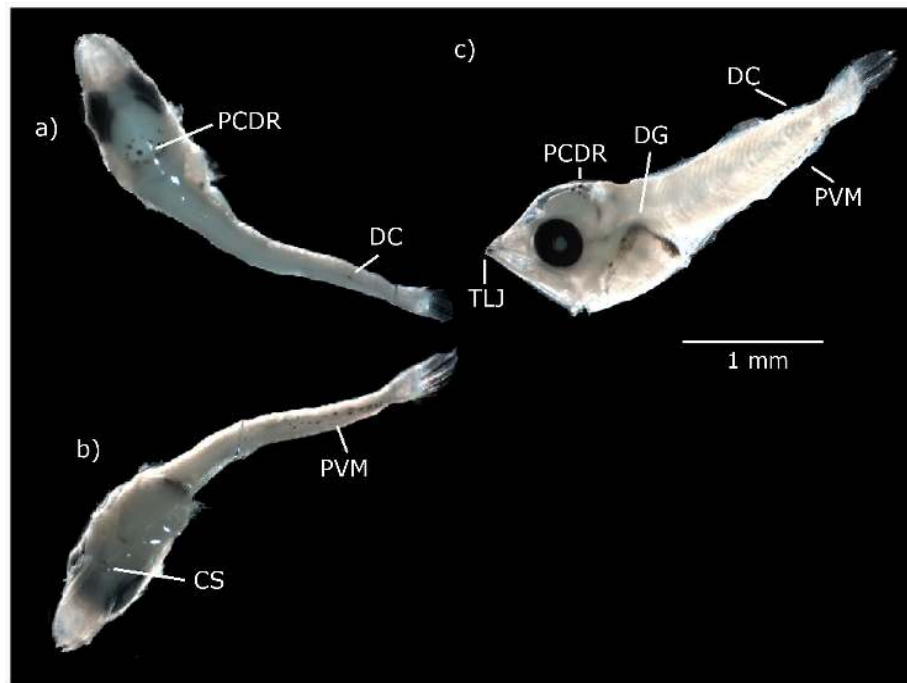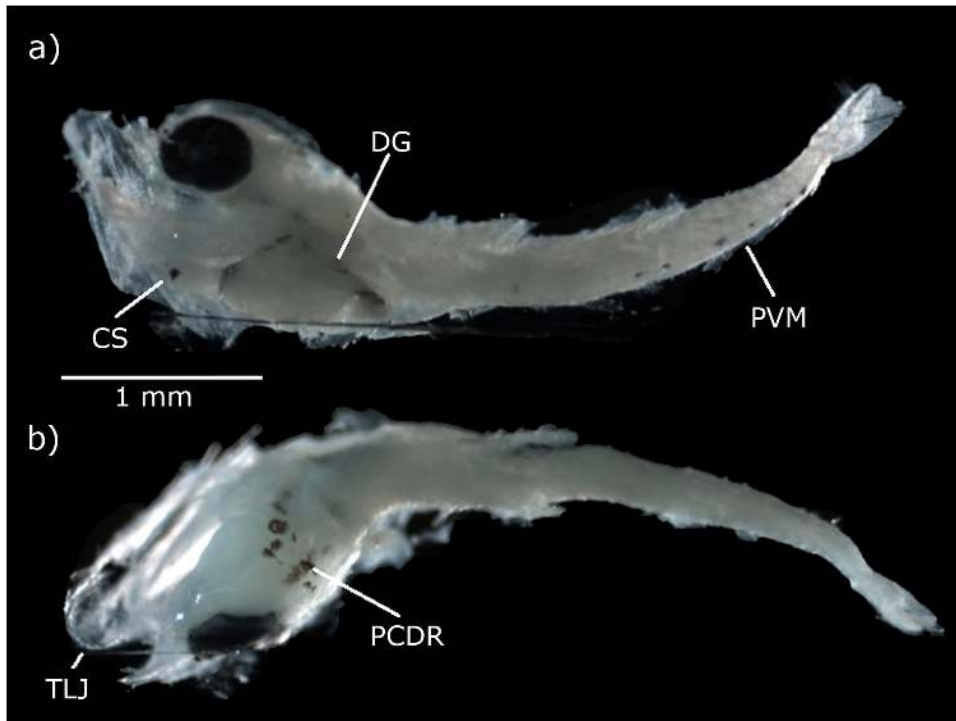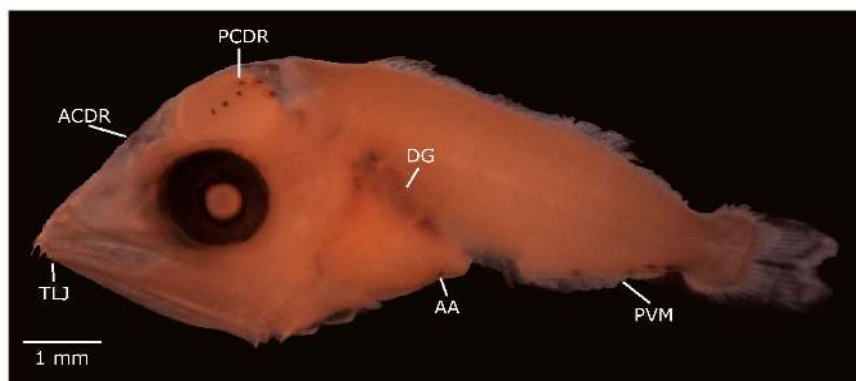

