Abstract.—Restriction endonuclease analysis of mitochondrial cytochrome *b* and 12S ribosomal RNA (rRNA) gene fragments amplified by polymerase chain reaction (PCR) was applied to thirteen western Atlantic snapper species (the genera *Lutjanus*, *Ocyurus* and *Rhomboplites*), in order to assess reliability of this method for genetic species and stock identification studies. Eight species (*L. apodus*, *L. buccanella*, *L. campechanus*, *L. cyanopterus*, *L. griseus*, *L. synagris*, *L vivanus* and *R. aurorubens*) could be identified by haplotype analysis on either single or both fragments, while the others (*L. analis*, *L. jocu*, *L. mahogoni*, *L. purpureus* and *O. chrysurus*) were not separated from one another because of overlapping or identical haplotypes observed between species. High nucleon diversity estimates (h = 40–80%) observed within four species (*L. campechanus*, *L. cyanopterus*, *L. griseus* and *L. jocu*) indicated that sufficient intraspecific polymorphism could be detected by the PCR-[RFLP] restriction fragment length polymorphism analysis making it a useful method for investigating genetic stock structure.

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PCR-RFLP analysis on thirteen western Atlantic snappers (subfamily Lutjaninae): a simple method for species and stock identification

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In order to manage fisheries resources, it is essential to know the recruitment mechanisms of a given species. Lutjanidae is one of the largest teleostean families, comprising 4 subfamilies, 17 genera, and 103 species (Allen, 1985). More than a half of the lutjanid species belong to the subfamily Lutjaninae in which 14 species under three genera (*Lutjanus*, *Ocyurus* and *Rhomboplites*) have been described in the western Atlantic Ocean. All are fine food fishes and very important for commercial and recreational fisheries, and considerable concern has been focused on the need to understand recruitment in order to manage these reef species (Huntsman, 1981; Doherty, 1983; Roberts and Polunin, 1991). However, the close morphological similarity of larvae among lutjanid species, especially within the subfamily Lutjaninae, has made specific identification of larvae a difficult task (Richards, 1985; Leis, 1987; Richards and Lindeman, 1987). Allozyme electrophoresis has proven useful for identifying some fish species even at their embryonic or larval stages (Morgan, 1975; Smith and Crossland, 1977; Mork et al., 1983; Graves et al., 1989). Since DNA is virtually the same in any cell type of an individual and can be extracted from ethanol-preserved specimens, restriction fragment analyses of DNA is becoming a preferred method to investigate variation and relationships between fish species (Billington and Hebert, 1991). Distinct restriction fragment patterns between three bass species of the genus *Paralabrax* could be detected in ethanol-preserved individual eggs and larvae *via* Southern blot analysis (Graves et al., 1990). Although conventional mitochondrial DNA (mtDNA) methods are powerful for detecting variation in restriction fragment length, intensive DNA analysis would be difficult especially on very small samples, such as fish embryos or larvae, which offer a very small amount of DNA. To overcome this problem, the use of the polymerase chain reaction (PCR) method, which can amplify DNA sequences more than 10-million fold (Saiki et al., 1988), is neccessary. In this paper, we report amplification of two mitochondrial genes (cytochrome *b* and 12S rRNA) using fresh or frozen samples, ethanol-preserved embryos and larvae, and alcohol-preserved museum samples of thirteen western Atlantic snapper species, and results of restriction fragment length polymorphism (RFLP)

analysis on these two DNA fragments within and between species.

Materials and methods

Sample collection

The species used and sources of collection are listed in Table 1. All of the fresh or frozen specimens were collected in the Miami and Key West, FL, area during 1990 and 1991. Fertilized eggs and hatched larvae of *Lutjanus synagris* were raised at 28°C in 380-L tanks and fed a combination of cultured rotifers and wild-caught zooplankton. The eggs (4 to 6 hours after insemination) and larvae (3 and 9 days old) were fixed with 95% ethanol and kept for two months at room temperature. Two eggs and two larvae were randomly chosen. All of the museum specimens were obtained from the Ichthyology Museum at the University of Miami. The time since preservation ranged from 5 to 42 years and it is likely that most were fixed with formaldehyde first and then transferred to ethanol or isopropyl alcohol (C. R. Robins, Professor, Rosenstiel School of Marine and Atmospheric Science, Division of Marine Biology and Fisheries, Univ. Miami, 4600 Rickenbacker Causeway, Miami, FL 33149-1098, pers. commun.). However, exact records on preservation methods were not available. Samples are labelled as to source, for example, Lcy1 and LcyM1 designate *Lutjanus cyanopterus* fresh or frozen specimen No. 1 and *L. cyanopterus* museum specimen No. 1, respectively.

