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Identification of the larval and juvenile stages of the Cubera Snapper, *Lutjanus cyanopterus*, using DNA barcoding

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Abstract

The larvae and newly-settled juveniles of the Cubera Snapper, *Lutjanus cyanopterus*, are identified by DNA barcoding. Four larvae and three small juveniles of *L. cyanopterus* were detected among a large collection of pelagic larvae and a smaller collection of settled juveniles from the Caribbean coast of Panama. The mtDNA COI barcode sequences from the larvae and juveniles were virtually identical to sequences from adults sampled from the spawning aggregation in St. Thomas, USVI. Barcode sequences for the eleven regional species of *Lutjanus* species (*sensu lato*) were obtained and they exhibited deep interspecific divergences that allowed for efficient discrimination among the western Atlantic snapper species. The nearest neighbor species, the Mutton Snapper *L. analis*, was more than 11% divergent from *L. cyanopterus*. Cubera Snapper larvae are characterized by prominent melanophores along the outer spinous-dorsal-fin membranes and along the outer third of the longer pelvic-fin membranes. They are morphologically distinct from the late-stage larvae of the other regional snappers by their relatively wider caudal peduncle and their relative dorsal-spine lengths. Juveniles retain the black outer portion of the dorsal and pelvic-fin membranes and have a smaller body-depth than other regional snapper juveniles. The size at settlement is about 18 mm SL, relatively large for lutjanids. Daily otolith increments from *L. cyanopterus* larvae and juveniles indicate a pelagic larval duration of about 29 days with back-calculated spawning and settlement dates around the new moon. Although smaller adult Cubera Snappers can appear very similar to the Gray Snapper, *L. griseus*, the larvae and juveniles are quite different. In this case, the early life history stages reflect the deep genetic divergence between the two species while the adult forms converge in appearance.

Key words: snapper, Cubera, Lutjanidae, *Lutjanus*, *cyanopterus*, larvae, juvenile, identification, DNA barcoding, BOLD, fish, phylogenetics, pelagic larval duration, PLD, pargo, gray, *griseus*, Caribbean, western Atlantic

Introduction

There are at least ten species of snappers in the genus *Lutjanus* in the western Atlantic and, as one of the dominant predators on and around reefs, they are an important component of the tropical marine fauna and support major fisheries in the region (Allen 1985). Their early life history stages can be difficult to identify since most species share meristics and overlap in morphometrics. In such circumstances, DNA barcodes are especially helpful (Packer *et al.* 2009), particularly when rare and common species coexist and share a similar appearance, a frequent problem in larval identification for many fish taxa. To facilitate DNA barcode identification of fishes, regional working groups are coalescing under the Fish Barcode of Life (FISH-BOL) initiative (e.g. Swartz *et al.* 2008), which seeks to establish a barcode reference sequence library for all fishes

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(Ward *et al.* 2009). Our study contributes to, and certainly derives benefit from, this international barcode campaign.

The Cubera Snapper *Lutjanus cyanopterus* is the largest lutjanid species in the western Atlantic, reaching 125 pounds and over four feet in length (Kadison *et al.* 2006). Indeed, the Cubera Snapper is the third largest Caribbean reef fish, after the Goliath and Warsaw Groupers. Unfortunately, the Cubera Snapper has become rare and is presently classified as threatened or vulnerable throughout its range (Huntsman 1996). Spawning occurs in large aggregations at the deep edges of the reef shelf in late spring and summer and has been observed in Florida, Cuba, Belize, and the US Virgin Islands (Domeier & Colin 1997; Claro & Lindeman 2003; Heyman *et al.* 2005; Kadison *et al.* 2006). The subsequent larval stages are undescribed and little is known about the life of juvenile Cubera Snappers after settlement, other than their relative rarity and association with soft-bottom and mangrove habitats (Lindeman 1997; Lindeman & DeMaria 2005). Smaller adults of the Cubera Snapper are difficult to distinguish from the common Gray Snapper, *Lutjanus griseus*; the relative shape of the vomerine tooth patch is typically cited as the sole reliable criterion (Randall 1996). Given the arcane nature of this distinction and the orders of magnitude greater abundance of Gray Snappers, little is certain concerning Cubera Snappers until they outgrow their congeners and reach their distinctive size. The application of DNA barcoding to identify species at all life-history stages is clearly useful for progress in understanding the ecology and assessing the stocks and population dynamics of this particularly vulnerable species.

Materials and methods

Larval *Lutjanus* spp. were collected from the waters over the reef as part of a long-term sampling program at Ukubtupo reef at the Smithsonian Tropical Research Institute field station in the San Blas Islands of Panama between 1980 and 1986 (details of method and dates in Victor (1986); 330 lutjanid larvae were collected). Larvae were attracted to a light suspended over the shallow reef, captured in a dipnet, and preserved in 90% ethanol. Tissue samples from subsets of the *Lutjanus* spp. larvae were submitted for DNA barcoding to the BOLD project (Ratnasingham & Hebert 2007). Four larvae shared a distinct set of markings (black pelvic-fin edges) that did not match any known snapper larvae and tissues from these individuals were submitted for sequencing. Juvenile snappers were collected from mangrove habitats near Isla Grande, Panama in May 2007.

