Use of Single-stranded Conformational Polymorphisms (SSCP) to Detect Species Relationship and Population Structure in the Atlantic Sharpnose Shark (*Rhizoprionodon terraenovae*) and the Caribbean Sharpnose Shark (*R. porosus*)

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ABSTRACT

The Atlantic sharpnose shark, *Rhizoprionodon terraenovae,* and the Caribbean sharpnose shark, *R. porosus*, are small coastal-temperate and tropical sharks of the continental shelves that overlap in distribution along the Gulf of Mexico, Florida and around the Yucatan Peninsula to the Caribbean Sea. In order to properly distinguish between the two species, current methods require counting caudal and precaudal vertebrate. These species used to be highly abundant within their prospective ranges, but current fishery pressures have caused declines in landing. Assessment of population size and structure is necessary to determine future management plans for this species. Use of SSCP is a viable method to differentiate between the two species and to measure population structure. SSCP analysis was able to detect 11 unique haplotypes in the Atlantic sharpnose shark and 3 haplotypes in the Caribbean sharpnose. Sequencing confirmed the variation between the species with an estimated nucleotide divergence as high as 1.08%.

KEY WORDS: Mitochondrial DNA, population genetics, sharks

Uso de los Polimorfismos Solo-trenzados de Conformational (SSCPs) de Detectar la Relación de la Especie y la Estructura de la Población en los de *Rhizoprionodon terraenovae* y *R. porosus*

Los tiburones atlánticosde terraenovae el Rhizoprionodon de Sharpnose, y el tiburón del Caribe Sharpnose son tiburones costero-templados y tropicales pequeños de las plataformas continentales que se traslapan en la distribución a lo largo del golfo de México, la Florida y alrededor de la península de Yucatan al mar del Caribe. Para distinguir correctamente entre las dos especies, los métodos actuales hay que contar el vertebrado caudal y precaudal. Estas especies eran altamente abundantes dentro de sus gamas anticipadas, pero las presiones actuales de la industria pesquera

han causado declinaciones en el aterrizaje. El gravamen del tamaño y de la estructura de la población es necesario determinar los planes futuros de la gerencia para esta especie. El uso de SSCPs es un método viable a distinguir entre las dos especies y para medir la estructura de la población. El análisis de SSCP podía detectar los haplotypes únicos indicativos a una especie particular. El uso de ordenar confirmó la variación entre la especie con una divergencia estimada del nucleotide de hasta 1,08%. El análisis de AMOVA que comparaba tiburones atlánticos del sharpnose recogió en la bahía de Campeche con las muestras obtenidas a partir de cuatro otros sitios a través del golfo de México y a lo largo del Océano Atlántico del noroeste encontró que los once haplotypes observados fueron distribuidos uniformemente a través de la gama (ST = 0,022, p = 0.191). Las diferencias al menos significativas fueron observadas en en parejas análisis entre la bahía de Campeche y de otros sitios. Estas diferencias desaparecieron cuando las muestras fueron examinadas basaron en los datos temporales que sugerían que el bottlenecking genético está ocurriendo en los terraenovae del R. a lo largo de la costa mejicana.

PALABRAS CLAVES: SSCPs, Tiburón de atlántico y del Caribe del sharpnose, *Rhizoprionodon terraenovae*, *R. porosus*

INTRODUCTION

Sharks have been subjected to intense directed fisheries in recent years that have caused shark populations to decline. These declines are often attributed to sharks evolving a "k-selected" life history in which few young are born, growth is slow with maturity occurring after several years, and long reproductive cycles (Stone et. al. 1998). Thus, the probability of a stock under exploitation collapsing is high even with moderate fishing pressure. A collapse of shark stocks in the Gulf of Mexico would jeopardize the fisheries and entail significant economic and social costs affecting livelihoods for hundreds of families in the region (Bonfil 1997, Marquez-Farias and Castillo-Geniz 1998). The need to properly assess a particular fishery is of utmost importance in order to create proper management plans if this is to be prevented. Population size, fecundity, immigration, emigration, mortality (both natural and anthropogenic), and genetic composition must be assessed in order to determine susceptibility to fishery pressures. With advances in molecular biology, methods are now available that can aid in the assessment of a fishery, particularly in regards to migration and gene flow. The use of Single-Stranded Conformational Polymorphisms (SSCP) is a relatively new method that can yield a tremendous volume of data regarding population structure at a fraction of the cost using other methods (Orita et al. 1989, Hongyo et al. 1993).

