Research Article

Inclusion of South American samples reveals new population structuring of the blacktip shark (*Carcharhinus limbatus*) in the western Atlantic

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Abstract

Carcharhinus limbatus has a cosmopolitan distribution and marked genetic structuring, mainly because of its philopatric behavior. However, analysis of this structuring has not previously included South American populations. In the present study, we analyzed a sample of adult individuals collected on the northern coast of Brazil and compared the sequences of the mitochondrial control region with those of populations already genotyped. Relatively high haplotype diversity (12 haplotypes, genetic diversity of 0.796) was observed, similar to that in other populations but with a much larger number of private alleles. In contrast to populations studied previously, which were represented by neonates, the pronounced allelic variability found in the South American individuals may have resulted from migrations from other populations in the region that have yet to be genotyped. This population was also genetically distinct from the other Atlantic populations ($F_{st} > 0.8$), probably because of female philopatry, and apparently separated from the northwestern Atlantic group 1.39 million years ago. These findings indicate that the *C. limbatus* population from northern Brazil is genetically distinct from all other populations and should be considered as a different management unit for the protection of stocks.

Key words: Carcharhinus limbatus, control region, fishery stocks, mitochondrial DNA, population genetics.

Received: January 13, 2012; Accepted: May 24, 2012.

Introduction

Sharks are vulnerable to overexploitation by fisheries because of their low fecundity and slow maturation (Musick *et al.*, 2000; Myers and Worm, 2005; Tsai *et al.*, 2010; Tillett *et al.*, 2011) and their limited potential for recruitment. This combination of traits has resulted in a sharp decline in populations in many parts of the world (Baum and Myers, 2004; Bradshaw *et al.*, 2008; Ferretti *et al.*, 2010). Given these considerations, the effective management of shark species should also consider good knowledge of the ecological characteristics and population structure of current stocks (Vaudo and Heithaus, 2009). Identification of the genetic structuring of populations is fundamental for determining whether and to what degree reproductive isolation exists, and can identify subpopulations that need to be treated as separate management units (Palsboll *et al.*, 2007). The lack of information on the migration and dispersion patterns of sharks and the difficulties in obtaining an adequate sample of the distribution of a species hinder the evaluation of fishery stocks and potentially contribute to the application of management policies that are incompatible with the biological characteristics of each species, leading to possible overexploitation of stocks (Feldheim *et al.*, 2001; Hueter *et al.*, 2004; Gore *et al.*, 2008).

The large populations, ample geographic distribution and dispersal capacity of many marine species, together with the general lack of physical barriers to dispersion in the world's oceans, all contribute to long-distance gene flow, characterized by a reduced genetic structuring of populations (Palumbi, 1994; Ward *et al.*, 1994; Rosenbaum *et al.*, 2009). This is emphasized by the fact that only a few migrants are needed per generation to avoid the effects of genetic drift, which results in segregation of the genetic characteristics of populations at different localities (Waples, 1998). On the other hand, traits such as the lack of mobile larvae or philopatric behavior may limit gene flow and result in the genetic divergence of populations of marine organisms, even when they have a continuous distribution (Meylan *et al.*, 1990).

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The blacktip shark, *Carcharhinus limbatus*, has a cosmopolitan distribution, and is typically found in tropical and subtropical coastal waters (Compagno *et al.*, 2005). In the western Atlantic, the species is found from Massachusetts to southern Brazil (Compagno, 1984). This shark is highly mobile, being able to travel distances of over 2,000 km, and migrates seasonally (Kohler *et al.*, 1998; Carlson and Brusher, 1999).

The blacktip shark uses shallow coastal waters as nurseries where the juveniles spend the first few months of their lives (Hueter *et al.*, 2005). These areas are normally relatively productive, with abundant dietary resources, and are also well protected from predators (Simpfendorfer and Milward, 1993; Heupel *et al.*, 2004; Heupel *et al.*, 2007; Faunce and Layman, 2009; Heupel and Simpfendorfer, 2011). An analysis of the genetic structure of different nurseries of *C. limbatus* in North America revealed that the females do not disperse randomly but are philopatric, returning to reproduce in the nurseries where they were born. This behavior can lead to marked genetic heterogeneity among nurseries (Keeney *et al.*, 2003).

