Stock Identification in Nassau Grouper, *Epinephelus striatus*, Using Microsatellite DNA Analysis

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ABSTRACT

The Nassau grouper, *Epinephelus striatus*, is a large serranid which is widely distributed throughout the western tropical Atlantic. This protogynous species forms large spawning aggregations at spatially and temporally predictable locations along the edge of insular and continental shelves. Larvae may remain in the plankton for over a month, but the extent to which they are dispersed during this period is unknown, and little is known about possible population subdivision within the range of this species. The purpose of this study was to identify any stock separation which may exist among localized populations of Nassau grouper within the western tropical Atlantic from four spawning aggregations in Belize, Central America as well as from Florida, Cuba, and the Bahamas. Microsatellite markers were developed using a DNA cloning procedure. Sample sites were then compared based on genotypes obtained by PCR amplification and electrophoresis of two microsatellite regions of Nassau grouper DNA. Tests for deviations from Hardy-Weinberg equilibrium and heterogeneity among allele and genotype frequency distributions revealed no conclusive evidence for stock separation among sample sites. These results imply that spawning aggregations are not exclusively self-recruiting, and that larvae are at least occasionally dispersed over great distances.

KEY WORDS: Nassau Grouper, genetic variation, population structure

INTRODUCTION

The Nassau grouper, *Epinephelus striatus*, is a large serranid inhabiting the coral reefs of the western tropical Atlantic. It is common throughout the West Indies and along the coast of Central and South America to Venezuela. It is rare in the Gulf of Mexico (Hoese and Moore, 1977; Sadovy and Eklund, 1994), but

is found in the waters of southern Florida and Bermuda. *Epinephelus striatus* is commercially harvested in many Caribbean countries, and is among the most economically important finfish species of the Caribbean (Munro, 1973; Thompson and Munro, 1978; Colin, 1992).

This species is protogynous, and for most of its adult life leads a solitary existence as a benthic predator among the crevices and caves of coral reefs (Carter *et al.*, 1994). However, once a year large numbers of individuals gather to form spawning aggregations at specific locations along the reef edge. These spawning aggregations may consist of several thousand to 100,000 individuals (Smith, 1972). The locations and timing of *E. striatus* spawning aggregations are very predictable, usually occurring around the full moon of January or February (see Colin *et al.*, 1987). Many of these aggregations have been known to local fishermen for decades, and have been heavily exploited since as early as the 1920's (Craig, 1969). Declines in Nassau grouper landings have been noted for aggregations in the Bahamas, Belize, Cuba, Honduras, and Mexico; while some spawning aggregations in Belize, Bermuda, Puerto Rico, and the U.S. Virgin Islands have disappeared entirely (Sadovy and Eklund, 1994). Species which form spawning aggregations may be more susceptible to heavy fishing pressure, and sex ratios in populations of this and other grouper species which are heavily fished are often skewed (Colin *et al.*, 1987; Claro *et al.*, 1990; Colin, 1992; Carter *et al.* 1994; Coleman *et al.* 1996). In U.S. waters, Nassau grouper have been severely overfished, and any harvest has been prohibited (South Atlantic Fishery Management Council 1991).

Our knowledge of the reproductive biology and early life history of *E. striatus* is, at best, incomplete. Smith (1972) reported an observation of spawning off Cat Cay in the Bahamas and gave the first account of reproductive behavior in the species. Other aggregations have been document in the Caribbean over the past two decades (cf. Olsen and LaPlace, 1978; Colin *et al.*, 1987; Carter, 1988; Carter *et al.* 1994), as well as the group dynamics and environmental conditions under which these aggregations form (Colin *et al* ,1987; Colin, 1992). Shenker *et al.*, (1993) reported that the larvae recruit to shallow waters about 35 - 40 days after spawning, which is consistent with what is known of the pelagic egg and larval stage of this species (Colin, 1992; J. Tucker, pers. comm.). The extended pelagic stage coupled with the vagaries of Caribbean currents (cf. Wust, 1964; Kinder, 1983) creates something of a conundrum regarding the disappearance of some, and depletion of many, spawning aggregations in the Caribbean Basin. If dispersal from spawning sites is extensive, as we might infer from what is known of the species biology, then why have depleted aggregations not recovered via recruitment from other areas? Certainly, two possibilities exists. First, dispersal may not be as extensive as we might be led to believed from the existing data. Second, the occupation of

particular spawning sites might be a learned behavior and not genetically programmed. These explanations are not necessarily mutually exclusive as dispersal could be restricted and spawning sites fidelity a learned behavior. However, one or the other must hold in order to explain the empirical observation regarding the disappearance of spawning groups throughout the Caribbean.

The primary objective of this study was to identify any stock separation which may exist among localized populations of Nassau grouper within the western tropical North Atlantic. The existence of population structuring would tend to refute the notion that extensive dispersal and, consequently, high gene flow are among spawning aggregations. We have selected genetic analysis of microsatellites markers as the primary means to characterize genetic differences as other methodological approaches have either proven to be singularly uninformative in this species (allozyme electrophoresis, Hatley, in press) or unlikely to be informative based upon previous studies of similar large serranid-like species (eg. analysis of mtDNA variation, Richardson and Gold, 1993, Chapman, 1987, 1989, 1990, Sedberry *et al.*, inpress).

