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DNA Microsatellite Loci and Genetic Structure of Red Snapper in the Gulf of Mexico

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Abstract.—Five polymorphic microsatellite loci were developed from genomic DNA of red snapper *Lutjanus campechanus* and used to evaluate genetic variation among 194 red snapper sampled from three locations in the northern Gulf of Mexico and one location off the northern Yucatan Peninsula in Mexico. From 5 to 13 alleles were observed per locus, and expected heterozygosities ranged from 0.143 to 0.779. No significant departures from expectations of Hardy–Weinberg equilibrium were found at any locus either within samples or when samples were pooled across localities. Locus-by-locus tests of allele-frequency homogeneity over the four localities were nonsignificant. Weir and Cockerham's Θ at each locus ranged from -0.003 to 0.012, and Statkin's R_{ST} at each locus ranged from -0.014 to 0.008. None of the estimates of Θ and R_{ST} differed significantly from zero, and magnitudes of Θ and R_{ST} did not appear to vary with geographic distance between localities. These results are consistent with the hypothesis that red snapper at these localities constitute a single population. There are, however, a number of caveats to this hypothesis. Detection of population structure of red snapper in the Gulf of Mexico may require more samples and larger sample sizes, and the importance of local reproduction to recruitment needs to be investigated via examination of larvae settling onto reefs. A positive but nonsignificant correlation existed between mean number of repeat units per microsatellite allele and heterozygosity of the locus.

Red snapper *Lutjanus campechanus* represent a valuable finfish resource in the northern Gulf of Mexico (Gulf) that is exploited by both commercial and recreational fishers (Goodyear 1992). Despite intensive management since 1990 when the Reef Fish Fishery Management Plan of the Gulf of Mexico Fishery Management Council (GMFMC) became operative, the red snapper fishery in the northern Gulf remains overfished (Goodyear 1995; Schirripa and Legault 1997) and the subject of considerable debate. Confounding the issue is that juvenile red snapper represent a significant component of shrimp trawl bycatch (Gutherz and Pellegrin 1988).

Assessment and management of red snapper in the northern Gulf currently follow a unit (single) stock hypothesis (GMFMC 1989, 1991). The single-stock model is based largely on the observations that postlarval stages (i.e., juveniles, subadults, and adults) of red snapper are sedentary, nonmigratory, and associated with specific substrates or structures (Bradley and Bryan 1975; Beaumariage and Bullock 1976; Szedlmayer and Shipp 1994; Szedlmayer 1997). Red snapper eggs and larvae, however, are pelagic during the 28–30-d presettlement period (Leis 1987) and could be dispersed hydrodynamically (Goodyear 1992).

Previous studies of genetic diversity in red snapper, relative to the issue of stock structure in the northern Gulf, have yielded conflicting results. One study of allozyme variation (Johnson 1987) and two studies of mitochondrial DNA (mtDNA) variation (Camper et al. 1993; Gold et al. 1997) have been consistent with the unit stock hypothesis; whereas another study (Bortone and Chapman 1995) reported highly significant differences in frequencies of several mtDNA haplotypes among red snapper sampled from various localities in the northern Gulf. Bortone and Chapman (1995) acknowledged that their results were at odds with the potential for gene flow in red snapper and with other genetic studies. They hypothesized that samples from fry traps might have contained schools of closely related individuals, and thus allele frequencies might not have been representative of adult stocks.

In this paper, we report (i) the isolation and op-

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TABLE 1.—Polymerase chain reaction (PCR) primers used to amplify microsatellite loci in red snapper from the Gulf of Mexico.

Locus	Repeat	Primer	Sequence
Lca20	$(CA)_9$	*Lca20U*	5′-CAACCCTCTGGCTAGTGTCA
		Lca20L	5′-ATCCTGAAGCCCTGGTTTAC
Lca22	$(CA)_{18}$	*Lca22U2*	5′-TCCACAGGCTTTCACTCTTTCAG
		Lca22L2	5′-TGCTCTTTTCTTTCCGTCATTCC
Lca43	$(CA)_{8,5}$[a]	*Lca43U*	5′-ACTGAAATGCTGCTCTCCTT
		Lca43L	5′-CACTGTTTACTTCTTCTGTT
Lca64	$(CA)_{12}$	*Lca64U*	5′-CTCCAATCCTCCTCTCACCT
		Lca64L	5′-AGTGCCCCTGATACACTTGC
Lca91	$(GT)_8$	*Lca91U*	5′-GCATCCACCCTAAACATTTT
		Lca91L	5′-CTTCATCAGAGCAGCATCCT