Genetic structuring has been recorded among nurseries of *C. limbatus* in North America, the Gulf of Mexico and the Caribbean (Keeney *et al.*, 2005). These authors concluded that females copulate with males from different regions and then return to their natal nurseries to breed. In addition to this local structuring, a global analysis by Keeney and Heist (2006), which included new samples from Africa and the Indo-Pacific region, demonstrated pronounced structuring between these populations and those of the eastern Atlantic. However, this analysis did not include any South American populations.

Although *C. limbatus* is common along the coast of Brazil, little is known about its population structure or reproductive behavior in this region. The occurrence of pregnant females and juveniles off the coast of the Brazilian states of Rio Grande do Norte and Paraná indicates that nurseries exist in these areas (Yokota and Lessa, 2006; Bornatowski, 2008).

In the present study, sequences of the control region of mitochondrial DNA were analyzed to evaluate the genetic variability in *C. limbatus* on the northern coast of Brazil. The data were also used to assess the degree of divergence in relation to other populations (Keeney *et al.*, 2003, 2005) and the possible existence of distinct genetic lineages within the geographic distribution of the species.

Materials and Methods

The specimens of *C. limbatus* analyzed were all adults collected during a boat expedition on the coast of the Brazilian states of Pará (0°28'38.82" N, 48°18'55.38" W) and Amapá (01°31'56.8" N, 49°42'14.1" W) and were identified based on Compagno (1984) (Figure 1). A sample of muscle tissue was extracted from each specimen and stored in 95% ethanol at -20 °C. Total DNA was extracted using a standard protocol (Sambrook *et al.*, 1989) in which the tissue was digested with ribonucleases for 40 min at 37 °C and then dissolved in a solution of SDS and proteinase K at 55 °C. The material was subsequently washed
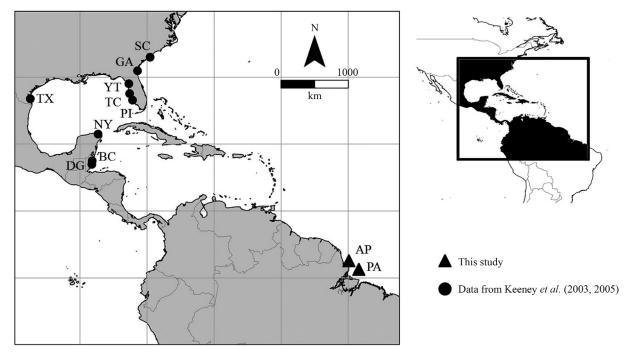

Figure 1 - Sampling localities for this phylogeographic study. Circles represent populations from Keeney *et al.* (2003, 2005) (BC – Belize City; DG – Dangriga; GA – Georgia; NY – Laguna Yalahau, northern Yucatan; PI – Pine Island Sound, Florida; SC – Bulls Bay, South Carolina; TC – Terra Ceia Bay, Florida; TX – Padre Island, Texas; YT – Yankeetown, Florida) and triangles represent locations sampled in this study (AP – Amapá and PA – Pará).

in phenol-chloroform and precipitated with sodium acetate and isopropanol. The resulting total DNA was hydrated and frozen.

The control region of the mitochondrial DNA of 25 specimens of *C. limbatus* was amplified using the polymerase chain reaction (PCR). The primers and the amplification protocol used were described by Keeney *et al.* (2003). The PCR products were purified using an ExoSAP-IT kit (Amersham Pharmacia Biotech. Inc., UK) and then sequenced using the dideoxynucleotide method (Sanger *et al.*, 1977) with a Big Dye reagent kit (ABI PrismTM Dye Terminator Cycle Sequencing Ready Reaction; Applied Biosystems, Foster City, CA, USA). The sequences were aligned automatically with CLUSTAL W (Larkin *et al.*, 2007) run in the BIOEDIT sequence editor (Hall, 1999), for visual inspection and possible corrections. The nucleotide composition, number of transitions and transversions were calculated using MEGA 4.0 (Tamura *et al.*, 2007).