MATERIALS AND METHODS

Sampling and DNA Isolation.

Samples were collected from four Nassau grouper spawning aggregations off the coast of Belize, Central America during the winter of 1993-94 and 1994-95. These four aggregations were located at Caye Glory on the barrier reef and at the northeastern corners of Lighthouse Reef atoll, Turneffe Islands atoll, and Glovers Reef atoll (Figure 1). Samples were also collected from the waters around Key West, Florida throughout the sampling period (Table 1). Since the Nassau grouper has become quite rare in U.S. waters, and no spawning aggregation is currently known in this area, these samples were difficult to obtain. They were therefore collected whenever possible; however, most Florida samples were collected during the winter spawning season. In addition, several samples were obtained from Cuba and from the Bahamas (Figure 2). Belizean samples were obtained from local fishermen, who caught them using handlines, spears, and fish traps. Fish from Florida, Cuba, and the Bahamas were speared or caught on hook and line (Table 1). Sample size depended upon availability.

Samples consisted of approximately 0.5 cm^3 of heart muscle tissue from each individual. The heart tissue was immediately placed in a 1.5 ml centrifuge tube containing 1 ml of an SDS/urea lysis solution (1% sodium dodecyl sulfate/6M urea). The samples were then transported to the lab and stored at room temperature. Total genomic DNA isolations were then performed on all tissue samples using two PCI (phenol/chloroform/isoamyl alcohol, 25:24:1)

extractions and a subsequent chloroform extraction. Samples were then dialyzed at 4° C using Spectra/Por 4 (MWCO:12,000-14,000) dialysis membrane tubing (Baxter) for 24 - 48 hours against TE buffer (10 mM Tris pH 8.0 / 1 mM EDTA) to remove any remaining phenol or chloroform. Groups of 20 - 25 samples were dialyzed in 2 l of buffer, which was changed twice during the dialysis period. Five microliters of each DNA isolation was then electrophoresed on a 0.8% agarose gel in TAE buffer to confirm the presence of an adequate amount of high molecular weight DNA.

Cloning

In general, the methods used in this study for cloning follow those established by Ausubel *et al.*, (1987). One DNA sample was digested with the Dpn II restriction enzyme and ligated into Bam HI-digested M13 vector. Recombinant M13 DNA was then transfected into *E. coli* cells and cultured on YT agar plates. Clones were screened for the presence of inserts containing GT-repeat microsatellites. Clones testing positive for GT-repeats were isolated and the inserts were sequenced using the Sequenase 2.0 (US Biochemical) commercial sequencing kit. DNA sequence data obtained for regions flanking GT-repeat microsatellites were analyzed with the DNASIS (Hitachi Software Engineering Co., LTD.) software package to design primers for use in PCR amplification. Primers were synthesized by the Biochemistry Department at the Medical University of South Carolina.

Microsatellite PCR

The primer sets designed from microsatellite DNA sequence data were used to amplify total genomic DNA obtained from Nassau grouper heart tissue samples (see above). Several other microsatellite primer sets designed for use in other studies were also tested. Optimum cycling parameters for these microsatellite primer sets were 30 seconds at 94°C, 30 seconds at 50°C, and 30 seconds at 72°C for 35 cycles. The optimum PCR reaction concentrations were: 0.2 mM for each dNTP, 1X Kocher buffer (0.067 M Tris, 0.017 M ammonium sulfate, 0.01 M -mercaptoethanol, pH 8.8), 2.5 mM magnesium sulfate, and 0.25 µM for each primer, with 2.5 units of Taq polymerase and 2 µl of a total genomic DNA extraction in a total volume of 100 µl. Amplification products were screened for length polymorphisms by electrophoresis on 15% polyacrylamide TBE-buffered gels (BioRad Protean II, 4 - 6 hours at 20 mA per gel) stained with ethidium bromide. Genotypes for each individual were determined by comparison with both a molecular weight standard and a Nassau grouper sample for consistency.

