MITOCHONDRIAL DNA ANALYSES FOR SPECIES IDENTIFICATION OF SNAPPERS FROM CARIBBEAN WATERS

by

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ABSTRACT

This study examined phylogenetic relationships among fifteen species of the Lutjanidae family occurring within the Caribbean Basin, based on mitochondrial 12S rDNA analysis. Previous investigations have limited their scope to species occurring in the western Atlantic (WA) and Cuba, or to several species within the Lutjaninae subfamily. This is the first phylogenetic study that includes all 3 subfamilies of lutjanids occurring in the Caribbean. We identified diagnostic polymorphisms within the mitochondrial DNA (mtDNA) for 15 lutjanid species in the Caribbean. Specimens were obtained from local catches at La Parguera, Puerto Real and Rincón, western Puerto Rico and Cataño, at the north. DNA variation was quantified through the use of polymerase-chain-reaction (PCR) amplification of fragments corresponding to 450 bp of the mtDNA 12S rRNA gene followed by sequencing. Intraspecific variation was not found within any species for the adult specimens analyzed during this study. Assessment of phylogenetic relationships of species was conducted using Neighbor Joining (NJ) and Bayesian Inference (BI) analyses. Phylogenetic relationships within the subfamily Lutjaninae remained rather unresolved for some species. Nevertheless, our study suggests that even if not strongly supported, the groups found are associated according to morphology, habitat or feeding preferences. In addition, the consistency in the sequence data for each species in this study demonstrates that the 12S rRNA gene is a reliable tool for taxonomic identification within this family. These sequences constitute a sort of molecular key for all the 15 species of lutjanids studied, useful for identification of early stages and processed tissues or fillets for fisheries management regulations.

Lutjanidae is one of the largest teleostean families, commonly known as snappers and is one of the most important in Caribbean fisheries. For appropriate management, description of dispersion patterns for each species is needed. However, specific identification of lutjanid larvae is still difficult despite published larval descriptions and is one of the main bottlenecks in our understanding of their early life history. To address this problem, we identified diagnostic polymorphisms within the mitochondrial DNA (mtDNA) for 15 lutjanid species in the Caribbean. Adult specimens were obtained from local catches and larvae from plankton tows using a 202 µm mesh net. DNA variation was quantified through the use of polymerase-chain-reaction (PCR) amplification of fragments corresponding to 450 bp of the mtDNA 12S rRNA gene followed by sequencing. Phylogenetic trees were constructed from DNA sequence data including adults and larvae. Seven species were identified among the collected larvae: *Lutjanus apodus, Lutjanus synagris, Lutjanus analis, Lutjanus griseus, Lutjanus mahogoni, Ocyurus chrysurus and Rhomboplites aurorubens.* Identification of these larvae increases our understanding of early larval stages taxonomy. In addition, this information is useful for the design of research leading to the description of spawning, dispersal and recruitment patterns, as well as habitat selection for these species. These analyses are of vital relevance for assessments regarding the establishment of Marine Protected Areas (MPAs) as a management option to restore diminishing stocks of fish populations.

RESUMEN

En este estudio se examinaron las relaciones filogenéticas entre quince especies de la familia Lutjanidae que habitan en la cuenca del Mar Caribe, por medio de análisis de un fragmento del gen 12S rRNA del ADN mitocondrial. Investigaciones previas limitaron su enfoque a especies del Atlántico occidental y Cuba, o a algunas especies de la subfamilia Lutjaninae. Este es el primer estudio filogenético que incluye las tres subfamilias de lutjánidos del Caribe. Se identificaron polimorfismos diagnósticos dentro del ADN mitocondrial para las quinces especies analizadas. Los especimenes se obtuvieron en pescaderías locales de La Parguera, Puerto Real, Rincón, al oeste de Puerto Rico y en Cataño, al norte. La variación en el ADN se cuantificó por medio de la reacción de polimerasa en cadena (PCR) de un fragmento correspondiente a 450 pb del gen mitocondrial 12S rRNA, seguido de secuenciación. No se encontró variación intraespecífica entre los individuos de las especies analizadas en este estudio. La relaciones filogenéticas fueron investigadas utilizando análisis de Neighbor Joining (NJ) e Inferencias Bayesianas (BI). Los resultados de nuestro estudio sugieren que, aunque no apoyado robustamente, los grupos encontrados en los árboles filogenéticos se asociaron de acuerdo a morfología, hábitat o hábitos de alimentación. Además, la consistencia en las secuencias de cada especie en este estudio demuestra que el gen 12S rRNA se puede utilizar como una herramienta confiable para la identificación de especies dentro de esta familia. Las secuencias encontradas constituyen un tipo de clave molecular para las quince especies de lutjánidos estudiados. Estas secuencias pueden ser útiles para la identificación de larvas de lutjánidos, así como de filetes de especies protegidas por regulaciones de manejo.

La familia Lutjanidae es una de las más grandes de entre los peces óseos, se conocen como pargos o chillos, y es una de las mas importantes en las pesquerías del Caribe. Para su manejo es necesario describir los patrones de dispersión de las larvas de cada especie. Sin embargo, la identificación de las larvas de lutjánidos es aún muy difícil a pesar de la existencia de descripciones publicadas y representa uno de los mayores obstáculos en el entendimiento de sus ciclos de vida. Para atender este problema, identificamos polimorfismos diagnósticos en el ADN mitocondrial (mtADN) para 15 especies de lutjánidos del Caribe. Los especimenes se obtuvieron en pescaderías locales de La Parguera, Puerto Real, Rincón, al oeste de Puerto Rico y en Cataño, al norte. Las larvas fueron obtenidas mediante arrastres de plancton utilizando redes con malla de 202 µm. Se cuantificaron variaciones en el mtADN amplificando fragmentos correspondientes a 450 pb del gen de 12S rRNA por medio de la reacción de polimerasa en cadena (RPC) seguido de secuenciación. Se construyeron árboles filogenéticos utilizando las secuencias de ADN de los adultos y larvas. Siete especies de lutjánidos fueron identificadas dentro de las muestras de larvas: *Lutjanus apodus, Lutjanus griseus, Lutjanus synagris, Lutjanus analis, Lutjanus mahogoni, Ocyurus chrysurus* y *Rhomboplites aurorubens*. La identificación de estas larvas puede facilitar el entendimiento de investigaciones sobre patrones de dispersión en las que fueron identificadas sólo a nivel de familia. Esta información también será de gran valor para diseñar investigaciones mas detalladas dirigidas a describir patrones de dispersión y reclutamiento, así como la selección de lugares de desove y habitáculo para estas especies. Estos análisis son de vital importancia para realizar evaluaciones para el establecimiento de Áreas Marinas Protegidas (AMPs) como opciones de manejo dirigidas a reestablecer los abastecimientos de poblaciones de peces de arrecife de coral en disminución.

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To my family . . .

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1 GENERAL INTRODUCTION

Fishes of the Lutjanidae family are widely distributed in tropical and subtropical waters. The family has 123 species, 21 genera and 5 subfamilies (Anderson, 2003; Froese and Pauly, 2006). Of the above, 5 genera and 18 species are believed to be present in the western Atlantic, while 5 genera, 15 species and 3 subfamilies (Lutjaninae, Apsilinae and Etelinae) are reported for the Caribbean (Robins and Ray, 1986; Anderson, 2003). The largest subfamily is the Lutjaninae with 73 species and the smallest is the subfamily Paradicichthyinae with two species. The subfamilies Etelinae and Apsilinae have 18 and 10 species, respectively (Allen, 1985).

The subfamily Lutjaninae represents about two thirds of the species in the family which is the best known; however, the others also deserve attention and are relevant aquatic resources in many regions of the world (FAO, 2005). The species in the subfamily Lutjaninae constitute an important component of the reef fisheries in tropical and subtropical latitudes throughout their geographical range, while the deep-water subfamilies Apsilinae and Etelinae represent by far the most important component of the deep-bottom fishery in the Pacific, Atlantic, Indian oceans and in the Caribbean Sea (Cummings and Matos-Caraballo, 2003).

Landings of snappers are of significant volume and economic value due to the excellent quality of the meat and high demand, making them some of the most appreciated species in the market today. However, there is concern about the status of several fisheries. In the Gulf of Mexico alone, red snapper (*Lutjanus campechanus*) and vermilion snapper (*Rhomboplites aurorubens*) are currently over-fished (Coleman *et al.,,* 1999). Cubera snappers (*Lutjanus cyanopterus*) and mutton snapper (*Lutjanus analis*) are listed as vulnerable by the International Union for Conservation of Nature, and considered at risk of extinction (IUCN, 2007).

Snappers are of important economic and ecological value in the tropical western Atlantic and the Caribbean. Lutjanids are heavily exploited by extractive fisheries, with their stocks declining. Efforts on the protection and management of fishes of this family are imperative. Decreases in natural populations of snappers have motivated broad attention in comprehensive studies on reproduction, species identification, early life histories, larval identification, diversity, population structure and phylogenies (Sarver et al., 1996; Lindeman et al., 2007; Moura and Lindeman, 2007; Liu, 2007; Zhu, 2006; Chow and Walsh, 1992; Chow et al., 1993; Miller and Cribb, 2007; Loftus, 1992). Approaches to determine stock assessments commonly include the assembly and review of all available fishery data and life history information.

Exploration of the taxonomic identification, early life history and phylogenetic relationships of lutjanids is far from complete and continually under review (Rivas, 1949; Rivas, 1966; Vergara, 1980; Johnson, 1980; Lee and Tsoi, 1988; Chow and Walsh, 1992; Sarver et al., 1996; Leis, 2005, 2007; Miller and Cribb, 2007). New species have been identified recently (Moura and Lindeman, 2007) and species previously described as valid have been recognized as natural intergeneric hybrids of lutjanids (Loftus, 1992; Domeier and Clarke, 1999).

Phylogenetic studies of lutjanids have intended to increase the understanding of relationships of the Lutjanidae with related families (Johnson, 1980, 1993; Carpenter, 1990), among lutjanid subfamilies and of closely related species in the subfamily Lutjaninae (Miller and Cribb, 2007; Zhu et al., 2006; Sarver et al., 1996). Detailed descriptions of larvae have also led to inferences of relationships among lutjanid subfamilies (Leis, 2005).

Phylogenetic inferences could provide a way to establish the present value of species. The close relationship between taxonomic and genetic diversity is clearly expressed by a phylogenetic tree. If it is considered that each species has diverged genetically from its relatives

by an amount roughly proportional to the time since their common ancestor, branch lengths scaled to observed genetic divergence between species provide a quantitative measure of diversity within a clade (Erwin, 1991; Krajewski, 1991). From this perspective, old, monotypic lineages often make large contributions to diversity, thus, their conservation should be a high priority. Phylogenetic systematics, in combination with conservation genetics, provide a critical framework for understanding diversity (Féral, 2002) and predict vulnerability to exploitation of tropical reef fishes (Jennings et al., 1999).

Dynamics of larval dispersal also constitutes a critical feature of control on fish communities and populations. Many unresolved issues in the ecology and evolution of marine populations center on how far planktonic larvae disperse away from their parents (Levin, 2006). Regardless of the importance of the ecological processes affected by larval fish dynamics, the inability of unambiguous taxonomic identification of early life stages of many taxa is still a major burden that impairs the proficient management of these populations. Early larval stages of lutjanids are extremely similar and difficult to distinguish to genus and species level (Chow et al., 1993; Clarke et al., 1997; Victor, 2008). Effective management of valuable snapper fisheries depends upon the availability of life history information concerning the biology, habitat requirements and spatial distribution of individual species. The ability to identify individuals of snapper species throughout ontogeny is critical for a better understanding of the early life history and population dynamics of these species under natural conditions. Therefore, is it imperative to develop alternate methods for the identification of each species at their early life stages.

Conventionally, phylogeny, ontogenetic descriptions and species identification of lutjanids relied on morphological features (Rivas, 1949, 1966; Vergara, 1980; Johnson, 1980). However, the development of molecular techniques has helped enliven studies of fish

systematics and evolution (Lecointre, 1996; Kosher, 1997; Sotka and Palumbi, 2006). The realm of methods developed for molecular systematics (Hillis et al., 1996; Richardson, 2007) offers new sets of characters to explore relationships among fishes. Molecular methods can provide keys for species in cases where morphological methods are worthless (Zhang, 2004; Victor, 2008).

Mitochondrial DNA (mtDNA) has become a standard molecule of choice amongst most ichthyologists and herpetologists doing comparative molecular genetics. Animal mitochondria own several properties which make them attractive to work with: (1) they are passed on from generation to generation directly from mother to offspring, thus providing a direct chain of ancestry across generations; (2) they are independent units and numerous within a cell and therefore, they are easy to extract and separate from genomic DNA (Avise, 1994). Since different regions of mtDNA evolve at different rates, specific mtDNA genes have been targeted for phylogeny reconstruction (Hillis et al., 1996), species identification and assays of intraspecific variation (Chow et al., 1993). Analysis using conserved genes like mtDNA 12S ribosomal RNA (rRNA) is a very useful tool for molecular taxonomic studies and is a frequently used marker in genetic studies (Ward et al., 2005; Zhang and Liu, 2006). Given that the substitution rate of the 12S rRNA gene is half that of the protein-coding genes (Brown et al., 1982), it is more appropriate to identify species (Féral, 2002).

The main objectives of this study are: (1) to describe mitochondrial DNA (mtDNA) sequence motifs in the 12S rRNA gene that are diagnostics to species of lujanids (Family Lutjanidae); (2) to reassess previous molecular phylogenetic analyses of Caribbean lutjanids using diverse methods; (3) to address the complexity in the identification of lutjanid larvae by means of mtDNA sequence motifs in the 12S rRNA gene diagnostics to lutjanid species.

This dissertation is written in a manuscript format and consists of two major chapters, each with its own abstract, introduction, materials and methods, results and discussion. In the first chapter, the phylogenetic relationships of Caribbean snappers (Family Lutjanidae) based on mitochondrial DNA sequences are explored. In the second chapter, mitochondrial DNA analyses are applied to species identification of snapper larvae from Caribbean waters.

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2 Phylogenetic relationships of Caribbean snappers (Family Lutjanidae) based on mitochondrial DNA sequences

INTRODUCTION

Fishes of the Lutjanidae family are widely distributed in tropical and subtropical waters. The family has 123 species, 21 genera and 5 subfamilies (Anderson, 2003; Froese and Pauly, 2006). Of the above, 5 genera and 18 species are believed to be present in the Western Atlantic while 3 subfamilies (Lutjaninae, Apsilinae and Etelinae), 5 genera, 15 species and are reported for the Caribbean (Robins and Ray, 1986; Anderson, 2003).

Within those assemblages there are several of the most important components of Caribbean fisheries. Species in the subfamily Lutjaninae constitute an important component of the reef fisheries in tropical and subtropical latitudes throughout their geographical range, while the deep-water subfamilies Apsilinae and Etelinae represent by far the most important component of the deep-bottom fishery in the Pacific, Indian and Atlantic oceans, and the Caribbean.

Despite the importance of this family, substantial gaps exist on the systematic and ecological information of each species. Most of the Caribbean *Lutjanus* species currently recognized as valid (Allen 1985, 1987; Anderson 2003; Loftus 1992) were described in the 18th and 19th centuries (Bloch, 1790; Poey, 1860). Following these earlier descriptions and subsequent taxonomic reassessments (Jordan and Swain, 1884; Jordan and Fesler, 1893; Ginsburg, 1930) Rivas (1949, 1966), Anderson (1967, 2003), and Vergara (1977) reviewed some of the Lutjanidae taxonomy. Allen (1987) provided an identification key for twelve *Lutjanus* species in the western Atlantic. Several new species of snappers and even genera have been described (Anderson, 1987;

Randall et al., 1987, 1993; Allen, 1985; Moura and Lindeman, 2007). Species described as valid by Poey (1860), such as *Lutjanus ambiguus* and *Lutjanus lutjanoides,* have been reevaluated. Loftus (1992), Domeier and Clake (1992) and Williams and Rodríguez, (*unpubl. data*) provide evidence to suggest that those species are indeed natural hybrids of lutjanids. Nevertheless, there is still debate within the scientific community about the validity of some of these species (Moura and Lindeman, 2007). The high similarity of morphology and interspecific crossbreeding (Domeier and Clarke, 1992) within lutjanids increases taxonomic uncertainty and hampers inference of phylogenetic relationship assessments.