Genetic (H) and nucleotide (π) diversity indices were determined with ARLEQUIN 3.1 (Excoffier *et al.*, 2005). Pairwise genetic divergence between populations was estimated by F_{st} (Excoffier *et al.*, 1992), with the significance being tested by 1000 permutations. Analysis of molecular variance (AMOVA), run in ARLEQUIN 3.1, was used to assess the genetic variability among populations. For this, the data provided by Keeney *et al.* (2003, 2005) and Keeney and Heist (2006) were added to those collected during this study. The data from previous studies were grouped into seven populations [BC – Belize City, Belize; DG – Dangriga, Belize; EA/IP – eastern Atlantic/Indo-Pacific (Africa, India, Australia, Philippines, Hawaii, USA); EG – eastern Gulf of Mexico (Pine Island, Terra Ceia and Yankeetown, USA); NA – northwestern Atlantic (South Carolina and Georgia; USA); NY – northern Yucatan (Laguna Yalahau northern Yucatan; Mexico); WG – western Gulf of Mexico (Padre Island, USA)], in addition to the northern Brazilian samples (Table 1). The haplotype network was constructed using the median joining (MJ) method in the NETWORK program, version 4.0 (Bandelt *et al.*, 1999).

Fu's *Fs* neutrality test (Fu, 1996) was applied in ARLEQUIN 3.1 to assess possible deviations from neutrality and provide inferences on the demographic history of the samples analyzed. The mismatch distribution (Rogers and Harpending, 1992) was constructed in DNAsp 5.0 (Librado and Rozas, 2009). The coalescence approach available in LAMARC 2.0 (Kuhner, 2006) was used to estimate demographic parameters such as the exponential growth rate (g) and the θ parameter. The estimates were based on runs of 2,000,000 steps, sampled at every 100 generations, with 10% of the samples discarded as burn-in. The results were evaluated using TRACER v. 1.4 (Rambaut and Drummond, 2007).

Divergence between populations was estimated using PAUP*, version 4.0b10 (Swofford, 2003), based on the evolutionary model chosen according to the Akaike crite-

rion, as determined by jModelTest (Posada, 2008). To estimate the divergence time between samples, we followed the approach of Keeney and Heist (2006), who assumed that the eastern Atlantic/Indo-Pacific and northwestern Atlantic were isolated from one another by the rise of the Isthmus of Panama (3.2 million years ago – Mya) to arrive at a mutation rate of 0.43% per million years.

Results

Sequences of the control region 1067-1070 base pairs in length were obtained for 25 specimens of *C. limbatus*. Thirteen polymorphic sites were identified, nine of which were transitions, with one transversion and three deletions/insertions that resulted in 12 haplotypes (GenBank access number JX025760-71); the most common haplotype was found in 11 individuals (Table 1). Haplotype diversity was relatively high (H = 0.7967), whereas nucleotide diversity was low (π = 0.0021). These values were consistent with those reported for other populations and nurseries of *C. limbatus* and other shark species (Table 2).

With regard to population structuring, the F_{st} values (data not shown) between the western Atlantic populations analyzed by Keeney *et al.* (2005) and the Brazilian samples were high (> 0.80) and significant, effectively the largest observed. The arrangement that included the six western Atlantic groups analyzed by Keeney *et al.* (2005) (northern Atlantic, eastern Gulf of Mexico, western Gulf of Mexico, northern Yucatan, Belize City and Dangriga) and the Brazilian samples was tested using AMOVA (only samples from the western Atlantic were used here because of the small number of individuals in the samples from the other populations). This analysis indicated that around 60% of the total variation (F_{ct} = 0.6064, p < 0.05) derived from that between groups, while the remaining 40% (F_{st} = 0.6008, p < 0.05) resulted from variation within populations. Variation between populations of the same group was not significant (F_{sc} = -0.0143, p > 0.05).

A plot of the mismatch distribution that included all of the samples genotyped worldwide (Keeney *et al.*, 2003, 2005; Keeney and Heist, 2006) and the Brazilian samples resulted in a multimodal curve (Figure 2A) that could be accounted for by structuring of the different groups of haplotypes. However, when only the western Atlantic samples were included, the curve was bimodal (Figure 2B), indicating the existence of two groups of haplotypes that corresponded to the populations genotyped by Keeney *et al.* (2003, 2005) and the Brazilian samples, respectively. This finding further emphasized the differentiation of these two groups.