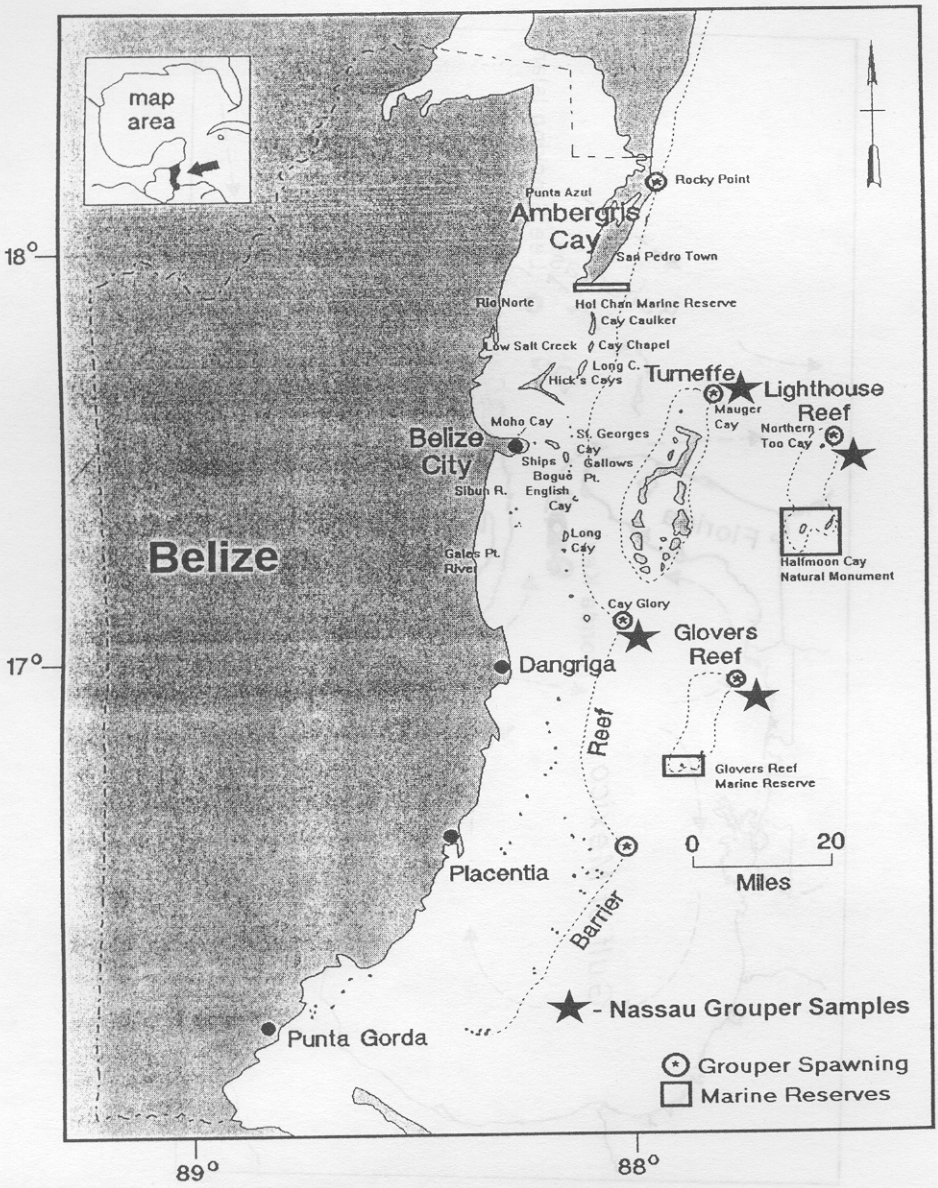

Figure 1. *Epinephelus striatus*. Map showing the locations of sample sites in Belize.

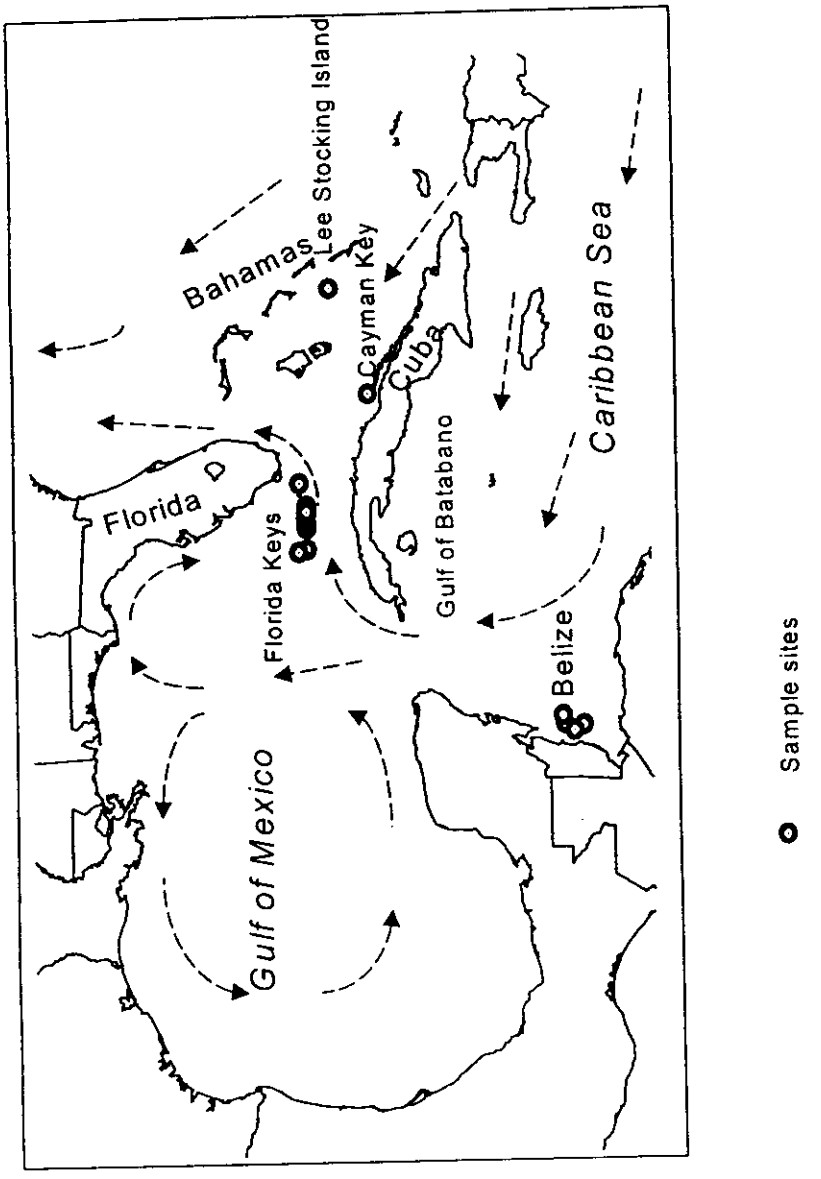

Figure 2. *Epinephelus striatus*. Map showing sampling sites throughout the Caribbean in relation to typical pattern of surface currents in the western tropical Atlantic for the month of January (from Wust, 1964).

