Description of a Distinct Snapper Larvae and Species Identification Based on Mitochondrial DNA Analyses

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ABSTRACT

Lutjanidae is one of the largest teleostean families. Distinguishing lutjanid larvae from each other is difficult despite published larval descriptions. Taxonomic identification of these larvae to the species level represents one of the main bottlenecks in our understanding of their early life cycle. Such information is of vital relevance in the assessment of marine protected areas (MPAs) as management options to restore the declining stocks of commercially exploited fishes. To address this problem, we are in the process of identifying mitochondrial DNA (mtDNA) restriction fragment length polymorphisms (RFLP) diagnostics to lutjanids. Adult specimens were obtained from catches of local fishermen at the Parguera Fishing Village, southwest Puerto Rico. Larvae were obtained from plankton tows using a 202 µm mesh net. We quantified mtDNA variation through the use of polymerase-chain-reaction (PCR) amplification of fragments corresponding to 450 bp of the 12S rRNA gene, followed by digestion with restriction enzymes. Seven haplotypes have been observed; four of them have been found in only one species of the adult specimens. One of the distinct haplotypes also corresponded for an unknown larvae. The haplotype of the unknown larvae matched with that of the adult of *Lutjanus griseus*. We consistently found and described a morphologically distinct type of lutjanid larva not previously described with a molecular haplotype and occurrence which might be compatible with *Lutjanus apodus*. Thus far, the procedure that we are undertaking is producing results that suggest that specific haplotypes will be identified for most of the species.

KEY WORDS: Snapper larvae, mitochondrial DNA (mtDNA), MPAs

Descripcion de una Larva de Pargo Distinta y su Identificación Basada en el Análisis de ADN Mitocondrial

La familia Lutjanidae es una de las familias más grande de peces óseos. La distinción de las larvas de lutjánidos unas de otras es muy difícil aun utilizando las descripciones publicadas. La identificación taxonómica de estas larvas a nivel de especie representa uno de los mayores obstáculos en el entendimiento de su ciclo de vida. Dicha información es de vital importancia en el reconocimiento de Areas Marinas Protegidas (AMPs) como opción de manejo para restaurar abastecimeintos de peces comercialmente explotados. Para atender este problema, estamos en el proceso de identificar polimorfismos de patrones de restriccion (RFLP) en el ADN mitocondrial (mtDNA) diagnósticos para lutjánidos. Los especímenes de peces adultos han sido obtenidos de capturas de pescadores locales. Las larvas fueron obtenidas de arrastres de plankton utilizando una malla de 202 µm. Cuantificamos la variación en el mtDNA a través del uso de la reacción de polimerasa en cadena (PCR), amplificando un fragmento correspondiente a 450 pb del gen 12S rRNA, seguido de digestión con enzimas de restricción. Se han observado siete haplotipos, cuatro en una sola especie y en una una larva desconocida. El haplotipo de la larva desconocida corresponde al de los adultos de *Lutjanus griseus*. Consistentemente, encontramos y describimos un tipo de larva morfológicamente distinta, no descrita previamente, cuyo haplotipo molecular y época de ocurrencia pueden ser compatibles con el de *Lutjanus apodus*. Hasta ahora, el proceso que estamos llevando a cabo está produciendo resultados que sugieren que se identificarán haplotipos específicos para la mayoría de las especies.

PALABRAS CLAVES: Larva de pargo, ADN mitocondrial (mtDNA), Áreas Marinas Protegidas (AMPs)

INTRODUCTION

Most coral reef fishes have a pelagic larval stage that differs morphologically from juveniles and adults, making the identification keys based on adult fish meristics useless. Taxonomic identification of coral reef fish larvae to the species level still represents one of the main bottlenecks in our understanding of the early life cycle of many reef fishes. Such information is of vital relevance in assessment of marine protected areas (MPA) as management options to restore the declining stocks of commercially exploited coral reef fishes.

Fisheries studies require accurate identification of subject species. Identification of the developmental stages of fishes is complicated by the small size of the specimens, their fragility, and the relatively great changes in their structure and pigmentation. Experience has shown that

major changes can occur over very small growth increments and these can only be documented by a continuous growth series (Moser *et al.* 1984). Traditionally identification of larval fishes was based on morphological characteristics. Character sets, whether morphological or molecular, are the primary data collected to study fish systematics or population structure and dynamics. Morphological character sets require little equipment to gather and can be very informative when used to address population problems suited to their level of sensitivity. Molecular characters have proven to be particularly informative in studies of closely related taxa (Graves *et al.* 1990, Bembo *et al.* 1995, Chow and Inoue 1993).