The phylogeny of the Lutjanidae family was originally based only on morphological characteristics (Rivas, 1966; Vergara, 1980; Johnson, 1980). Based on morphology, three phenetic groups within western Atlantic *Lutjanus* was hypothesized. Phylogenetic relationships of several western Atlantic species were examined using biochemical and molecular data. Chow and Walsh (1992) analyzed phylogenetic relationships between seven species within the Lutjaninae subfamily by both enzyme electrophoresis and skull morphometry and suggested at least two distinct groups within the genus *Lutjanus* and a close relationship between these and the monotypic *Ocyurus chrysurus.* Sarver et al., (1996) explored the relationships of fourteen western Atlantic species using mitochondrial DNA (mtDNA) sequences including species from two subfamilies: Lutjaninae and Etelinae. Their data strongly supported one clade composed of *L. griseus*, *L. apodus* and *L. jocu*; moderate support was found for the sister relationship of *L. campechanus and L. vivanus*, while the relationships and placement of the remaining species were not fully resolved. Sequences of mtDNA of 3 western Atlantic lutjanines included with those of Indo-Pacific snappers formed a well defined clade within the former group. Although

considerable efforts to verify systematics in this family have been made, the phylogenetic relationships between species of the Lutjanidae, or even among the *Lutjanus sp*., are still unclear.

While some efforts to elucidate phylogenetic relationships of snappers found in Cuba and the western Atlantic were done, none have included all members of the Lutjanidae family in the Caribbean. The distribution of many snapper species overlaps among these regions; however, some of the species in the Caribbean are not reported for the western Atlantic or Cuba and vice versa (Anderson, 2003). Here we include fifteen species of lutjanids reported to date for the area of Puerto Rico, most with a Caribbean-wide distribution. In this study we present the results of phylogenetic analyses based on molecular sequence data from a 12S rRNA mtDNA gene fragment. This gene has been used to examine phylogenetic relationships of morphologically similar perciform taxa (Sarver et al., 1996, 1992; Kocher, 1997; Miller and Cribb, 2007; Zhang and Liu, 2006). Sarver et al., (1996) analyzed a fragment of the 12S rRNA gene as well; our analyses are based in a sequence somewhat corresponding to theirs but extended at both ends. We included 2 additional species that were not previously incorporated: *Apsilus dentatus* (subfamily Apsilinae) and *Pristipomoides aquilonaris* (subfamily Etelinae). Thus, our phylogenetic study is, so far, the first to include all the 3 subfamilies of lutjanids occurring in the Caribbean.

We aim to reassess previous molecular phylogenetic analyses of Caribbean lutjanids using diverse methods. Assessments of phylogenetic relationships of species in this study were conducted using Neighbor Joining (NJ) and Bayesian Inference (BI) analyses.

MATERIALS AND METHODS

Study Site

La Parguera is located in southwestern Puerto Rico, where the shelf extends offshore to approximately 11 km before dropping abruptly from 20 to 3,800 m. To the south, the shelf break defines the end of the insular platform, while to the north a deeper sandy fringe borders the inner boundary of the shelf edge reef (Figure 2.1).

Sample collection

Samples from 15 species of lutjanids (52 individuals) were collected from local markets at La Parguera, Puerto Real and Rincón, western Puerto Rico (Fig. 2.1). Species from 3 subfamilies were included: Lutjaninae (*Lutjanus analis, Lutjanus apodus, Lutjanus bucanella, Lutjanus cyanopterus, Lutjanus jocu, Lutjanus griseus*, *Lutjanus mahogoni*, *Lutjanus synagris*, *Lutjanus vivanus, Ocyurus chrysurus and Rhomboplites aurorubens*), Apsilinae (*Apsilus dentatus*) and Etelinae (*Etelis oculatus, Pristipomoides macrophtalmus and Pristipomoides aquilonaris*) (Table 2.1). Additional samples of *L. jocu* and *O. chrysurus* were collected from local markets at Cataño, northern Puerto Rico. The Marine Forensic Team, Center for Coastal Environmental Health and Biomolecular Research, National Centers for Coastal Ocean Science (NCCOS), NOAA, Charleston, SC provided 2 samples of *L. cyanopterus.* Muscle or liver tissue was dissected from fresh specimens and preserved frozen at -20 °C.

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from 25 mg of tissue using the QIAamp® DNA Mini Kit (QIAGEN, Inc.), according to manufacturer's protocol. A fragment of the 12S rRNA gene of

~450 bp was amplified with the primers 5'-TCAAACTGGGATTAGATACCCCACTAT-3' and 5'-TGACTGCAGAGGGTGACGGGCGGTGTGT-3' (Kocher et al., 1989). Polymerase chain reaction (PCR) was conducted in a total volume of 50 µl with 80 ng of template DNA, 0.75 µl of each primer (20 µM), 1.5 µl (25µM) MgCl2, 5 µl 10X reaction buffer, 8 µl dNTP's (each 2.5 mM), and 2 µl (2 units) of RED *Taq*™ genomic DNA polymerase (Sigma Chemical Co.). Amplifications were carried out in an Eppendorf® Mastercycler with an initial denaturation step at 95 °C for 2 min., followed by 30 cycles of 95 °C denaturation for 30 secs., 55 °C annealing for 1 min. and 72°C extension for 1.5 min., and a final extension step at 72°C for 10 min. Amplified DNA was purified using the QIAquick® PCR purification kit (QIAGEN, Inc.), according to manufacturer's protocol. Cycle sequencing was conducted using the same primers utilized for PCR amplification. Automated sequencing was performed at external facilities[1]. Corresponding fragments of the 12S rRNA gene sequence from *Cyprinus carpio* and *Caranx melanpygus* were acquired from GenBank to be used as outgroups (Accession numbers: X61010 and AP004445).

Phylogenetic analyses

Sequences from the 12S rRNA gene fragment from the above species as well as those from *C. carpio* and *C. melampygus* outgroups were aligned and edited with *MEGA4* (Tamura, 2007).

[1] Sequences were performed at Nevada Genomics Center: INBRE Grant # 2P2RR016463, UPR – Sequencing and Genotyping facility (IMBRE NCRR – NIH grant P20 RR0 16470, NSF – CREST – CATEC, S.C.O.R.E. grant S06GM8102) and UPR – Mayaguez NSF-MRI # 0503541.

Alignment was done under the following parameters: pairwise alignment parameters = gap opening 10.00, gap extension 0.10, DNA weight matrix IUB; multiple alignment parameters = gap opening 10.00, gap extension 0.20, delay divergent sequences 30%, DNA weight matrix IUB. All sequences aligned unambiguously.

The resulting alignment was visually verified and then exported to NEXUS format for further analysis in other programs. The ends of the aligned sequences were trimmed afterwards to match the length of the shortest. Data was analyzed by Neighbor Joining (NJ) and Minimum evolution using MEGA4.

Analyses of Maximum parsimony (MP) and maximum likelihood (ML) were done, using PAUP* version 4.0b (Swofford, 2003), and Bayesian inferences (BI) using MrBayes version 3.1.1 (Ronquist and Huelsenbeck, 2003). Mean uncorrected pairwise distances were calculated. Pairwise comparisons of uncorrected sequence divergence were calculated with gaps treated as missing data. Site saturation was examined by plotting transitions (s) and transversions (v) against sequence divergence. Modeltest version 3.7 (Posada and Crandall, 1998) was used to estimate the best substitution model and parameters for MP, ML, ME and BI analyses. Maximum parsimony analyses used heuristic searches with all characters equally weighted. Nodal support was inferred by bootstrap analysis. Bayesian inference analysis was run over 1,000,000 (ngen = 1,000,000) via simultaneous Metropolis-coupled Monte Carlo Markov (MCMC) chains and every 100^{th} tree was saved (samplefreq = 100). Posterior probabilities estimates were conducted for nodal support in BI analyses. Tree topologies from the various analyses (NJ, ME, MP, ML and BI) were compared for clade arrangements and nodal support.

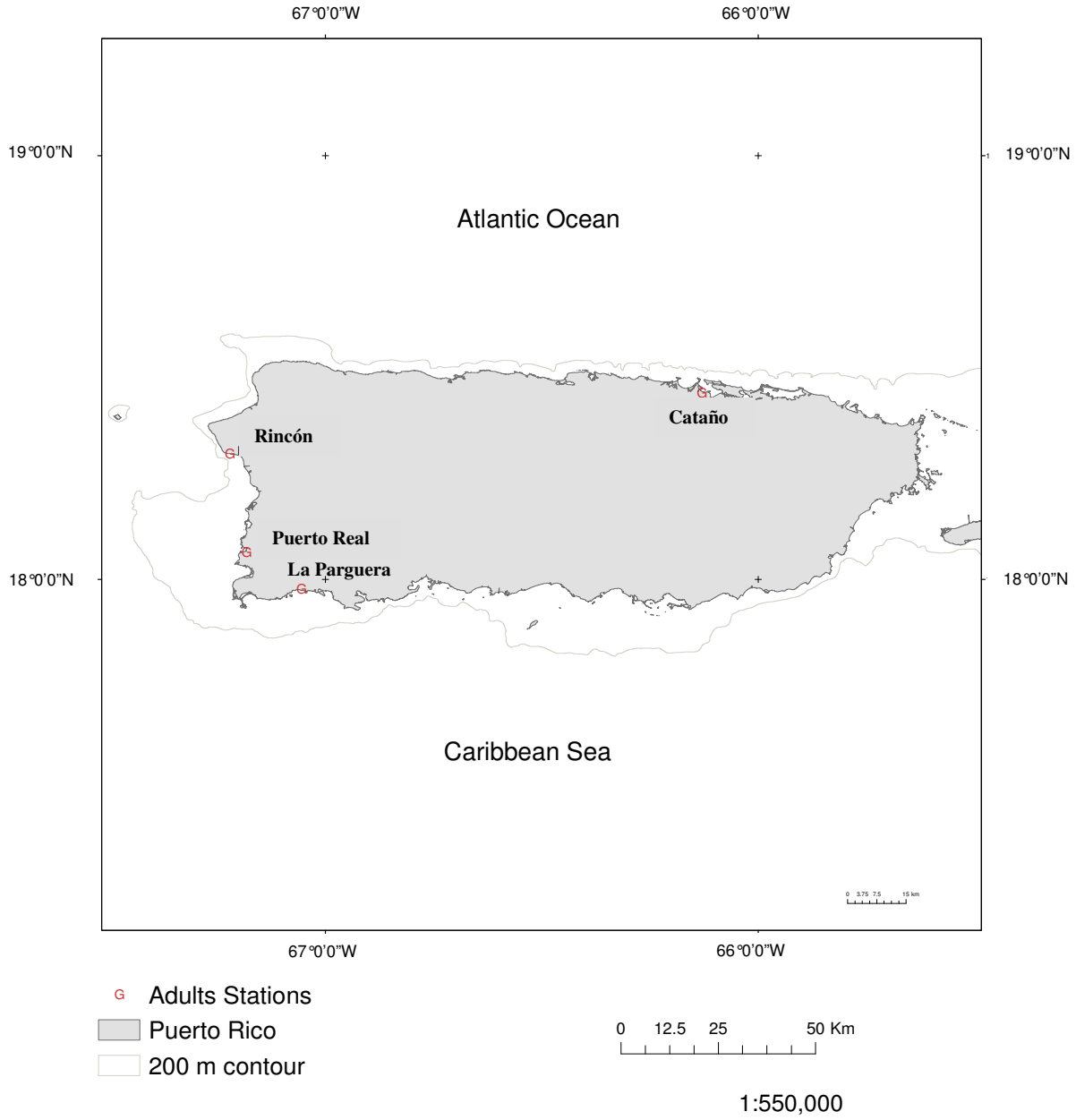

Figure 2.1. Sampling sites for adult vouchers

Table 2.1. Lutjanid reference sample collection location and sample size**.**

Species name	Common name	Catch location (no. of samples), date collected	Sample size	Sample numbers
Apsilus dentatus (A. d)	Black snapper (Chopa negra)	Parguera (1), 2001; Puerto Real (1), 2004, Rincon (1), 2004	3	A070, A092, A116
Etelis oculatus (E. o)	Queen snapper (Cartucho)	Puerto Real (2), 2002; Rincon (2), 2004	4	A067, A068, A093, A094
Lutjanus analis (L. ana)	Mutton snapper (Sama)	Parguera (3), 2001	3	A009, A010, A020
Lutjanus apodus (L. apo)	Schoolmaster (Pargo amarillo)	Parguera (3), 2000, 2001, 2002	3	A007, A019, A032
Lutjanus bucanella (L. b)	Blackfin snapper (Negrita)	Parguera (2), 2000, 2001; Rincon (1), 2004	3	A005, A069, A108
Lutjanus cyanopterus (L. c)	Cubera snapper (Cubera)	Parguera (1), 2000; Florida Keys (1) , 1999; St. Petersburg, FL, 2001 (1)	3	A003, Lcya001*, Lcya004*
Lutjanus griseus (L. g)	Gray snapper (Pargo gris/prieto)	Parguera (4), 2000, 2001	4	A004, A016, A017, A018
Lutjanus jocu (L. j)	Dog snapper (Pargo perro)	Parguera (1), 2000, Puerto Real (1), 2004; Cataño (1), 2007		A008, A101, A126
Lutjanus mahogoni (L. m)	Mahogani snapper (Pargo ojón/Manchego)	Parguera (3) 2002	3	A073, A078, A079
Lutjanus synagris (L. s)	Lane snapper (Arrayao)	Parguera (5), 2001	5	A011, A012, A013, A014, A015
Lutjanus vivanus (L. v)	Silk snapper (Chilla rubia)	Parguera (2) 2000, 2002; Puerto Real (1), 2004	3	A006, A071, A100
Ocyurus chrysurus (O. c)	Yellowtail snapper (Colirrubia)	Parguera (6) 2000, 2002, 2007; Cataño (2), 2007	8	A001, A026, A029, A030, A051, A128, A129, A130
Pristipomoides macrophtalmus (P. m)	Cardinal snapper (Muniama)	Rincón (2), 2004	2	A095, A096
Pristipomoides aquilonaris (P. a)	Wenchman (Muniama limosnera)	Rincón (2), 2005	2	A106, A108
Rhomboplites aurorubens (R. a)	Vermillion snapper (Tunaro)	Puerto Real (3), 2001, 2004	3	A066, A083, A084

*Tissue sample provided by The Marine Forensic Team, Center for Coastal Environmental Health and Biomolecular Research, National Centers for Coastal Ocean Science (NCCOS), NOAA, Charleston, SC.

RESULTS

Sequencing of the 12S rRNA mtDNA gene fragment produced an average of approximately 415 bp for all lutjanid taxa. Multiple alignments resulted in a consensus length of 405 characters (base pairs and gaps) available for analysis (Fig. 2.2). Mean uncorrected sequence divergence for all taxa (including outgroups) was 7.5%. Mean uncorrected divergence among lutjanid species was 4.3%. The largest sequence divergence among lutjanids species was between *P. aquilonaris* and *R. aurorubens* at 11.1%. The smallest sequence divergence of 0.26% was observed for two pairs of species within the Lutjaninae subfamily: *L. bucanella* - *O. chrysurus* and *L vivanus* - *O chrysurus.* The second smallest sequence divergence of 0.53% was observed for the pairs: *L. mahogoni* and *O. chrysurus, L. analis and L. synagris,* and *L. vivanus* - *L. bucanella* (Table 2.2).

Saturation of nucleotide substitutions can be inferred from nonlinearity in plots of number of transitions or transversions relative to sequence divergence. Evidence for saturation effects at sequence divergence levels of 25% was not observed in a plot of transitions and transversions vs sequence divergence (Fig. 2.3), indicating that there is phylogenetic signal in the data set.

Analysis of the sequence set using Modeltest indicates that the best substitution model to be applied is the general time reversal (GTR) model, incorporating estimates of invariable (I) sites with among-site variation (G) or GTR + I + G; I =0.4875, G =0.4810.

The inferred evolutionary relationships of the 17 taxa examined produced an optimal tree with a total branch length = 0.624 using the Neighbor-Joining (NJ) method (Fig. 2.4). The topology of the NJ tree place outgroups and three major groups within lutjanids as monophyletic

taxa, with low to moderate support. The groups formed by lutjanids correspond to subfamilies already recognized using morphological characters. The most basal group was formed by the genera *Pristipomoides* and *Etelis*. Strong support was observed for *Pristipomoides* species, but not for *Etelis oculatus*. *Apsilus dentatus*, the only species of Apsilinae occurring in the study area, was located as a basal taxon to the Etelinae and Lutjaninae. The most speciose group corresponds to the Lutjaninae subfamily. A clade formed by *L. jocu, L apodus* and *L. griseus* (griseus group) was strongly supported within lutjanines. Moderate support was observed for a clade formed by *L. analis, L. mahogoni* and *L. synagris* (black spot group). A clade including a group of *O. chrysurus*, *L. vivanus* and *L. bucanella* (a deep water group) was poorly supported. The relationship of *L. cyanopterus* and *R. aurorubens* as paraphyletic taxa to the griseus group was weakly supported.