Cubera Snapper adults were caught by hook-and-line at the spawning aggregation south of St. Thomas, US Virgin Islands in May 2007. We removed small fin clips for DNA extraction and the snappers were released alive. Tissues of other snapper species were variously obtained from adults collected by spear and from fish markets in the US Virgin Islands (2006) and from Florida Gulf-coast commercial fishers (2005). Additional specimens sequenced included newly-settled snappers from Glover's Reef, Belize (2006), Barbados (2005), and Panama (1981–2007)(Table 1).

DNA extractions were performed with the NucleoSpin96 (Machery-Nagel) kit according to manufacturer specifications under automation with a Biomek NX liquid-handling station (Beckman-Coulter) equipped with a filtration manifold. A 652-bp segment was amplified from the 5′ region of the mitochondrial COI gene using a variety of primers (Ivanova *et al.* 2007). PCR amplifications were performed in 12.5 µl volume including 6.25 µl of 10% trehalose, 2 µl of ultra pure water, 1.25 µl of 10× PCR buffer (10mM Kcl, 10mM $(NH_4)_2SO_4$, 20mM Tris-HCl (pH8.8), 2mM Mg SO_4, 0.1% Triton X-100), 0.625 µl of $MgCl_2$ (50mM), 0.125 µl of each primer (0.01mM), 0.0625 µl of each dNTP (10mM), 0.0625 µl of *Taq* DNA polymerase (New England Biolabs), and 2 µl of template DNA. The PCR conditions consisted of 94°C for 2 min, 35 cycles of 94°C for 30 s, 52°C 40 s, and 72°C for 1 min, with a final extension at 72°C for 10 min. One of the larval specimens had degraded DNA (after 25 years in ethanol) and one of us (JH) applied a "minibarcode" technique to obtain a shorter mtDNA sequence that spanned 249 bp of the COI barcoding sequence (Hajibabaei *et al.* 2006).

TABLE 1. Specimens sequenced for the neighbor-joining tree, following the order in Fig. 1. SIO=Scripps Institution of Oceanography Marine Vertebrate Collection.

Species	Source	GenBank Accession #	Museum Number
Pristipomoides aquilonaris	Colon, Panama	FJ998490	SIO–09–192
Lutjanus cyanopterus	San Blas, Panama (larva)	GQ329867	SIO–09–168
	San Blas, Panama (larva)	GQ329868	SIO–09–169
	Colon, Panama (juvenile)	FJ998467	SIO–09–167–1
	Colon, Panama (juvenile)	FJ998469	SIO–09–167–2
	Colon, Panama (juvenile)	FJ998468	SIO–09–166
	St. Thomas, USVI (adult)	FJ998470	SIO–09–171
	St. Thomas, USVI (adult)	GQ329866	SIO–09–172
Lutjanus buccanella	St. Thomas, USVI	FJ998464	SIO–09–173–1
	St. Thomas, USVI	FJ998463	SIO–09–173–2
	St. Thomas, USVI	FJ998462	SIO–09–173–3
Lutjanus analis	Glover's Reef, Belize	FJ998454	SIO–09–174
	Florida	FJ998455	SIO–09–175
	St. Thomas, USVI	FJ998456	SIO–09–176
	Colon, Panama	FJ998457	SIO–09–177
Lutjanus mahogoni	Glover's Reef, Belize	FJ998477	SIO–09–178
	Barbados	FJ998478	SIO–09–179
	St. Thomas, USVI	FJ998480	SIO–09–181
	Colon, Panama	FJ998479	SIO–09–180
Lutjanus synagris	St. Thomas, USVI	FJ998482	SIO–09–183
	St. Thomas, USVI	FJ998483	SIO–09–184
	Colon, Panama	FJ998481	SIO–09–182
Ocyurus chrysurus	St. Thomas, USVI	FJ998487	SIO–09–185–1
	St. Thomas, USVI	FJ998488	SIO–09–185–2
	Colon, Panama	FJ998489	SIO–09–186
Lutjanus campechanus	Florida	FJ998466	SIO–09–187
Lutjanus vivanus	St. Thomas, USVI	FJ998485	SIO–09–188–1
	St. Thomas, USVI	FJ998484	SIO–09–188–2
	St. Thomas, USVI	FJ998486	SIO–09–188–3
Lutjanus griseus	Colon, Panama	FJ998471	SIO–09–166
	Glover's Reef, Belize	FJ998472	SIO–09–178
	Florida	FJ998473	SIO–09–187
Lutjanus apodus	Glover's Reef, Belize	FJ998461	SIO–09–174
	Barbados	FJ998460	SIO–09–189
	St. Thomas, USVI	FJ998459	SIO–09–181
	San Blas, Panama	FJ998458	SIO–09–190
Lutjanus jocu	Carrie Bow Cay, Belize	FJ998475	SIO–09–191
	Colon, Panama	FJ998474	SIO–09–167
	St. Thomas, USVI	FJ998476	SIO–09–188

Specimen information and barcode sequence data from this study were compiled using the Barcode of Life Data Systems (BOLD, www.barcodinglife.org, Ratnasingham & Hebert 2007). All sequence data is publicly accessible on BOLD (project code BVCS) and is also available on GenBank (Table 1). Sequence divergence was calculated using BOLD with the Kimura 2-parameter (K2P) model generating a mid-point rooted neighbour-joining (NJ) phenogram to provide a graphic representation of the species-divergence.