DNA extraction

Fresh or frozen specimens Mitochondria-enriched or total genomic DNA samples were prepared from fresh or frozen specimens by the method of Chapman and Powers (1984) with slight modification.

Museum specimens A piece of white muscle (0.1–0.2 g) was dissected from each individual, shredded and rinsed in TEK buffer (50 mM Tris, 10 mM EDTA,

Table 1
Sample species of snappers (Lutjaniae: *Lutjanus*, *Ocyurus* and *Rhomboplites*) and source of collection.

Species	Abbreviation	Fresh or frozen samples locality and date	Museum samples label, locality, and date
L. analis	La	Miami, FL, '90,'91 (6)*	
L. apodus	Lap	Miami, FL, '90,'91 (7)	
L. buccanella	Lb	Miami, FL, '91 (4)	
L. campechanus	Lc	NA	UMML 27092, 28°N, 80°W, '66 (M2)**
			UMML 27093, 28°N, 80°W, '66 (M1)
			UMML 27096, 11°N, 60°W, '66 (M3)
			UM/M2422, Gulf of Mexico, '58 (M4-5)
L. cyanopterus	Lcy	Key West, FL, '91 (1)	UMML15923, Virgin Isl., '59 (M1)
			UM/M4868, Bahama, '56 (M2)
L. griseus	Lg	Miami, FL, '90,'91 (9)	
L. jocu	Lj	NA	Cat.No. 1944, Puerto Rico, '57 (M1)
			Cat.No. 620, Cutler, FL, '49 (M2-4)
			UMML34424, Miami, FL, '86 (M6)
			UM/M5660, Miami, FL, '64 (M7)
L. mahogoni	Lm	Key West, FL, '91 (2)	UMML7523, Knight Key, FL, '57 (M1)
			UM/M4865, Miami,FL '50 (M2)
L. purpureus	Lp	NA	UM/M6093, Venezuela, '65 (M1-2)
			St.5708, Venezuela, '65 (M3)
			St.5658, Venezuela, '65 (M4)
			UM/M6086, Venezuela, '65 (M5-6)
L. synagris	Ls	Miami, Key West, FL, '90 (7)	
L. vivanus	Lv	Miami, FL, '90,'91 (5)	STA5739, Panama, '65 (M1)
O. chrysurus	Oc	Miami, Key West, FL, '90 (8)	
R. aurorubens	Ra	Miami, FL, '90 (6)	

NA=not available.
*=number of individuals examined.
**=museum specimens were labelled, for example, as LcM1 designating *Lutjanus campechanus* museum specimen No. 1. All museum samples were obtained from the Ichthyological Museum of the University of Miami. UMML is the catalogue number for each individual; values in parentheses indicate lots in which several individuals were contained.

1.5% KCl, pH 7.5) for 10 minutes at room temperature. The muscle fibrils were finely minced and homogenized in 5–10 mL of ice-cold TEK buffer by means of a motor-driven, glass-teflon homogenizer. Five or more strokes of the homogenizer were neccessary to grind the toughest muscle tissue. The homogenate was transferred to a plastic centrifuge tube (50-mL capacity), and SDS and proteinase K were added as described in Chapman and Powers (1984). The sample was then incubated at 65°C for 2 hours or more and vigorously shaken at intervals. This crude sample was phenol-chloroform extracted and ethanol precipitated as described in Chapman and Powers (1984). The amount of DNA was usually too small to be seen as a pellet, but was rehydrated in 500 µl of TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0) and transferred to a 1.5 mL microcentrifuge tube. To concentrate this DNA sample, ethanol precipitation was repeated and the pellet was rehydrated in 10 µl of TE buffer.

Embryos and larvae Each specimen was transferred to a 1.5-mL microcentrifuge tube. TEK buffer was added and decanted several times to rinse specimens and to remove ethanol. The specimen was then homogenized in 500 µl of TEK buffer and total DNA was isolated as described above. The pellet was rehydrated in 10 µl of TE buffer.