Figure 5. Pigment spot patterns of *Auxis rochei* flexion larvae at 3.61 mm NL. a) dorsal view; b) ventral view; c) lateral view. TLJ: tip of lower jaw, PCDR: posterior cephalic dorsal region, CS: cleithral symphysis, DG: dorsal gut, PVM: postanal ventral midline, DC: dorsal caudal / Patrones de pigmentación de larva de *Auxis rochei* en estadio de flexión a los 3,61 mm NL. a) vista dorsal; b) vista ventral; c) vista lateral. TLJ: punta de la mandíbula inferior, PCDR: región cefálica posterior dorsal, CS: sínfisis cleitral, DG: dorsal del estómago, PVM: línea media ventral postanal, DC: dorsal caudal

Figure 6. Pigment spot patterns of *Auxis thazard* flexion larvae at 4.00 mm NL. a) ventral view; b) dorsal view. TLJ: tip of lower jaw, PCDR: posterior cephalic dorsal region, CS: cleithral symphysis, DG: dorsal gut, PVM: postanal ventral midline, DC: dorsal caudal / Patrones de pigmentación de larva de *Auxis thazard* en estadio de flexión a los 4,00 mm NL. a) vista ventral; b) vista dorsal. TLJ: punta de la mandíbula inferior, PCDR: región cefálica posterior dorsal, CS: sínfisis cleitral, DG: dorsal del estómago, PVM: línea media ventral postanal

Figure 7. Pigment spot patterns of *Auxis rochei* postflexion larvae at 10.58 mm NL. Patterns start to fade due epidermis tissue thickening. TLJ: tip of lower jaw, PCDR: posterior cephalic dorsal region, ACDR: anterior cephalic dorsal region, DG: dorsal gut, AA: anterior to anus, PVM: postanal ventral midline / Patrones de pigmentación de larva de *Auxis rochei* en estadio de postflexión a los 10,58 mm NL. Los patrones comienzan a desvanecerse debido al engrosamiento de la epidermis. TLJ: punta de la mandíbula inferior, PCDR: región cefálica posterior dorsal, ACDR: región cefálica anterior dorsal, DG: dorsal del estómago, AA: anterior al ano, PVM: línea media ventral postanal

A higher variation was observed in the number of pigment spots on the postanal ventral midline (PVM). In larvae of *A. rochei* such pigment spots ranged from 10-15 in preflexion, 9 in flexion, and 9-17 in postflexion. Larvae of *A. thazard* showed 7 pigment spots in a preflexion larva, from 7-11 in flexion, and 10-14 in postflexion. Dorsally in caudal peduncle (DC), from 0-2 pigment spots were observed in both species at all developmental stages.

Morphometry: A lower number of larvae at different developmental stages were used in morphometric analysis (body and head measurements), because several larvae were degraded or deformed (twisted or bent) by the ethanol dehydration. Thus, measurements were obtained of the best-preserved larvae genetically identified: 5 larvae of *A. rochei* (1 in preflexion and 4 in postflexion) and 4 larvae of *A. thazard* (1 in preflexion, 2 in flexion, and 1 in postflexion). Ratio of PAL/NL was lower in preflexion larva of *A. rochei* (0.31), in comparison to preflexion larva of *A. thazard* (0.44), but it was similar in postflexion larvae of both species. Ratio of HL/NL was similar in both species at preflexion and postflexion stages. Ratio of UJL/HL was higher in preflexion larva of *A. rochei* (0.89) in comparison to preflexion larva of *A. thazard* (0.66), but it was similar in both species at postflexion stage. Finally, ratio of ED/HL was similar in both species at postflexion stage (Table 3).