Estimates of the pelagic larval duration (PLD) and age since settlement were obtained from analyses of otolith microstructure (Victor 1991; Thorrold & Hare 2002). Otolith increments have been shown to be daily in other *Lutjanus* spp. (mostly unpublished studies referred to in Zapata and Herron (2002) and Denit and Sponaugle (2004)); in this species we assumed they were daily. Preparation, techniques and interpretations mostly followed Campana (1992). The three pairs of otoliths of the young Cubera Snappers were extracted under a dissecting microscope and then examined with transmitted light and a polarizing filter under a compound microscope at magnifications from 200 to 600X. Areas of the otolith that are white under reflected light are dark with transmitted light (more proteinaceous matrix than carbonate crystals). In this paper, dark and light will refer to the appearance under the transmitted light of the compound microscope (higher magnifications). On a finer scale, each daily increment is made up of a translucent accretion band dominated by carbonate crystals and a relatively more opaque "discontinuous" zone made up primarily of an organic matrix of protein. The translucent light band is formed mainly during the day while the narrower darker line is formed mostly at night.

Results and discussion

Barcoding. Sequences were obtained from eleven species of *Lutjanus* (including *Ocyurus chrysurus*, often considered *Lutjanus chrysurus*) and the outgroup *Pristipomoides aquilonaris*. Table 1 lists the specimen data for the 39 sequences used for the phenogram in Fig. 1. The barcode sequences of three juvenile specimens from mangrove habitats in Panama were identical to the sequences from the reference adult Cubera Snappers. The full barcode sequence was successfully obtained from the 18.8 mm SL larva that shared the distinctive black pelvic-fin edges and it differed by a single bp from the other Cubera Snappers. The minibarcode of 249 bp from the 17.7 mm SL larval specimen matched exactly the corresponding minibarcode sequence for the Cubera Snappers (the two other larvae did not successfully sequence). Since the closest other sequence among the western Atlantic snapper species (the Mutton Snapper *L. analis*) is more than 11% divergent from the Cubera Snappers, the 99.8% sequence match confirms the species identification (Fig. 1).

The matching of larvae to adults by barcode sequences is one of the more valuable applications of DNA barcoding technology (Hebert *et al.* 2003) and the technique can be particularly useful for marine fishes. Marine fishes tend to have widely-dispersed and little-known pelagic larvae and knowledge of their early life-history is critical to understanding the ecology and population dynamics of these important commercial fishes. For reef fishes in particular, very limited information is available on their pelagic stage and the larvae of most reef-associated species have not yet been identified. Barcoding promises to rapidly resolve most identification questions.

This case study illustrates the utility of the technique for marine fishes. The genus *Lutjanus* represents an important ecological and economic component of the reef fish fauna and the early life-history stages have not been described for most species; indeed, half of the Caribbean species remain undescribed in the most-recently published review (Lindeman *et al.* 2005). We sampled all of the tropical western Atlantic *Lutjanus* except *L. alexandrei* and *L. purpureus* for reference sequences. *L. alexandrei* is endemic to Brazil. *L. purpureus* has the same DNA sequences as the allopatric northern red snapper *L. campechanus* and therefore likely represents the southern population of the red snapper (Gomes *et al.* 2008). The results indicate the genus is ideal for barcode matching because there are numerous species with deep divergences in sequence between species (from 2.7–11.4% between closest neighbors) and minimal variation within species, even when sampled from a wide geographic range. Many individuals we collected from opposite ends of the

Caribbean (Florida, USVI, Belize, Panama, and Barbados) exhibited identical 652 bp COI barcodes (Fig. 1). The within-species variation in sequences was typically below 0.5% in our larger samples of ten to thirty individuals (unpublished data).