Amplification of mitochondrial cytochrome *b* and 12S rRNA genes

The two pairs of primers used that targeted cytochrome *b* and 12S rRNA genes were abbreviated forms of those described by Kocher et al. (1989). The nucleotide sequences of each set of primers were as follows: cytochrome *b*, 5'-GCTTCCATCCAACATCTCAGCATGATG-3' and 5'-GCAGCCCCTCAGAATGATATTTGTCCTC-3'; 12S rRNA, 5'-TCAAACTGGGATTAGATACCCCACTAT-3' and 5'-TGACTGCAGAGGGTGACGGGCGGTGTGT-3'. These primers were synthesized by R. K. Werner in the Department of Biochemistry and Molecular Biology at the University of Miami.

Polymerase chain reaction was carried out in a final volume of 50 µl in a reaction mixture described by Kocher et al. (1989). This reaction mixture was preheated at 94°C for 3 minutes followed by 30–35 cycles of amplification (93°C for 1 min, 44°C for 1 min, and 72°C for 1 min). The same cycle was applied to amplify both cytochrome *b* and 12S rRNA genes. After amplification, the reaction mixture was separated from covering oil and transferred to a 1.5-mL microcentrifuge tube. TE buffer (0.5 mL) was added to the reaction mixture, followed by chloroform: isoamylalcohol (24:1) extraction and ethanol precipitation. After centrifugation at 12,000 × g for 10 minutes, the pellet was dried under reduced vacuum, rehydrated with 10 to 50 µl of TE, and stored at 4°C.

Endonuclease digestion for amplified DNA fragments

Nine restriction endonucleases used were *Alu* I, *Cfo* I, *Dde* I, *Hae* III, *Hin* fI, *Mbo* I, *Msp* I, *Rsa* I, and *Taq* I, all recognizing symmetric 4-base pair sequences. One unit of each enzyme was applied to 1 to 2 µl of amplified PCR product in a final reaction volume of 5 µl. The digested samples were electrophoresed through 2% NuSieve (3:1) (FMC BioProducts, Rockland, ME) agarose gels in TBE buffer (90 mM Tris-boric acid, and 2 mM EDTA). DNA bands were visualized and photographed following electrophoresis and staining with ethidium bromide.

Data analysis The size of fragment amplified and restricted was estimated in comparison with a size standard (1 kb ladder, BRL). Nucleotide sequence divegence (p) (Upholt, 1977) was calculated by using the proportion of shared restriction fragments between specimens. Restriction patterns by each endonuclease were designated A, B or C for composite haplotype analysis, and the number of haplotype was used to estimate nucleon diversity (h) (Nei and Tajima, 1981) within species.

Results

Gene amplification

Each pair of primers successfully amplified 355±5 bp and 450±5 bp fragments of the cytochrome *b* and 12S rRNA genes, respectively. There were no apparent differences in the fragment size among species. Both DNA fragments in all of the fresh and frozen specimens and in ethanol-preserved embryos and larvae were well amplified. In contrast, the amount of DNA was usually much less in museum specimens, to the extent that PCR did not always amplify one or both of the gene fragments.

Restriction fragment analysis

Figure 1, A and B, shows representative restriction patterns with diagnostic enzymes for amplified fragments of the cytochrome *b* and 12S rRNA genes. The restricted fragment distributions for nine enzymes of 72 individuals in cytochrome *b* and 59 individuals in 12S rRNA genes are shown in Tables 2 and 3.