Table 1. *Epinephelus striatus.* Location, date, and number of Nassau grouper samples collected from 1993-1995.

Sample Site	Collection Date	Number
Belize		
Caye Glory	1/94	32
	1/95	25
Turneffe Is	**12/93**	**45**
(Mauger Caye)	1/95	64
Lighthouse Rf.	12/93	33
(North. Too Cay)	1/95	74
Glovers Atoll	1/94	74
	1/95	49
United States		
Key West, Florida	1/94	30
	11/94	5
	12/94	12
	1/95	12
	2/95	6
	3/95	12
	4/95	2
	5/95	10
	7/95	5
	8/95	2
	10/95	1
Cuba		
Cayman Key	1/95	7
	10/95	14
Bahamas		
Lee Stocking Island	4/94	7
	7/94	3
	8/94	13

Statistical Analysis

Chi-square tests were performed to assess conformity of genotype distributions with Hardy-Weinberg expectations, except in cases where any expected value was less than 1 or when greater than 20% of the expected values were less than 5. Log-likelihood ratio tests for contingency tables (G-tests *sensu* Zar 1984) were performed on allele class distributions to assess heterogeneity among sample sites. Roff-Bentzen chi-square tests (Roff and Bentzen, 1989) were also performed on genotype distributions. F-statistics (Wright, 1965; Nei, 1965)

were calculated for both loci in order to assess the relative contributions of within-site and among-site components of genetic variance.

RESULTS

Microsatellite Loci.

The cloning effort to identify Nassau grouper microsatellite loci resulted in the design of primer sets for use in the amplification of two Nassau grouper microsatellite loci (Table 2). One of these primer sets, Est 127, amplified a large (>500 bp) region of DNA. This region did not appear to be polymorphic, and was not useful for stock identification purposes. The other primer set, Est 534, was used in this study. Several additional microsatellite primer sets designed for use in other fish species, including gag (*Mycteroperca microlepis*, Serranidae), wreckfish (*Polyprion americanus*, Polyprionidae), and European sea bass (*Dicentrarchus labrax*, Serranidae), were also tested on Nassau grouper DNA samples (Table 2). With the exception of the *D. labrax* primers, all of these successfully amplified Nassau grouper DNA samples. While most of these amplified monomorphic regions of DNA, some regions were quite polymorphic. One of these primer sets (Gag 010), designed by Eleby and Ball (unpubl.) for gag grouper, was used in this study.

Both the Est 534 and Gag 010 microsatellite primer sets consistently amplified DNA from almost all samples. However, Est 534 amplified only one of the samples obtained from the Bahamas, while Gag 010 amplified all of them. Other sample size differences between the two data sets are the result of the failure of either primer set to amplify DNA from a few individuals.

Both microsatellite loci used in this study were highly polymorphic, with more than 10 alleles at each locus. At the Est 534 locus, alleles ranged from approximately 50 bp in length to over 110 bp. At the Gag 010 locus, alleles ranged from approximately 104 - 174 bp. Due to the resolution limitations of the electrophoresis protocol employed and the small sample sizes for some location, alleles were combined into allele classes based upon size intervals. The size intervals were chosen by dividing the database approximately into fourths. The Est 534 alleles were combined into the following 4 allele classes: A (<80 bp), B (82-88 bp), C (90-100 bp), and D (>100 bp)(see Table 3). Alleles at the Gag 010 locus were also combined into 4 allele classes: A (<130 bp), B (130-140 bp), C (142-156 bp), and D (>156 bp)(see Table 4). The overall and individual site genotype distributions for these two loci were calculated using the allele class designations of each individual (Tables 5 and 6).

Table 2. *Epinephelus striatus.* Microsatellite and mtDNA primer sets tested on Nassau grouper DNA samples in this study, along with the test results (P = polymorphic, M = monomorphic, -- = no amplification or inconsistent amplification) and the source of the primer sets. Asterisks indicate primer sets used in this study for stock identification.