Of particular interest in the Gulf of Mexico and the Caribbean Sea, is the susceptibility of the Atlantic sharpnose, *Rhizoprionodon terraenovae* and the Caribbean sharpnose, *R. porosus*, to fishing pressures. These small coastal sharks are the most common sharks in the western Atlantic, with the Atlantic sharpnose occurring from New Brunswick to Florida in the northwestern Atlantic and

throughout the Gulf of Mexico, being replaced by the Caribbean sharpnose south of the Yucatan peninsula to Brazil (Springer 1964, Compagno 1984). During the early 1990s, landings of the Atlantic sharpnose shark in Mexico and the United States had increased to unprecedented levels. While the United States implemented a management plan in 1995 to regulate the fishery, stocks in Mexican coastal waters remained unprotected. Demographic models predicted that if fishing pressures remained constant, a crash in the population was inevitable (Cortez 1995, Bonfil 1997, Marquez-Farias and Castillo-Geniz 1998). Due to the wide distribution of these species, the need to assess the population structure is important. However, both species are morphologically similar when examined externally. Current methods for differentiating between the two species require counting of precaudal vertebrae (Springer 1964, Compagno 1984). Questions have also been posed whether the two species are separate, or should be considered subspecies to each other. The purpose of this study is to characterize the genetic relationship between *R. terraenovae* and *R. porosus* using SSCP by testing the hypothesis that two distinct species exist.

MATERIALS AND METHODS

Fin clippings of *R. terraenovae* representing three regions and R. porosus were obtained from six different sampling sites (Figure 1). Thirteen samples originating from off the coast of Tabasco and Campeche, Mexico, were obtained through Texas Parks and Wildlife (TPWD). Thirty-two samples were collected off the Texas-Louisiana coast and Dauphin Island from recreation and commercial fishermen between September 2000 and November 2001. Finally, Thirty-two samples were collected with the cooperation of the Virginia Institute of Marine Science (VIMS) during shark longlining cruises in the Chesapeake Bay during July 2001 or originated from Georgia/South Carolina Coast. Twelve fin clippings from positively identified *R. porosus* were obtained from the NSU Oceanographic Center in Florida, where ten originated from Belize, and two were collected off Brazil. All fin clippings collected were preserved in 95 % ethanol and stored at room temperature.

The mtDNA was isolated by cutting the fin clippings into small pieces, and treating the fragments with Proteinase K. The samples were passed through Qiagen DNAEZ extraction columns following the manufacturers instructions. The Polymerase Chain Reaction (PCR) was then performed to isolate the d-loop and the Cytochrome b (Cyt b) gene from the mtDNA. Primers (5'-TTGGGTTTCTCGTATGACCG-3') and (5'-AGAGCGTCGGTCTTGTAAACC-') were used to isolated the D-loop region, and primers (5'- GCCATAAATCGAAAAACCACCCA –3') and (5'-AAGTATCATTCGGGTTTGATATG –3') were used to isolate the Cyt b gene. DNA amplification was performed using the Gibco/BRL^{0} Platinum High Fidelity PCR supermix using 100 ng DNA-Template and 0.5 μM of each primer. Amplification occurred for 30 cycles (30 s at 95° C, 30 s at 50° C, 60 s at 72 C°). The PCR products were then purified using the Qiagen^{0} PCR purification kit. 600

ng of PCR product were then digested with either Rsa I (d-loop) or Alu I (Cyt b) overnight in a total volume of 30 µL. SSCP analysis was performed following Hongyo et al. (1993). Samples were loaded on to a 20.0 X 20.0 X 0.1 cm 0.5 X MDE® polyacrylamide gel ran at 10° C for six hours at 15 watt constant. The gel was stained with a 0.5 µg/ml ethidium bromide in 1 X TBE solution for 20 minutes, followed by destaining in distilled water for five minutes. Fragment size for each sample was determined by comparing results to a 123 bp DNA ladder and reporting migration values as relative base pairs (bp). Samples determined to have the same migration patterns were then assigned a haplotype designation. Two of each haplotype identified was then sequence using the Beckman-Coulter CEQ2000® Capillary Sequencing system and aligned using the Omiga®1.1 Sequence Alignment program. AMOVA and Öst pairwise analysis was then performed to look for statistical differences between the two species using Arlequin statistical analysis program (Excoffier et al. 1992).