DISCUSSION

Molecular analysis with COI of the mtDNA demonstrated the existence of *A. thazard* and *A. rochei* larvae in the Gulf of California. To a large extent, our results were in agreement with those cutoff criterion established by Avise (2000) and Hebert *et al*. (2003), because some larvae of *Auxis rochei* showed intraspecific divergences higher than 1% when compared to adults of the same species. Such intraspecific variation could be the natural variation in species, because larvae and adults of *A. rochei* were in the same clade in the NJ tree, with a high statistical support (99%). Previous studies, with COI fraction of mtDNA in larvae or juveniles of *Auxis* genera, have found statistical support higher than 97% of bootstrap to discriminate species of *Auxis* (Chow *et al*. 2003, Ji *et al*. 2011, Kumar *et al*. 2013). In addition, in the Gulf of California were confirmed the same 8 variable sites that were found to discriminate between *Auxis rochei* and *A. thazard* from the Mediterranean Sea (Puncher *et al*. 2015).

In this study, such as Azmir *et al*. (2017), DNA barcoding was a better method for deeper taxonomic levels identification and was able to validate the accuracy of traditional larval fish identification. However, as mentioned Azmir *et al*. (2017), the success of using barcoding for species identification strongly depends on the presence of high-quality reference sequences. In our

Table 3. Minimum, maximum (between parentheses), and mean values, of body ratios of *Auxis rochei* and *A. thazard* larvae. Standard length (NL), preanal length (PAL), head length (HL), upper jaw length (UJL), horizontal eye diameter (ED), and number of larvae (N) / Valores promedio, mínimo y máximo (entre paréntesis), de proporciones corporales de larvas de *Auxis rochei* y *A. thazard*. Longitud estándar (NL), longitud preanal (PAL), longitud cefálica (HL), longitud de la mandíbula superior (UJL), diámetro horizontal del ojo (ED), y número de larvas (N)

Body ratio	*Auxis rochei*		*Auxis thazard*			*Euthynnus lineatus*
	Preflexion	Postflexion	Preflexion	Flexion	Postflexion	Postflexion
	N= 1	N= 4	N= 1	N= 2	N= 1	N= 1
PAL/NL	0.31	(0.40-0.47) 0.43	0.44	(0.37-0.39) 0.38	0.42	0.53
HL/NL	0.26	(0.26-0.33) 0.28	0.28	(0.25-0.25) 0.25	0.30	0.33
UJL/HL	0.89	(0.55-0.80) 0.70	0.66	(0.67-0.80) 0.73	0.71	0.80
ED/HL	--	(0.28-0.38) 0.33	0.44	(0.40-0.44) 0.42	0.36	0.35

--, No data

study, such reference sequences were obtained from adults specimens well identified and GenBank database.

Matsumoto (1959) suggested that the localities where *Auxis* larvae of about 3 mm occur probably represent actual spawning sites. The molecular identification of early *Auxis* larvae suggests the presence of spawning sites of both species in the Gulf of California during spring and summer. However, because sampling was not carried out throughout the year, results of spawning season were not conclusive. In other study, a combine data from DNA barcoding, comparative morphology and analysis of color pattern, link a serranid fish larvae to its adult, suggesting connectivity in this species across the Caribbean Sea (Baldwin & Johnson 2014).

Since the late 50's there are antecedents which report difficulties in classification of tuna larvae, particularly of small tuna larvae of *Auxis* genera worldwide (Jones 1949, 1960; Uchida 1963, 1981). Moser (1996) proposed that to improve classification of such larvae a combination of methods, such as the number of myomeres, the body shape, head spination, and melanophores distribution, are required. In this study, molecular identification, pigment spot patterns, and morphometric were included.

Nevertheless, the pigmentation did not show clear patterns that could help to differentiate between *A. thazard* and *A. rochei* larvae. One or 2 pigment spots could be present at the tip of the snout in both species of *Auxis* genera. Further, such characteristic is present in other genera of Scombridae, like *Katsuwonus* and *Thunnus* (Richards 2006), and appears in larvae from the flexion stage (Jones 1960). In the case of *Euthynnus lineatus* larva, 3 pigment spots were observed on the left side of the lower jaw, not at the tip. The pigment spots at the anterior cephalic dorsal region could be present in flexion and postflexion larvae of *A. thazard* and *A. rochei*, respectively, and were even present in postflexion larva of *E. lineatus*. These pigment spots were recorded by Uchida (1963), who reported the appearance of the first anterior pigment in larvae of 8 mm of length. In this study, the first pigment spot in the anterior cephalic region was recorded in a flexion larva of *A. thazard* (3.6 mm of standard length) and a postflexion larva of *A. rochei* (5.05 mm of standard length). The pigment spot at cleithral symphysis was present in both species. This characteristic has been recorded in larvae of *Auxis*, but this is also present in larvae of *Euthynnus* (Jones 1949, 1960; Uchida 1963, 1981).