Bayesian Inference (BI) analysis yielded a strict consensus tree produced form 1001 trees after "burning" (Fig. 2.5). Species included as outgroups: *C. carpio* and *C. melampygus* were resolved as basal to the subfamilies Apsilinae, Etelinae and Lutjaninae. Subfamilies Apsilinae and Etelinae resolved as paraphyletic to Lutjanidae with *A. dentatus* basal to Etelinae. Within etelines, the clade formed by *P. aquilonaris*, *P. macrophtalmus* and *E. oculatus* was recovered, as did in the NJ tree. Likewise, the relationship *P. aquilonaris*, *P. macrophtalmus* was resolved with high posterior probability and *E. oculatus* as sister taxa (paraphyletic) with moderate posterior probability. The clade *L. jocu, L apodus* and *L. griseus* (griseus group) was also recovered with high posterior probabilities. As in the NJ tree, the BI tree showed moderate support for a clade of *L. bucannella*, *O. chrysurus* and *L. vivanus*, except that *R. aurorubens* was also included as sister taxa in BI tree. Placement of *L. analis, L. mahogoni*, *L. synagris* and *L.*

cyanopterus were not resolved by BI, but as sister taxa to the other *L. sp*. Nevertheless, *L. cyanopterus* position was basal to all other lutjanines. Both NJ and BI trees showed relatively low node support for many of the ingroup Lutjaninae taxa. This lack of resolution and support for some species suggest that they may form a genetically and morphologically plastic group, with affinities with diverse groups, as the griseus or deep water group.

Following comparisons of tree topologies from each analysis (NJ, ME, MP, ML and BI), we decided to include and discuss only those produced by NJ and BI. Trees produced by ME, MP and ML showed comparable topologies to both NJ and BI, thus the latter were chosen as representatives of the possible arrangements formed with our data. Intraspecific variation was not found within any species for the adult specimens analyzed during this study.

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                          10        20        30        40        50        60
                     ....|....|....|....|....|....|....|....|....|....|....|....|
C. carpio            TGTC-------CGCCAGGGTACTACGAGCATTAGCTTAAAACCCAAAGGACCTGACGGTG
C. melampygus        CATCAAACATCCGCCTGGGAATTACGAACATTAGTTTAAAACCCAAAGGACTTGGCGGTG
P. aquilonaris       TACC------CCGCCCGGGTACTACGAGCATTAGCTTGAAACCCAAAGGACTTGGCGGTG
P. macrophtalmus     TACC------CCGCCCGGGTACTACGAGCATCAGCTTGAAACCCAAAGGACTTGGCGGTG
E. oculatus          TACC------C-GCCCGGGTACTACGAGCATTAGCTTAAAACCCAAAGGACTTGGCGGTG
A. dentatus          TACC-------CGCCTGGGTACTACGAGCATCAGCTTGAAACCCAAAGGACTTGGCGGTG
L. apodus            TATC-------CGCCCGGGGACTACGAGCATCAGCTTAAAACCCAAAGGACTTGGCGGTG
L. analis            TATC-------CGCCCGGGGACTACGAGCATTAGCTTGAAACCCAAAGGACTTGGCGGTG
L. bucanella         TATC------CTGCCCGGGGACTACGAGCATCAGCTTGAAACCCAAAGGACTTGGCGGTG
L. cyanopterus       TATC-------CGCCCGGGGACTACGAGCATCAGCTTGAAACCCAAAGGACTTGGCGGTG
L. griseus           TATC-------CGCCCGGGGACTACGAGCATCAGCTTAAAACCCAAAGGACTTGGCGGTG
L. jocu              TATC-------CGCCCGGGGACTACGAGCATCAGCTTAAAACCCAAAGGACTTGGCGGTG
L. mahogoni          TATC-------CGCCCGGGGACTACGAGCATCAGCTTGAAACCCAAAGGACTTGGCGGTG
L. synagris          TATC-------CGCCCGGGGACTACGAGCATCAGCTTGAAACCCAAAGGACTTGGCGGTG
L. vivanus           TATC------CTGCCCGGGGACTACGAGCATCAGCTTGAAACCCAAAGGACTTGGCGGTG
O. chrysurus         TATC------C-GCCCGGGGACTACGAGCATCAGCTTGAAACCCAAAGGACTTGGCGGTG
R. aurorubens        TATCT-----CTGCCCGGGGACTACGAGCATCAGCTTAGAACCCAAAGGACTTGGCGGTG

                              70        80        90       100       110       120
                     ....|....|....|....|....|....|....|....|....|....|....|....|
C. carpio            TCTCAGACCCCCCTAGAGGAGCCTGTTCTAGAACCGATAACCCCCGTTCAACCTCACCAC
C. melampygus        CTTAACATCCACCTAGAGGAGCCTGTTCTAGAACCGATAATCCCCGTTTAACCTCACCCC
P. aquilonaris       CTTTAGACCCACCTAGAGGAGCCTGTTCTAGAACCGATAACCCCCGTTCAACCTCACCTT
P. macrophtalmus     CTTTAGACCCACCTAGAGGAGCCTGTTCTAGAACCGATAACCCCCGTTCAACCTCACCTT
E. oculatus          CTTTAGATCCACCTAGAGGAGCCTGTTCTAGAACCGATAACCCCCGTTCAACCTCACCTT
A. dentatus          CTTTAGATCCACCTAGAGGAGCCTGTTCTAGAACCGATAACCCCCGTTCAACCTCACCTT
L. apodus            CTTTAGATCCACCTAGAGGAGCCTGTTCTAGAACCGATTACCCCCGTTCAACCTCACCTT
L. analis            CTTTAGATCCACCTAGAGGAGCCTGTTCTAGAACCGATTACCCCCGTTCAACCTCACCTT
L. bucanella         CTTTAGATCCACCTAGAGGAGCCTGTTCTAGAACCGATTACCCCCGTTCAACCTCACCTT
L. cyanopterus       CTTTAGATCCACCTAGAGGAGCCTGTTCTAGAACCGATTACCCCCGTTCAACCTCACCTT
L. griseus           CTTTAGATCCACCTAGAGGAGCCTGTTCTAGAACCGATTACCCCCGTTCAACCTCACCTT
L. jocu              CTTTAGACCCACCTAGAGGAGCCTGTTCTAGAACCGATTACCCCCGTTCAACCTCACCTT
L. mahogoni          CTTTAGATCCACCTAGAGGAGCCTGTTCTAGAACCGATTACCCCCGTTCAACCTCACCTT
L. synagris          CTTTAGATCCACCTAGAGGAGCCTGTTCTAGAACCGATTACCCCCGTTCAACCTCACCTT
L. vivanus           CTTTAGATCCACCTAGAGGAGCCTGTTCTAGAACCGATTACCCCCGTTCAACCTCACCTT
O. chrysurus         CTTTAGATCCACCTAGAGGAGCCTGTTCTAGAACCGATTACCCCCGTTCAACCTCACCTT
R. aurorubens        CTTTAGATCCACCTAGAGGAGCCTGTTCTAGAACCGATTACCCCCGTTCAACCTCACCTT
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Figure 2.2. Sequence alignment of the 12S rRNA gene fragment for lutjanids and outgroups.

```
                          130       140       150       160       170       180
                 ....|....|....|....|....|....|....|....|....|....|....|....|
C. carpio        TTCTAGCCACCCCAGCCTATATACCGCCGTCGTCAGCTTACCCTGTGAAGGTAATAAAAG
C. melampygus    CCCTAGCTTTTTCCGCCTATATACCACCGTCGCCAGCTTACCCTGTGAAGG-ACTAATAG
P. aquilonaris   TTCTTGTTTAACCCGCCTATATACCACCGTCGCCAGCTTACCCTGTGAAGG-CCTCATAG
P. macrophtalmus TTCTTGTTTAACCCGCCTATATACCACCGTCGCCAGCTTACCCTGTGAAGG-CCTCATAG
E. oculatus      TTCTTGTTTAACCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGAAGG-CCTCATAG
A. dentatus      TCCTTGTTTTCCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGAAGG-CCTTATAG
L. apodus        TCCTTGTTTCTCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGAAGG-ACTTATAG
L. analis        TCCTTGTTTCCCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGAAGG-ACTCATAG
L. bucanella     TCCTTGTTTTTCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGAAGG-ACTCATAG
L. cyanopterus   TCCTTGTTTTTCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGAAGG-GCTCATAG
L. griseus       TCCTTGTTTCTCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGAAGG-GCTGATAG
L. jocu          TCCTTGTTTCCCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGAAGG-ACTTATAG
L. mahogoni      TCCTTGTTTCCCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGAAGG-ACTCATAG
L. synagris      TCCTTGTTTCCCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGGAGG-ACTTATAG
L. vivanus       TCCTTGTTTTCCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGAAGG-ACTCATAG
O. chrysurus     TCCTTGTTTTCCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGAAGG-ACTCATAG
R. aurorubens    TCCTTGTTTTTCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGAAGG-ACTAATAG

                          190       200       210       220       230       240
                 ....|....|....|....|....|....|....|....|....|....|....|....|
C. carpio        TAAGCAAAATGGGCACAACCCAAAACGTCAGGTCGAGGTGTAGCGCATGAAGTGGGAAGA
C. melampygus    TAAGCACAATCGGCACAGCCCAGAACGTCAGGTCGAGGTGTAGTGAATGGGAGGGGAAGA
P. aquilonaris   TAAGCAGAATCGGCACAGCCCAGAACGTCAGGTCGAGGTGTAGCGTATGGAAAGGGAAGA
P. macrophtalmus TAAGCAGAATCGGCACAGCCCAGAACGTCAGGTCGAGGTGTAGCGTATGGAAAGGGAAGA
E. oculatus      TAAGCAAAATTGGCACAGCCCAAAACGTCAGGTCGAGGTGTAGCGTATGAAAAGGGAAGA
A. dentatus      TAAGCAAAATTGGCACAGCCCAAAACGTCAGGTCGAGGTGTAGCGTATGGAAAGGGAAGA
L. apodus        TAAGCAAGATTGGCATAGCCCAAAACGTCAGGTCGAGGTGTAGCGTATGGAAGGGGAAGA
L. analis        TAAGCAAGATTGGCACAGCCCAAAACGTCAGGTCGAGGTGTAGCGTATGGAAAGGGAAGA
L. bucanella     TAAGCAAGATTGGCACAGCCCAAAACGTCAGGTCGAGGTGTAGCGTATGGAAAGGGAAGA
L. cyanopterus   TAAGCAAGATTGGCACAGCCCAAAACGTCAGGTCGAGGTGTAGCGTATGGAAAGGGAAGA
L. griseus       TAAGCAAGATTGGCATAGCCCAAAACGTCAGGTCGAGGTGTAGCGTATGGAAAGGGAAGA
L. jocu          TAAGCAAGATTGGCATAGCCCAAAACGTCAGGTCGAGGTGTAGCGTATGGAAGGGGAAGA
L. mahogoni      TAAGCAAGATTGGCACAGCCCAAAACGTCAGGTCGAGGTGTAGCGTATGGAAAGGGAAGA
L. synagris      TAAGCAAGATTGGCACAGCCCAAAACGTCAGGTCGAGGTGTAGCGTATGGAAAGGGAAGA
L. vivanus       TAAGCAAGATTGGCACAGCCCAAAACGTCAGGTCGAGGTGTAGCGTATGGAAAGGGAAGA
O. chrysurus     TAAGCAAGATTGGCACAGCCCAAAACGTCAGGTCGAGGTGTAGCGTATGGAAAGGGAAGA
R. aurorubens    TAAGCAAGATTGGCACAGCCCAAAACGTCAGGTCGAGGTGTAGCGAATGGAAAGGGAAGA
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Figure 2.2. Continued.

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                          250        260        270        280        290        300
                    ....|....|....|....|....|....|....|....|....|....|....|....|
C. carpio           AATGGGCTACATTTTCTAAT-ATAGAATA-TTACGAAC-ATGCACCATGAAACA-ATGC-
C. melampygus       AATGGGCTACATTCGCTGCCCACAGCGAA--CACGAATGCTACAC--TGAAACATGTAG-
P. aquilonaris      AATGGGCTACATTCTCTGCT-ATAGAGAA--CACGAATGATACGT--TGAAACACGTGTA
P. macrophtalmus    AATGGGCTACATTCTCTGTT-ATAGAGAA--CACGAATGATACGT--TGAAACACGTGTA
E. oculatus         AATGGGCTACATTCTCTAAT-ACAGAGAA--TACGAACGATACGC--TGAAACACGTATA
A. dentatus         AATGGGCTACATTCCCTAACTATAGAGAA--TACGAACGATACAC--TGAAATACGTAT-
L. apodus           AATGGGCTACATTCCCTAAC-ACAGTGAA-ATACGAACGATGCAC--TGAAATACACAT-
L. analis           AATGGGCTACATTCCCTAAT-ATAGTGTA-ATACGAACGATACAC--TGAAATACGTAT-
L. bucanella        AATGGGCTACATTCCCTAAT-ATAGTGAA-ATACGAACGATACAC--TGAAATACGTAT-
L. cyanopterus      AATGGGCTACATTCCCTAAC-ACAGTGAA-ATACGAACGATACAC--TGAAATACGTAT-
L. griseus          AATGGGCTACATTCCCTAAC-ATAGTGAA-ATACGAACGATGCAC--TGAAATACGCAT-
L. jocu             AATGGGCTACATTCCCTAAC-ATAGTGAATATACGAACGATGCAC--TGAAATACGCAT-
L. mahogoni         AATGGGCTACATTCCCTAAT-ATAGTGAA-ATACGAACGATACAC--TGAAATACGTAT-
L. synagris         AATGGGCTACATTCCCTAAT-ATAGTGAA-ATACGAACGATACAC--TGAAATACGTAT-
L. vivanus          AATGGGCTACATTCCCTAAT-ACAGTGAA-ATACGAACGATACAC--TGAAATACGTAT-
O. chrysurus        AATGGGCTACATTCCCTAAT-ATAGTGAA-ATACGAACGATACAC--TGAAATACGTAT-
R. aurorubens       AATGGGCTACATTCCCTAGC-ATCGGGCATATACGAACGATACAC--TGAAATACGTAT-

                          310        320        330        340        350        360
                    ....|....|....|....|....|....|....|....|....|....|....|....|
C. carpio           TTGAAGGAGGATTTAGTAGTAAAAGGGAAGTAGAGTGTCCCTTTTGAACCCGGCTCTGAG
C. melampygus       CTGAAGGAGGATTTAGCAGTAAGCAGAAAGCAGAGTGTTCCGCT-GAAGCCGGCTCTTAA
P. aquilonaris      CTGAAGGAGGATTTAGCAGTAGGCAGGAAATAGAGTGTTCTGCC-GAAGCCGGCCCTGAA
P. macrophtalmus    CTGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGTGTTCTGCC-GAAGTTGGCCCTGAA
E. oculatus         CTGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCCGCT-GAAATCGGCCCTGAA
A. dentatus         CCGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCCGCT-GAAATCGGCCCTGAA
L. apodus           CTGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCTGCT-GAAACCGGCCCTGAA
L. analis           CTGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCCGCT-GAAACCGGCCCTGAA
L. bucanella        CCGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCCGCT-GAAACCGGCCCTGAA
L. cyanopterus      CTGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCCGCT-GAAACCGGCCCTGAA
L. griseus          CTGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCTGCT-GAAACCGGCCCTGAA
L. jocu             CTGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCTGCT-GAAACCGGCCCTGAA
L. mahogoni         CTGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCCGCT-GAAACCGGCCCTGAA
L. synagris         CTGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCCGCT-GAAACCGGCCCTGAA
L. vivanus          CCGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCCGCT-GAAACCGGCCCTGAA
O. chrysurus        CCGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCCGCT-GAAACCGGCCCTGAA
R. aurorubens       CCGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCCGCT-GAAACCGGCCCTGAA
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Figure 2.2. Continued.

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                          370        380        390        400
                     ....|....|....|....|....|....|....|....|....|
C. carpio            ACGCGTACACACCGCCCGTCACTCTCCCCTGTCAA--------AA
C. melampygus        GCGCGCACACACCGCCCGTCACCCTCCCCAAGCAACTGGACCTAA
P. aquilonaris       GCGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
P. macrophtalmus     GCGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
E. oculatus          GCGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
A. dentatus          GCGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
L. apodus            GCGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
L. analis            GCGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
L. bucanella         GCGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
L. cyanopterus       GCGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
L. griseus           GCGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
L. jocu              GCGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
L. mahogoni          GCGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
L. synagris          GCGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
L. vivanus           GCGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
O. chrysurus         GCGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
R. aurorubens        GCGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
```

Figure 2.2. Continued.