The unimodal curve obtained for the Brazilian samples (Figure 2C) suggested a process of expansion (Slatkin and Hudson, 1991; Rogers and Harpending, 1992). This conclusion was supported by the sum of the squared deviations, which did not reject this hypothesis (0.0117,

Table 1 - Position of the polymorphic sites and geographic distribution of the haplotypes. The haplotype numbers (Hap) are listed in the first column, and N indicates the number of individuals in which the haplotype was observed. Nucleotides identical to haplotype 1 are indicated by dots (BC – Belize City; BR – northern Brazil; DG – Dangriga; EA/IP – eastern Atlantic/Indo-Pacific; EG – eastern Gulf of Mexico (Pine Island, Terra Ceia and Yankeetown); NY – northern Yucatan (Laguna Yalahau northern Yucatan); WA – western Atlantic (Bulls Bay and Georgia); WG – western Gulf of Mexico (Padre Island). Note that the different haplotype groups are shaded in different tones of gray.

Hap	N	WA	EG	WG	NY	BC	DG	EA/IP	BR
H1	125	35	53	14	23				
H2	52	12	34	6					
H3	21		15	6					
H4	1		1						
H5	29		11	13	5				
H6	17		4	7	6				
H7	3		3						
H8	1		1						
H9	4		4						
H10	13		2	11					
H11	2		2						
H12	3		3						
H13	2		2						
H14	1				1				
H15	9			1	8				
H16	5				5				
H17	1			1					
H18	1					1			
H19	1			1					
H20	1			1					
H21	16				7	7	9		
H22	12				2	2	10		
H23	3					3			
H24	20							20	
H25	1							1	
H26	1							1	
H27	2							2	
H28	2							2	
H29	1							1	
H30	1							1	
H31	1							1	
H32	1							1	
H33	5							5	
H34	2							2	
H35	1							1	
H36	1							1	
H37	2							2	
H38	1								1
H39	1								1
H40	1								1
H41	11								11
H42	1								1
H43	1								1
H44	1								1
H45	4								4
H46	1								1
H47	1								1
H48	1								1
H49	1								1

Table 2 - Diversity indices for the mitochondrial control region in different populations of *C. limbatus* and other shark species: π = nucleotide diversity, H = haplotype diversity, N = sample size (individuals) and HAP = number of haplotypes recorded.Note that samples from the eastern Atlantic and Indo-Pacific are excluded here because of their small size. Localities are coded as in Figure 1 and Table 1.

Species	π	H	N	HAP	Source
Carcharhinus limbatus (BR)	0.0021	0.796	25	12	Present study
Carcharhinus limbatus (SC)	0.0035	0.371	34	2	Keeney *et al.* (2003)
Carcharhinus limbatus (GA)	0.0004	0.461	12	2	Keeney *et al.* (2003)
Carcharhinus limbatus (PI)	0.0120	0.785	45	10	Keeney *et al.* (2003)
Carcharhinus limbatus (TC)	0.0106	0.720	45	8	Keeney *et al.* (2003)
Carcharhinus limbatus (YT)	0.0134	0.796	45	9	Keeney *et al.* (2003)
Carcharhinus limbatus (TX)	0.0016	0.813	49	8	Keeney *et al.* (2003)
Carcharhinus limbatus (NY)	0.0029	0.790	49	7	Keeney *et al.* (2003)
Carcharhinus limbatus (BC)	0.00077	0.680	13	4	Keeney *et al.* (2005)
Carcharhinus limbatus (DG)	0.00049	0.526	19	2	Keeney *et al.* (2005)
Cetorhinus maximus	0.0013	0.720	62	6	Hoelzel *et al.* (2006)
Carcharias taurus	0.003	0.717	26	4	Stow *et al.* (2006)
Sphyrna lewini	0.013	0.800	271	24	Duncan *et al.* (2006)
Rhincodon typus	0.011	0.974	69	44	Castro *et al.* (2007)
Triakis semifasciata	0.0067	-	169	5	Lewallen *et al.* (2007)
Galeorhinus galeus	0.0071	0.92	116	38	Chabot *et al.* (2009)

p = 0.707), and by Fu's *Fs* value, which indicated a significant deviation from neutrality (Fs = -5.7438, p = 0.001). Since these results supported a population expansion model, the coalescent model with constant exponential population growth provided by LAMARC 2.0 was chosen to estimate the demographic parameters of the Brazilian samples. The value of theta (θ) was 0.005859 and the exponential growth rate (g) was 606.02, indicating a relatively high rate of demographic growth.