The pigment spots dorsally on gut were present in most larvae of both species and the number and the size

of such pigments seems to be random. The pigment spot anterior to anus was present in some larvae of both species. In the study of Rebollar-Prudente (2000), such pigment spots were useful to separate larvae of *Auxis* genera, but in the present study did not support differentiation between both species.

The pigment spots on postanal ventral midline showed some differences in number between preflexion larvae of *A. rochei* and a larva of *A. thazard*. However, such pigment spots in flexion and postflexion larvae were uninformative for differentiation between the 2 species. Even the *E. lineatus* larva showed similar numbers in pigment spots on postanal ventral midline, but such pigment spots were paired, while in *Auxis* larvae those were unpaired. The pigment spots dorsally on caudal peduncle were also uninformative, because they were present only in some larvae of both species.

Thus, the pigment spots at the tip of the snout, cephalic, cleithral symphysis, anterior to anus, postanal ventral midline (in flexion and postflexion), and dorsally on caudal peduncle were uninformative for separating larvae of *Auxis* genera, because they were present only in some larvae of both species, and did not show clear patterns of differentiation. Almost all those pigment spots were shared with the *E. lineatus* larva. There were some differences in the number of pigment spots on postanal ventral midline between preflexion larvae of *A. rochei* and a larva of *A. thazard*. However, because there was only 1 larva of *A. thazard* in preflexion, such differences should be corroborated in a large number of larvae.

There is a group of pigment spots that were used by previous studies to classify 2 larval types of *Auxis* (Matsumoto 1959, Uchida 1981). Larvae of type I (*A. thazard*) showed 2 or more lines of pigment spots in the caudal peduncle (dorsal, ventral, and lateral) and larvae of type II (*A. rochei*) only showed 1 or 2 lines of pigment spots in the caudal peduncle (dorsal and ventral). Richards (2006) employed this classification, but such classification did not separate larvae in preflexion and flexion stages, because the greatest changes happen after the 7 mm of larval length (Matsumoto 1959, Uchida 1981, Richards 2006). Ji *et al*. (2011) reviewed the differences in pigment spot patterns of caudal peduncle and found that only some larvae of *Auxis rochei* showed the dorsal line of pigment spots. In the present study, most larvae of both *Auxis* species did not show the dorsal line of pigment spots, probably because almost all larvae were smaller than 7 mm.

Morphometrics showed some differences in PAL/NL and UJL/HL ratios between preflexion larvae of *A. thazard* and *A. rochei*. However, because only 1 larva each in preflexion was available for both species, such ratios should be confirmed in large sample sizes. The rest of the body ratios were uninformative for differentiating between *A. thazard* and *A. rochei* larvae. Rebollar-Prudente (2000) used morphometric and meristic to classify 2 larval types of *Auxis*. He employed a discriminant analysis to separate preflexion larvae of the 2 types and developed classification functions using the measures of standard length, eye diameter, head depth, and snout length. In the present study, similar to that of Rebollar-Prudente, the preanal, upper jaw and standard length were informative to differentiate preflexion larvae of *A. thazard* and *A. rochei*.

As in the present study, Chow *et al*. (2003) also highlighted the division of *Auxis* species with COI fraction in the west Pacific, but they were not able to relate it to morphologic characteristics of larvae. They concluded that could be separated using gill rakers, however such characteristic could be useful in more developed larvae or juveniles.

In conclusion, this study rejects the taxonomic hypothesis of morphological differentiation of *Auxis* spp. larvae and confirms the constraints to identifying larvae in preflexion, flexion, and postflexion of *Auxis* spp, using pigmentation patterns and morphometric, as proposed early by Jones (1949, 1960) and Uchida (1963, 1981). Even though there are some differences in the pigment spots on postanal ventral midline and PAL/NL and UJL/HL ratios of preflexion larvae of *Auxis* spp., such characteristics are not conclusive. At the moment, to identify larvae of *Auxis* collected in the Gulf of California, we suggest the use of molecular identification with COI fraction of mtDNA or morphological identification should be more conservative and use *Auxis* spp. This finding by no means try to undermine the work of taxonomists, because DNA barcoding rely on sequences obtained from voucher specimens well identified through their morphology or curated in the case of misidentifications (Hubert *et al*. 2015, Azmir *et al*. 2017, Collet *et al*. 2017). Hence, the taxonomic studies of larval fish should continue using combination of both conventional (morphology) and new (DNA barcode) methods (Azmir *et al*. 2017). In future ecologic studies, molecular identification will help to identify spawning sites of both *Auxis* species, increasing our understanding of spawning habitat selection as well as the interactions of both species.

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