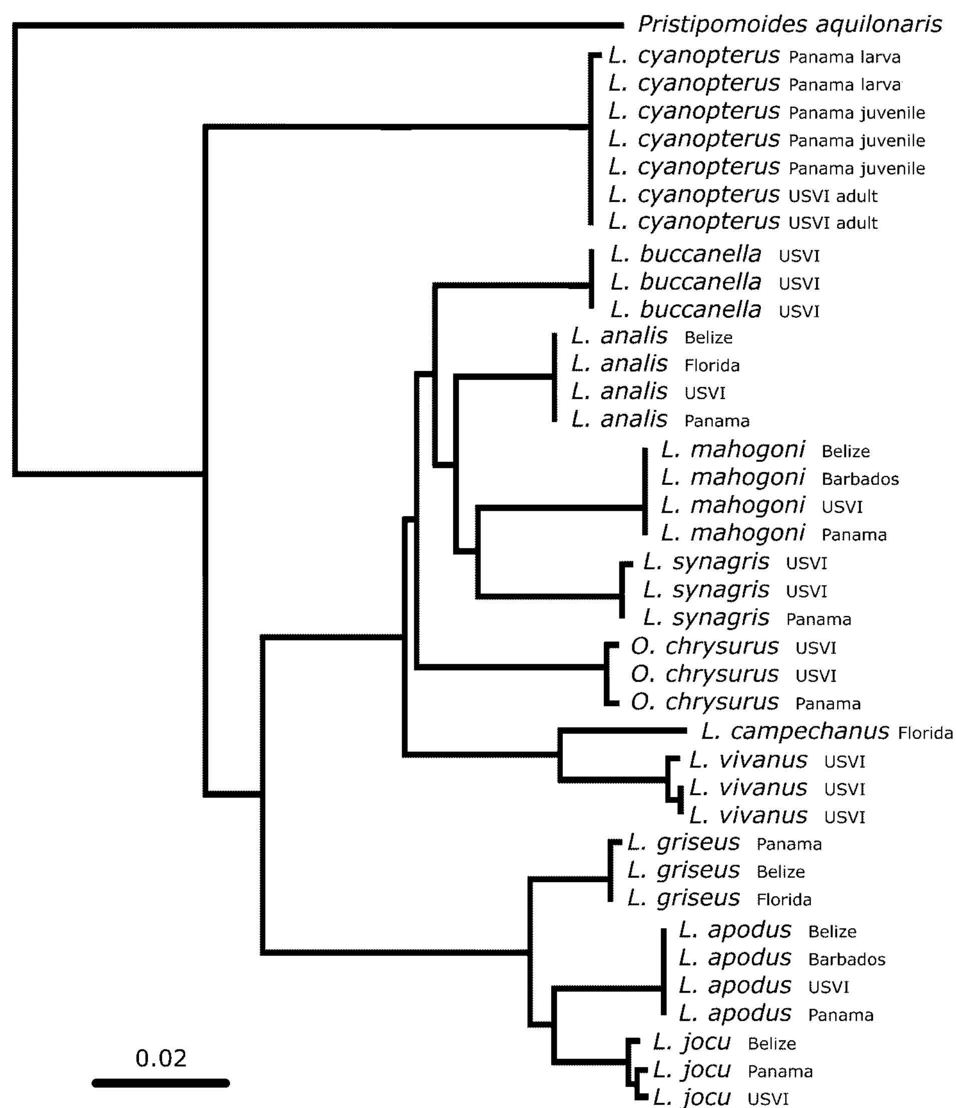

FIGURE 1. Neighbor-joining tree of the Caribbean *Lutjanus* spp. (including *Ocyurus chrysurus*; with *Pristipomoides aquilonaris* as the outgroup) based on the mtDNA barcode region of COI; distances are calculated using the Kimura two-parameter (K2P) model of base-substitution.

An additional benefit of barcoding is that it permits the separation of rare from common species. Many reef-fish genera include abundant species with close relatives that can be rare, or even undescribed (e.g. Victor 2007). In the past, the larvae and juveniles of the rare species probably were included in the large samples of the common species, with no straightforward method to distinguish them. Indeed, Cubera Snapper larvae and juveniles have probably been lumped with other snappers in previous surveys.

The phylogenetic relationships of western Atlantic snappers of the genus *Lutjanus* have been explored using other independent gene sequences. Sarver *et al.* (1996) sequenced two mtDNA gene segments, 12S rDNA and cytochrome *b*, and generated trees somewhat different from ours. One difference was that in their study *L. cyanopterus* unexpectedly fell even further from the other regional *Lutjanus*, actually beyond the outgroup snappers (*Rhomboplites*, *Pristipomoides* and *Etelis*). Another difference was that the shallow-water spot-snappers, *L. synagris*, *L. mahogani*, and *L. analis*, were not consistently separated into a single clade in

their two trees (Sarver *et al.* 1996). One point of agreement is that *Ocyurus chrysurus* falls well within the clade of *Lutjanus* spp. Further studies that include nuclear gene sequences and a more rigorous phylogenetic analysis should help to refine the relationships between snapper species in the region.

FIGURE 2. Larval Cubera Snapper, *Lutjanus cyanopterus*, from the San Blas Islands of Panama, 18.8 mm SL.

Larval description. The four late-stage larvae of *L. cyanopterus* measured from 17.7 to 18.8 mm SL and were pre- and early-transitional (mostly unmarked with a translucent body and then developing some body pigmentation). The larvae were typical lutjanid larvae, having the characteristic non-serrated large spine at the preopercular angle (this spine is reduced during the transitional stages and absent on juvenile snappers). In basic form they closely resemble other described lutjanid larvae (Leis & Trnski 1989; Lindeman *et al.* 2005), with a wide and relatively thick body, long sloping forehead, large round eye, large terminal mouth, long dorsal-fin base and short anal-fin base (Fig. 2). The dorsal, anal, and pelvic fins have stout spines and a standard *Lutjanus* fin-ray formula of D-X,14 A-III,8 Pect-16.