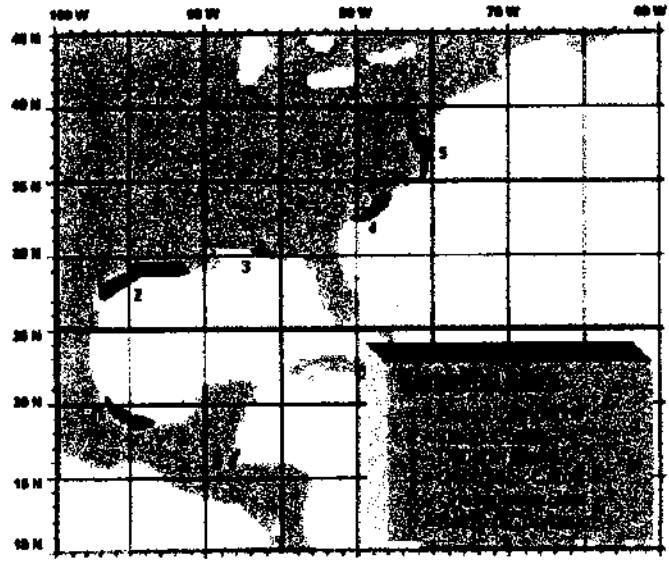

Figure 1. Location of sites for collection of the Atlantic sharpnose shark, *R. terraenovae*, and the Caribbean sharpnose shark, *R.porosus*.

RESULTS

Cyt b and the D-loop region of mtDNA were successfully amplified from both species and produced distinct bands per haplotype. A total of 11 haplotypes were identified in *R. terraenovae* (n = 69) and 3 haplotypes observed in *R. porosus* (n = 12) (Table 1). Both species never shared a common haplotype. The ability to discern between haplotypes was quite high. A typical gel illustrating the differences

between haplotype is shown (Figure 2). Haplotype diversity in the Atlantic sharpnose was high (h=0.8549 ± 0.053) compared to the Caribbean sharpnose (h=0.667 ± 0.091). AMOVA analysis was performed comparing the six samples sites grouped as two distinct populations (Table 2). Genetic variation observed between the two species was moderate (21.42%), and indicated that genetic material was not being shared between the two species (Φ_{ST} = 0.113, p<0.001). The hypothesis that *R. terraenovae* and *R. porosus* are distinct and separate species is validated since it seems that both species are reproductively isolated. Further evidence supporting this conclusion was obtained using pairwise Φ_{ST} analysis (Table 3). Comparision between individual populations within each group produced Φ_{ST} values that were highly significant (p < 0.001) in all cases.

Table 1. Haplotypes designations and haplotype frequencies observed in mtDNA isolated from the Atlantic sharpnose shark, *R. terraenovae*, and the Caribbean sharpnose shark, *R. porosus*.

Haplotype	Northwestern Atlantic (28)	Northern Gulf of Mexico (31)	Bay of Campeche (13)	Caribbean (12)
AX	**12** 0.429	**13** 0.419	**2** 0.154	–
AY	**2** 0.071	**1** 0.032	–	–
BX	**1** 0.036	**4** 0.129	–	–
BY	**6** 0.214	**2** 0.065	**1** 0.077	–
BZ	**1** 0.036	–	**2** 0.154	–
CX	**2** 0.071	**6** 0.193	**2** 0.154	–
CY	–	**1** 0.032	**3** 0.231	–
CZ	**2** 0.071	**1** 0.032	**1** 0.077	–
DX	**1** 0.036	**2** 0.065	**1** 0.077	–
EX	**1** 0.036	**1** 0.032	–	–
FZ	–	–	**1** 0.077	–
GW	–	–	–	**4** 0.333
GV	–	–		**6** 0.500
HU	–	–	–	**2** 0.167
h	0.831 ± 0.053	0.811 ± 0.055	0.923 ± 0.050	0.667 ± 0.091

Bold number indicates number of individuals with observed haplotype at a site.
Lower number indicates frequency of the haplotype at a site.

Lower number indicates frequency of the haplotype at a site.

Table 2. AMOVA design and results with significance tests based on 1023 permutations comparing differences between the Atlantic sharpnose shark, *R. terraenovae*, and the Caribbean sharpnose shark, *R. porosus*.

Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of Variation
Between Population of Species	1	2.845	0.1132 Va	21.42
Sample sites within Population of Species	2	1.169	0.0078 Vb	1.48
Between individuals at a sample site	80	41.457	0.4073 Vc	77.10
Total	83	36.595	0.5282	

V_c and Φ_{ST} : P (Random Value < Observed) = < 0.001
P (Random Value = Observed) = < 0.001
P (Random Value = Observed) = < 0.001

V_b and Φ_{SC} : P (Random Value > Observed) = 0.134
P (Random Value = Observed) = < 0.001
P (Random Value = Observed) = 0.135 ± 0.009

V_a and Φ_{CT} : P (Random Value > Observed) = < 0.001
P (Random Value = Observed) = 0.235
P (Random Value = Observed) = 0.235 ± 0.011

Table 3. Pairwise Φ_{st} analysis values and significance levels out of 110 permutations comparing difference between the Atlantic sharpnose shark, *R. terraenovae*, and the Caribbean sharpnose shark, *R. porosus*.

Sample Site	NW Atl	N. GoM	Campeche	Caribbean
NW Atl	0.000 -	-	-	-
N. GoM	-0.001 0.378 ± 0.04	0.000 -	-	-
Campech e	0.036 0.082 ± 0.03	0.036 0.982 ± 0.01	0.000 -	-
Caribbean	0.240 < 0.001*	0.250 < 0.001*	0.203 < 0.001*	0.000 -

* = Significant Difference

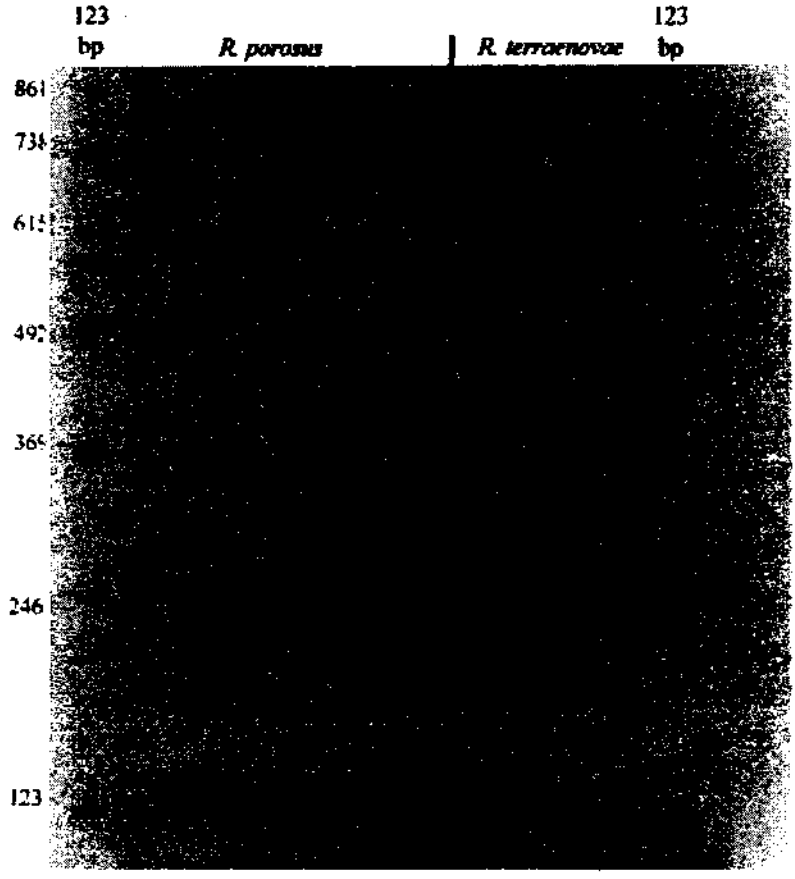

Figure 2. SSCP comparing Cyt b between *R. porosus* and *R. terraenovae* on a 0.5% MDE gel

Sequencing of each observed haplotype indicated that a relative low amount of nucleotide diversity between the two species existed. 729 bp of the D-loop region (Figure 3) and 650 bp of Cyt b (Figure 4) were successfully sequenced. 92 % of all nucleotide substitutions involved a transversion from a purine to a pyramidine residue. Fifteen nucleotides separated the most distantly related samples when comparing both genes between species, while five nucleotides separated the most closely related. This corresponded to a nucleotide divergence ranging from 0.36 % to 1.08 %. Phylogenetic inference based on parsimony analysis of the sequencing data was estimated where it was determined that 24 evolutionary steps were required for the observed haplotypes to have occurred (Felsenstein 1985)(Figure 5).

