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Applying genetic techniques to study remote shark fisheries in northeastern Madagascar

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Abstract

Background and aims. The shark fisheries of Madagascar remain largely unstudied. Remoteness makes fisheries monitoring challenging while the high value of shark fins combined with the extreme poverty in Madagascar creates intensive pressure on shark resources.

Materials and methods. We use DNA barcoding and species-specific PCR assays to characterize shark fisheries in Antongil Bay in northeastern Madagascar.

Results. The 239 samples taken from individuals collected in 2001 and 2002 correspond to 19 species. The four most common species were *Sphyrna lewini*, *Rhizoprionodon acutus*, *Carcharhinus brevipinna*, and *C. sorrah*. Antongil Bay may be a breeding area for *C. brevipinna*, *C. leucas*, and *S. lewini*.

Conclusion. Local names are generally not a useful proxy for monitoring the species harvested in the fishery. Conservation efforts should characterize species exploitation at present, create spatial and temporal fishing restrictions to protect endangered species, and restrict large mesh gillnets.

Keywords: Cox1, *conservation, ITS2, shark fins, species identification*

Introduction

Sharks are an important component of marine biodiversity that are increasingly threatened by extensive and often unregulated fisheries. Up to 73 million sharks are harvested annually to fuel the lucrative global market for flesh and fins (Clarke et al. 2006a). As top predators in marine ecosystems, the removal of sharks can have far-reaching impacts on marine ecosystems (Stevens et al. 2000; Myers et al. 2007). Given that many sharks have life history characteristics including slow growth, late maturity, and low fecundity, even limited fisheries can have a significant effect. Since shark fisheries can occur in remote areas across the globe, gathering data on the species harvested and the potential impact of the fishery on ecosystem function and biodiversity is difficult, resulting in a lack of baseline data on these important fisheries.

Antongil Bay in northeastern Madagascar is an area that has been studied with respect to its whales, corals, and fisheries and surrounding terrestrial biodiversity (Kremen et al. 1999; Ersts and Rosenbaum 2003; McClanahan 2006; Doukakis et al. 2007). Both targeted and by-catch sharks fisheries exist in the area with flesh consumed locally and fins exported for use in shark-fin soup (Smale 1998; Doukakis et al. 2007).

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Limited information exists regarding the sustainability of these fisheries, but area fishers indicate that the fisheries catch has declined in recent years (Smale 1998; Doukakis et al. 2007). As these fisheries provide a vital source of income and sustenance, especially because many of the terrestrial resources are protected by the Masoala National Park, managing them is of primary importance.

The shark fauna in and around Antongil Bay was characterized in a short-term study that determined over 50 species that could potentially occur in the area and confirmed the presence of at least six of these species (Smale 1998). Vulnerable (*Negaprion acutidens*) and endangered (*Sphyrna lewini*) species were observed in the fishery harvest (Smale 1998; IUCN 2010). As one of the few fairly large shallow-water habitats on the eastern coast of Madagascar, the Bay has been recognized as a potentially important shark breeding area (Smale 1998; Doukakis et al. 2007). How the fishery may impact this function cannot be determined without more detailed fishery information.

As in many shark fisheries, the fisheries in Antongil Bay often land only fins rather than whole sharks, making data collection at the species-level challenging, even when using trained port observers. In light of this, herein we use an approach to characterize the species composition of the shark fisheries in Antongil Bay based upon genetic techniques including DNA barcoding, a standardized approach for the genetic identification of animals (Hebert et al. 2003). We base this work on existing studies using DNA-based species identifications for sharks (e.g. Shivji et al. 2002; Clarke et al. 2006b; Powers et al. 2010) and the growing barcode reference library in the Barcode of Life Data System (BOLD; Ratnasingham and Hebert 2007), which has recently garnered significant discriminatory coverage for many species of sharks (e.g. Ward et al. 2007, 2008; Holmes et al. 2009; Wong et al. 2009). Beyond providing baseline information regarding the species harvested in the Bay, we explore the potential importance of Antongil Bay as a breeding area and the utility of local names as a monitoring tool. We conclude with suggestions on research and conservation initiatives for Bay shark fisheries.

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Materials and methods

Samples

Fin snip samples from 280 individual sharks were collected through cooperative programs with fishers and through market surveys in and around Antongil Bay, Madagascar, in 2001 and 2002 (see Doukakis et al. 2007). Sharks are captured in traditional and artisanal fisheries that employ small and large mesh gillnets, respectively, as well as beach seines in the traditional fishery. In all cases, the collection location, size and sex of the animal, the local name, and anything remarkable about the animal (e.g. pregnancy) were collected at the time of fin snip sampling. Fin snips were preserved in 95% v/v ethanol. Fins were examined for species identification through mitochondrial 5′ cytochrome *c* oxidase subunit I (*Cox1*) "barcode" sequencing ($n = 177$) or ribosomal internal transcribed spacer 2 (ITS2) species-specific PCR primer tests ($n = 197$), or both ($n = 109$), as described below.