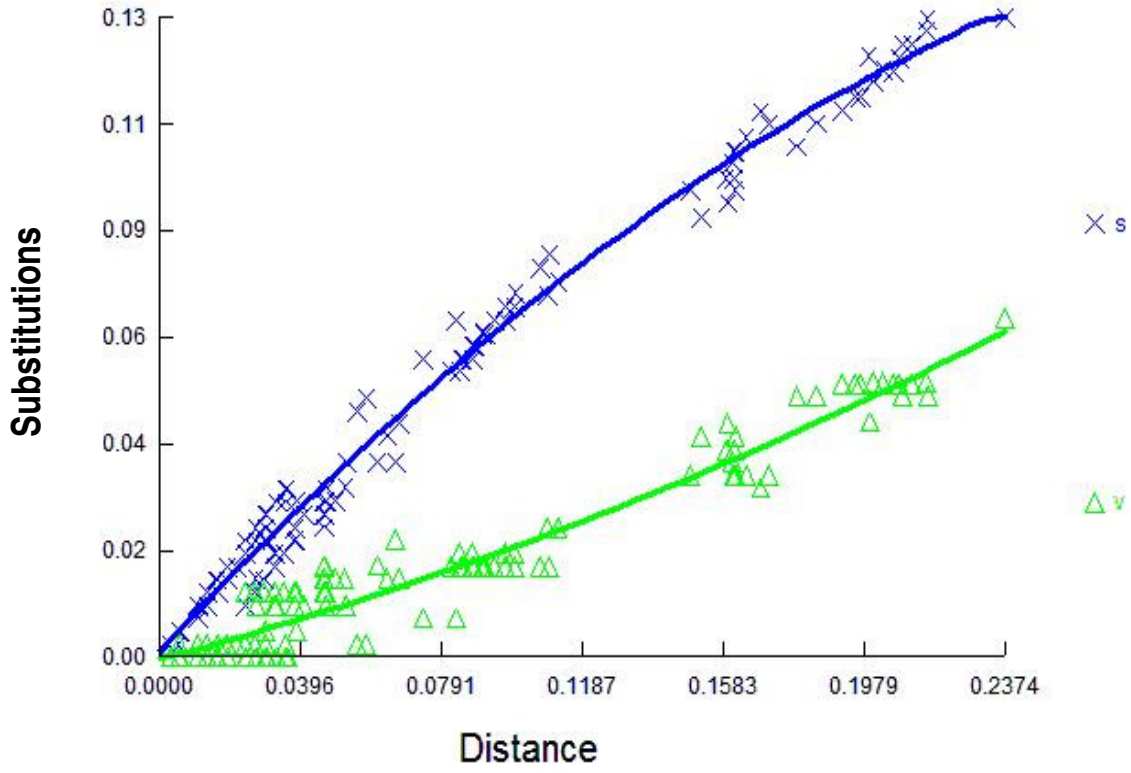

Figure 2.3. Plot of the number of transitions (s) and transversions (v) versus nucleotide divergence for the 12S rRNA data set.

Table 2.2. Estimates of evolutionary divergence (Tajima and Nei, 1993) among sequences of 15 lutjanids and outgroups (*C. carpio* and *C. melampygus*) based on 12S rRNA data.

	C. c	*C. m*	*P. a*	*P. m*	*E. o*	*A. d*	*L. apo*	*L. ana*	*L. b*	*L. c*	*L. g*	*L. j*	*L. m*	*L. s*	*L. v*	*O. c*	*R. a*
C. c																	
C. m	0.2482																
P. a	0.2116	0.1514															
P. m	0.2221	0.1572	0.0160														
E. o	0.1850	0.1584	0.0580	0.0549													
A. d	0.2010	0.1538	0.0812	0.0723	0.0380												
L. apo	0.2051	0.1565	0.1101	0.1067	0.0643	0.0524											
L. ana	0.1885	0.1571	0.0856	0.0883	0.0471	0.0382	0.0352										
L. b	0.2064	0.1533	0.0883	0.0851	0.0498	0.0298	0.0324	0.0133									
L. c	0.2138	0.1472	0.0913	0.0880	0.0470	0.0326	0.0269	0.0187	0.0106								
L. g	0.2091	0.1631	0.1009	0.0976	0.0614	0.0468	0.0134	0.0298	0.0269	0.0215							
L. j	0.1987	0.1627	0.1008	0.0974	0.0671	0.0467	0.0106	0.0297	0.0323	0.0324	0.0134						
L. m	0.1961	0.1565	0.0854	0.0821	0.0470	0.0326	0.0296	0.0053	0.0079	0.0133	0.0242	0.0241					
L. s	0.1999	0.1601	0.0915	0.0882	0.0527	0.0326	0.0297	0.0107	0.0133	0.0187	0.0270	0.0242	0.0053				
L. v	0.2064	0.1533	0.0913	0.0880	0.0470	0.0298	0.0324	0.0133	0.0053	0.0106	0.0324	0.0323	0.0079	0.0133			
O. c	0.2030	0.1565	0.0883	0.0851	0.0498	0.0271	0.0351	0.0106	0.0026	0.0133	0.0297	0.0296	0.0053	0.0106	0.0026		
R. a	0.2135	0.1506	0.1110	0.1075	0.0678	0.0444	0.0441	0.0355	0.0245	0.0301	0.0384	0.0440	0.0328	0.0356	0.0300	0.0273	

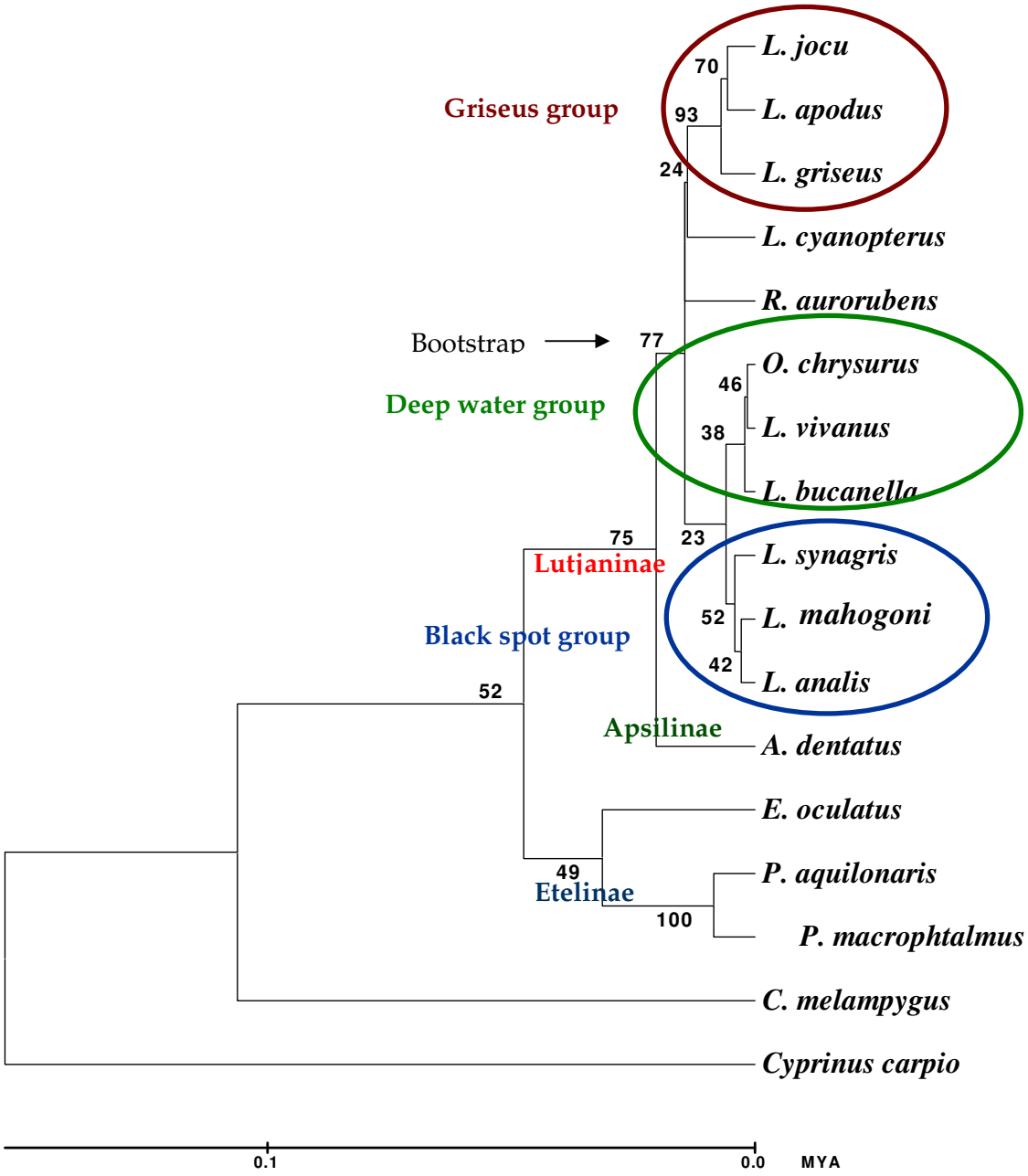

Figure 2.4. Molecular phylogeny produced by the Neighbor-Joining method for Caribbean lutjanids and outgroups inferred from 12S rRNA mtDNA. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (5000 replicates) is shown next to the branches.

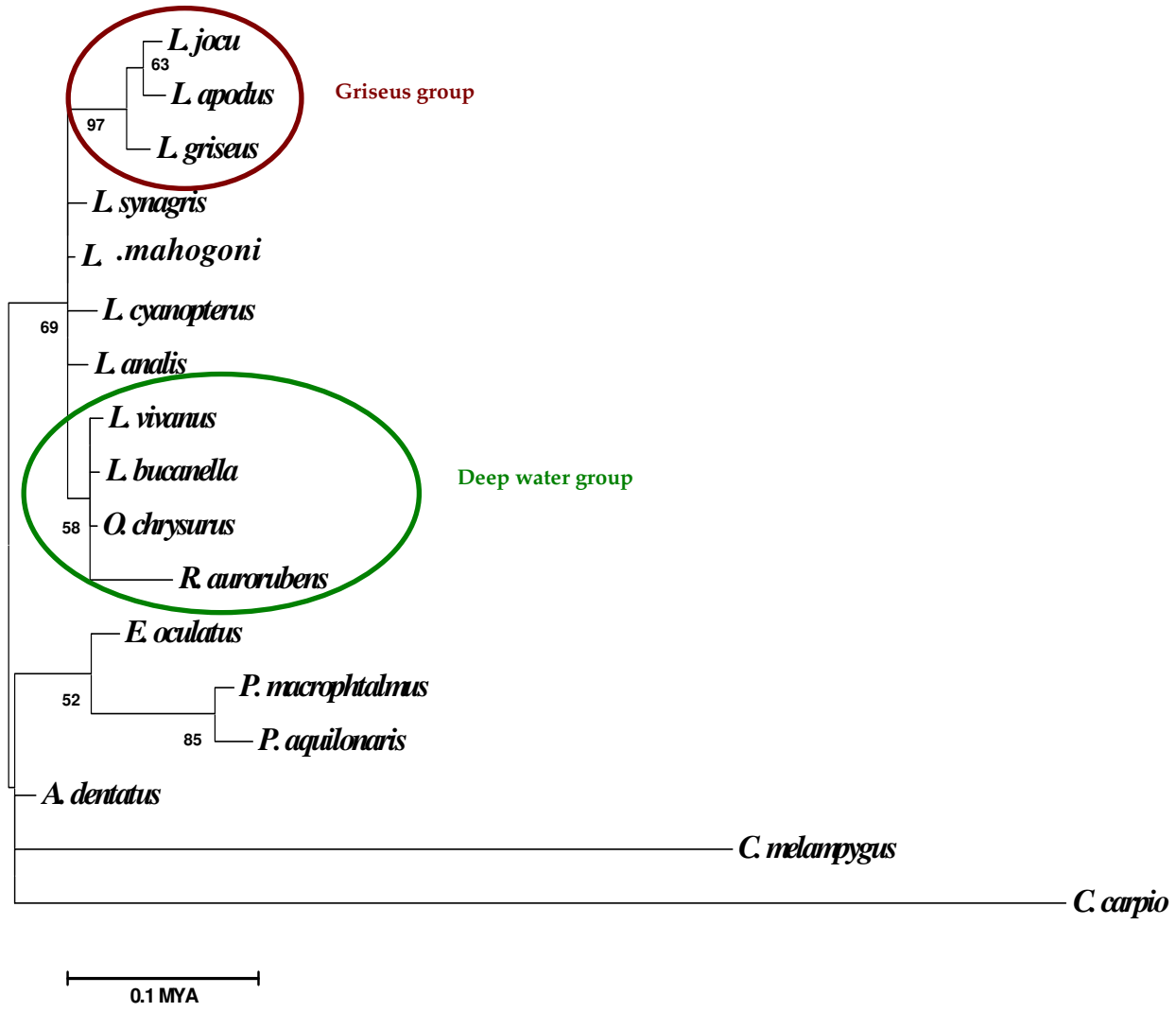

Figure 2.5. Molecular phylogeny produced using Bayesian Inference for Caribbean lutjanids and outgroups inferred from 12S rRNA mtDNA data**.** Posterior probabilities are indicated at the nodes with probabilities <50% not shown.

DISCUSSION

This study examined phylogenetic relationships among all species of lutjanids occurring within the Caribbean Basin, based on mitochondrial 12S rDNA analysis. Previous investigations have limited their scope to species occurring in the western Atlantic (WA) and Cuba, or to several species within the Lutjaninae subfamily (Rivas 1966; Vergara, 1980; Chow et al., 1992 and Sarver et al., 1996). Those studies were based on morphological, biochemical or molecular data. In a molecular study, Sarver et al., (1996) investigated the relationships of 14 western Atlantic snappers using 12S rRNAmt and cytochrome b (cyt *b*) data. Phylogenetic relationships of lutjanids using molecular data have been explored for the Indo-Pacific and China as well (Miller and Cribb, 2007; Zhang and Liu, 2006 and Zhu et al., 2006).

Based on morphology, Rivas (1966) and Vergara (1980) hypothesized three phenetic groups within western Atlantic Lutjanus: the *griseus* group (*L. griseus*, *L. apodus*, and *L. jocu* and *L. cyanopterus*); the *synagris* or *mahogoni* group (*L. mahogoni* and *L. synagris*); and the *analis* or *vivanus* group (*L. analis*, *L. campechanus*, *L. purpureus* and *L. vivanus*). Vergara and Rivas disagreed on the placement of *L. bucanella*: Vergara placed it in the *mahogoni* group, whereas Rivas placed it in the *analis* group. Based on isozyme and morphological data, Chow and Walsh (1992) analyzed six species within the Lutjaninae subfamily. They suggested only two well defined groups, *griseus* and *analis*, with a third group (*synagris*) that had affinities to both those well defined groups.

This study complements information found in previous investigations based on DNA sequences of lutjanid species investigations (Sarver et al., 1996). Mitochondrial 12S rRNA data from the 15 species of lutjanids reported to date in the Caribbean were included, 13 studied by

Sarver et al., (1996) plus 2 additional species not reported for the western Atlantic specificity. We included an additional species of Pristipomoides (*P. macrophtalmus*) and *A. dentatus*, the only representative of Apsilinae, both limited to the Caribbean.

Different phylogenetic tree arrangements were found using NJ and BI, based on mitochondrial 12SrDNA analysis of Lutjanidae species. It should be noted that the NJ method is based on distances, while Bayesian inference of phylogeny is based on likelihood functions, utilizing Markov Chain Monte Carlo (MCMC) simulation (Metropolis et al., 1953) in combination with the chosen model and data to produce a posterior probability distribution of trees. Thus, since NJ is the less susceptible to inconsistencies between candidate trees of both methods, it inferred a tree with additional and more diverse groups.

The NJ and BI trees, both showed a topology with a well defined group formed by *L. apodus, L. jocu* and *L. griseus* as in Sarver et al., (1996). The NJ tree just weakly supported the inclusion of *L. cyanopterus* and *R. aurorubens* as sister taxa to the *griseus* clade. Rivas (1966) and Vergara (1980) also included *L. cyanopterus* in the *griseus* group. Chow and Walsh (1992) also found a well defined clade of *L. griseus* and *L. apodus*; however, *L. jocu*, *L. bucanella* and *L. cyanopterus* were not examined in their study. In addition, the NJ method moderately grouped *L. analis*, *L. mahogoni* and *L. synagris* while these 3 species were placed as sister taxa to the other clades in the BI tree. The NJ tree produced a third clade that grouped *O. chrysurus*, *L .bucanella* and *L. vivanus* and excluded *R. aurorubens*, while the latter species was clustered with the other those species in the BI tree. In agreement with the one of the phylogenetic trees analyzed by Sarver et al., (1996), we found moderate support for the inclusion of *R. aurorubens* in the "deep water" group in our BI tree. *Rhomboplites aurorubens* is a deep water habitant; therefore

placement in the deep water group is more suitable than closer to the griseus group, as in the NJ tree. In contrast to the present work, *L. cyanopterus* was placed basal to all snappers by Sarver et al., Examination of the *L. cyanopterus* sequence used by Sarver et al., with those of other lutjanid sequences deposited in GenBank (Blast), showed too many dissimilarities, thus suggesting that the authors mistakenly incorporated a sequence from other species in their analysis. Another possibility may have been the existence of a variant haplotype exceptionally distinct from specimens from Puerto Rico. Instraspecific variability was not found for any of the *L. cyanopterus* specimens in our study (including samples from U.S.); therefore intraspecific variation was rejected as a possible explanation for such incongruence.