Primer Designation	Sequence	Result	Source
Est127F	5'-GATCTCTGTGTGTGTGTG	M	current
Est127R	5'-CAATCAACAGAAGAGC		study
Est534F*	5'-TGTTTTGCTGCTTCACTCTATG	P	current
Est534R*	5'-AGCTGTAAACACACACAC		study
Gag007F	5'-CTGTAATAGACAACCCACTGTAC	P	Eleby
Gag007R	5'-CCTGTAGCATCTTCACTAGCTG		and Ball (unpubl.)
Gag010F*	5'-CTAGAGGATCATTTGACAATGTAG	P	"
Gag010R*	5'-CCTGACTAATCCACAGTAATTGC		
Pam002F	5'-GATCAGAGGCAGAGCGAGTGG	M	"
Pam002R	5'-ACAGCCTCGCAGGTCTCCTC		
Pam006F	5'-CTGATGGTTAAGCTGGTGC	M	"
Pam 006R	5'-CAATGTGTCTAACATTCGCC		
Pam010F	5'-GTGGCCTTGGTGGAAGCAG	M	"
Pam010R	5'-GCGCACTAGGTGCCAAATATC		
Pam013F	5'-GATCTGCCAGATGGAAAGAC	M	"
Pam013R	5'-CCACTCACTGGTGCAGAAAC		
Pam017F	5'-CTGACTTTGTATGCATGTCCG	M	"
Pam017R	5'-CTGACAGCTGTCAAGAGAATG		
Pam021F	5'-GATCTGACAATGACCACTTTACT	P	"
Pam021R	5'-CTCTATAGGAATGCTGCTTTTG		
Pam025F	5'-CAAATAACATATGCACACATCAGC	M	"
Pam025R	5'-CTTCTCTGGCATGAATGTTTG		
Pam035F	5'-GGCTCGCTCTGGGCATTAC	M	"
Pam035R	5'-ACAACGTGAGCTATACCCGCC		
Pam039F	5'-CTTAGTTTCTCTTGGGAGTGTC	M	"
Pam039R	5'-CCTAACTATAGACCAAATGTTG		
Dla003F	5'-AAACAGTCTTTCAAGTGGTC	--	Garcia de Leon
Dla003R	5'-ATGGACAACTGCTGTCATAG		*et al.*,
Dla006F	5'-ACAGCAAAGATAAACATCTG	--	1995
Dla 006R	5'-TTCATGATGTTTCACCAGG		
Dla008F	5'-TGAGGAAGGTTTGAGAGAC	--	"
Dla 008R	5'-TTCTGCTCCTTAGATGAAC		
Dla009F	5'-TACAGCACCTCTTGAGAAGGG	--	"
Dla009R	5'-GGCGTACTGCAGGAAAACAG		

Table 3. *Epinephelus striatus*. Allele class frequency distributions for each sample site at the Est 534 microsatellite locus (A: <80bp, B: 82 - 88bp, C: 90 - 100bp, D: >100bp).

Sample Site	Allele Class				Sample Size
	A	B	C	D	(2N)
Belize					
Caye Glory	0.19	0.36	0.24	0.21	104
Glovers Atoll	0.24	0.28	0.26	0.22	238
Lighthouse Reef	0.22	0.27	0.28	0.23	202
Turneffe Island	0.19	0.28	0.27	0.26	184
Florida	0.18	0.33	0.24	0.24	176
Cuba	0.29	0.24	0.12	0.36	42
TOTAL	0.21	0.29	0.25	0.24	946

Table 4. *Epinephelus striatus*. Allele class frequency distributions for each sample site at the Gag 010 microsatellite locus (A: <130bp, B: 130 - 140bp, C: 142 - 156bp, D: >156bp).

Sample Site	Allele Class				Sample Size
	A	B	C	D	(2N)
Belize					
Caye Glory	0.09	0.42	0.25	0.24	104
Glovers Atoll	0.11	0.28	0.28	0.33	234
Lighthouse Reef	0.12	0.32	0.29	0.27	202
Turneffe Island	0.10	0.28	0.32	0.31	200
Florida	0.11	0.23	0.36	0.29	166
Cuba	0.14	0.26	0.38	0.21	42
Bahamas	0.11	0.35	0.24	0.30	46
TOTAL	0.11	0.30	0.30	0.29	994

Table 5. *Epinephelus striatus*. Genotype distributions for each sample site at the Est 534 microsatellite locus.

Sample Site	Genotypes									
	AA	AB	AC	AD	BB	BC	BD	CC	CD	DD
Belize										
Caye Glory	3	5	6	3	10	4	8	6	3	4
Glovers Atoll	8	12	14	14	8	27	12	8	5	11
Lighthouse Rf.	4	13	12	11	3	21	15	6	11	5
Turneffe Is.	4	10	11	6	7	14	14	5	14	7
Florida	3	6	11	9	15	9	13	5	13	4
Cuba	0	5	1	6	0	3	2	0	1	3
TOTAL	22	51	55	49	43	78	64	30	47	34

Table 6. *Epinephelus striatus*. Genotype distributions for each sample site at the Gag 010 microsatellite locus.

Sample	Site	Genotypes								
	AA	AB	AC	AD	BB	BC	BD	CC	CD	DD
Belize										
C. G.	1	3	14	6	7	8	1	10	2	0
Glovers	19	14	20	6	12	20	7	10	6	3
Light.	9	14	17	5	12	13	8	15	4	4
Turneffe	10	21	15	5	10	17	5	8	8	1
Florida	9	14	10	6	17	7	5	8	6	1
Cuba	2	1	4	0	3	6	3	0	1	1
Bahamas	2	4	4	2	1	4	1	3	2	0
TOTAL	52	71	84	30	62	75	30	54	29	10