[a] The sequence CCCCTG occurs between the 8-CA and 5-CA repeats at *Lca43*.

timization of five polymorphic microsatellite (DNA) loci in red snapper and (ii) the results of a preliminary survey of allelic variation at these loci among red snapper from the northern Gulf. In brief, microsatellites are short stretches of nuclear DNA composed of di-, tri-, and tetranucleotide arrays that are embedded in unique DNA, inherited in a Mendelian fashion, and distributed throughout euchromatic regions of chromosomes (Weber and May 1989; Weber 1990; Wright 1993). Variants at microsatellite loci are thought to arise much more rapidly than mtDNA variants, meaning that recently diverged subpopulations (stocks) may be detected more easily with microsatellites than with mtDNA. This discriminatory power has been demonstrated recently in both Atlantic cod *Gadus morhua* (Bentzen et al. 1996; Ruzzante et al. 1996) and in Atlantic salmon *Salmo salar* (McConnell et al. 1997).

Methods

Samples used in this study were among those obtained in 1991 (Gold et al. 1997) and included individuals taken offshore from the following localities (sample sizes in parentheses): Panama City, Florida ($N = 48$); Dauphin Island, Alabama ($N = 53$); Galveston, Texas ($N = 47$); and Merida, Mexico ($N = 44$). Tissue samples (heart) were collected and stored as described in Gold et al. (1997). Genomic DNA was isolated from one frozen red snapper heart as described in Gold and Richardson (1991), then digested with restriction enzyme *Dpn* II and electrophoresed against a 100-base-pair (bp) size standard in 1.4% TAE (0.04 M tris-acetate 0.001 M ETDA) agarose gels. Fragments spanning 400–1,000 bp were excised from the gel, extracted via electroelution, concentrated by ethanol precipitation, and ligated into a polycloning site within the ampicillin resistance gene of a pUC18 vector that had been dephosphorylated and digested with *Bam*H I. Ligation was carried out using T4 DNA ligase, and the reaction was used to transform *E. coli* DH5α cells. Ampicillin selection was used to identify red snapper recombinant colonies. A total of 2,304 colonies was screened with the aid of a Beckman Biomek 2000 workstation. Each colony was spotted twice to screen for false positives. Radiolabeled ($\gamma^{32}P$) hybridization probe was prepared from a mixture of repetitive oligonucleotides [$(CA)_{15}$, $(GA)_{15}$, $(ATT)_7$, $(CCT)_7$, $(GACA)_8$; A = adenine, C = cytosine, G = guanine, T = thymine] using PNK (polynucleotide kinase). Positive colonies were retrieved from the library and grown overnight in LB-AMP. Positive clones were sequenced from both ends using standard M13 sequencing primers on an IBI 373 or 377 automated sequencer. Primers for PCR within the region flanking the repeat motifs were selected using the Oligo 4.0 software package (Rychlik 1992). Polymerase chain reaction (PCR) was performed under a variety of conditions to optimize production of high yields of target sequence and minimize additional fragments.

Once appropriate PCR conditions were obtained, amplification products of five microsatellite loci were obtained from genomic DNA of 194 individual red snappers sampled from four localities in the Gulf (Table 1). The PCR reactions contained approximately 1–10 ng genomic DNA, 0.1 units *Taq* DNA polymerase, 0.5 μM each primer, 200 μM each dNTP (deoxynucleotide triphoshate), 2 mM $MgCl_2$, and 1× *Taq* buffer (50 mM KCl, 10 mM tris, 0.1% Triton X-100, pH 9.0). One primer was radio-labeled with $\gamma^{32}P$ before amplification. Amplification consisted of a 2-min denature step at 94°C, 25 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s, followed by a single 5-min extension step at 72°C. Alleles at individual

loci were separated on denaturing polyacrylamide gels and visualized via autoradiography using the cloned fragment at each locus as a size standard. Known DNA sequences were used to confirm spacing of alleles. Genotypic frequencies at each locus were tested for deviations from Hardy–Weinberg equilibrium by pooling rare genotypes and applying the EXACTP option of the HDYWBG step in the BIOSYS-1.7 package of Swofford and Selander (1981). Homogeneity of allele frequencies was tested using the randomization procedure of Roff and Bentzen (1989). We also estimated Weir and Cockerham's (1984) Θ (an unbiased estimator of Wright's genetic variance statistic FST; Wright 1969) and Slatkin's (1995) R_{ST}. The former was derived by using Arlequin 1.1 (Schneider et al. 1997) as described in Michalakis and Excoffier (1996); the latter was derived by using RST CALC (Goodman 1997). The R_{ST} considers the squared size differences between alleles and is more appropriate for loci that evolve via stepwise mutations. Statistical significance of Θ and R_{ST} was assessed by random permutations (1,000 trials per comparison). We also estimated the (weighted) mean number of repeats per locus by assuming the diversity of allele sizes was attributable solely to differences in number of repeat units. Estimates of the mean number of repeats per locus were weighted by the relative frequency of each allele at that locus.