As observed in our results and those of others (Chow and Walsh, 1992; Sarver et al., 1996; Zhu et al., 2006; Zhang et al., 2006), phylogenetic relationships within the subfamily Lutjaninae remains rather unresolved for some species. Nevertheless, our study suggests that even if not strongly supported, the groups found are associated also according to morphology, habitat or feeding preferences.

There is consistent agreement between some morphological characters of the species studied and our molecular analyses. The griseus group, with stronger nodal support in our study, has been also well supported by morphometric examination (Rivas, 1966). In agreement with Miller and Cribb (2007) and Johnson (1980), our NJ tree grouped species with a large black spot above the lateral line and below the anterior portion of the soft dorsal fin: *L. analis*, *L. mahogoni* and *L. synagris*, the black spot group. Habitat and feeding behavior are common to *L. vivanus*, *O. chrysurus* and *L. bucanella* and were grouped in both our NJ and BI trees (plus *R. aurorubens* in the BI tree). These species are deep water dwellers, sharing adaptations for a pelagic

environment, and tend to have slender bodies and forked caudal fin. Even though *O. chrysurus* is not a deep water inhabitant, the present analysis includes it in the "deep water" group. This result supports studies by Domeier and Clarke (1992) who suggested that *O. chrysurus* may have acquired morphological characters common with pelagic species, probably due to adaptations for swimming and feeding in the water column as this species primarily feeds on zooplankton (Randall, 1967). The taxonomic status of *Ocyurus* has been controversial for a long time. Evermann and Marsh (1900) and Vergara (1980) separated *Ocyurus* from Lutjanus based on minor morphological differences. Domeier and Clarke (1992) and Loftus (1992) have argued that *Ocyurus* should be reclassified as *Lutjanus* because of the ability of *Ocyurus* to hybridize with other species of Lutjanus. Both our NJ and BI trees place *Ocyurus* in clades close to *Lutjanus* species. These results support suggestions by others (Chow and Clarke, 1992; Sarver et al*.,* 1996) that *Ocyurus* should be synonymized with Lutjanus. On the other hand, in this study *R. aurorubens* clustered with *Lutjanus* but showing the greatest distance among all pairwise comparisons. The status of *Rhomboplites* as a monotypic genus, is supported by our results and by morphologic, electrophoretic and morphometric data (Johnson 1980, 1993; Chow and Walsh, 1992). *Rhomboplites* may be the offshoot of the three genera in the Lutjaninae, as already suggested by Johnson (1980).

The subfamilies Etelinae and Apsilinae were resolved as monophyletic sister taxa to the Lutjaninae by NJ. Bayesian inference analyses set the Etelinae and Apsilinae as paraphyletic, with Apsilinae as basal to all other lutjanids. Thus, in the present work, the exact placement of *Apsilus* remains uncertain. This difference may be attributed to intrinsic ambiguity in *Apsilus* as a natural group. Based on morphological characters, Johnson (1980) hypothesized that Etelinae

must be the earlier group in the Lutjanidae family, while Johnson stated "that *Apsilus* on the other hand, has never been recognized as a natural group". *Apsilus* has been variously placed as a lutjanine or eteline. *Apsilus* shares with etelines primitive characters such as the abductor mandibulae while the skull is believed to be in a state of transition to the Lutjaninae condition. The fact that *Apsilus* hasnot been included in any comprehensive phylogenetic study hampers a well informed hypothesis about this relationship. Furthermore, the relationship among some genera of the Etelinae and Apsilinae has been questioned based on larval morphology (Leis, 2005). For the Etelinae our trees show moderate support for the inclusion of *E. oculatus* with the well supported clade of *Pristipomoides*, suggesting that *Etelis* may be also in a transitional state between the Etelinae and Apsilinae.

Reduced resolution for some groups in our study may be attributed to several limitations inherent to the data used. The 12S rRNA gene is of conserved nature among species, owed to evolutionary constrains, limiting the degree of mutations at certain positions. The apparent limited freedom for mutations can be explained by substitution reversions, which mask ancestral steps and generate inconsistency in phylogenetic trees. Nevertheless, our data set did not show evidence of substitution saturation; hence adequate phylogenetic signal was present. Adding that we used a relatively short fragment of the gene, a longer fragment will possibly provide an enhanced phylogenetic scheme.

Even when BI analyses showed lower resolution within lutjanines, NJ produced clades that relate with morphology and habitat preferences. Our investigation of the phylogenetic relationships of the Lutjanidae using a fragment of the 12S rRNA gene generally supports the phylogenetic hypothesis based on adult morphology proposed by Johnson (1980) and Rivas

(1966). The employment of additional mitochondrial or nuclear genes to explore genetic variation among lutjanid taxa will provide a more complete picture of the evolution of this important family of fishes. Even when relationships of lutjanids were not fully resolved, our phylogenetic study is, so far, the first to include all the 3 subfamilies of lutjanids occurring in the Caribbean. However, as intraspecific variation was not observed, species were characterized unambiguously. The consistency in the sequence data for each species in this study demonstrates that the 12S rRNA gene is a reliable tool for taxonomic identification within this family. These sequences constitute a sort of molecular key for all the 15 species of lutjanids studied, useful for identification of early stages and processed tissues or fillets for fisheries management regulations.

Identification of lutjanid larvae to the species level is still very difficult due to the high similarity among species. Morphological characterization of larvae is still ambiguous for many species, especially for closely related members of the Lutjaninae subfamily. Sequence data from this study may be used as a key for comparison with DNA from unknown lutjanid larvae. Efforts to use a segment of DNA (mtDNA C oxidase subunit I gene or COI) as a barcode of species identity have been successful for various taxa, including fish larvae (Paine et al., 2007; Ward et al., 2005).

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3 Mitochondrial DNA Analysis for Species Identification of Snapper larvae (Pisces: Lutjanidae) from Caribbean waters

INTRODUCTION

Nearly all marine teleost fishes possess a pelagic larval stage that is morphologically distinct from the adults and many orders of magnitude smaller (Moser et al., 1984). Understanding the processes affecting both the survival and transport of larval stages is one of the principal challenges in marine fish ecology, as these processes will influence the spatial distribution, population dynamics, migration strategies and evolution of a species (Cowen et al., 2006). While many indirect methods have been developed over the years to evaluate larval ecology and transport (Doherty, 1981; Swearer et al., 1999; Jones et al., 1999; Limouzy-Paris et al., 1994, 1997) a comprehensive understanding of these issues still requires the sampling of eggs and larvae in their natural environment. Ichthyoplankton collections are a powerful tool for addressing many other important questions in fish ecology and fisheries management including the identification of spawning locations, the extension of larval dispersal (Cowen, 2002; Leis and Mc Cormick, 2002; Mora and Sale, 2002; Ramírez and García, 2003), and the quantification of population levels or biomass of fished species (Ralston et al., 2003). However, these collections are generally underutilized since larval fishes are not frequently identified to species due to their small size and limited morphological development.

Dispersal dynamics of fish larvae, a major key for the design and implementation of Marine Protected Areas (MPAs), have been studied for decades (Palumbi, 2003, 2004). Most fish larvae are planktonic thus, potentially capable of long distance dispersal (Sale, 1980; Leis, 1991). Nevertheless, under certain conditions larvae of coral reef fishes may be retained near

natal grounds (Jones et al., 1999; Swearer et al., 1999; Sponaugle, 2002). The balance between long distance dispersion and retention influences the level of genetic and ecological connectivity among fish populations (Palumbi and Sotka, 2006). Thus, the dynamics of larval dispersal constitutes a critical feature of control on fish communities and populations. Regardless of the importance of the ecological processes affected by larval fish dynamics, the inability of unambiguous taxonomic identification of early life stages of many taxa is still a major burden that impairs the proficient management of these populations. Therefore, at present there is the need to design ways for the identification of each species at their early life stages.

The Lutjanidae (snappers) is one of the largest teleostean families with exceptional importance for Caribbean fisheries. Effective management of valuable snapper fisheries depends upon the availability of life history information concerning the biology, habitat requirements, and spatial distribution of individual species. The ability to identify individuals of the various snapper species throughout ontogeny is critical for a better understanding of the early life history and population dynamics of these species under natural conditions.

Early larval stages of the various lutjanids are extremely similar and difficult to distinguish to genus and species level. Clarke et al., (1997) found that within some Caribbean lutjanids there are some subtle differences in pigmentation that may facilitate identification of pre-flexion larvae to the species level. Ontogeny among species in the family Lutjanidae is known for a few species and is very similar among taxa. Developmental series of western Atlantic snapper larvae have been described for *Rhomboplites aurorubens* (Laroche, 1977), *Lutjanus campechanus* (Collins et al., 1980; Rabalais et al., 1980), *Lutjanus griseus* (Richards and Saksena, 1980) and *Ocyurus chrysurus* (Riley et al., 1995). Clarke et al., (1997) described

developmental series of artificially spawned and laboratory reared specimens of three species: *Lutjanus analis*, *Lutjanus synagris* and *L. griseus*. Pre-transitional larvae (usually from 12-17 mm Standard length (SL) may perhaps be distinguished for some species; however indistinctness persists specially for species in the sub family Lutjaninae (Victor, 2008).

Comparative studies using larval characters to reliably identify field collected specimens are scarce due to the limited available information concerning the co-occurrence of many species. Descriptions of lutjanid larvae are available for various Indo-Pacific species (Reader and Leis, 1996; Leis and Carson-Ewart, 2004; Leis, 2005, 2007). A guide for the identification of the early life stages of lutjanid fish of the western central Atlantic has made the identification of some of these species easier to some extent (Lindeman et al., 2005). Nevertheless, due to the extreme similarity among small larvae (pre-flexion stage), specific identification of other co-occurring species in the western Atlantic is dependent on descriptions of reared series of these larvae.

Victor (2008) is working on a comprehensive photographic guide to the larvae of coral reef fishes. After extensive efforts, he concludes that morphological differences among some species within the Lutjaninae subfamily are too subtle to unambiguously identify them to the species level. He proposes that the only certain way to distinguish among those species is by DNA analysis.

The use of DNA sequencing is the most recent approach used for the identification of fish larvae, thus rapidly becoming a standard for larval identification. Once a library of sequences is developed for a group of species, then individual larvae can be sequenced and matched with sequences of known species (Ward, 2005). This approach is sometimes called molecular key identification (Richardson et al., 2006).

To address the complexity in the identification of lutjanid larvae, we described mitochondrial DNA (mtDNA) sequence motifs diagnostics to Lutjanid species in a fragment of the 12S rRNA gene. Afterwards, these motifs were used to unambiguously identify lutjanid larvae to the species level.

MATERIALS AND METHODS

Study Site

La Parguera is located in southwestern Puerto Rico, where the shelf extends offshore to approximately 11 km before dropping abruptly from 20 to 3,800 m. To the south, the shelf break defines the end of the insular platform, while to the north a deeper sandy fringe borders the inner boundary of the shelf edge reef (Figs. 2.1 and 3.1).

Collections of larvae and voucher tissue

Larval fish samplings

Ichthyoplankton sampling was designed to cover the period when mutton snapper (*Lutjanus analis*) historically aggregates at known spawning sites in La Parguera (Rojas, 1960; Domieier et al., 1996). Plankton tows using a 300 µm mesh net were performed at the shelf edge, specifically at two sites commonly known as “El Hoyo” and “La Cuarta Mella” (Fig. 3.1). These sites are within the area where Ramírez and García (2003) found high abundance of lutjanid larvae and suggested that the area is an important source of snapper larvae. They found higher abundance of snapper larvae during the period February – May, which corresponds to the season of spawning aggregations for these taxa, and covers the period of our samplings.

Plankton samples were obtained by oblique tows encompassing most of the water column down to maximum depths of 25 m. Samples were collected after the full moons of April and May, 2006, and April 2007, following the massive spawning event of *Lutjanus analis* and *Ocyurus chrysurus* (Yellowtail snapper) as documented by local commercial landings. An additional set of samples were collected earlier, during August, 2002 at "El Hoyo", aimed to assess lutjanid larvae occurrence during a season when no spawning aggregations were reported (Figuerola and Torres, 2001). Samples were preserved in 20% ethanol and seawater in the field.

Entire samples were examined under a binocular microscope and lutjanid larvae were sorted out. Larval snappers (Lutjanidae) were identified according to meristic and morphometric characters (Lindeman et al., 2005). Photographs and SL measurements were taken for individual larva and classified as pre-flexion, flexion or post-flexion based on the upward flexion of the urostyle, which comes before the formation of the caudal fin. Each larva was stored in 95% ethanol for subsequent DNA extraction.

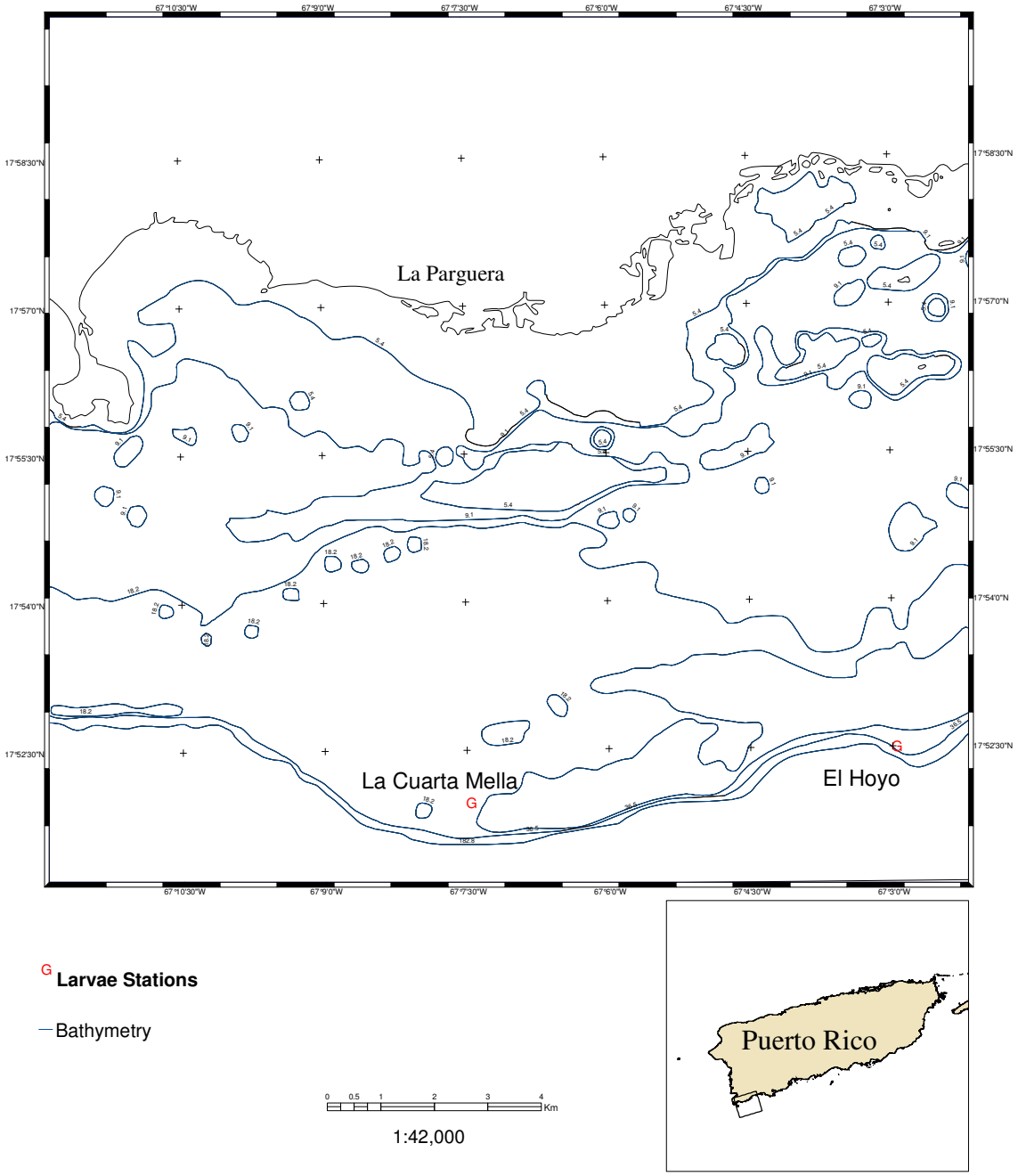

Figure 3.1. Sampling sites for larval collections**.**

Voucher samples from adult species

Voucher samples for 15 species of lutjanids were collected from local markets at La Parguera, Puerto Real and Rincón, Puerto Rico (Fig. 2.1) including: *Lutjanus analis, Lutjanus apodus, Lutjanus bucanella, Lutjanus cyanopterus, Lutjanus jocu, Lutjanus mahogoni, Lutjanus griseus*, *Lutjanus synagris*, *Lutjanus vivanus, Ocyurus chrysurus, Rhomboplites aurorubens*, *Apsilus dentatus*, *Etelis oculatus, Pristipomoides macrophtalmus and Pristipomoides aquilonaris*. Additional samples of *Lutjanus jocu* and *Ocyurus chrysurus* were collected from local markets at Cataño, northern Puerto Rico. The Marine Forensic Team, Center for Coastal Environmental Health and Biomolecular Research, National Centers for Coastal Ocean Science (NCCOS); NOAA, Charleston, SC provided 2 samples of *L. cyanopterus*. Muscle or liver tissue was dissected from fresh specimens and preserved frozen at -20 °C.