Hardy-Weinberg Equilibrium

Chi-square tests for conformity to Hardy-Weinberg expectations (HWE) were first performed on genotype distributions of individuals from each sample site (Table 7). Sample sizes from Caye Glory, Cuba, and the Bahamas were too small to permit valid chi-square analysis. The only case in which an individual sample site did not conform to HWE was the Glovers Reef site at the Est 534 locus ($\chi^2 = 17.45$, $p = 0.04$). When all of the Belizean sample sites were combined, they conformed to HWE at both loci, and when the non-Belizean sites were combined (Florida, Cuba, and the Bahamas), they also conformed to HWE. When all sample sites were combined, they conformed to HWE in the case of the Est 534 locus. However, they differed significantly from HWE at the Gag 010 locus ($\chi^2 = 19.58$, $p = 0.02$). When the sites for which sample sizes were small (Caye Glory, Cuba, and the Bahamas) were removed and the others combined, the result was the same: at the Est 534 locus, no significant difference was detected, while at the Gag 010 locus, the result was again significant ($\chi^2 = 19.23$, $p = 0.02$).

Heterogeneity Tests

The Belizean samples were collected at two distinct time intervals (Winter 1993 - 1994 and Winter 1994 - 1995), and sample sizes for both years were large enough that it was possible to test for differences between sample periods. G-tests of the first year allele class frequency distributions versus the second year allele class frequency distributions yielded no significant results, indicating that there was no significant shift in allele class frequency distribution between the two sample years. Therefore, all samples from each sample site were pooled. G-tests were performed on a contingency table containing four different sets of allele class frequency distributions: (1) frequency distributions from all sample sites, (2) only the distributions from the individual Belizean sample sites, (3) the combined Belizean sample sites distribution plus the Florida, Cuba, and (in the case of the Gag 010 data set) Bahamas distributions, and (4) the combined Belizean distribution and the combined frequency distribution of the other sample sites. None of the tests produced a significant result at either the Est 534 or Gag 010 locus (Table 8).

Roff-Bentzen chi-square tests were then performed on contingency tables containing genotype frequency distributions. Again, tests were performed on: (1) the overall data sets, (2) the Belizean site data sets, (3) the combined Belizean data set vs. Florida, Cuba, and the Bahamas, and (4) the combined Belizean data set vs. the combined data set from other sample sites (Table 9). For the Gag 010 data set, none of the tests were significant. However, for the Est 534 data set, there was significant heterogeneity in the overall data set ($\chi^2 = 65.20$, $p = 0.03$), and in the combined Belize sites vs. Florida and Cuba data set ($\chi^2 =$

32.87, p = 0.02). Tests on Belizean sample sites and combined Belize vs. combined other were not significant (Table 9).

F-Statistics

F-Statistics calculated for both Est 534 and Gag 010 data sets showed that in the case of the Est 534 data set, F_{IS} (0.021) was greater than F_{ST} (0.005) by a factor of 4 (Table 10). For the Gag 010 data set, the difference was greater than an order of magnitude, with $F_{IS} = 0.106$ and $F_{ST} = 0.007$.

Table 7. *Epinephelus striatus.* Summary of tests for Hardy-Weinberg equilibrium at both microsatellite loci(* indicates significant values, NA indicates that the sample size was too small to perform this test).

	Locus	
Individual Sample Sites:	**Est 534**	**Gag 010**
Caye Glory	NA	NA
Turneffe Is.	$\chi^2 = 2.10$ $p = 0.99$	$\chi^2 = 1.99$ $p = 0.99$
Lighthouse Reef	$\chi^2 = 6.27$ $p = 0.71$	$\chi^2 = 12.30$ $p = 0.20$
Glovers Atoll	$\chi^2 = 17.45$ $p = 0.04^*$	$\chi^2 = 9.73$ $p = 0.37$
Florida	$\chi^2 = 9.42$ $p = 0.40$	$\chi^2 = 12.11$ $p = 0.21$
Cuba	NA	NA
Bahamas	NA	NA
Combinations of Sample Sites:		
Belize	$\chi^2 = 9.01$ $p = 0.44$	$\chi^2 = 13.79$ $p = 0.13$
Florida,Cuba, and Bahamas	$\chi^2 = 5.63$ $p = 0.78$	$\chi^2 = 7.25$ $p = 0.61$
All	$\chi^2 = 6.05$ $p = 0.74$	$\chi^2 = 19.58$ $p = 0.02^*$
All except Caye Glory Cuba, and Bahamas	$\chi^2 = 5.70$ $p = 0.77$	$\chi^2 = 19.23$ $p = 0.02^*$

Table 8. *Epinephelus striatus.* Summary of G-tests for heterogeneity among allele class frequency distributions for both microsatellite loci.

Data Set	Locus Est 534	Gag 010
Overall	G = 13.61 p = 0.56	G =18.70 p = 0.41
Belize	G = 4.29 p = 0.89	G = 10.1 p = 0.34
Belize (combined) vs. Florida, Cuba, and Bahamas	G = 9.32 p = 0.16	G = 8.31 p = 0.50
Belize (combined) vs. Other (combined)	G = 2.39 p = 0.50	G = 5.05 p = 0.17

Table 9. *Epinephelus striatus.* Summary of Roff-Bentzen chi-square tests for heterogeneity among genotype frequency distributions for both microsatellite loci (* denotes significant values).