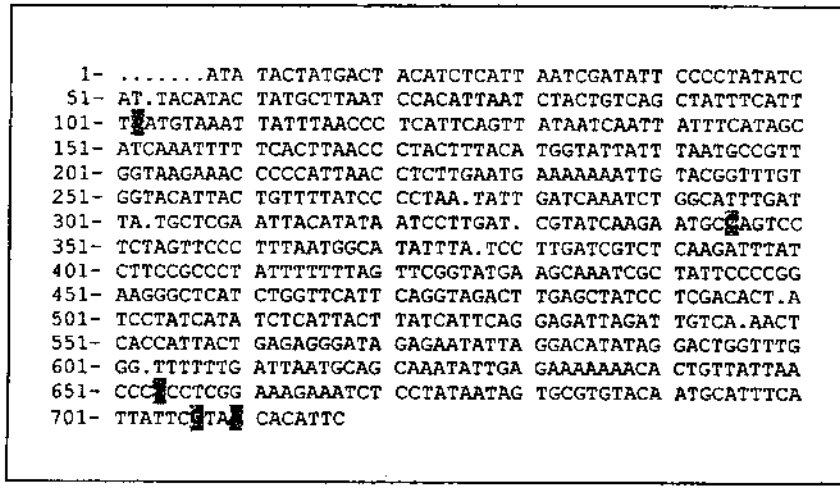

Figure 3. Nucleotide sequence of a 729 bp fragment of theD-loop region isolated from *R. terraenovae*. Highlighted nucleotide indicates a site where variability may occur in *R. porosus*.

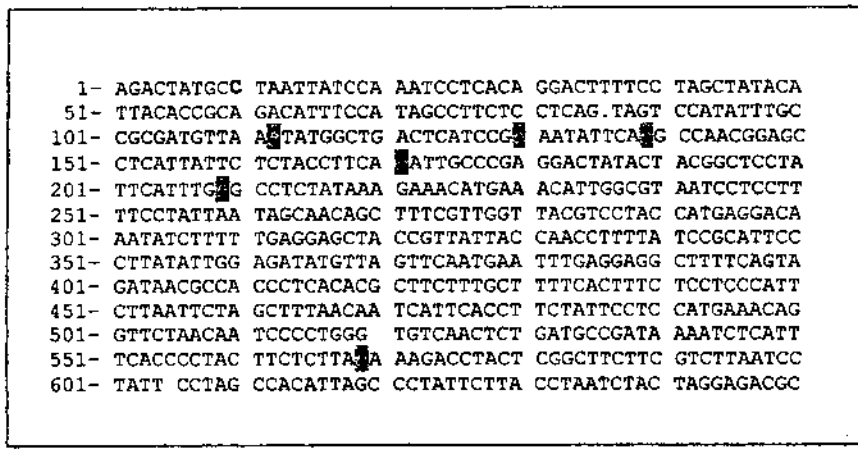

Figure 4. Nucleotide sequence of a 650 bp fragment of Cyt B isolated from *R. terraenovae*. Highlighted nucleotide indicates a site where variability may occur in *R. porosus*.