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from voucher samples and from individual larvae, using the QIAamp® DNA Mini Kit (QIAGEN, Inc.), according to manufacturer's protocol. A fragment of the 12S rRNA gene of ~450 bp was amplified with the primers: 5'-TCAAACTGGGATTAGATACCCCACTAT-3' and 5'-TGACTGCAGAGGGTGA CGGGCGGTGTGT-3' (Kocher et al., 1989). Polymerase chain reaction (PCR) was conducted in a total volume of 50 µl with 80 ng of template DNA, 0.75 µl of each primer (20 µM), 1.5 µl (25µM) MgCl2, 5 µl 10X reaction buffer, 8 µl dNTP's (each 2.5 mM), and 2 µl (2 units) of RED *Taq*™ genomic DNA polymerase (Sigma Chemical Co.). Amplifications were carried out in an Eppendorf® Mastercycler with an initial denaturation step at 95 °C for 2 min., followed by

30 cycles of 95 °C denaturation for 30 secs., 55 °C annealing for 1 min and 72°C extension for 1.5 min., and a final extension step at 72°C for 10 min. Amplified DNA was purified using the QIAquick® PCR purification kit (QIAGEN, Inc.), according to manufacturer's protocol. Cycle sequencing was conducted using the same primers utilized for PCR amplification using an automated sequencer at external facilities[2]. Corresponding fragments of the 12S rRNA gene sequences from *Cyprinus carpio* and *Caranx melanpygus* were acquired from GenBank to be used as outgroups (Accession numbers: X61010 and AP004445).

Phylogenetic analyses

Sequences from the 12S rRNA gene fragment from voucher samples and larvae as well as those from *C. carpio* and *C. melampygus* outgroups were aligned and edited with *MEGA4* (Tamura, 2007). Alignment was done under the following parameters: pairwise alignment parameters = gap opening 10.00, gap extension 0.10, DNA weight matrix IUB; multiple alignment parameters = gap opening 10.00, gap extension 0.20, delay divergent sequences 30%, DNA weight matrix IUB. All sequences aligned unambiguously.

The resulting alignment was visually verified, the ends of the aligned sequences were trimmed afterwards to match the length of the shortest. Voucher and larvae sequences were used to calculate mean uncorrected pairwise distances. Pairwise comparisons of uncorrected sequence divergence (SDv) were calculated with gaps treated as missing data. A sequence identity (100-SDv) matrix was constructed with BioEdit©.

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[2] Sequences were performed at Nevada Genomics Center: INBRE Grant # 2P2RR016463, UPR – Sequencing and Genotyping facility (IMBRE NCRR – NIH grant P20 RR0 16470, NSF – CREST – CATEC, S.C.O.R.E. grant S06GM8102) and UPR – Mayagüez NSF-MRI # 0503541.

Intraspecific variation was not found for the adult voucher specimens analyzed during this study. Thus, the consensus sequence of any species was always identical to any sequence of the species. Consensus sequences were aligned with sequences obtained from larvae to evaluate equal matches. The consensus sequences were used as a molecular key that allowed identification of each larva to the species level. A phylogenetic tree was produced by Neighbor Joining (NJ) using *MEGA4* (Tamura, 2007). The tree included one voucher sequence of each lutjanid species and one larval sequence representing each species.

RESULTS

A total of 89 lutjanid larvae were sorted from plankton samples, with 57 being successfully sequenced. Sequencing of the 12S rRNA mtDNA gene fragment produced an average of approximately 415 bp for all lutjanid taxa and larvae. Multiple alignments resulted in a consensus length of 400 characters (base pairs and gaps) available for analysis (Fig. 3.2). The phylogenetic tree produced by NJ clustered each larva with the respective species identified. Bootstrap values for several larva-species matches were low; this was due to the inherent close phylogenetic relationship of the species, all belonging to Lutjaninae subfamily (Fig. 3.3).

Seven species were identified within the lutjanid larval collection: *Ocyurus chrysurus*, *Rhomboplites aurorubens*, *Lutjanus griseus*, *Lutjanus apodus*, *Lutjanus analis, Lutjanus mahogoni* and *Lutjanus synagris* accounting for 64% of the total larvae examined (Table 3.1). The SL range of these larvae was 3.1 – 6.3 mm (Table 3.2). Identified lutjanid larvae in this study included 47 % of the 15 reported species for the Caribbean. All seven species are within

the Lutjaninae subfamily, representing 3 genuses (*Lutjanus*, *Ocyurus* and *Rhomboplite*s). Lutjaninae is the more speciose subfamily in the area, with 11 species, from which we found 63%. Most of these species are heavily fished coral reef fishes, while *Rhomboplites* is part of deep water fisheries in the area.

Most of identified larvae shared 100% identity with adult voucher consensus sequences (Table 3.3). Ten larvae shared 99.7% sequence identity with *O. chrysurus* (Yellowtail snapper) and were arbitrarily labeled as *O. chrysurus 2*. This substitution in position 261 (Fig. 3.2) was unique for these larval specimens. *Ocyurus chrysurus* was the most abundant lutjanid larva. Larvae of *O. chrysurus 2* represented 18% of the identified lutjanid larvae (Fig. 3.4). For that reason we incorporated more voucher specimens of *O. chysusrus* than for any other species in the study. However, the *O. chrysurus 2* haplotype was never found among voucher specimens. Even while *O. chrysurus* and *L. vivanus* haplotypes shared 99.4 % of sequence identity (Table 3.3), the possibility that those particular specimens were variants of *L. vivanus* was rejected. Both *O. chysusrus* and *O. chrysurus 2* shared variations in positions 7 and 258 not shared with *L. vivanus* (Fig. 3.2).

The majority of the larvae collected during spring 2006 were found at El Hoyo, while during the spring of 2007 most were collected at La Cuarta Mella (Fig. 3.5). Although a similar number of lutjanid larvae was collected in springs 2006 and 2007; a higher percent of the 2007 larvae was identified (Fig. 3.6). Higher success of identification of the 2007 samples was achieved by optimized preservation procedures and enhanced DNA quantifications with a NanoDrop™ spectrophotometer that allowed the use of more precise amounts of starting DNA material in the PCRs.

Table 3.1. Relative abundance of species of identified lutjanid larvae.

Identified Species	Year collected	# of Individuals	Relative frequency (% from total lutjanids collected)	Relative frequency (% from total Identified lutjanids)
O. chrysurus (O. c)	Fall 2002; Spring 2006 and 2007	19	21	33
O. chrysurus 2 (O. c 2)	Fall 2002; Spring 2006 and 2007	10	11	18
O. chry. + O. chry2	Fall 2002; Spring 2006 and 2007	29	33	51
L. apodus (L. apo)	Spring 2006 and 2007	11	12	19
L. analis (L. ana)	2006 and 2007	8	9	14
L. synagris (L. s)	Spring 2006 and 2007	6	7	11
L. griseus (L. g)	Fall 2002	1	1	2
L. mahogoni (l. m)	Spring 2007	1	1	2
R. aurorubens (R. a)	Fall 2002	1	1	2
Total lutjanid larvae identified		**57**	**64**	
Total lutjanid larvae collected		**89**		

Table 3.2. Identified lutjanid larvae.

Date	Sampling Site	Stage	SL (mm)	Sample Id	Species
August 5, 2002	El Hoyo	Pre	3.8	L20	*O. chrysurus*
August 5, 2002	El Hoyo	Flexion	4.2	L21	*O. chrysurus 2*
August 30, 2002	El Hoyo	Flexion	4.9	L24	*R. aurorubens*
August 30, 2002	El Hoyo	Pre	4.1	L23	*L. griseus*
April 24, 2006	El Hoyo	Post	5.0	L111	*O. chrysurus 2*
April 26, 2006	El Hoyo	Pre	4.3	L126	*O. chrysurus 2*
April 26, 2006	El Hoyo	Pre	4.8	L128	*O. chrysurus 2*
May 3, 2006	El Hoyo	Post	5.7	L107	*O. chrysurus*
May 3, 2006	El Hoyo	Flexion	4.6	L108	*O. chrysurus 2*
May 3, 2006	El Hoyo	Pre	5.0	L109	*O. chrysurus*
May 3, 2006	Cuarta Mella	Pre	3.1	L112	*L. apodus*
May 3, 2006	El Hoyo	Pre	3.5	L125	*L. apodus*
May 3, 2006	El Hoyo	Pre	4.0	L122	*L. apodus*
May 3, 2006	El Hoyo	Post	5.7	L116	*L. analis*
May 3, 2006	El Hoyo	Pre	4.0	L159	*O. chrysurus 2*
May 3, 2006	El Hoyo	Flexion	5.1	L160	*O. chrysurus*
May 3, 2006	El Hoyo	Flexion	4.6	L161	*L. analis*
May 3, 2006	El Hoyo	Flexion	4.6	L162	*O. chrysurus*
May 3, 2006	El Hoyo	Flexion	4.3	L163	*O. chrysurus*
May 3, 2006	El Hoyo	Flexion	4.0	L164	*L. analis*
May 3, 2006	Cuarta Mella	Post	5.4	L165	*L. analis*
May 3, 2006	Cuarta Mella	Flexion	4.9	L167	*O. chrysurus*
May 25, 2006	El Hoyo	Post	6.3	L136	*L. apodus*
June 5, 2006	Shelf Edge	Post	6.0	L135	*L. synagris*
April 18, 2007	Cuarta Mella	Flexion	4.5	L175	*O .chrysurus*
April 18, 2007	Cuarta Mella	Flexion	4.5	L179	*O. chrysurus*
April 18, 2007	Cuarta Mella	Flexion	5.0	L181	*O .chrysurus*
April 18, 2007	Cuarta Mella	Flexion	5.0	L182	*O .chrysurus*
April 18, 2007	Cuarta Mella	Flexion	5.2	L184	*O. chrysurus 2*
April 18, 2007	Cuarta Mella	Flexion	5.4	L186	*O. chrysurus*
April 18, 2007	Cuarta Mella	Flexion	5.3	L187	*O. chrysurus*
April 18, 2007	Cuarta Mella	Pre	4.5	L188	*O. chrysurus 2*
April 18, 2007	Cuarta Mella	Pre	4.9	L189	*O. chrysurus*
April 18, 2007	Cuarta Mella	Post	5.8	L191	*O. chrysurus*
April 18, 2007	Cuarta Mella	Flexion	5.2	L192	*O. chrysurus*
April 18, 2007	Cuarta Mella	Flexion	4.4	L193	*L. apodus*
April 18, 2007	Cuarta Mella	Flexion	5.0	L194	*O .chrysurus 2*
April 18, 2007	Cuarta Mella	Flexion	4.9	L195	*O. chrysurus 2*
April 18, 2007	Cuarta Mella	Flexion	4.9	L196	*O. chrysurus*
April 18, 2007	Cuarta Mella	Flexion	5.1	L199	*O. chrysurus*
April 18, 2007	Cuarta Mella	Flexion	4.4	L200	*L. apodus*
April 18, 2007	Cuarta Mella	Flexion	4.5	L201	*L. apodus*
April 18, 2007	Cuarta Mella	Flexion	3.5	L203	*L. apodus*
April 18, 2007	Cuarta Mella	Flexion	4.1	L204	*L. apodus*
April 18, 2007	Cuarta Mella	Flexion	4.5	L205	*L. apodus*
April 18, 2007	Cuarta Mella	Flexion	3.8	L207	*L. synagris*
April 18, 2007	Cuarta Mella	Flexion	3.1	L208	*L. synagris*
April 18, 2007	Cuarta Mella	Flexion	4.0	L209	*L. analis*
April 24, 2007	El Hoyo	Flexion	4.0	L211	*L. synagris*
April 24, 2007	El Hoyo	Flexion	3.5	L212	*L. synagris*
April 24, 2007	Cuarta Mella	Post	5.8	L213	*L. synagris*
April 24, 2007	Cuarta Mella	Post	6.1	L214	*L. mahogoni*
April 24, 2007	Cuarta Mella	Pre	4.0	L215	*L. analis*
April 24, 2007	Cuarta Mella	Post	5.0	L216	*O. chrysurus*
April 24, 2007	Cuarta Mella	Post	6.0	L217	*L. apodus*
April 24, 2007	Cuarta Mella	Flexion	4.7	L218	*L. analis*
April 24, 2007	Cuarta Mella	Post	6.0	L219	*L. analis*

Table 3.3. Sequence Identity Matrix for lutjanids species and larvae.

	C. c	*C. m*	*P. a*	*P. m*	*E. o*	*A. d*	*L. apo*	L125	*L. ana*	L116	*L. b*	*L. c*	*L. g*	L23	*L. j*	*L. m*	L214	*L. s*	L207	*L. v*	*O. c*	L20	L21 (*O. c* 2)	*R. a*	L24
C. c																									
C. m	0.770																								
P. a	0.807	0.842																							
P. m	0.799	0.837	0.984																						
E. o	0.827	0.839	0.942	0.945																					
A. d	0.814	0.843	0.916	0.924	0.958																				
L. apo	0.813	0.839	0.893	0.896	0.934	0.945																			
L125	0.813	0.839	0.893	0.896	0.934	0.945	1.000																		
L. ana	0.826	0.839	0.914	0.911	0.950	0.958	0.966	0.966																	
L116	0.826	0.839	0.914	0.911	0.950	0.958	0.966	0.966	1.000																
L. b	0.811	0.839	0.911	0.914	0.945	0.963	0.966	0.966	0.984	0.984															
L. c	0.808	0.846	0.909	0.911	0.950	0.963	0.973	0.973	0.981	0.981	0.986														
L. g	0.811	0.834	0.901	0.903	0.937	0.950	0.986	0.986	0.971	0.971	0.971	0.979													
L23	0.811	0.834	0.901	0.903	0.937	0.950	0.986	0.986	0.971	0.971	0.971	0.979	1.000												
L. j	0.817	0.832	0.898	0.901	0.929	0.948	0.986	0.986	0.968	0.968	0.963	0.966	0.984	0.984											
L. m	0.821	0.839	0.914	0.916	0.950	0.963	0.971	0.971	0.994	0.994	0.989	0.986	0.976	0.976	0.973										
L214	0.821	0.839	0.914	0.916	0.950	0.963	0.971	0.971	0.994	0.994	0.989	0.986	0.976	0.976	0.973	1.000									
L. s	0.819	0.836	0.909	0.911	0.945	0.963	0.971	0.971	0.989	0.989	0.984	0.981	0.973	0.973	0.973	0.994	0.994								
L207	0.819	0.836	0.909	0.911	0.945	0.963	0.971	0.971	0.989	0.989	0.984	0.981	0.973	0.973	0.973	0.994	0.994	1.000							
L. v	0.811	0.839	0.909	0.911	0.948	0.963	0.966	0.966	0.984	0.984	0.994	0.986	0.966	0.966	0.963	0.989	0.989	0.984	0.984						
O. c	0.816	0.839	0.911	0.914	0.947	0.968	0.966	0.966	0.989	0.989	0.994	0.986	0.971	0.971	0.968	0.994	0.994	0.989	0.989	0.994					
L20	0.816	0.839	0.911	0.914	0.947	0.968	0.966	0.966	0.989	0.989	0.994	0.986	0.971	0.971	0.968	0.994	0.994	0.989	0.989	0.994	1.000				
L21 (*O. c* 2)	0.816	0.841	0.911	0.914	0.947	0.968	0.963	0.963	0.986	0.986	0.992	0.984	0.968	0.968	0.966	0.992	0.992	0.986	0.986	0.992	0.997	0.997			
R. a	0.802	0.837	0.888	0.891	0.925	0.945	0.950	0.950	0.958	0.958	0.971	0.963	0.955	0.955	0.953	0.961	0.961	0.958	0.958	0.966	0.966	0.966	0.966		
L24	0.802	0.837	0.888	0.891	0.925	0.945	0.950	0.950	0.958	0.958	0.971	0.963	0.955	0.955	0.953	0.961	0.961	0.958	0.958	0.966	0.966	0.966	0.966	1.000	