The divergence among the haplotypes recorded in the northwestern Atlantic, northern Brazil and eastern Atlantic/Indo-Pacific was evaluated using the model (HKY+I+G: Lset Base = (0.3178 0.2024 0.1352) Nst = 2 TRatio = 8.1339 Rates = gamma, Shape = 0.5869 Pinvar = 0.9469) selected by jModelTest. The mean corrected divergence values for the three comparisons were 1.47% (0.6-2.7%) for northwestern Atlantic *vs.* northern Brazil, 2.62% (1.4-4.4%) for northwestern Atlantic *vs.* eastern Atlantic/Indo-Pacific and 3.68% (2.2-5.7%) for northern Brazil *vs.* eastern Atlantic/Indo-Pacific. The mean within-group divergence among the haplotypes was similar in the three groups, at 0.3% in the northwestern Atlantic and northern Brazil and 0.37% in the eastern Atlantic/Indo-Pacific.

The haplotype network analysis revealed a clear separation of the Brazilian samples from the other populations of *C. limbatus* (Figure 3), given both the lack of shared haplotypes and the large number of mutations that typically separated the Brazilian haplotypes from those of other populations (five mutation steps from other western Atlantic populations). Based on the rate of 0.43% per million years,

the most conservative estimate for the separation of the Brazilian and northwestern Atlantic populations would be 1.38 Mya.

Discussion

Despite the intense exploitation of stocks throughout the world and the relative vulnerability of the sharks to such pressure (Baum and Myers, 2004; Myers *et al.*, 2007), studies of many populations worldwide, including those of *C. limbatus* analyzed here, have found relatively high levels of haplotypic diversity. Furthermore, the number of haplotypes recorded in the northern Brazilian sample was relatively large when compared with the populations analyzed by Keeney *et al.* (2003, 2005), which could be accounted for by a process of expansion. However, as only one Brazilian population was genotyped, it is possible that the large number of unique haplotypes resulted from migrations among different South American populations not yet sampled but distinct from those sampled by Keeney *et al.* (2003, 2005). New data, including new sampling strategies and nuclear markers, would be necessary to test this hypothesis.

In contrast to the results of this study, recent analyses of shark populations from the coastal regions of Southern and Central America have not detected any differentiation between these areas (Mendonça *et al.*, 2011; Karl *et al.*, 2012). Mendonça *et al.* (2011) found no evidence of population structure in *Rhizopriodon porosus* from the Caribbean and northern Brazil. According to these authors, this lack of structuring may be related to the high migration rates across this region, particularly the high rates of pri-
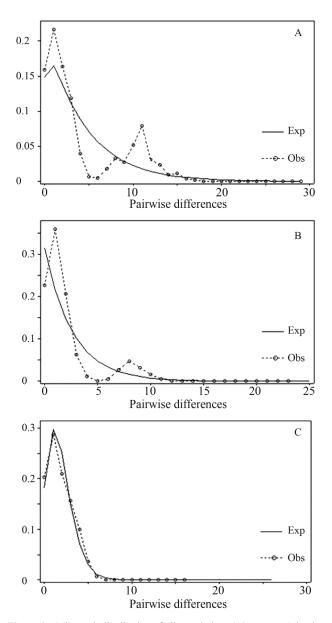
Figure 2 - Mismatch distribution of all populations (A), eastern Atlantic populations (B) and Brazilian samples (C). The solid line represents the curve expected (Exp) based on the expansion model. Obs – observed.

mary production in the delta of the Amazon River, which attracts the sharks to this area.

This interpretation is supported by the fact that, in contrast to the studies of Keeney *et al.* (2003, 2005), the sample analyzed in the present study consisted of adults and is thus likely to have included potential migrants. Given this situation, a more reliable evaluation of this question will depend on the genotyping of other populations from South and Central America and the inclusion of juveniles from nursery grounds.

Obviously, it is difficult to sample the full geographic variation of cosmopolitan marine species such as the blacktip shark, so the inclusion of the present samples from Brazil is an important contribution to understanding the

level of structuring that currently exists among populations of the western Atlantic. Keeney *et al.* (2003, 2005) investigated the genetic structuring of *C. limbatus* from the northwestern Atlantic, Gulf of Mexico and Caribbean by analyzing mitochondrial (control region) and nuclear (microsatellite) markers in neonates and found significant heterogeneity among nurseries. The differences observed in the frequency of mitochondrial haplotypes and microsatellites indicated the existence of philopatric behavior in the females, given the greater differentiation in maternally-mediated genetic traits in comparison with biparental traits.