Results

Of 2,304 red snapper clones screened, 141 were positive in initial screens for microsatellite repeat motifs. Of these, 87 were sequenced. Five of the clones were false positives, that is, did not contain a microsatellite-like repeat; two contained the same repeat (and flanking regions), and 25 were unusable for various reasons. These reasons included (i) repeat too close to vector, (ii) repeat too short, (iii) flanking regions enriched in AT base pairs, or (iv) repeat contained multiple short or imperfect repeats. The majority (>50%) of the repeats were dinucleotide motifs. Primer sets were developed for 26 loci and used to screen a subset of 12 individuals from the sample from Dauphin Island, Alabama. Five loci, all dinucleotide repeats (Table 1), were selected for use in subsequent assays of allelic variation among samples of red snapper. The remaining loci were either monomorphic (four loci), judged to be too polymorphic for extended assay (three loci), or set aside for further primer optimization (11 loci) or additional sequencing (three loci).

A total of 194 individuals from the four geographic localities were genotyped. Allele frequencies at each locus by locality (Table 2) demonstrate the general pattern of one or two (three for locus *Lca64*) common alleles, plus several low-frequency alleles. Observed number of alleles per locus ranged from five to thirteen (43 alleles total), and there were five private alleles (i.e., alleles found only in one sample). Three private alleles were found in the sample from Merida, Mexico, and two were found in the sample from Galveston, Texas. Genotype frequencies at each locus in each sample and pooled across samples did not differ significantly ($P > 0.05$) from expectations of Hardy–Weinberg equilibrium. Expected heterozygosity at each locus (pooled over samples) was 0.143 (*Lca20*), 0.741 (*Lca22*), 0.436 (*Lca43*), 0.779 (*Lca*64), and 0.571 (*Lca91*). Tests of homogeneity of allele frequencies among localities at all five loci, including tests of whether estimated Θ and R_{ST} values differed from zero, were uniformly nonsignificant (Table 3). Estimates of Θ and R_{ST} between pairs of samples at each locus (and across all loci) also did not differ significantly from zero, and pairwise values of either Θ or R_{ST} did not vary with geographic distance between localities (data not shown).

The distribution of alleles at each locus for all samples was fairly uniform among the five loci (Table 2). Except for locus *Lca22,* the next most common alleles at each locus were only one or two repeat units different in size from the most common allele. The (weighted) mean number of repeats per locus was 8.5 for *Lca91,* 8.9 for *Lca20,* 9.0 for *Lca43,* 13.4 for *Lca64,* and 14.7 for *Lca22*; the shortest number of repeats observed was four (*Lca43*) and the largest was 22 (*Lca22*). Interestingly, the two loci (*Lca64* and *Lca22*) that averaged ten or more repeats had higher expected heterozygosities (see above) than did the three remaining loci that averaged fewer than ten repeats, a finding consistent with the correlations observed by Weber (1990) and Schug et al. (1997) between repeat number and levels of polymorphism and repeat number and mutation rate, respectively. In our data set, the correlation between arcsine-transformed, expected heterozygosities and (weighted) mean number of repeats was marginally nonsignificant ($r_{[3]} = 0.789$, $0.10 < P < 0.05$). However, the power of the test of significance was limited because of the small number of loci surveyed. Had we used any of the three loci judged in preliminary screening to be too polymorphic for routine use, the correlation may well have been significant as

TABLE 2.—Allele frequencies (number) at each locus among samples of red snapper from the Gulf of Mexico. Allele number represents the size in base pairs of the fragment amplified by the PCR primers employed.