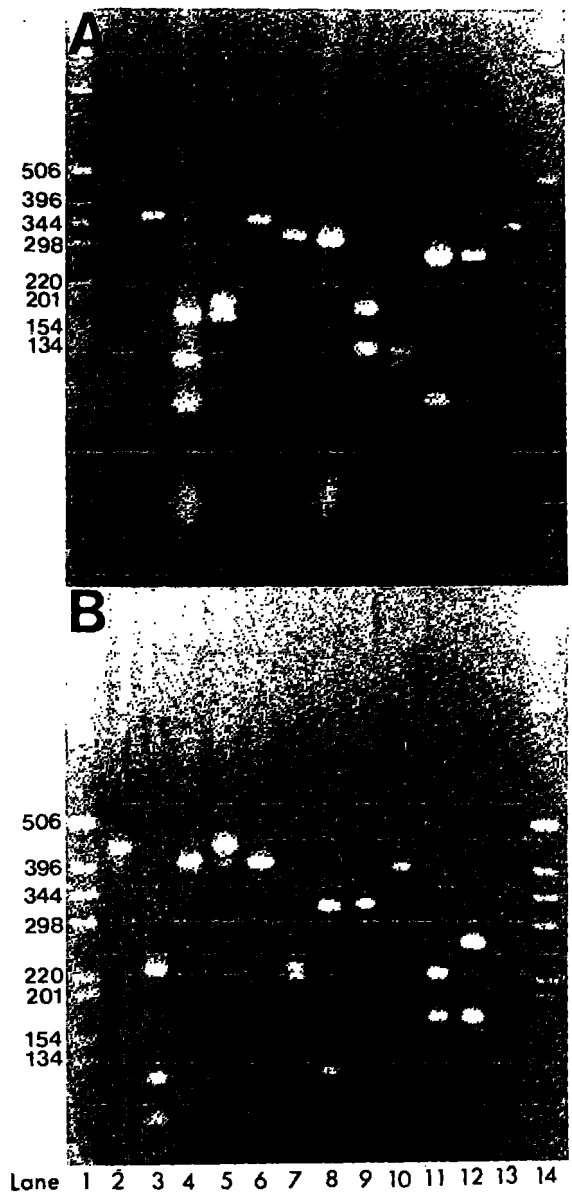

Figure 1

Restriction profiles of cytochrome *b* (**A**) and 12S rRNA (**B**) gene fragments amplified by PCR. (A) lanes 1 and 14 (size standard), 2 (blank), 3 and 4 (La and Ra digested by *Alu* I), 5 and 6 (Lap and Lv digested by *Cfo* I), 7 and 8 (La and Ra digested by *Dde* I), 9 and 10 (Ra and Ls digested by *Hae* III), 11 and 12 (Lap and Ra digested by *Hin* fI), and 13 (La no digestion); (B) lanes 1 and 14 (size standard), 2 (La no digestion), 3 (La digested by *Alu* I), 4 (Lb digested by *Cfo* I), 5 (Ls digested by *Dde* I), 6 (Lg digested by *Hae* III), 7 (Oc digested by *Hin* fI), 8 (Ls digested by *Mbo* I), 9 and 10 (Lg and La2 digested by *Msp* I), 11 and 12 (Lg and La digested by *Taq* I), and 13 (blank).

For the 355 bp cytochrome *b* fragment, four enzymes (*Mbo* I, *Msp* I, *Rsa* I and *Taq* I) had no restriction sites in any of the species. *Dde* I had restriction site(s) in all species examined, without apparent size difference in restricted fragments within and between species. Variation on the restricted fragment length within and between species was observed for four of the nine enzymes examined. *Alu* I appeared to have no restriction sites in this fragment of all *Lutjanus* spp. and *O. chrysurus* (Oc), but two restriction sites producing three fragments (170, 110 and 74 bp) were evident in *R. aurorubens* (Ra). *Cfo* I, *Hae* III and *Hin* fI digestions revealed polymorphism within species; *L. griseus* (Lg) for all three enzymes, *L. analis* (La) for *Cfo* I and *Hin* fI, *L. campechanus* (Lc) for *Hae* III and *Hin* fI, and *O. chrysurus* (Oc) for *Cfo* I only.

All nine enzymes had recognition site(s) in the 450 bp 12S rRNA fragment of at least one species. No length variation of the restricted fragments between species was observed for *Cfo* I and *Hae* III digestions. *Taq* I digestion produced 230 and 170 bp fragments in *L. griseus* (Lg), while 280 and 170 bp fragments were observed in the other species. Intraspecific polymorphism was observed for the other six enzymes; *Alu* I (for Lc, Lcy, Lg, and Lj), *Dde* I (for Lc, Lcy, and Lg), *Hin* fI (for La, Lap, Lcy, and Lj), *Mbo* I (for La, Lc, and Lj), *Msp* I (for La, Lcy, and Lj), and *Rsa* I (for Lc, Lcy, and Lj).