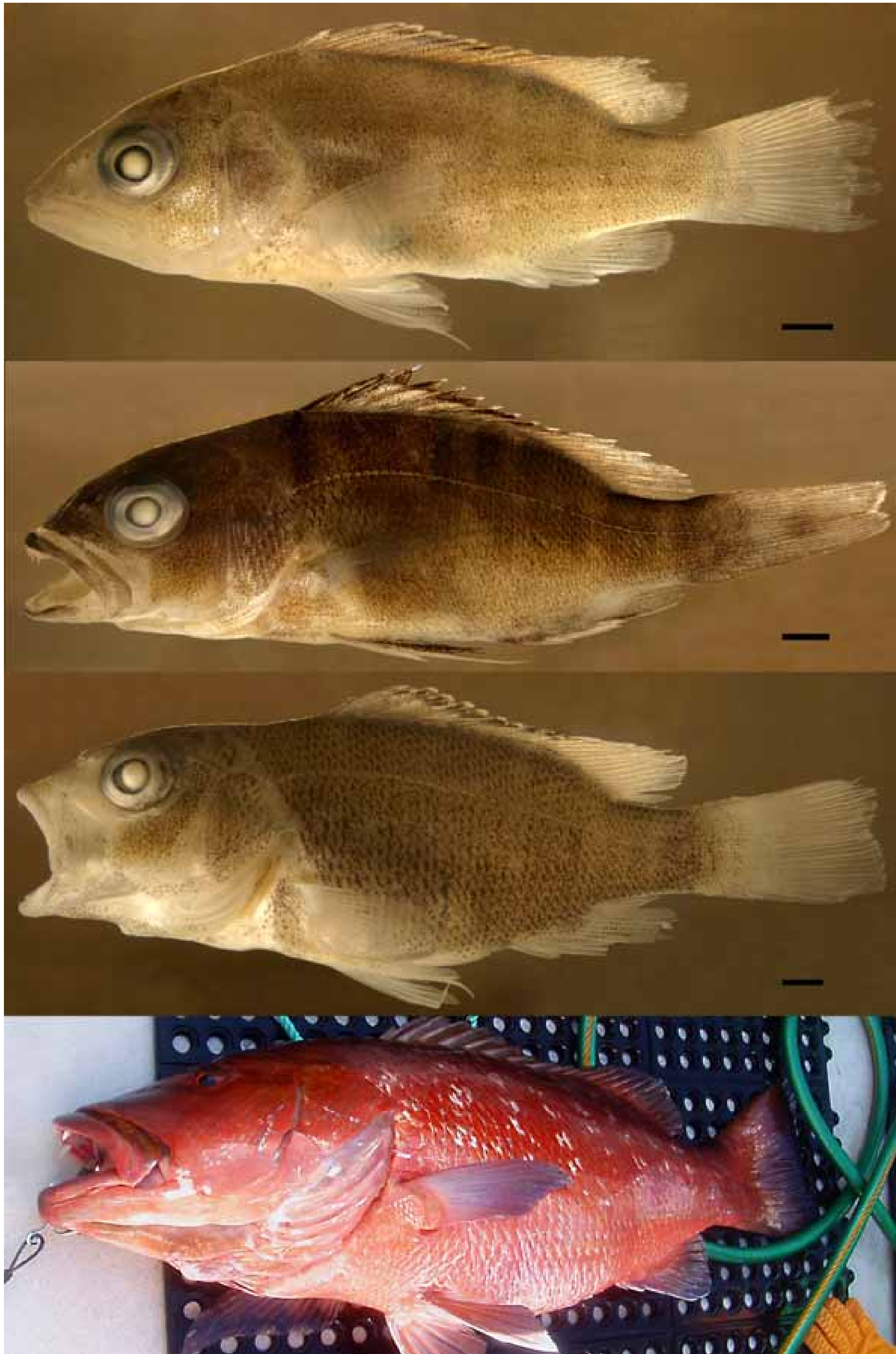

FIGURE 3. *Lutjanus cyanopterus*, from top: 25.8 mm SL, 30.2 mm SL, 34 mm SL (juveniles), and 76 cm SL adult. The three juvenile snappers above are from Isla Grande, Panama (scale bar=2 mm) and the adult is from the spawning aggregation in St. Thomas, USVI.