	Sample site			
Allele	Merida	Galveston	Dauphin Island	Panama City
		Lca20		
207	0.011 (1)	0.000	0.000	0.000
211	0.000	0.021 (2)	0.000	0.000
213	0.045 (4)	0.064 (6)	0.047 (4)	0.042 (4)
215	0.920 (81)	0.883 (83)	0.953 (92)	0.938 (92)
217	0.023 (2)	0.032 (3)	0.000	0.021 (2)
		Lca22		
231	0.000	0.011 (1)	0.000	0.021 (2)
233	0.000	0.021 (2)	0.000	0.011 (1)
235	0.432 (38)	0.404 (38)	0.491 (52)	0.426 (40)
236[a]	0.034 (3)	0.021 (2)	0.019 (2)	0.032 (3)
237	0.023 (2)	0.043 (4)	0.028 (3)	0.000
239	0.205 (18)	0.266 (25)	0.208 (22)	0.202 (19)
241	0.091 (8)	0.074 (7)	0.075 (8)	0.138 (13)
243	0.068 (6)	0.085 (8)	0.057 (6)	0.043 (4)
245	0.057 (5)	0.043 (4)	0.038 (4)	0.043 (4)
247	0.068 (6)	0.000	0.019 (2)	0.043 (4)
249	0.011 (1)	0.000	0.028 (3)	0.021 (2)
251	0.011 (1)	0.032 (3)	0.038 (4)	0.011 (1)
253	0.000	0.000	0.000	0.011 (1)
		Lca43		
175	0.045 (4)	0.011 (1)	0.047 (5)	0.000
179	0.011 (1)	0.000	0.000	0.000
183	0.068 (6)	0.098 (9)	0.142 (15)	0.184 (18)
185	0.773 (68)	0.804 (74)	0.745 (79)	0.663 (65)
187	0.068 (6)	0.054 (5)	0.047 (5)	0.122 (12)
189	0.011 (1)	0.000	0.000	0.020 (2)
191	0.023 (2)	0.022 (2)	0.019 (2)	0.010 (1)
193	0.000	0.011 (1)	0.000	0.000
		Lca64		
158	0.000	0.011 (1)	0.010 (1)	0.022 (2)
160	0.000	0.011 (1)	0.010 (1)	0.000
162	0.000	0.022 (2)	0.010 (1)	0.011 (1)
164	0.295 (26)	0.319 (30)	0.230 (23)	0.287 (27)
166	0.216 (19)	0.202 (19)	0.210 (21)	0.287 (27)
168	0.216 (19)	0.330 (31)	0.310 (31)	0.255 (24)
170	0.160 (14)	0.074 (7)	0.100 (10)	0.074 (7)
172	0.080 (7)	0.011 (1)	0.110 (11)	0.032 (3)
174	0.023 (2)	0.022 (2)	0.000	0.011 (1)
176	0.011 (1)	0.000	0.010 (1)	0.022 (2)
		Lca91		
131	0.011 (1)	0.000	0.000	0.000
133	0.000	0.011 (1)	0.010 (1)	0.000
135	0.023 (2)	0.011 (1)	0.038 (4)	0.000
137	0.511 (45)	0.511 (48)	0.481 (51)	0.402 (37)
139	0.375 (33)	0.414 (39)	0.452 (48)	0.543 (50)
141	0.057 (5)	0.053 (5)	0.019 (2)	0.033 (3)
143	0.011 (1)	0.000	0.000	0.011 (1)
145	0.011 (1)	0.000	0.000	0.011 (1)

[a] Allele 236 differed from alleles 235 and 237 by only a single base. It was observed in 10 individuals and in all samples, and hence it was assumed to be real.

TABLE 3.—Tests of spatial homogeneity in allele distributions among samples of red snapper from the Gulf of Mexico; P_{RB} is the probability based on the randomization procedure of Roff and Bentzen (1989); Θ and R_{ST} are estimates of population subdivision based on Weir and Cockerham (1984) and Slatkin (1995), respectively.

		Θ		R_{ST}	
Locus	P_{RB}	Estimate	P	Estimate	P
Lca20	0.32	−0.001	0.87	−0.007	0.76
Lca22	0.21	−0.003	0.77	−0.009	0.90
Lca43	0.08	0.012	0.08	0.008	0.26
Lca64	0.28	0.001	0.27	0.008	0.12
Lca91	0.43	0.004	0.22	−0.014	0.85

all three loci possessed between 17 and 20 repeats in the cloned allele.

Discussion

Results of this study are consistent with the hypothesis of a single population (stock) of red snapper in the northern Gulf of Mexico (Gulf). Frequencies of alleles at five microsatellite loci were homogeneous across sample localities and two estimates of population subdivision (Θ and R_{ST} did not differ significantly from zero). Sample localities ranged from offshore of Panama City, Florida, and Dauphin Island, Alabama, in the eastern Gulf to offshore of Galveston, Texas, and to Merida, Mexico, in the western Gulf. In addition, there was no apparent relationship between genetic divergence and geographic distance in pairwise comparisons of sample localities, indicating the absence of a significant isolation-by-distance effect relative to genetic divergence. These results are concordant with the previous studies of geographic variation in restriction sites of red snapper mtDNA (Camper et al. 1993; Gold et al. 1997) but not with a study of geographic variation of restriction sites in the mitochondrially encoded 16S rRNA gene (Bortone and Chapman 1995).