Data of restriction fragment patterns were summarized for haplotype analysis for species discrimination (Table 4). Straightforward diagnostic restriction profiles were observed in the 12S rRNA fragment of *L. griseus* (Lg) digested by *Taq* I and in the cytochrome *b* fragment of *R. aurorubens* (Ra) digested by *Alu* I, without RFLP within species. Haplotype analysis on cytochrome *b* fragment separated *L. cyanopterus* (Lcy) and *L. vivanus* (Lv), and that on 12S rRNA gene fragment separated *L. campechanus* (Lc). *L. apodus* (Lap), *L. buccanella* (Lb) and *L. synagris* (Ls) were descriminated by haplotype analysis on both fragments. In contrast, identical haplotypes were found in five individuals of *L. analis* (La1, 5, 52–54), all of *L. mahogony* (Lm1, 2, M2) and in seven of *O. chrysurus* (Oc1–7), and in two individuals of *L. jocu* (LjM2, M3) and in one of *L. purpureus* (LpM2). One individual of *L. analis* (La2), two of *L. jocu* (LjM1, M7), and one of *O. chrysurus* (Oc50) had their own distinct haplotype.

Mean percent nucleotide sequence divergence (p) within species ranged from 0 to 2.43 with an average of 0.65. The nucleotide sequence divergence for those between species within the genus *Lutjanus* ranged from 0.11 to 3.84 with an average of 1.69, for those between

Table 2

Restriction profiles of 355 bp cytochrome *b* gene fragment of 13 snapper (Lutjanidae) species. 1=present; 0=absent.

		Species and sample number																
		La		Lap	Lb	Lc		Lcy	Lg		Lj	Lm	Lp	Ls	Lv	Oc		Ra
Enzymes and fragments		1,5, 52–54	2	1–7	1–4	M1, M2,M3	M4	1, M1,M2	2–5, 8–10, 13	6	M1–M3	1,2,M2	M1–M6	1–6, 9	1–5, M1	1–7	50	1–6
Alu I	355	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
	170	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
	110	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
	74	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Cfo I	355	1	0	0	1	1	1	0	0	1	1	1	1	0	1	1	0	1
	185	0	1	1	0	0	0	1	1	0	0	0	0	1	0	0	1	0
	170	0	1	1	0	0	0	1	1	0	0	0	0	1	0	0	1	0
Dde I	330	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hae III	190	0	0	0	1	1	0	1	0	1	1	0	1	0	0	0	0	1
	120	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	100	1	1	1	0	0	1	0	1	0	0	1	0	1	1	1	1	0
Hin fI	355	1	0	0	0	0	1	0	1	0	1	1	1	1	0	1	1	0
	275	0	1	1	1	1	0	1	0	1	0	0	0	0	1	0	0	1
	80	0	1	1	1	1	0	1	0	1	0	0	0	0	1	0	0	1
Mbo I	355	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Msp I	355	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rsa I	355	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Taq I	355	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Table 3

Restriction profiles of 450 bp 12S rRNA gene fragment of 13 snapper (Lutjanidae) species. 1=present; 0=absent.

		Species and sample number																			
		La		Lap		Lb	Lc		Lcy		Lg		Lj			Lm	Lp	Ls	Lv	Oc	Ra
Enzymes and fragments		1,5, 52–54	2	1–3	7	1–4	M1, M4	M5	1, M1	M2	2,3,5 6,8,9	4	M1	M2, M3	M7	1,2,M2	M2	1–6,9	1–5,M1	1–7,50	1–3
Alu I	450	0	0	0	0	0	0	1	0	1	0	1	1	0	0	0	0	0	0	0	0
	230	1	1	1	1	1	1	0	1	0	1	0	0	1	1	1	1	1	1	1	1
	120	1	1	1	1	1	1	0	1	0	1	0	0	1	1	1	1	1	1	1	1
	100	1	1	1	1	1	1	0	1	0	1	0	0	1	1	1	1	1	1	1	1
Cfo I	420	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dde I	450	1	1	1	1	1	1	0	1	0	1	1	0	1	1	1	1	1	1	1	1
	420	0	0	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0
Hae III	410	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hin fI	450	0	1	1	0	0	1	1	0	1	0	0	1	0	1	0	0	1	0	0	1
	230	1	0	0	1	1	0	0	1	0	1	1	0	1	0	1	1	0	1	1	0
	220	1	0	0	1	1	0	0	1	0	1	1	0	1	0	1	1	0	1	1	0
Mbo I	450	0	1	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0
	320	1	0	1	1	1	1	0	1	0	1	1	0	1	1	1	1	1	1	1	1
	130	1	0	1	1	1	1	0	1	0	1	1	0	1	1	1	1	1	1	1	1
Msp I	450	0	0	0	0	0	1	1	0	1	0	0	1	0	0	0	0	0	0	0	0
	400	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	320	1	0	1	1	1	0	0	1	0	1	1	0	1	1	1	1	1	1	1	1
Rsa I	450	1	1	1	1	1	1	0	1	0	1	1	1	1	0	1	1	1	1	1	1
	250	0	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0
	120	0	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0
Taq I	280	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1
	230	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0
	170	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Table 4