During the past decade, the use of mitochondrial DNA (mtDNA) to study the phylogeny and population genetics of fishes, amphibians and reptiles has greatly proliferated, and mtDNA has become the standard molecule of choice among most ichthyologists and herpetologists doing comparative molecular genetics.

Analysis of the distribution of eggs and larvae for life history and recruitment studies of the fishery has been hampered by the inability to identify eggs and larvae of most snapper species. The close morphological similarity of larvae among lutjanid species has made identification of larvae a difficult task (Richards 1985, Leis 1987, Richards and Lindeman 1987). Many of the lutjanid larvae in their pre-flexion stages have been unidentifiable at the species level, using the conventional morphological observations. Very small snapper larvae may be confused with other percoid families and show substantial geographical variability in reproduction (Lindeman 2005). In addition, morphological characters are very similar within the family making the life history of this group very difficult to study. Eggs, larvae, and early juveniles are only known for various species (Lindeman 2005). Lindeman *et al.* (2005) assembled information for the identification of early life history of snappers, but diagnoses for some species are still incomplete.

In a previous study, restriction endonuclease analysis of mitochondrial DNA amplified by polymerase chain reaction was applied to snapper species in order to assess reliability of this method for genetic species and stock identification studies (Chow *et al.* 1993). It is highly probable that some snapper species contain a large number of maternal lineages within the population, which can be detected by Polymerase Chain Reaction – Restriction Fragment Length Polymorphisms (PCR-RFLP) analyses as demonstrated in this study. Results from Chow *et al.* (1993) indicate that PCR-RFLP analysis is much simpler and less expensive than conventional mtDNA and nucleotide sequence analyses. With the PCR-RFLP method (Whitmore 1990), not only are quantitative analyses of species composition at early life stages possible (Whitmore 1992), but also intensive genetic analysis. Since relatively little is known about the spawning habits, dispersal, and migration of these snappers species, this study aims to describe diagnostic motifs of PCR-RFLP that can be used to identify fish larvae of the Lutjanidae family. The approach is based on comparisons of genetic profiles of a fragment of the 12S rRNA gene in the mtDNA between adults lutjanids and their early life stages.

MATERIALS AND METHODS

Sample Collection

Adult specimens from fourteen species (*Lutjanus griseus*, *Lutjanus apodus*, *Lutjanus cyanopterus*, *Lutjanus jocu*, *Lutjanus vivanus*, *Lutjanus bucanella*, *Lutjanus synagris*, *Lutajnus analis*, *Lujanus mahogoni*, *Ocyurus chrysurus*, *Rhomboplites aurorubens*, *Pristipomoides macrophtlamus*, *Etelis oculatus* and, *Apsilus dentatus*) were obtained from catches of local fishermen fisherman village either fresh or frozen, depending on availability.

From one to two grams of muscle or liver from each specimen were brought to the laboratory in separate bags on ice. Once in the laboratory 25 mg were used for DNA extraction and the remaining of the samples were stored at –80°C. Larvae were obtained from plankton tows using a 202 µm mesh net, preserved either in formalin or DMSO.

Morphological Observations

Identification of larvae to the family level, measurements and, morphological descriptions were made under a binocular dissecting microscope following the guide by Leis and Carson (2000).

DNA Extraction

Total genomic DNA of adult individuals was extracted and purified from 25 mg (fresh or frozen) of tissue using the QIAamp DNA Mini Kit from QIAGEN, Inc.

Total genomic DNA was extracted and purified from each individual larva using the QIAamp DNA Mini Kit from QIAGEN, Inc.

Amplification of Mitochondrial 12S rRNA

The pair of primers used to target 12S RNA were abbreviated forms of those described by Kocher *et al.* (1989). The nucleotide sequence of each set of primers were as follows for the 12S rRNA fragment:

TCAAACTGGGATTAGATACCCCACTAT and
TGACTGCA GAGGGTGACGGGCGGTGTGT.

Polymerase chain reaction was carried in a final volume of 25 µL in a reaction mixture described by Kocher *et al.* (1989). This reaction mixture was preheated at 94°C for 2.5 minutes followed by 30 cycles of amplification (94° C for 30 sec., 45°C for 1.5 min., and 72°C for 1.5 min.) with a final elongation of 10 minutes at 72°C.