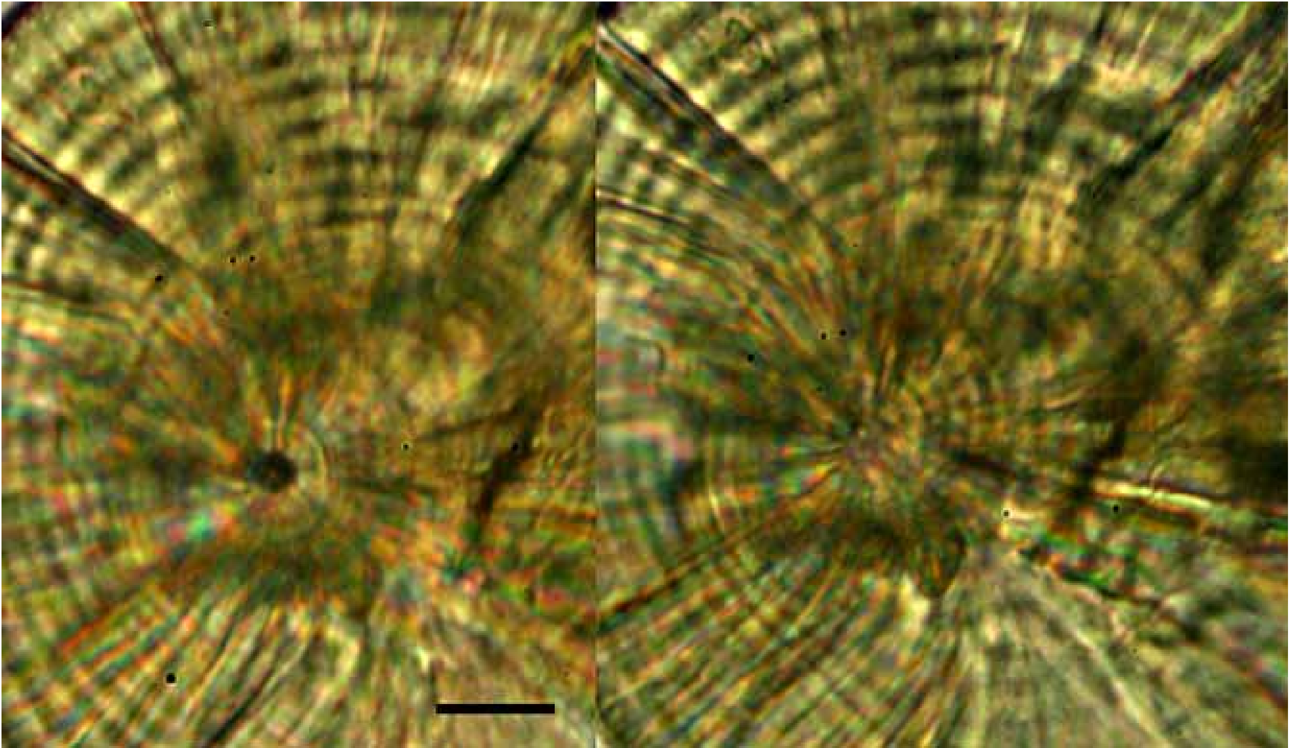

FIGURE 4. Central portion of the larval otolith (lapillus) at two focal planes under transmitted and polarized light at 400X magnification. The primordium is the dark round spot at the center of the otolith view on the left, followed by the core (outlined by a bright line), and then a central zone of about 12 daily increments followed by wider late-larval daily increments (scale bar=50 microns).

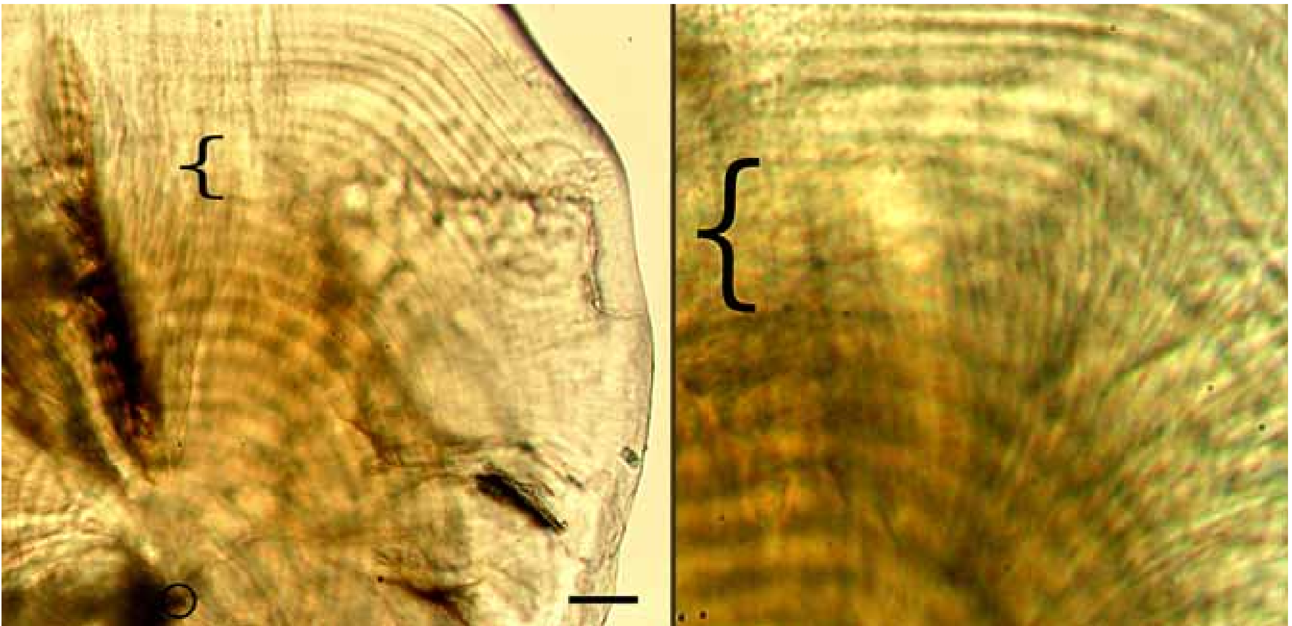

FIGURE 5. The otolith (lapillus) of a juvenile *L. cyanopterus* with prominent late-larval daily increments followed by a settlement transition (bracket) and then narrower post-settlement daily increments. At left, the black circle surrounds the primordium; the early larval increments are not in focus at this plane (scale bar=30 microns). Higher magnification at right.

The pretransitional larvae of *L. cyanopterus* are lightly marked (Fig. 2). On the body, thin lines of melanophores develop on each side adjacent to the base of the spinous dorsal fin, from the base of the third to

sixth and then from the eighth to tenth spines. The melanophore rows continue along the base of the soft portion of the dorsal fin, widening to cover the outer pterygiophore segments and intensifying beneath the fourth to eighth and then the last two rays. The rows merge and extend along the dorsal midline of the caudal peduncle as a single band of melanophores ending at the start of the procurrent caudal-fin rays. A more pigmented early transitional larva has started to develop short melanophore streaks lining some of the myomere edges along the mid-upper body. A central patch of surface melanophores develops on the end of the caudal peduncle, filling in progressively from ventral to dorsal. At the end of the lateral midline of the caudal peduncle there are also a few deep melanophores. The ventral midline is lightly marked; often only a single large melanophore underlying the base of the last few anal-fin rays, which, after a space, extends as a single band of melanophores along the ventral midline of the caudal peduncle up to the first procurrent caudal-fin rays. There can also be one or two inconspicuous melanophores at the base of the other anal fin rays or spines.