DNA extraction, PCR amplification, and DNA sequencing of Cox1 *barcodes*

DNA was extracted and PCR amplified using one of two procedures. In the first procedure, samples were extracted using DNeasy Kits (Qiagen, Valencia, CA, USA) with minor modifications and 24-h digestions. A 652-bp fragment from the 5′ region of the *Cox1* gene was PCR amplified using the forward and reverse primer cocktail pair C_VF1LF1t1 and C_VR1LR1t (Ivanova et al. 2007). When this cocktail failed to produce clean sequences, C_FishF1t1 and C_FishR1t1 were used (Ivanova et al. 2007). The PCR reactions used puRetaq Ready-to-go beads (GE Healthcare, Piscataway, NJ, USA), reconstituted to a 25-μl final volume (1 μl genomic DNA, 2 μl primer cocktail, 22 μl water, one bead). Each PCR reaction mixture therefore consisted of 200 μM in 10 mM Tris–HCl of each dNTP, 50 mM KCl, and 1.5 mM $MgCl_2$. The second procedure followed the extraction protocols detailed by Ivanova et al. (2006) and employed PCR primers C_VF1LF1t1 and C_VR1LR1t1 (Ivanova et al. 2007) appended with M13 tails (Messing 1983). Each PCR reaction mixture consisted of 6.25 μl of 10% trehalose, 3.0 μl ultrapure ddH_2O, 1.25 μl of 10 × PCR buffer for Platinum Taq (Invitrogen, Inc., Foster City, CA, USA), 0.625 μl of 50 mM $MgCl_2$, 0.125 μl each primer (10 μM), 0.0625 μl of 10 mM dNTP mix, 0.06 μl Platinum Taq DNA polymerase (Invitrogen, Inc.), and 0.5–2.0 μl template DNA. In both procedures, PCR amplification reactions were conducted on Eppendorf Mastercycler gradient thermal cyclers (Brinkmann Instruments, Inc., Westbury, NY, USA) with reaction conditions consisting of 2 min at 94°C, followed by 35 cycles of 30 s at 94°C, 40 s at 52°C, and 1 min at 72°C, followed by 10 min at 72°C and a hold at 4°C.

For sequencing products without the M13 tail, PCR products were labeled using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA, USA). Each cycle sequencing reaction mixture consisted of 1.0 μl primer (3.2 μM; M13For or M13R), 5 μl PCR product, 1 μl sequencing buffer, and 1 μl BigDye. The initial denaturation of 94°C for 2 min was followed by 35 cycles of 94°C for 30 s, 50°C for 1 min, and 60°C for 4 min. Contig assembly was carried out in Sequencer 4.7 (Gene Codes Corporation, Inc., Ann Arbor, MI, USA). Products with the M13 tail were sequenced in a similar manner, with PCR products labeled using the BigDye

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Terminator v3.1 Cycle Sequencing Kit and each cycle sequencing reaction mixture consisted of 5.0 μl of 10% trehalose, 0.917 μl ultrapure ddH_2O, 1.917 μl of 5 × buffer (400 mM Tris–HCl ph 9.0 and 10 mM $MgCl_2$), 1.0 μl primer (10 μM; M13F or M13R), 0.167 μl BigDye, and 1.5 μl PCR product and the same cycling conditions. Bi-directional sequencing reactions were carried out with the M13 primers and resolved using an ABI 3730 × l DNA Analyzer (Applied Biosystems, Inc). Contig assembly was carried out using SeqScape 2.1.1 (Applied Biosystems, Inc). All contig assemblies and underlying electropherogram trace files were deposited in BOLD. Sequences were also submitted to GenBank (accession numbers HQ171607–HQ171777) using BOLD.

Species-specific PCR of the ITS2 region

The species-specific primers were designed to specifically amplify portions of the ITS2 region. The primers used are those that selectively amplify bigeye (*Alopias supercilious*), pelagic thresher (*Alopias pelagicus*), common thresher (*Alopias vulpinus*), Java (*Carcharhinus amboinensis*), gray reef (*Carcharhinus amblyrhynchos*), spinner (*Carcharhinus brevipinna*), silky (*Carcharhinus falciformis*), bull (*Carcharhinus leucas*), sandbar shark (*Carcharhinus plumbeus*), spottail (*Carcharhinus sorrah*), tiger (*Galeocerdo cuvier*), shortfin mako (*Isurus oxyrinchus*), longfin mako (*Isurus paucus*), blue sharks (*Prionace glauca*), scalloped (*S. lewini*), smooth (*Sphyrna zygaena*), and great hammerhead (*Sphyrna mokarran*) sharks and a complex including dusky (*Carcharhinus obscurus*), Galapagos (*Carcharhinus galapagensis*), and oceanic whitetip (*Carcharhinus longimanus*) sharks. The methods and primers are described elsewhere (Pank et al. 2001; Shivji et al. 2002; Abercrombie 2004; Abercrombie et al. 2005).