Haplotypes of 74 individuals of thirteen snapper (Lutjanidae) species, and percent sequence divergence (p) and nucleon divergence (h). Eleven columns represent 9 endonucleases: *Alu* I (1 and 5); *Cfo* I (2); *Dde*I (6); *Hae*III (3); *Hin*fI (4 and 7); *Mbo*I (8); *Msp*I (9); *Rsa*I (10); *Taq*I (11).

Gene specimen	cytochrome *b*				12S rRNA							p*	h**
	1	2	3	4	5	6	7	8	9	10	11		
La1, 5, 52–54	A	A	B	A	B	A	B	B	C	A	B		
La2	A	B	B	B	B	A	A	A	B	A	B	1.2	33.3
Lap1–3	A	B	B	B	B	A	A	B	C	A	B		
Lap4–6	A	B	B	B	—	—	—	—	—	—	—		
Lap7	A	B	B	B	B	A	B	B	C	A	B	0.25	28.6
Lb1–4	A	A	A	B	B	A	B	B	C	A	B	0	0
LcM1	A	A	A	B	B	A	A	B	A	A	B		
LcM2,3	A	A	A	B	not amplified								
LcM4	A	A	B	A	B	A	A	B	A	A	B		
LcM5	not amplified				A	B	A	A	A	B	B	1.54	70.0
Lcy1,M1	A	B	A	B	B	A	B	B	C	A	B		
LcyM2	A	B	A	B	A	B	A	A	A	B	B	2.43	66.7
Lg2,3,5,8,9	A	B	B	A	B	A	A	B	C	A	C		
Lg4	A	B	B	A	A	A	A	B	C	A	C		
Lg6	A	A	A	B	B	A	A	B	C	A	C		
Lg10,13	A	B	B	A	—	—	—	—	—	—	—	0.7	41.7
LjM1	A	A	A	A	A	B	A	A	A	A	B		
LjM2,M3	A	A	A	A	B	A	B	B	C	A	B		
LjM7	not amplified				B	A	A	B	C	B	B	2.18	83.3
Lm1,2,M2	A	A	B	A	B	A	B	B	C	A	B	0	0
LpM1,3–6	A	A	A	A	not amplified								
LpM2	A	A	A	A	B	A	B	B	C	A	B	0	0
Ls1–6,9	A	B	B	A	B	A	A	B	C	A	B	0	0
Lv1–5,M1	A	B	B	B	B	A	B	B	C	A	B	0	0
Oc1–7	A	A	B	A	B	A	B	B	C	A	B		
Oc50	A	B	B	A	B	A	B	B	C	A	B	0.13	25.0
Ra1–3	B	A	A	B	B	A	A	B	C	A	B		
Ra4–6	B	A	A	B	—	—	—	—	—	—	—	0	0

— = PCR was not attempted.

*Mean percent sequence divergence (Upholt, 1977): individuals possessing incomplete haplotype were excluded.

**Percent nucleon diversity (Nei and Tajima, 1981): individuals possessing an incomplete haplotype were included in the most common haplotype.

Lutjanus and *Ocyurus* ranged from 0.7 to 3.07 with an average of 1.21, for those between *Lutjanus* and *Rhomboplites* it ranged from 1.24 to 2.81 with an average of 2.16, and for that between *Ocyurus* and *Rhomboplites* it was 2.41. Nucleon diversity estimates (h) within species ranged from 0 to 83.3%.

Discussion

Eight out of thirteen western Atlantic snapper species examined may be identified by PCR-RFLP analysis of the two mitochondrial genes, although further analysis for the intraspecific polymorphism is neccessary for

complete discrimination. Snappers are an important component of both the recreational and commercial fisheries in the Southeastern United States, Gulf of Mexico, and Caribbean. Analysis of the distribution of eggs and larvae for life history and recruitment studies of the fishery has been hampered by the inability to identify eggs and larvae of most snapper species. Our results significantly increase the number of eggs and larvae that can be unambiguously identified. Incomplete separation by our methods for all thirteen species is due to sharing of identical haplotypes between some species, which could be caused by possible occurrence of natural hybrids between snapper species (Domeier and Clarke, 1992). The lack of between species resolution may also be attributed to the fact that the amplified fragments are too short for RFLP analysis, since the number of restriction sites is subjected to the fragment length.