On the head there is a dense patch of melanophores overlying the braincase with a scattering developing between the braincase and the first dorsal spine. A patch of small melanophores develops at the tip of the upper jaw and then extends upward along the snout. The lower jaw is mostly unmarked, with only a few small melanophores near the tip. The opercular area is covered in iridescence extending down to the pelvic-fin insertion. The inner cleithral surface of the gill cavity is darkly pigmented and there are internal melanophores lining the dorsal aspect of the peritoneum and retroperitoneum extending down to the vent. The abdominal cavity is covered by a silvery camouflage layer.

The fin spines notably do not have the serrations commonly found among many other larval snappers and groupers. The outline of the dorsal fin is somewhat distinctive; the second dorsal-fin spine is the longest, with the subsequent spines becoming progressively and evenly shorter such that the profile of the spinous tips forms a straight downward-sloping line. The anal-fin spines are stout, the second and third longer than the first, but otherwise the relative lengths are variable. Melanophores on the dorsal-fin membranes are present along most of the length of the membrane just behind the second dorsal-fin spine and then densely on the outer third of all of the subsequent spinous dorsal-fin membranes. The soft dorsal fin is unmarked. There are a few melanophores at the base of the caudal-fin segmented rays, primarily on the uppermost of the ventral set of fin-rays. The anal fin membranes are unmarked. The pelvic fins have dense melanophores along the outer third of the fin membranes of the longest two or three rays.

The pretransitional larvae of *L. cyanopterus* can be distinguished from similar stages of the other regional *Lutjanus* spp. All of the shallow-water species and some of the deeper water species have late-larval descriptions: five species in Lindeman *et al.* (2005) and the others described in Victor (2009). Morphological differences include a wider caudal peduncle in larval *L. cyanopterus* than in other *Lutjanus* (body depth at last dorsal ray less than 2.4 times into maximum body depth). The relative lengths of the dorsal fin spines are also distinctive; late larvae of other *Lutjanus* mostly do not have the evenly-shorter array of spines after the second spine with the tips forming a straight line. Larvae of the snapper clade comprising *L. apodus*, *L. jocu*, and *L. griseus* have anterior serrations on the anal-fin spines not seen in *L. cyanopterus*. Late-larval *L. analis* can closely resemble *L. cyanopterus* in morphology, but have a narrower caudal peduncle.

Marking differences include the pattern of melanophores concentrated on the outer third of the pelvic fin membranes (vs. uniformly along the fin or absent in many other *Lutjanus*). The distinctive band of melanophores concentrated at the outer edge of the membranes of the spinous dorsal fin is also not found in most other *Lutjanus.* The mostly-unmarked anal-fin base can be shared with pretransitional *L. analis* larvae, while other *Lutjanus* have a row of melanophores along the anal fin base and on some of the membranes. *L. cyanopterus* larvae also have a densely-speckled upper jaw and snout with a mostly unmarked lower jaw, while in other species the markings are often equivalent. Several snapper species also develop a lateral spot early in transition, which is not present on *L. cyanopterus*. Some other species develop caudal-fin base melanophores on the dorsal half of the fin from early stages, while melanophores are limited to the ventral caudal-fin rays in pre-transitional *L. cyanopterus*.

It is interesting to note that despite the similarity in adult form and markings, larval Cubera Snappers are

quite different from larval Gray Snappers, *L. griseus*. They differ in morphology: Cubera Snapper larvae have distinctly-wider caudal peduncles, evenly-graded dorsal-fin-spine lengths, and non-serrated dorsal and anal fin spines. In addition, there is little overlap in markings: Cubera Snapper larvae have melanophores at the outer portion of the dorsal-fin-spine membranes vs. around the base and lower portion in Gray Snappers; on the pelvic-fin membranes vs. none on the pelvic fins; at the center and rear of the caudal peduncle vs. along the lower half; and sparing the anal fin entirely vs. along the anal-fin base and on the first membranes. In addition, Gray Snappers settle at a relatively small size for snappers, averaging 13.2 mm SL in Panama and 12.6 mm SL in Belize (Victor 2008) and 13.4 in North Carolina (Tzeng *et al.* 2003), while Cubera Snapper larvae are relatively large at transition (18–19 mm SL). In this pairwise case, at least, it appears that the early-life-history stages reflect the phylogenetic distance while the adult forms converge in appearance.

Juvenile Description. Juveniles of *Lutjanus cyanopterus* develop indistinct vertical dark bars (Fig. 3). The black outer portion of the dorsal-fin membranes persists in juveniles, often with an abruptly-white edging. The black markings on the outer third of the pelvic-fin membranes also persist well into the juvenile stage. Darker individuals develop a black cap across the eyeball, melanophores along the full length of the pelvic-fin membranes, and intensified black bars and a black edging to the spinous dorsal fin.