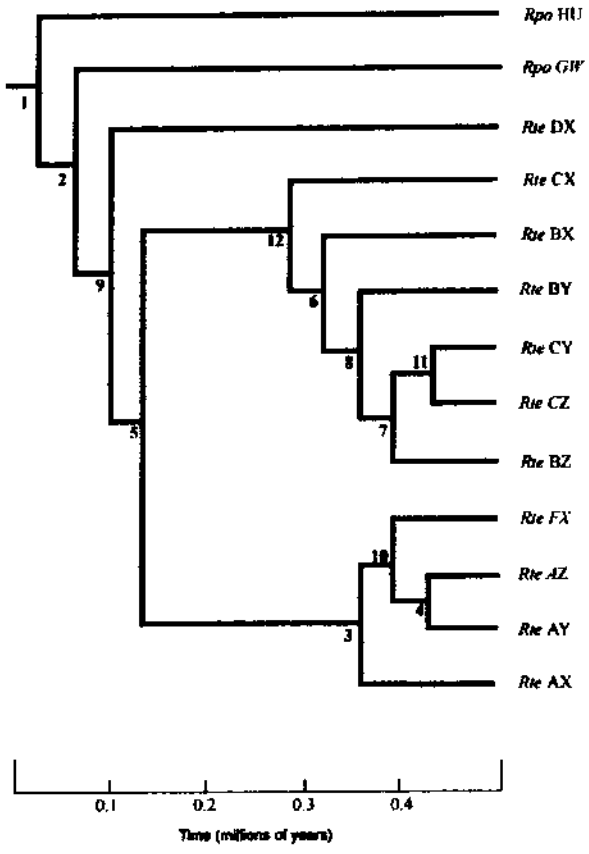

Figure 5. Phylogenetic inference based on parsimony analysis of the sequencing data using PHYLIP. Number at each node represents number of mutational steps required for haplotype to be observed.

DISCUSSION

The genus *Rhizoprionodon* currently exists on both coasts of North and South America. Roughly three million years before present (BP) the Plieocene rise of the Isthmus of Panama was completed (Bermingham et al. 1997). This event would have led to the geographical isolation of this Genus between the Atlantic and Pacific oceans. Thus, the ancestor of *R. terraenovae* and *R. porosus* would begin to change from its Pacific counterpart since it was now reproductively isolated. The behavior of organisms living in the region would also be affected due to ecological changes resulting from the differences in geography. This geological event caused northward projection of the equatorial current that now contributes to the formation of the Gulf Stream. Nursery ground, feeding grounds and possibly mating grounds would most

Stream. Nursery ground, feeding grounds and possibly mating grounds would most

likely have been affected. Organisms in the area would begin to undergo speciation and radiate out along the new coastlines entering new niches. Further speciation probably could have occurred due to glacial-interglacial oscillations. Major glacial-interglacial oscillations tend to occur on a 100,000 year time scale. Evidence exists that at the peak of interglacial or glacial intervals, the enhanced changes in temperature from average levels affect the hydrologic cycles (McManus et al. 1999). One consequence suggests that as temperatures fall, the flow of the Gulf Stream would intensify. If this does occur, it may be possible that the current may be intense enough to cause a temporary barrier to be formed geographically isolating members of the same species. This may be the mechanism that caused speciation between the Atlantic sharpnose and the Caribbean sharpnose. Examination of the distribution between the two species seems to follow the boundaries of the Gulf Stream (Compagno 1984). A difference of five nucleotides separated samples of *R. porosus* collected from Belize with those of *R. terraenovae*. Intrinsic rates of mutations in shark mtDNA have been estimated to occur at a divergence rate of 2.3% per million years (Martin et al. 1992, Martin 1995). This suggests that divergence between the two species could have occurred in a period of 90,000 years. In contrast, the two samples collected from Brazil was high (1.08 %) compared to the Atlantic sharpnose and Caribbean sharpnose collected in Belize. However, the size of sample pool to represent this area is small. Further studies are needed to characterize the population structure of the Caribbean sharpnose.

A high amount of nucleotide diversity was observed in the Atlantic sharpnose covering throughout the range of this organism. A previous study performed by Heist (1994) identified a total of seven haplotypes using Restrictrion Fragment Length Polymorphisms (RFLP) after screening the entire mtDNA genome (~16.5 kb in length) of the Atlantic sharpnose shark in contrast to eleven haplotypes bing identified from just two genes (~1.6 kb in length). This indicates that SSCP has higher resolving power compared to RFLP. The frequencies of haplotypes tend to indicate a panmictic population may exist. This tends to support the finding of Heist (1994). However, nucleotide diversity in the Bay of Campeche tends to be lower compared to other sites. Nucleotide diversity in the Caribbean sharpnose was also low relative to populations in the Northern Gulf of Mexico and in the northwest Atlantic. In both cases, the low diversity may be the result of overfishing. Management plans are in effect off the coast of the United States that may help maintain the gene pool in the corresponding population. In contrast, many fish populations within other parts of the Gulf of Mexico and Caribbean do not benefit from a comprehensive fishery management plan. Further studies are required to ascertain the health of populations along coastal Gulf of Mexico to see if genetic bottlenecking is occurring at localized areas.

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