An analysis of a wider spread of samples, including the Pacific, concluded that the subdivision found in the Atlantic was not repeated in the Pacific. In addition, shallow population structuring was found between the lineages of the eastern Atlantic and the Pacific. The addition of new Atlantic populations, such as the samples from northern Brazil, revealed additional structuring, as demonstrated by AMOVA, although these samples were genetically more divergent than those genotyped by Keeney *et al.* (2003, 2005). This was clearly shown by the mismatch plots in which the general multimodal curve was replaced by a bimodal arrangement when only the western Atlantic samples were included. Despite the relative differentiation of the Caribbean populations (Belize City and Dangriga) in relation to the other northwestern Atlantic samples (as emphasized by the haplotype network), these populations were still part of the northwestern Atlantic mismatch curve and shared common haplotypes. This observation confirmed that the samples from northern Brazil represent a diverse (separated by five mutations) and completely independent unit in relation to the remaining Atlantic populations, probably as a consequence of philopatric behavior, as previously noted in literature (Keeney *et al.*, 2003, 2005; Hueter *et al.*, 2004, 2005; Keeney and Heist, 2006).

The divergence time between the northeastern Brazilian and northwestern Atlantic populations was estimated at 1.38 Mya. However, as the mutation rate of the control region of *C. limbatus* is still unknown and since this value was based on the proposition of Keeney and Heist (2006), this divergence time may have been underestimated. The data nevertheless indicate that at least one separation event has occurred between northern and southern Atlantic populations since the formation of the Isthmus of Panama (3.3 Mya). As *C. limbatus* is adapted to warmer tropical and subtropical waters, shifts in ocean temperatures during the Pleistocene may have contributed to the isolation of populations (Keeney and Heist, 2006). The possibility of contact between the eastern and western Atlantic populations through a southern corridor, including the Brazilian coast, is not supported by the data presented here.

The ability of sharks to travel long distances and the fact that the marine environment has few physical barriers to dispersal (Palumbi, 1994; White *et al.*, 2009) make it ex-
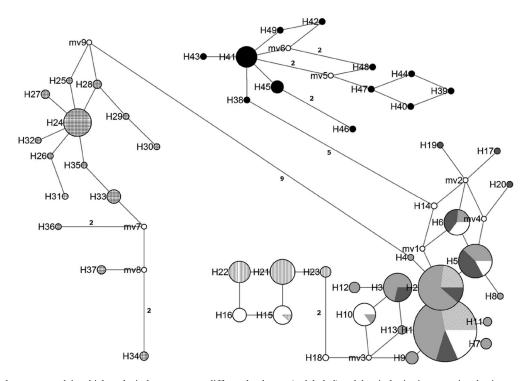
Figure 3 - Haplotype network in which each circle represents a different haplotype (as labeled) and the circle size is proportional to its recorded frequency (based on all samples). The length of the branch joining different haplotypes is proportional to the number of mutations that separate them. The number of mutations is indicated in each branch, except where only a single mutation is involved. The relative frequencies of the haplotypes in each population are represented by different types of shading:diagonal stripes = northwestern Atlantic (SC and GA); light gray = eastern Gulf of Mexico (YT, TC and PI); dark gray = western Gulf of Mexico (TX); white = northern Yucatan (NY); vertical stripes = Belize City (BC); horizontal stripes = Dangriga (DG); crossed hatching = eastern Atlantic and Indo-Pacific; black = northern Brazil.

tremely difficult to define the limits of a species geographic distribution. As shown here, although *C. limbatus* can disperse over distances > 2,000 km (Carlson and Brusher, 1999), its populations can be genetically highly differentiated. The samples analyzed here were taken from a stock distinct from those examined in previous studies and the differentiation can be accounted for not only by the migratory behavior of the females but also by their fidelity to breeding sites, a pattern confirmed in other studies.

The results of this study emphasize the marked variability of the Brazilian samples, which is characteristic of a population in expansion, as well as considerable differences in comparison with other populations of the western Atlantic. This suggests that the Brazilian population should be treated as a distinct management unit, although such conclusions should be considered tentative given the fact that the study was based on a single molecular marker and only one population. Obviously, more representative sampling and the analysis of additional markers would provide more conclusive evidence on the genetic characteristics of the South American populations of *C. limbatus*.

Acknowledgments

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Associate Editor: Fausto Foresti