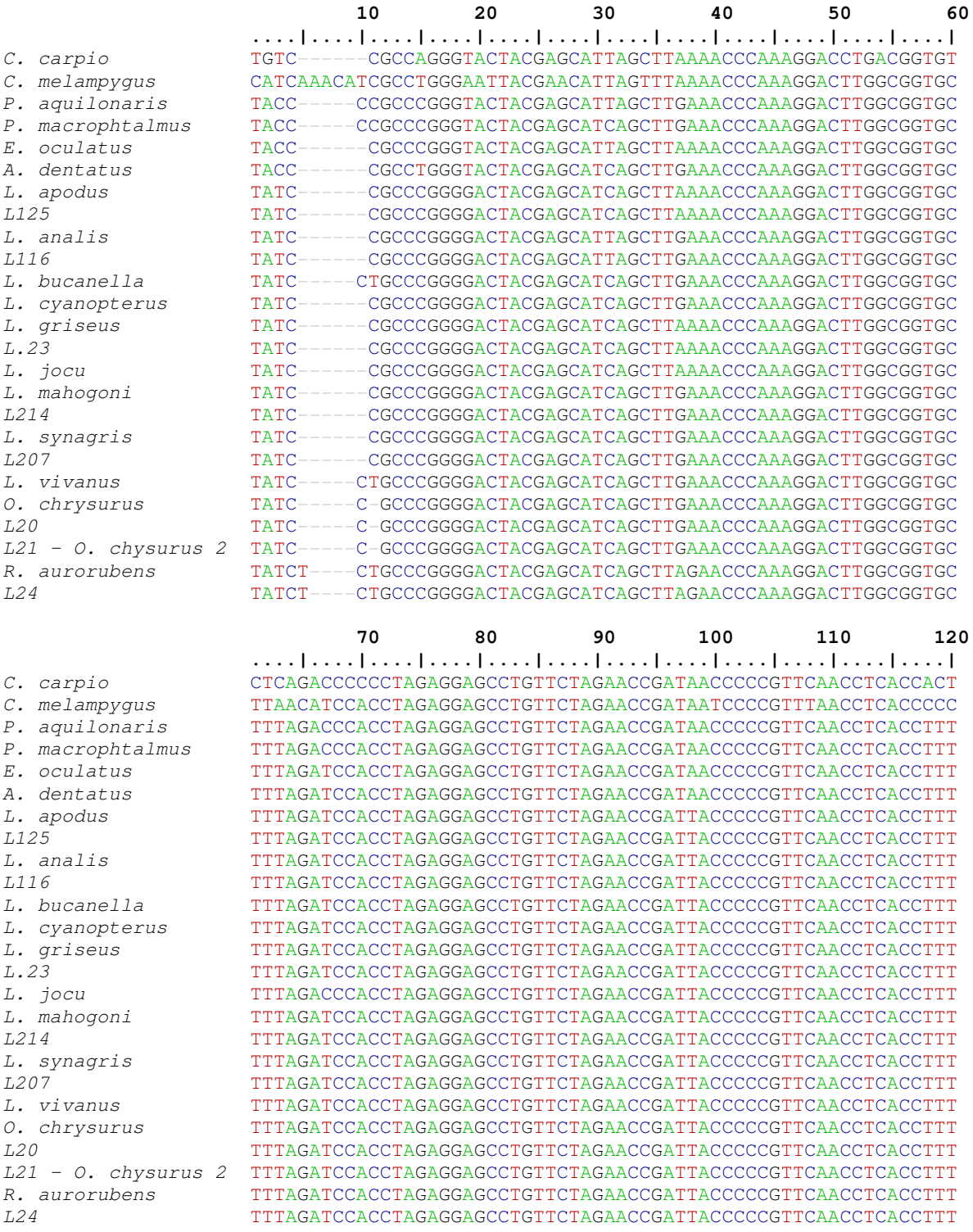

Figure 3.2. Sequence alignment of the 12SrRNA gene fragment for lutjanids and identified larvae.

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                          130       140       150       160       170       180
                    ....|....|....|....|....|....|....|....|....|....|....|....|
C. carpio           TCTAGCCACCCCAGCCTATATACCGCCGTCGTCAGCTTACCCTGTGAAGGTAATAAAAGT
C. melampygus       CCTAGCTTTTTCCGCCTATATACCACCGTCGCCAGCTTACCCTGTGAAGG-ACTAATAGT
P. aquilonaris      TCTTGTTTAACCCGCCTATATACCACCGTCGCCAGCTTACCCTGTGAAGG-CCTCATAGT
P. macrophtalmus    TCTTGTTTAACCCGCCTATATACCACCGTCGCCAGCTTACCCTGTGAAGG-CCTCATAGT
E. oculatus         TCTTGTTTAACCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGAAGG-CCTCATAGT
A. dentatus         CCTTGTTTTCCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGAAGG-CCTTATAGT
L. apodus           CCTTGTTTCTCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGAAGG-ACTTATAGT
L125                CCTTGTTTCTCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGAAGG-ACTTATAGT
L. analis           CCTTGTTTCCCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGAAGG-ACTCATAGT
L116                CCTTGTTTCCCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGAAGG-ACTCATAGT
L. bucanella        CCTTGTTTTTCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGAAGG-ACTCATAGT
L. cyanopterus      CCTTGTTTTTCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGAAGG-GCTCATAGT
L. griseus          CCTTGTTTCTCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGAAGG-GCTGATAGT
L.23                CCTTGTTTCTCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGAAGG-GCTGATAGT
L. jocu             CCTTGTTTCCCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGAAGG-ACTTATAGT
L. mahogoni         CCTTGTTTCCCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGAAGG-ACTCATAGT
L214                CCTTGTTTCCCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGAAGG-ACTCATAGT
L. synagris         CCTTGTTTCCCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGGAGG-ACTTATAGT
L207                CCTTGTTTCCCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGGAGG-ACTTATAGT
L. vivanus          CCTTGTTTTCCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGAAGG-ACTCATAGT
O. chrysurus        CCTTGTTTTCCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGAAGG-ACTCATAGT
L20                 CCTTGTTTTCCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGAAGG-ACTCATAGT
L21 - O. chysurus 2 CCTTGTTTTCCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGAAGG-ACTCATAGT
R. aurorubens       CCTTGTTTTTCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGAAGG-ACTAATAGT
L24                 CCTTGTTTTTCCCGCCTATATACCACCGTCGTCAGCTTACCCTGTGAAGG-ACTAATAGT

                          190       200       210       220       230       240
                    ....|....|....|....|....|....|....|....|....|....|....|....|
C. carpio           AAGCAAAATGGGCACAACCCAAAACGTCAGGTCGAGGTGTAGCGCATGAAGTGGGAAGAA
C. melampygus       AAGCACAATCGGCACAGCCCAGAACGTCAGGTCGAGGTGTAGTGAATGGGAGGGGAAGAA
P. aquilonaris      AAGCAGAATCGGCACAGCCCAGAACGTCAGGTCGAGGTGTAGCGTATGGAAAGGGAAGAA
P. macrophtalmus    AAGCAGAATCGGCACAGCCCAGAACGTCAGGTCGAGGTGTAGCGTATGGAAAGGGAAGAA
E. oculatus         AAGCAAAATTGGCACAGCCCAAAACGTCAGGTCGAGGTGTAGCGTATGAAAAGGGAAGAA
A. dentatus         AAGCAAAATTGGCACAGCCCAAAACGTCAGGTCGAGGTGTAGCGTATGGAAAGGGAAGAA
L. apodus           AAGCAAGATTGGCATAGCCCAAAACGTCAGGTCGAGGTGTAGCGTATGGAAGGGGAAGAA
L125                AAGCAAGATTGGCATAGCCCAAAACGTCAGGTCGAGGTGTAGCGTATGGAAGGGGAAGAA
L. analis           AAGCAAGATTGGCACAGCCCAAAACGTCAGGTCGAGGTGTAGCGTATGGAAAGGGAAGAA
L116                AAGCAAGATTGGCACAGCCCAAAACGTCAGGTCGAGGTGTAGCGTATGGAAAGGGAAGAA
L. bucanella        AAGCAAGATTGGCACAGCCCAAAACGTCAGGTCGAGGTGTAGCGTATGGAAAGGGAAGAA
L. cyanopterus      AAGCAAGATTGGCACAGCCCAAAACGTCAGGTCGAGGTGTAGCGTATGGAAAGGGAAGAA
L. griseus          AAGCAAGATTGGCATAGCCCAAAACGTCAGGTCGAGGTGTAGCGTATGGAAAGGGAAGAA
L.23                AAGCAAGATTGGCATAGCCCAAAACGTCAGGTCGAGGTGTAGCGTATGGAAAGGGAAGAA
L. jocu             AAGCAAGATTGGCATAGCCCAAAACGTCAGGTCGAGGTGTAGCGTATGGAAGGGGAAGAA
L. mahogoni         AAGCAAGATTGGCACAGCCCAAAACGTCAGGTCGAGGTGTAGCGTATGGAAAGGGAAGAA
L214                AAGCAAGATTGGCACAGCCCAAAACGTCAGGTCGAGGTGTAGCGTATGGAAAGGGAAGAA
L. synagris         AAGCAAGATTGGCACAGCCCAAAACGTCAGGTCGAGGTGTAGCGTATGGAAAGGGAAGAA
L207                AAGCAAGATTGGCACAGCCCAAAACGTCAGGTCGAGGTGTAGCGTATGGAAAGGGAAGAA
L. vivanus          AAGCAAGATTGGCACAGCCCAAAACGTCAGGTCGAGGTGTAGCGTATGGAAAGGGAAGAA
O. chrysurus        AAGCAAGATTGGCACAGCCCAAAACGTCAGGTCGAGGTGTAGCGTATGGAAAGGGAAGAA
L20                 AAGCAAGATTGGCACAGCCCAAAACGTCAGGTCGAGGTGTAGCGTATGGAAAGGGAAGAA
L21 - O. chysurus 2 AAGCAAGATTGGCACAGCCCAAAACGTCAGGTCGAGGTGTAGCGTATGGAAAGGGAAGAA
R. aurorubens       AAGCAAGATTGGCACAGCCCAAAACGTCAGGTCGAGGTGTAGCGAATGGAAAGGGAAGAA
L24                 AAGCAAGATTGGCACAGCCCAAAACGTCAGGTCGAGGTGTAGCGAATGGAAAGGGAAGAA
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Figure 3.2. Continued.

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                          250        260        270        280        290        300
                    ....|....|....|....|....|....|....|....|....|....|....|....|
C. carpio           ATGGGCTACATTTTCTAAT-ATAGAATA-TTACGAAC-ATGCACCATGAAACA-ATGC-T
C. melampygus       ATGGGCTACATTCGCTGCCCACAGCGAA--CACGAATGCTACAC--TGAAACATGTAG-C
P. aquilonaris      ATGGGCTACATTCTCTGCT-ATAGAGAA--CACGAATGATACGT--TGAAACACGTGTAC
P. macrophtalmus    ATGGGCTACATTCTCTGTT-ATAGAGAA--CACGAATGATACGT--TGAAACACGTGTAC
E. oculatus         ATGGGCTACATTCTCTAAT-ACAGAGAA--TACGAACGATACGC--TGAAACACGTATAC
A. dentatus         ATGGGCTACATTCCCTAACTATAGAGAA--TACGAACGATACAC--TGAAATACGTAT-C
L. apodus           ATGGGCTACATTCCCTAAC-ACAGTGAA-ATACGAACGATGCAC--TGAAATACACAT-C
L125                ATGGGCTACATTCCCTAAC-ACAGTGAA-ATACGAACGATGCAC--TGAAATACACAT-C
L. analis           ATGGGCTACATTCCCTAAT-ATAGTGTA-ATACGAACGATACAC--TGAAATACGTAT-C
L116                ATGGGCTACATTCCCTAAT-ATAGTGTA-ATACGAACGATACAC--TGAAATACGTAT-C
L. bucanella        ATGGGCTACATTCCCTAAT-ATAGTGAA-ATACGAACGATACAC--TGAAATACGTAT-C
L. cyanopterus      ATGGGCTACATTCCCTAAC-ACAGTGAA-ATACGAACGATACAC--TGAAATACGTAT-C
L. griseus          ATGGGCTACATTCCCTAAC-ATAGTGAA-ATACGAACGATGCAC--TGAAATACGCAT-C
L.23                ATGGGCTACATTCCCTAAC-ATAGTGAA-ATACGAACGATGCAC--TGAAATACGCAT-C
L. jocu             ATGGGCTACATTCCCTAAC-ATAGTGAATATACGAACGATGCAC--TGAAATACGCAT-C
L. mahogoni         ATGGGCTACATTCCCTAAT-ATAGTGAA-ATACGAACGATACAC--TGAAATACGTAT-C
L214                ATGGGCTACATTCCCTAAT-ATAGTGAA-ATACGAACGATACAC--TGAAATACGTAT-C
L. synagris         ATGGGCTACATTCCCTAAT-ATAGTGAA-ATACGAACGATACAC--TGAAATACGTAT-C
L207                ATGGGCTACATTCCCTAAT-ATAGTGAA-ATACGAACGATACAC--TGAAATACGTAT-C
L. vivanus          ATGGGCTACATTCCCTAAT-ACAGTGAA-ATACGAACGATACAC--TGAAATACGTAT-C
O. chrysurus        ATGGGCTACATTCCCTAAT-ATAGTGAA-ATACGAACGATACAC--TGAAATACGTAT-C
L20                 ATGGGCTACATTCCCTAAT-ATAGTGAA-ATACGAACGATACAC--TGAAATACGTAT-C
L21 - O. chysurus 2 ATGGGCTACATTCCCTAAT-ATAGCGAA-ATACGAACGATACAC--TGAAATACGTAT-C
R. aurorubens       ATGGGCTACATTCCCTAGC-ATCGGGCATATACGAACGATACAC--TGAAATACGTAT-C
L24                 ATGGGCTACATTCCCTAGC-ATCGGGCATATACGAACGATACAC--TGAAATACGTAT-C

                          310        320        330        340        350        360
                    ....|....|....|....|....|....|....|....|....|....|....|....|
C. carpio           TGAAGGAGGATTTAGTAGTAAAAGGGAAGTAGAGTGTCCCTTTTGAACCCGGCTCTGAGA
C. melampygus       TGAAGGAGGATTTAGCAGTAAGCAGAAAGCAGAGTGTTCCGCT-GAAGCCGGCTCTTAAG
P. aquilonaris      TGAAGGAGGATTTAGCAGTAGGCAGGAAATAGAGTGTTCTGCC-GAAGCCGGCCCTGAAG
P. macrophtalmus    TGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGTGTTCTGCC-GAAGTTGGCCCTGAAG
E. oculatus         TGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCCGCT-GAAATCGGCCCTGAAG
A. dentatus         CGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCCGCT-GAAATCGGCCCTGAAG
L. apodus           TGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCTGCT-GAAACCGGCCCTGAAG
L125                TGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCTGCT-GAAACCGGCCCTGAAG
L. analis           TGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCCGCT-GAAACCGGCCCTGAAG
L116                TGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCCGCT-GAAACCGGCCCTGAAG
L. bucanella        CGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCCGCT-GAAACCGGCCCTGAAG
L. cyanopterus      TGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCCGCT-GAAACCGGCCCTGAAG
L. griseus          TGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCTGCT-GAAACCGGCCCTGAAG
L.23                TGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCTGCT-GAAACCGGCCCTGAAG
L. jocu             TGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCTGCT-GAAACCGGCCCTGAAG
L. mahogoni         TGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCCGCT-GAAACCGGCCCTGAAG
L214                TGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCCGCT-GAAACCGGCCCTGAAG
L. synagris         TGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCCGCT-GAAACCGGCCCTGAAG
L207                TGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCCGCT-GAAACCGGCCCTGAAG
L. vivanus          CGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCCGCT-GAAACCGGCCCTGAAG
O. chrysurus        CGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCCGCT-GAAACCGGCCCTGAAG
L20                 CGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCCGCT-GAAACCGGCCCTGAAG
L21 - O. chysurus 2 CGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCCGCT-GAAACCGGCCCTGAAG
R. aurorubens       CGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCCGCT-GAAACCGGCCCTGAAG
L24                 CGAAGGAGGATTTAGCAGTAAGCAGAAAATAGAGCGTTCCGCT-GAAACCGGCCCTGAAG
```

Figure 3.2. Continued.

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                          370        380        390        400
                    ....|....|....|....|....|....|....|....|....
C. carpio           CGCGTACACACCGCCCGTCACTCTCCCCTGTCAA--------AA
C. melampygus       CGCGCACACACCGCCCGTCACCCTCCCCAAGCAACTGGACCTAA
P. aquilonaris      CGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
P. macrophtalmus    CGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
E. oculatus         CGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
A. dentatus         CGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
L. apodus           CGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
L125                CGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
L. analis           CGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
L116                CGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
L. bucanella        CGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
L. cyanopterus      CGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
L. griseus          CGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
L.23                CGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
L. jocu             CGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
L. mahogoni         CGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
L214                CGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
L. synagris         CGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
L207                CGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
L. vivanus          CGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
O. chrysurus        CGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
L20                 CGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
L21 - O. chysurus 2 CGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
R. aurorubens       CGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
L24                 CGCGCACACACCGCCCGTCACCCTCTGCAGTCAA--------AA
```

Figure 3.2. Continued.