Average sequence divergence observed between the snapper species is also low compared with those between congeneric species of other fish taxa where the entire mtDNA molecule was analyzed (see Billington and Hebert, 1991). Bartlett and Davidson (1991) using direct nucleotide sequence of 307 bp cytochrome *b* gene fragment found several distinct nucleotide substitutions between four tuna species of the genus *Thunnus*. Since many of the nucleotide substitutions observed between these tuna species are found to create or destroy palindromic sequences, PCR-RFLP analysis on the 355 bp cytochrome *b* gene fragment alone could identify these tuna species using only a few endonucleases (Chow and Inoue, 1993). They also found that RFLP analysis of cytochrome *b* and 12S rRNA gene fragments provided insufficient variation to separate all eight species of the genus *Thunnus*, but all tuna species could be separated using a 940 bp fragment of flanking region between ATPase and cytochrome oxidase subunit III genes. Similar analysis on the cytochrome *b* gene fragment could separate six billfish Pacific species and five Atlantic species, but separation of all species was incomplete (Chow[1]).

Although it appears that RFLP analysis may fail to pick up distinct nucleotide substitutions between species, analysis on longer fragments may overcome this disadvantage. Thus, differentiation between closely related species appears to vary among regions within the mtDNA and in a given gene may vary among taxa. Recently, Silberman and Walsh (1992) using PCR-RFLP analysis on the 28S ribosomal RNA gene simply and unambiguously distinguished three spiny lobster species of the genus *Panulirus*. Nuclear rDNA may be better suited for species descrimination, where little intraspecific polymorphism is expected (Dover, 1982).

We have observed relatively high intraspecific polymorphism in some snapper species, in which the nucleon diversity (h) of *L. campechanus*, *L. cyanopterus*, *L. griseus*, and *L. jocu* are 70, 67, 42, and 83%, respectively. Gyllensten and Wilson (1987), using restriction analysis on the entire mitochondrial DNA molecule, reported nucleon diversity of brown trout (*Salmo trutta*) to be 72%. Carr and Marshall (1991), using direct nucleotide sequence of a 298 bp cytochrome *b* gene, reported that of the western Atlantic and Norwegian populations of Atlantic cod (*Gadus morhua*) to be 36 and 88%, respectively, comparable with those of some snapper species. It is highly probable that some snapper species contain a large number of maternal lineages within the population, which can be detected by PCR-RFLP analyses as demonstrated in this study.

The present study indicates that PCR-RFLP analysis is much simpler and less expensive than conventional mtDNA and nucleotide sequence analyses and better suited for large-scale genetic surveys requiring large numbers of specimens. With this method, not only are quantitative analyses of species composition at early life history stages possible, but also intensive genetic analysis. Since relatively little is known about the spawning habits, dispersal, and migration of these snapper species, it would be very interesting to compare, for example, genetic structures between adults and their early life stages. Such genetic analyses would aid in evaluating the utility of management schemes, such as reserves and protected areas.

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Literature cited

Allen, G. R.

1985. FAO species catalogue. Snappers of the world. An annotated and illustrated catalogue of lutjanid species known to date. FAO Fish. Synop. No. 125, vol. 6, 208 p.

[1]Chow, S. 1992. Identification of billfish species using mitochondrial cytochrome *b* gene fragment amplified by polymerase chain reaction. ICCAT Working Doc. SCRS/92/66.

Bartlett, S., and W. S. Davidson.
1991. Identification of *Thunnus* tuna species by the polymarase chain reaction and direct sequence analysis of their mitochondrial cytochrome *b* genes. Can. J. Fish. Aquat. Sci. 48:309–317.

Billington, N., and P. D. N. Hebert.
1991. Mitochondrial DNA diversity in fishes and its implications for introductions. Can. J. Fish. Aquat. Sci. 48:80–94.

Carr, S. M., and H. D. Marshall.
1991. Detection of intraspecific DNA sequence variation in the mitochondrial cytochrome *b* gene of Atlantic cod (*Gadus morhua*) by the polymerase chain reaction. Can. J. Fish. Aquat. Sci. 48:48–52.