Endonuclease Digestion for Amplified DNA Fragments

Eleven restriction endonucleases used were: *Alu* I, *Bfa* I, *Hae* III, *Hin f* I, *Msp* I, *Nla* I, *Taq* I, *Hha* I, *Dpn* II, *Mse* II and *Ava* II all recognizing symmetric 4-base pair sequences. One unit of each enzyme was applied to 11 µL of the amplified PCR product in a final reaction volume of 15 µL. The digested samples were electrophoresed through 3% agarose gels. After electrophoresis and staining with ethidium bromide DNA bands were visualized and photographed.

RESULTS

Gene Amplification

The pair of primers used successfully amplified 455 ± 5 bp fragment of the 12S rRNA gene. There were no apparent differences in the fragment size among species.

Restriction Fragment Analysis

For the 455 bp 12S rRNA fragment, six enzymes (*Bfa* I, *Hae* III, *Hha* I, *Nla* I, *Taq* I and *Ava* II) had no restriction sites in any of the species. *Dpn* II and *Msp* I had restriction site(s) in all species examined, without apparent size difference in restricted fragments between species. *Alu* I produced two different cutting profiles. *Hin f* I had the same restriction sites for *Lutjanus vivanus*, *Lutjanus bucanella*, *Lutjanus analis* and *Ocyurus chrysurus.* *Mse* I digestion produced two different cutting profiles, with *Ocyurus chrysurus*, *Lutjanus analis* and *Lutjanus bucanella* designated as pattern 1. *Lutjanus apodus*, *Lutjanus cyanopterus* and *Lutjanus jocu* as well as the unknown larvae # 2 showed a cutting profile designated as pattern 2. *Mse* I did not cut that fragments from *Lutjanus vivanus, Lutjanus synagris* and *Lutjanus griseus* as well as for unknown larvae #1. One of the unknown larvae (#1) haplotype matched with that of *Lutjanus griseus*, being similar in shape to type 1 larvae (Figures 1 and 2).

Restriction patterns were summarized for haplotype designation for species discrimination. Seven haplotypes were observed for the species examined after digestion with eleven enzymes (Table 1).

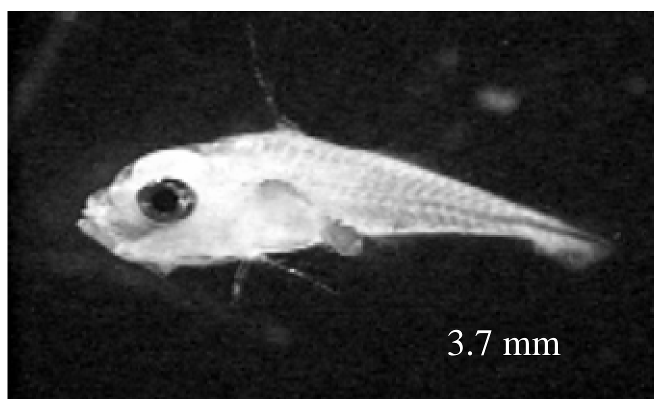

Figure 1. Larvae identified as *Lutjanus griseus* (Type 1).

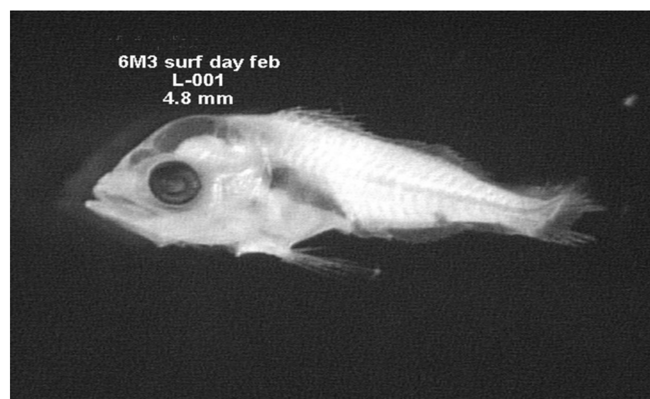

Figure 2. Type 1 larva.

Table 1. Summary of haplotypes for lutjanids.

	Enzymes			
Species	*Alu* I	*Hinf* I	*Mse* I	**Haplotype**
Ocyurus chrysurus	2	1	1	A
Lutjanus vivanus	1	1	0	B
Lutjanus cyanopterus	2	0	2	
Lutjanus jocu	2	0	2	C
Lutjanus apodus	2	0	2	
Lutjanus griseus	1	0	0	D
Unknown larvae # 1	1	0	0	
Lutjanus synagris	2	0	0	E
Lutjanus analis	1	1	1	F
Lutjanus bucanella	1	1	1	
Unknown larvae # 2	1	0	2	G

Morphological Observations

Three types of larvae have been differentiated morphologically (Figures 2 - 4). We consistently found a morphologically distinct type of lutjanid larva, apparently not previously described in the revised literature, which molecular haplotype and occurrence might be compatible with *Lutjanus apodus*. This larva was designated as Type 2 (Figure 3). Preliminary description includes:

Myomeres (vertebrae): 24

Fin Ray counts:
Dorsal fin: X-12 (ten spines, twelve soft rays)
Anal fin: III-7

Spines:
Third dorsal spine longer non serrated.