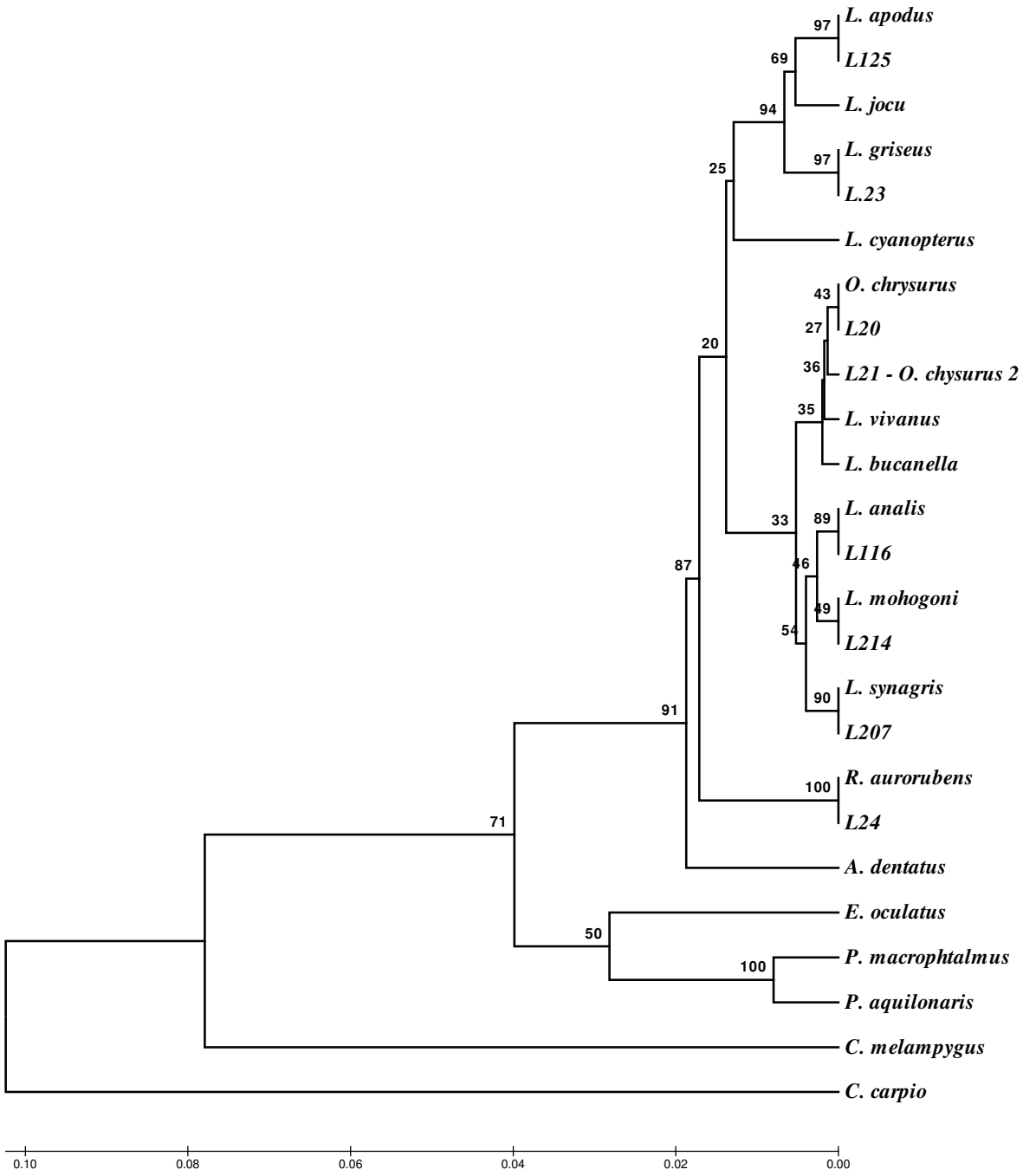

Figure 3.3. Neighbor Joining (NJ) tree for consensus 12r RNA sequences of Caribbean lutjanids and a consensus sequence of the identified larvae clustered with their respective species.

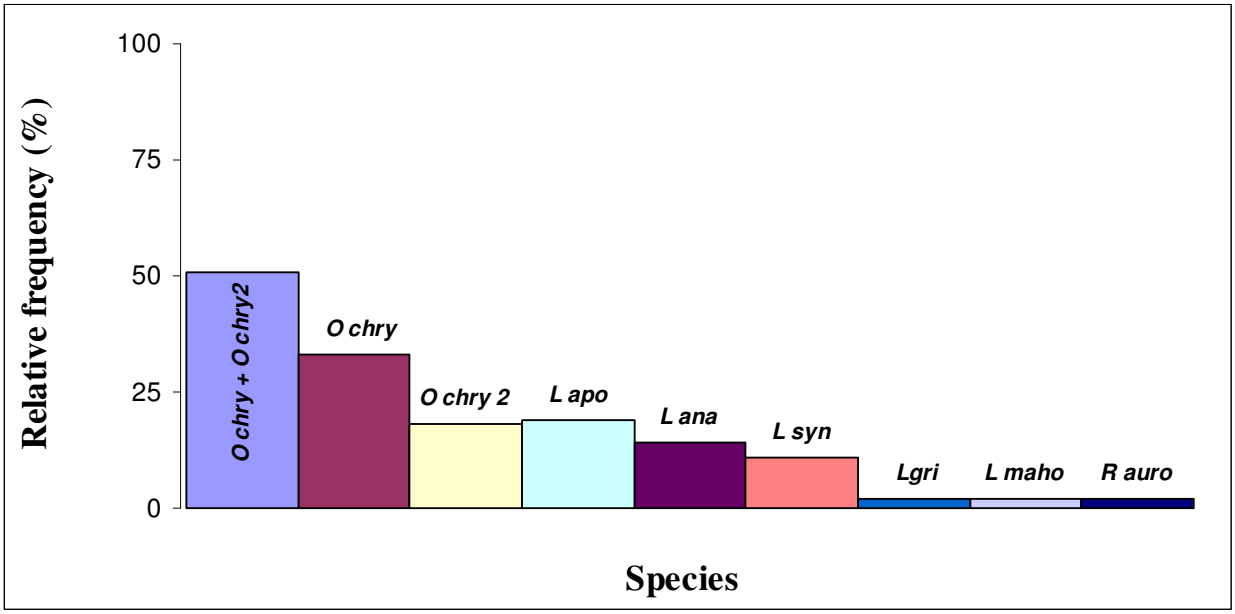

Figure 3.4. Relative frequency of species identified for lutjanid larvae**.**

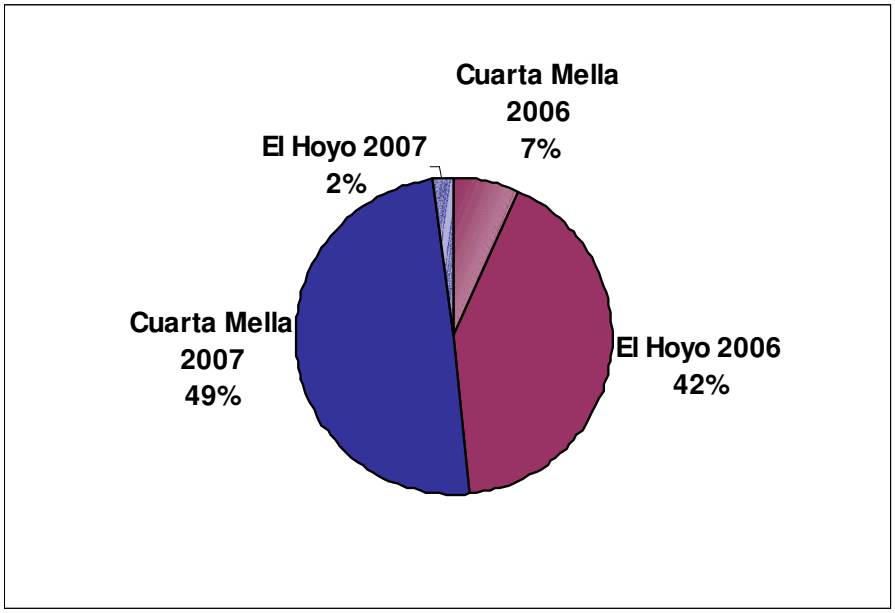

Figure 0.1. Total lutjanid larvae collected.

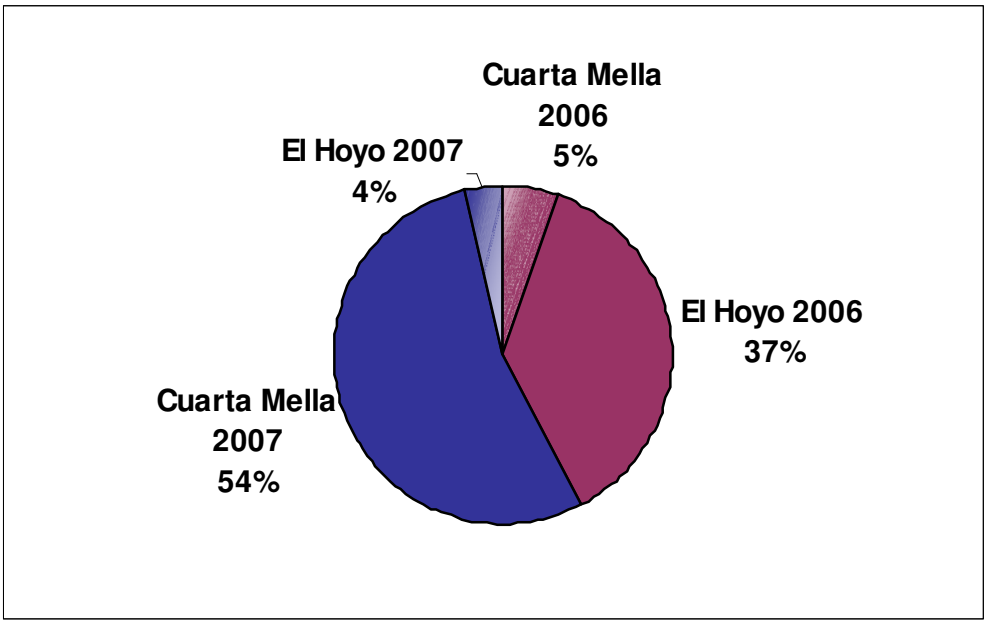

Figure 3.6. Total identified lutjanid larvae**.**

DISCUSSION

In this study sequencing of a fragment of mtDNA 12S rRNA proved to be useful for the identification of lutjanid larvae to the species level. This fragment met two requirements of a good molecular marker for Caribbean lutjanids: consistent interspecific differences and minimal instraspecific variation. All sorted larvae were assigned to a lutjanid species, except for a small uncertainty with the *O. chrysurus 2* larvae. Findings of some larvae with a haplotype similar to *O. chrysurus* may point toward certain degree of instraspecific variation within Yellowtail snapper populations in the area. As the distinct haplotype was not found in any of the adult voucher specimens, we recommend further screening to investigate polymorphism within species. Haplotype *O. chrysurus 2* came out 10 times, at each year we sampled, both at El Hoyo and La Cuarta Mella, thus representing a widespread variant.

Chow et al., (1993) used PCR-Restriction fragment length polymorphisms (RFLP) to access genetic species and stock discrimination of lutjanid larvae. They were not able to single out all species since the close phylogenetic relationship among lutjanids limits the resolution of RFLPs. In the present study, sequence analysis provided for better resolution and a reliable option for species identification of early life stages of fishes, in comparison to the limitations of morphological identification (Victor, 2008). It is argued that sequencing has drawbacks, including limitations imposed by cost and time, but this technology is being continuously improved making it evermore an attractive high resolution technique for species identification.

Even though the present study did not examine the total abundance of lutjanid larvae in the natural environment, i.e. not quantitative, there was a tendency towards higher frequencies of lutjanid larvae during spring samplings, than during the fall. Furthermore, higher frequencies of

snapper species were also found within larval specimens during the spring (Table 3.1). Six of the seven species identified in this study are part of the typical assemblage of coral reef fishes in La Parguera (Randall, 1968), most with reported spawning peaks during the spring (SAFMC, 2005). Ramírez and García (2003) found higher abundance of snappers during the period February – May, which corresponds to the months of massive spawning aggregations for this taxon within insular shelf waters.

Large groups of adult Yellowtail snappers, the species more frequently found in our study, were fished in "corridas", as termed by fishermen, about a month prior to our samplings in spring 2006 and 2007 at La Parguera shelf edge (fishermen interviews). Yellowtail snappers are known to exhibit schooling behavior (Thompson and Munro, 1974). Spawning aggregations (SA) of yellowtail snappers have not been reported in Puerto Rico, hence, it is uncertain if those aggregations observed in La Parguera were actually SAs. Large spawning aggregations are reported to occur seasonally off Cuba, the Turks and Caicos and the USVI (SAFMC, 2005). Large spawning aggregation occurs during May-July at Riley's Hump near the Dry Tortugas Key West, Florida (Muller et al., 2003).

Our study was designed to collect larvae after mutton snapper spawning aggregation events. Accordingly, we expected higher frequencies of mutton snapper larvae. In contrast we found more Yellowtail snapper larvae than of any other species. The present data suggests that a spawning peak of yellowtails was as well detected. Some aggregation sites may be used by various species, either simultaneously or at different times of the day, month or year. Others host a single species (Domeier and Colin, 1997). Another possibility is that larvae might have been exported or imported to the area as well, however the examination of larval transport was

outside the scope of this study, therefore this possibility was not examined further. However, Ramirez and García (2003) reported lutjanids as part of an assemblage of coral reef fish families that were concentrated within a relatively narrow belt fringing both the neritic and oceanic sides of the shelf edge. They found high abundance of lutjanids at a neritic station 10 km off the coast and suggested this corridor as an important source of snappers.

In conclusion, the 12S rRNA gene is appropriate for the identification for Caribbean lutjanids. The molecular key created in this study will facilitate further larval studies focusing on individual species. As ichthyoplankton surveys are still the most direct approach to investigate larval dynamics, specific identification of fish larvae is essential. In comprehensive plankton surveys thousands of fish larvae may be collected. Molecular analyses are becoming increasingly accessible, with costs reduced these may be feasible tools for studies were large quantities of larvae are collected (Richardson, 2006).

The need for taxonomic identification of early life stages of commercially important coral reef fishes have grown as efforts to develop stock assessment tools are becoming imperative. To date it is believed that a significant portion of reef fish larvae are retained to recruit back into their natal populations rather than being dispersed to other sites, therefore influencing the degree of connectivity among populations (Roberts, 1997; Sale, 2004; Cowen, 2000). Information on this connectivity among local populations is critically important for management, which is increasingly based on the use of marine protected areas (e.g. no-take zones) both to conserve, and to provide sustainable fisheries.

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4 CONCLUSIONS AND RECOMENDATIONS

Our investigation of the phylogenetic relationships of the Lutjanidae using a fragment of the 12S rRNA gene generally supports the phylogenetic hypothesis based on adult morphology proposed by Johnson (1980) and Rivas (1966). The employment of additional mitochondrial or nuclear genes to explore genetic variation among lutjanid taxa will provide a more complete picture of the evolution of this important family of fishes. Even when relationships of lutjanids were not fully resolved, our phylogenetic study is, so far, the first to include all the 3 subfamilies of lutjanids occurring in the Caribbean. However, as intraspecific variation was not observed, species were characterized unambiguously.

The consistency in the sequence data for each species in this study demonstrates that the 12S rRNA gene is a reliable tool for taxonomic identification within this family. These sequences constitute a sort of molecular key for all the 15 species of lutjanids studied, useful for identification of early stages and processed tissues or fillets for fisheries management regulations.

As a distinct haplotype of *Ocyurus chrysurus* was found within larval specimens but was not found in any of the adult voucher specimens, we recommend further screening of Yellowtail snapper populations to investigate polymorphism within species.

In conclusion, the 12S rRNA gene is appropriate for the identification for Caribbean lutjanids. The molecular key created in this study will facilitate further larval studies focusing on individual species. As ichthyoplankton surveys are still the most direct approach to investigate larval dynamics, specific identification of fish larvae is essential.