Chapman, R. W., and D. A. Powers.
1984. A method for the rapid isolation of mitochondrial DNA from fishes. Maryland Sea Grant Prog., Technical Report, UM-SG-TS-84-05, Univ. Md., College Park, 11 p.

Chow, S., and S. Inoue.
1993. Intra- and interspecific restriction fragment length polymorphisms in mitochondrial genes of *Thunnus* tuna species. Bull. Natl. Res. Inst. Far Seas Fish. 30 (in press).

Doherty, P.
1983. Recruitment surveys of coral reef fishes as tools for science and management. *In* J. T. Baker, R. M. Carter, P. W. Sammarco, and K. P. Stark (eds.), Proceedings of the inaugural Great Barrier Reef conference, August 28–September 2, 1983, p. 191–196.

Domeier, M. L., and M. E. Clarke.
1992. A laboratory produced hybrid between *Lutjanus synagris* and *Ocyurus chrysurus* and a probable hybrid between *L. griseus* and *O. chrysurus* (Perciformes: Lutjanidae). Bull. Mar. Sci. 50: 501–507.

Dover, G.
1982. Molecular drive: a cohesive mode of species evolution. Nature 299:111–117.

Graves, J. E., M. A. Simovich, and K. M. Schaefer.
1989. Electrophoretic identification of early juvenile yellowfin tuna, *Thunnus albacares*. Fish. Bull. 86:835–838.

Graves, J. E., M. J. Curtis, P. A. Oeth, and R. S. Waples.
1990. Biochemical genetics of southern California basses of the genus *Paralabrax*: species identification of fresh and ethanol-preserved individual eggs and early larvae. Fish. Bull., 88:59–66.

Gyllensten, U., and A. C. Wilson.
1987. Mitochondrial DNA of salmonids: inter- and intraspecific variability detected with restriction enzymes. *In* N. Ryman, and F. Utter (eds.), Population genetics and fishery management, p. 301–314. Washington Sea Grant, Univ. Washington Press, Seattle, WA.

Huntsman, G. R.
1981. Ecological considerations influencing the management of reef fishes. *In* Artificial reefs: proceedings of a conference held September 13–15, 1979, Daytona Beach, Florida, p. 167–175. Rep. Fl. Sea Grant No. 41.

Kocher, T. D., W. K. Thomas, A. Meyer, S. V. Edwards, S. Paabo, F. X. Villablanca, and A. C. Wilson.
1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci. U.S.A. 86:6196–6200.

Leis, J. M.
1987. Review of the early life history of tropical groupers (Serranidae) and snappers (Lutjanidae). *In* J. J. Polovina, and S. Ralston (eds.), Tropical snappers and groupers: biology and fisheries management, p. 189–237. Westview Press Inc., Boulder.

Morgan, R. P.
1975. Distinguishing larval white perch and striped bass by electrophoresis. Chesapeake Sci. 16:68–70.

Mork, J., P. Solemdal, and G. Sundnes.
1983. Identification of marine fish eggs: a biochemical genetics approach. Can. J. Fish. Aquat. Sci. 40:361–369.

Nei, M., and F. Tajima.
1981. DNA polymorphism detected by restriction endonucleases. Genetics 97:145–163.

Richards, W. J.
1985. Status of the identification of early life stages of fishes. Bull. Mar. Sci. 37:756–760.

Richards, W. J., and K. C. Lindeman.
1987. Recruitment dynamics of reef fishes: planktonic processes, settlement and demersal ecologies, and fisheries analysis. Bull. Mar. Sci. 41:392–410.

Roberts, C. M., and N. V. C. Polunin.
1991. Are marine reserves effective in management of reef fisheries? Rev. Fish. Biol. Fish 1:65–91.

Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.
1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–491.

Silberman, J. D., and P. J. Walsh.
1992. Species identification of spiny lobster phyllosome larvae *via* ribosomal DNA analysis. Mol. Mar. Biol. Biotech. 1:195–205.

Smith, P. J., and J. Crossland.
1977. Identification of larvae of snapper *Chrysophrys auratus* Forster, by electrophoretic separation of tissue enzymes. N. Z. J. Mar. Freshwater Res. 11:795–798.

Upholt, W. B.
1977. Estimation of DNA sequence divergence from comparison of restriction endonuclease digests. Nucl. Acids Res. 4:1257–1265.