The apparent absence of population structure among red snapper from the northern Gulf is consistent with the notion that genetic divergence is precluded by gene flow. Because mark–recapture and ultrasonic-tracking experiments (Beaumariage 1969; Fable 1980; Szedlmayer and Shipp 1994; Szedlmayer 1997) have generally indicated little movement of postlarval red snapper, the simplest hypothesis is that gene flow in red snapper occurs via hydrodynamic transport of pelagic eggs and larvae (Goodyear 1992; Gold and Richardson, in press). If so, an important question becomes how important is local reproduction (spawning) to local assemblages. Avise and Shapiro (1986), for ex-

ample, examined allozyme genotypes in juveniles of the sea bass *Anthias squamipinnis* that were settling onto coral reefs and concluded that pulses of larvae contained genetically unrelated individuals. By using microsatellite loci that possess many alleles at low frequencies, this possibility could be tested in red snapper by examination of young of the year collected from larval grounds. If larvae drift as related cohorts, juveniles settling onto substrate could share rare alleles at locally elevated frequencies. Alternatively, if larvae drift for considerable geographic distances, it becomes less likely that closely related individuals will remain associated. We are currently examining this hypothesis, in part, because of the potential importance of localized spawning to recruitment and, in part, because familial relatedness among individuals within a sample is one potential explanation for the results of Bortone and Chapman (1995).

There are, however, several caveats (listed in Gold and Richardson, in press) to the hypothesis of a single population of red snapper in the northern Gulf. The first, acknowledged by most authors (e.g., Camper et al. 1993), is that one cannot prove a null hypothesis. Studies with larger sample sizes and employing additional microsatellite loci might test the null hypothesis more rigorously. The second is that surface current patterns in the Gulf are not necessarily consistent with unrestricted, two-way transport of pelagic eggs and larvae between northern Florida and the Yucatan Peninsula, the extremes of our sample localities. The figure shown in Gold and Richardson (in press) indicates that the strong loop current passing between the Yucatan Peninsula and Cuba turns eastward through the Florida Straits and might assist egg and larval transport from the Yucatan to Florida but not the reverse. Passage of pelagic eggs and larvae along the northern Gulf where surface currents are not so strong or directional might be a possibility, but given that many of these currents often go in reverse directions, one might expect a ''stepping-stone'' pattern to gene flow, leading to an isolation-by-distance effect. The absence of such an effect with both mtDNA (Gold et al. 1997) and microsatellite (this paper) markers remains perplexing. Use of microsatellite loci that possess many alleles at low frequencies could test this possibility.

A third caveat is that observed genetic homogeneity among the samples of red snapper may reflect past rather than present-day circumstances. We have suggested elsewhere (Gold et al. 1997) that red snapper likely (re)colonized the continental shelf in the northern Gulf after the last glacial retreat and that there might have been insufficient time for allele frequency differences to accumulate between the eastern and western Gulf. Part of the rationale for this suggestion was that most mtDNA haplotypes observed in red snapper are within one or two (presumed) base pair changes of one other, indicating recent divergence from a shared ancestor. The observation in this study that common alleles at the microsatellite loci (*Lca22* excepted) are within one or two steps of one another (Table 2) also is consistent with recent divergence, at least under a stepwise mutation model. In addition, even though the absolute number of migrants necessary to homogenize allele frequencies is relatively small, an assumption underlying the theoretical relationship between measures of population subdivision and gene flow is that populations have reached equilibrium with respect to drift and migration. There also might have been insufficient time for present-day red snapper subpopulations to reach this equilibrium. In either case, present-day subdivision of the red snapper population in the northern Gulf might not be detectable via genetic means, at least not with the small number of loci employed to date.

We are currently in the process of developing additional microsatellite loci for an expanded study of population structure in Gulf red snapper. If differences in allele frequency exist in red snapper from the Gulf of Mexico, the magnitudes of those differences are likely to be small, meaning that several loci and large numbers of individuals will be required to detect significant differences. Development of additional loci is thus needed to more rigorously test the hypothesis of subpopulation (stock) structure in the Gulf and to examine the importance of local reproduction (spawning) to local assemblages.

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