	Locus Est534	Gag010
Overall	$\chi^2 = 65.20$ p = 0.03*	$\chi^2 = 58.75$ p = 0.31
Belize	$\chi^2 = 34.26$ p = 0.16	$\chi^2 = 34.40$ p = 0.15
Belize (combined) vs. Florida, Cuba, Bahama's	$\chi^2 = 32.87$ p = 0.02*	$\chi^2 = 23.95$ p = 0.64
Belize (combined) vs. Other (combined)	$\chi^2 = 10.78$ p = 0.31	$\chi^2 = 5.11$ p = 0.85

DISCUSSION AND CONCLUSIONS

The results of this study are relevant to a number of issues regarding the genetics, reproductive biology and zoogeography of tropical groupers. First, is the issue of genetic variation in the larger members of the lower percoid groups. Second, is the relationships among individuals of a spawning aggregation. Third, is the genetic relationships among spawning aggregations along the Belize barrier reef. Fourth, is the potential for population differentiation in the Caribbean. Finally, is the potential for dispersal in larval or adult stages and/or

learning behavior to underpin similarities of differences at each of these levels. We will treat each of these items separately, eventhough they are highly interdependent.

Genetic Variation

It is a common observation that many of the larger members of the lower percoids exhibit restricted genetic variation compared to fishes in general (cf. Grove *et al.*, 1975, Hateley, in press, Chapman, 1987,1989,1990, Wirgin, 1989, Gold *et al.* 1993, Sedberry *et al.*, 1996). From our own unpublished work, we have observed little or no variation in the mitochondrial genome of several grouper species including *E. striatus, E. itajara, E. tigris, M. microlepis*, and *E. bonaci* and yet the microsatellite loci used in this study are highly polymorphic in all of these species. In the absence of overt differences in mutation rates and sex ratios, we expect gene diversity to be lower in mtDNA than nuclear genes, because the effective population size of the mitochondrial genome will be smaller than that of the nuclear genome (Takahata, 1983, Takahata and Slatkin, 1983, Birky *et al.* 1989). These conclusions are modified somewhat in protogynous species where females may outnumber males by a considerable margin (Birky *et al.*, 1989). As sex ratios become more strongly biased toward females, the more mtDNA diversity to nuclear gene diversity we expect (Birky *et al.*, 1989). Taken at face value, the limited mtDNA variation noted in Nassau and many grouper species would suggest that N_{ef} (the effective female population size) is quite small and N_e should be smaller still, with a concomitant reduction in nuclear gene variation. Clearly this is not the case, as substantial variation has been noted at microsatellite loci studied here. The most plausible explanation is that the rates of mutation in microsatellites are sufficiently high, relative to mtDNA and allozymes, to overcome whatever limitations are imposed on genetic variation by the biology of these species.

Genetic Relationships within Spawning Aggregations

The effect of fishing pressure on Nassau grouper spawning aggregations has been documented by Carter (1990). In that study, it was shown that females were significantly more common, and both sexes were significantly smaller, in heavily exploited aggregations. The latter observation is typical in over fished stocks (Rothschild, 1986), while the former is typically observed in over harvested groupers (cf. Carter *et al.*, 1990, Coleman *et al.*, 1996). The altered sex ratios have been attributed to the removal of larger and more aggressive (most male) individuals by hook-and-line fishing (cf. Gilmore and Jones, 1992). This should lead to a diminished N_e and the potential exists for changes in gene frequencies between years or among year classes for this reason. However, no differences in gene frequencies were noted between the 1994 and 1995 at any of

the sampling locations. Gene frequency differences among year classes would be manifest in our data as deviations from HW expectations within locations. Such deviations have been observed in other species (cf. Gyllensten and Ryman, 1985, Chapman, unpub. data) and been attributed to the effects associated with small population sizes. We see no evidence of departures from HW within spawning aggregations of Nassau grouper and this may imply that the population sizes have not been so reduced as to result in temporal changes in gene frequencies.

If true, the above conclusion would be heartening as one of our major concerns is the loss of genetic variation in heavily exploited populations. However, the conclusion should be viewed with some reservations as the effects may be masked by the methods used to bin alleles in this study. The binning process undoubtedly results in the loss of some information (Weir, 1992), but was unavoidable due to the number of potential genotypes versus the samples sizes. While we cannot totally discount the possibility that temporal variation exists in Nassau grouper and may reflect reductions in N_e, the effects are not so sever that they have influenced the distribution of the most common allelic states. The upside of this is that current fishing pressures do not appear to have reduced genetic variation in this species. The downside, is that is that such changes may lag behind population bottlenecks (cf. Nei *et al.*, 1975) and will become manifest in the future.

Genetic Variation within the Belize Reef

Within the Belizian reef system, Nassau groupers spawn on the northeast points along the barrier reef (Cay Glory) and atolls (Glovers, Lighthouse and Turniffe). The atolls are separated from the barrier reef, and each other, by 10-20 km of open water that varies in depth from 400 to 1000 m. While there is no documented evidence that these expanses of deep, open water serve as a barrier to adults, we deem it unlikely that they would make such migrations on a routine basis. The depth is well below that normally occupied by Nassau grouper and, to our knowledge, the species has never been observed in open waters between the atolls. The distance alone is not so imposing, as the species is widely dispersed over the barrier reef and around the atolls during the non-spawning season. Individuals must, therefore, undertake movements of 20 km or more to reach the spawning areas. These movements take place along the reef rather than across open water (J. Carter, pers. comm.)