Pigmentation:
Pigments present on articular jaw and branchiostegal rays. Melanophore on dorsal part of the body just below the 1st and 2nd dorsal fins. Melanophore at the end of the anal fin, over the caudal peduncle. Internal melanophore over the lateral line (approximately over myomeres 7 - 8 counting from notochord to head). Pigments on first dorsal and pelvic fins.

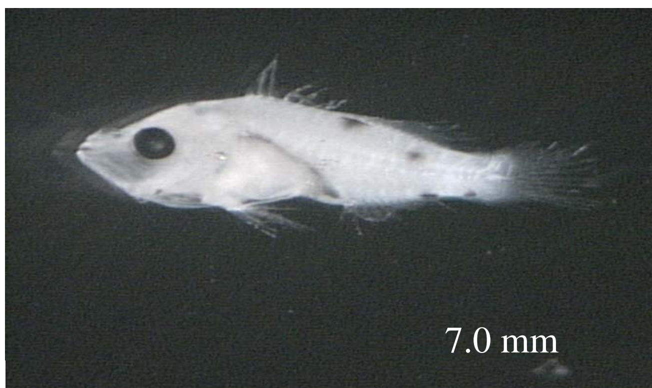

Figure 3. Type 2 larva.

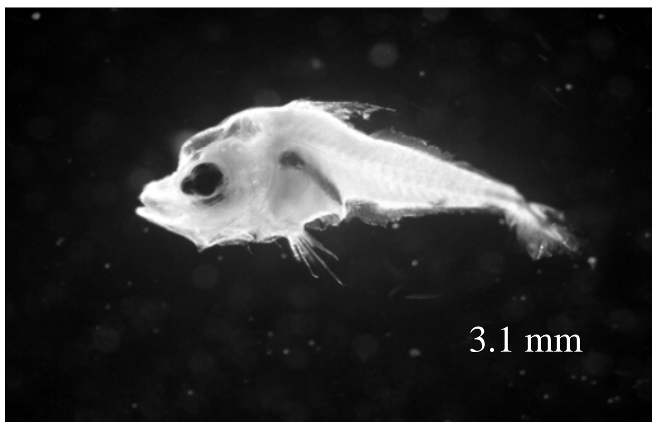

Figure 4. Type 3 larva.

DISCUSSION

Chow *et al.* (1993) were able to distinguish between eight of 13 Lutjanidae species, including six of nine species that were studied in this project, using nine restriction endonucleases for two fragments of the mitochondrial DNA (12S rRNA and *cyt* b. Of those nine enzymes, five coincide with the ones we used (Hae III, Msp I, Alu I, Taq I and Hinf I). As for them, our results show that *Hae* III did not produced length variation of the restricted fragments between species. We found a diagnostic restriction profile for *Lutjanus vivanus* in the fragment digested by *Alu* I, they observed intraspecific polymorphism for some species using this enzyme, but not for *L. vivanus*. They reported that *Taq* I digestion produced a straightforward diagnostic restriction profile in the 12S rRNA fragment of *Lutjanus griseus* but we did not detected any restriction sites for *Taq* I in our specimens. They observed intraspecific polymorphism for *Msp* I for *L. analis, L. cyanopterus* and *L. jocu*, we did not observed length variation in the restricted fragments in our subject species. The 12S rRNA fragment has been widely used for phylogenetic studies among distantly related species (Stock *et al.* 1991, Lecointre 1996).

So far, we were able to identify four species for the adult specimens: *Ocyurus chysurus*, *Lutjanus vivanus*, *Lutjanus analis and Lutjanus synagris* and one larva as *Lutjanus griseus* with our molecular data. As for the distinct larva which might be compatible with *Lutjanus apodus*, more extensive molecular analyses, including comparisons with adult species genetic profiles, are still needed to draw any conclusions on the identification.

Further analysis is necessary to validate these results as well as for intraspecific polymorphism for complete discrimination. In conclusion, the procedure that we are undertaking is producing results that suggest that specific haplotypes will be identified for most of the species.

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