Juvenile Cubera Snappers can be distinguished from other regional snapper juveniles primarily by the absence of the prominent dark stripe through the eye, no blue line under the eye, and no prominent spot on the side of the body. Juvenile Gray Snappers, *L. griseus*, have an obvious stripe through the eye and develop stripes across the body. The main morphological distinction for juveniles, the longer and narrower body of the Cubera Snapper, is distinctive; the other regional snappers have wider bodies both as juveniles and adults (the predorsal body depth of *L. cyanopterus* juveniles goes at least 2.8 times into SL vs. 2.4 or fewer in the other species).

Otolith microstructure and early life history. The lapilli and sagittae (two of the three pairs of otoliths) of Cubera Snappers closely resembled illustrations of those from other *Lutjanus* spp. (Allman & Grimes 2002; Zapata & Herron 2002; Denit & Sponaugle 2004). Although other workers used different preparation methods and examined different pairs of otoliths, the counts of otolith increments were remarkably similar.

We found the lapilli had clearer otolith increments than the largest pair of otoliths, the sagittae, and did not require sectioning (Allman and Grimes (2002) and Zapata and Herron (2002) used the same method). At the start of the array, not the geographic center of the otolith, there is a central primordium (the small dark spot in Fig. 4). Surrounding that is a round core without increments about 8 microns in diameter, often outlined by a ring that refracts light differently, appearing brighter, at certain focal planes, as in Fig. 4. The core broadly corresponds to the size of the lapillus at hatching for captive-raised Red Snapper, *Lutjanus campechanus* (Victor 2009). After the core, there is a zone of relatively narrow increments that progressively increase in width. This central zone comprises about ten to twelve increments and measures about 30–40 microns in diameter. The central zone usually appears lighter than the later portion of the larval otolith (see Fig. 5; in Fig. 4 the central zone is not lighter, but has less-distinct dark incremental lines). The lapilli of ten-day old Red Snapper larvae reared in captivity are also about 35 microns in diameter (Victor 2009), providing some validation and confirmation of the daily nature of the early increments present in the central zone of these otoliths.

After the central zone, there is a slightly darker region of conspicuous wide increments visible at all focal planes, often containing distinct sub-daily increment arrays which are limited to a single focal plane. These prominent late-larval increments are made up of a wide dark line followed by a similarly-wide lighter line. These increments extend to the edge of the otoliths of larvae, but are followed by a transition to a relatively lighter (more translucent) zone of increments in settled juveniles (as in Fig. 5). This indicates that the transition represents a settlement mark (Wilson & McCormick 1997). The increments after the transition are more contrasting than the earlier increments, each with a distinct narrow dark line followed by a wider and clear light line. The transition zone was sometimes an area of indistinct increments, about two or three wide (Fig. 5). This indistinct area was not present at the edge of our larval otoliths and it is difficult to confidently assign those increments to pre-or post settlement. This band likely represents some change in behavior around

settlement (perhaps a search for mangrove settlement habitats among the reef systems). We assigned them to the larval period primarily because they usually retained the overall darker background and prominent sub-daily arrays of the prior increments.

The late-larval specimens captured over the reef were likely coming in to settle. This pattern of ready-to-settle larvae entering reef waters and being captured at lights on moonless nights is well-established (Victor 1991). Two larvae with intact otoliths had 25 and 26 increments after the core up to the edge, indicating a likely pelagic larval duration (PLD) of 28 and 29 days if three days are added for fertilization, hatching, and development of the primordium and core of the otolith (Lindeman 1997; Lindeman *et al.* 2000). The pre-transition otolith increment counts for the settled juveniles ranged from 24–27 or a PLD of 27 to 30 days.

Various researchers have reported a variety of PLD counts for the regional *Lutjanus* (Lindeman *et al.* 2000; Allman & Grimes 2002; Zapata & Herron 2002; Denit & Sponaugle 2004). The majority of studies yield similar short PLDs of about one month with little variation, but some report widely variant PLDs. The source of the variation is unclear. There may be some natural variation within the region, but without much detail (and no illustrations) specifically on the interpretation of the otolith core and very early increments in these studies, methodological differences in counting are probably contributing to the variation (Victor 2008). The settlement transition has been described as a particularly dark and prominent increment on eastern Pacific *Lutjanus* otoliths (e.g. Zapata & Herron 2002); however this was not visible on our otoliths. Certainly, variation in the interpretation of when the settlement transition occurs could also contribute to observer error in PLD estimates.

Although our sample is small, some information on the timing of spawning and settlement in Panama can be extracted. The four larvae settled during the week before new moon, captured on June 24, 25, 26 and 30, 1981 (new moon on July 1). With a PLD of about one lunar month, spawning would have taken place the week before the June new moon. Similarly, back-calculated settlement dates for the three juveniles captured in 2007 were May 14–15, one or two days before the new moon and spawning would have taken place on the April new moon. The season of settlement is consistent with other geographic locations: spawning aggregations of *L. cyanopterus* have been observed during the spring and early summer months, March to August, in the USVI (Kadison *et al.* 2006), Florida and Cuba (Claro & Lindeman 2003), and Belize (Domeier & Colin 1997; Heyman *et al.* 2005). However, they observed spawning concentrated around the full moon (although with a broad range of days), which does not correlate with new-moon settlement. New-moon settlement peaks and back-calculated new-moon spawning has been found in the Gray Snapper (Tzeng *et al.* 2003). Better resolution of spawning and settlement cycles would require monitoring at the appropriate source and destination populations at the same time.

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