If the expanses of open water serve as a effective barrier to adult movements between the spawning aggregations, the lack of genetic differentiation among these aggregations would suggest two possibilities. First, the spawning aggregations may be recently derived from a common ancestral stock. This is unlikely because the atolls are quite ancient dating (Dillion and Vetter, 1973) and sea level fluctuations, since the Pliocene, have not been sufficient to make the

atolls any more accessible in the recent past than they are today. Thus, the possibility that adult migration among spawning aggregations was more extensive in the recent past seems unlikely, but cannot be totally dismissed. The second explanation for the lack of genetic differentiation among these spawning aggregations, is that gene exchange occurs through pelagic dispersal in egg or larval stages. As noted above, the juveniles recruit to lagoons some 35-40 days after the spawning peak (Shenker *et al.* 1993) and even modest currents would lead to some exchange among the various spawning aggregations in Belize.

Genetic Differentiation in the Caribbean Basin

The absence of substantial genetic differentiation among the widely dispersed spawning aggregations surveyed in this study, tends to reinforce the conclusions stated above regarding gene flow within the Belizian aggregations. In the present case, the restrictions on adult migration are even stronger because the distances between suitable habitats are even greater. The potential for genetic exchange among Nassau groupers sampled here (Belize, Cuba, Florida and the Bahamas) mediated by planktonic dispersal is greatly enhanced by the strong currents that flow from Belize through the Yucatan and Florida Straits (cf. Shulman and Bermingham, 1995). The effectiveness of planktonic dispersal must be viewed with some caution as recruitment occurs during a narrow time window (4-5 days, Shenker *et al.* 1993) and the likelihood of encountering a suitable lagoon at long range must be remote. While, the probability that an individual egg or larvae would survive this considerable trek is undoubtedly small, the effect on genetic differentiation is dependent upon N_e in the recipient populations. If the rate of migration, mediated thought larval drift, over such large distances is as small as we might imagine, our genetic data suggests that N_e is fairly large so that the product of $N_em > 1$ (cf. Wright, 1943). This, in turn, would suggest that migration has been sufficient to overcome the effects of genetic drift during the recent declines in Nassau grouper populations. Again, this conclusion is subject to the same reservations outlined above concerning the lags in gene frequencies changes discussed by Nei *et al.* (1975) and the possibility that the effects are masked by binning allelic classes (Weir, 1992).

Shulman and Bermingham (1995) also found limited genetic differentiation among populations of five reef associated species and reached the conclusion that neither egg type (pelagic vs. non-pelagic) nor length of pelagic stage was a simple predictor of genetic structure of populations. We have no quibble with this conclusion, as we doubt that there are any simple predictors of population structure. We do, however, find it disquieting that such a conclusion would be reached without a discussion of the relative N_e among the species studied, their relative fecundities, or mention of the fact that two of the species (*Halichoeres bivattatus* and *Thallosoma bifasciatum*) are protogynous. The relevance of

dispersal mechanisms to genetic population structure should be assessed in light of N_e (cf Wright, 1943).

The Extinction of Spawning Aggregations and High Gene Flow

Many spawning aggregations of Nassau grouper in the Caribbean Basin have disappeared over the past two decades including those at Rocky Point Belize, at several locations near Puerto Rico and in Bermuda. The latter is particularly disturbing because the species was common in Bermuda, but is rarely seen at present (B. Luckhurst pers. comm.). This tends to indicate that current migration rates from stronger populations are not sufficient to rebuild this depleted stock. It also begs the question, if current migration is so limited, how did the Bermuda population maintain itself without substantial self-recruitment? Further, if Nassau grouper populations can be self-recruiting, why is there no evidence of population subdivision in the data presented here? The only plausible explanation that occurs to us is that there must be a delicate balance between genetic drift (mediated by self-recruitment and N_e) and gene flow. In other words, most population sizes are large enough that limited migration compensates for genetic drift, but migration is not sufficiently high to rebuild a depleted stock, at least not in the short term. Bermuda might be a special case, in that it is the northern outpost of the Nassau grouper distribution, it is downstream from the center of the species range, and we know nothing about the genetic structure of the former population. Nonetheless, it serves as a warning to those involved in fisheries genetics. We have tacitly assumed that genetic homogeneity among stocks implied that the effects of over-exploitation on one stock would be dissipated among them all. The depletion of the Bermuda population may indicate that this assumption needs closer scrutiny, especially if there is a delicate balance between migration and drift.

The disappearance of the Rocky Point spawning aggregation is puzzling because adult and juvenile Nassau grouper have been observed in the vicinity and the location is not far removed from strong spawning aggregations. Thus, we cannot ascribe the loss of this aggregation to a limited supply of recruits. The failure of depleted populations to rebound by natural recruitment is a common occurrence in salmon and is due to imprinting of early life stages. Philopatry and, hence, population subdivision is strongly enforced among spawning populations by this mechanism. This is not likely to be a reasonable explanation in Nassau groupers as our data indicate that the spawning aggregations are not highly structured and it is difficult to imagine how imprinting would occur in pelagic dispersed larvae. The available data may indicate that occupation of a particular spawning area is a learned behavior. The disappearance of spawning aggregation could then be attributed to a lack of experienced adults to lead first time spawners to the spawning area.

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