Sequence analysis

Species identifications were made using the BOLD identification engine's "Full Database" option with the "Tree Based Identification" tool, taking into consideration the caveats discussed in Wong et al. (2009). When the BOLD analysis yielded ambiguous results or could not identify an individual to the species level, the sequences were compared with those in GenBank through BLAST (http://blast.ncbi.nlm.nih.gov/blast.cgi). ITS2 species-specific primers yielded positive results when PCR bands were of the expected size as detailed in the references noted above.

Once species identifications were obtained through the genetic methods, data on the length of individuals at capture were compared with known information (FishBase: http://www.fishbase.org; Smale 1998) on the size at birth to infer the use of Antongil Bay as a breeding and rearing area. Similarly, field notes regarding whether captured females were pregnant were used to reach the same conclusion.

Results

Of the 280 individuals examined, 239 were identified to the genus or species level (Table I). The barcoding approach resulted in more species identifications (171 of 177) as compared with species-specific PCR

Table I. Species identification assignments for samples collected in and around Antongil Bay Madagascar.

Species	*n*(*Cox1*)	*n*(ITS2)	*n*(total)	IUCN Red List	Fishery
Carcharhinus amboinensis	2	4	5	DD,u	a,t
Carcharhinus amblyrhynchos	1	8	8	NT,u	a
Carcharhinus albimarginatus	4	0	4	NT,u	a
Carcharhinus brevipinna	28	21	29	NT,u	a,t
Carcharhinus leucas	0	1	1	NT,u	a,t
Carcharhinus limbatus	1	1	1	NT,u	t
Carcharhinus obscurus⋆	6	5	6	V,d	a
Carcharhinus plumbeus	4	7	8	V,d	a,t
Carcharhinus sorrah	15	25	28	NT,u	a,m,t
Galeocerdo cuvier	9	4	11	NT,u	a,t
Hemipristis elongata	3	0	3	V,d	a,t
Loxodon macrorhinus	13	0	13	LC,u	a,m,t
Mustelus sp.	1	0	1		t
Prionace glauca	0	1	1	NT,u	a
Rhinobatos sp.	1	0	1		t
Rhizoprionodon acutus	40	0	40	LC,u	a,t
Sphyrna lewini	42	47	77	E,u	a,m,t
Sphyrna mokarran	0	1	1	E,d	m
Stegostoma fasciatum	1	0	1	V,d	a
Total	171	125	239		

Species identified using *Cox1* or ITS2 are indicated, with the total number of individuals identified indicated (*n*(total)) to clarify overlap in the methods applied. IUCN Red List listings are given along with population trajectories (IUCN 2010). IUCN status: DD, data deficient; E, endangered; NT, near threatened, and V, vulnerable. Trends: d, decreasing and u, unknown. Fishery indicates whether the species were recorded in the artisanal fishery (a), traditional fishery (t), or market survey (m). ⋆ITS2 primers only identified as dusky (*C. obscurus*), Galapagos shark (*C. galapagensis*), or oceanic whitetip shark (*C. longimanus*), but species identification was confirmed through *Cox1* analysis.

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(125 of 197). The species-specific PCR approach worked particularly well for hammerhead species but not as well for less commonly encountered species such as *Hemipristis elongata* and the guitarfish, *Rhinobatos*. The milk shark (*Rhizoprionodon acutus*) and largenose shark (*Loxodon macrorhinus*) could only be distinguished using species-specific PCR.

At least 19 species were found to be harvested in the fishery (Table I). For *Mustelus*, BLAST results indicated highest similarity to unidentified *Carcharhiniformes* species. The most common species encountered were the scalloped hammerhead (*S. lewini*; 32%), milk (*R. acutus*, 16.7%), spinner (*C. brevipinna*, 12%), spottail (*C. sorrah*, 11.7%), and largenose (*L. macrorhinus*, 5.4%) sharks. Ten species, including the four most common listed above, occurred in both the traditional and artisanal fisheries, while eight taxa were restricted to one of the fisheries or just found in the market (Table I). Six scalloped hammerhead, six milk, and one bull shark sample had total lengths corresponding to the range for length-at-birth for the species. Two individuals identified as *R. acutus* were pregnant at the time of capture. Local names were matched to species identification for 70 of the individuals (Table II). Strict correspondence between local names and species identifications was rarely found except in cases with fairly small sample sizes (Table II).

Discussion

Our survey adds a number of species to the six species found in Antongil Bay by Smale (1998). Smale (1998) did indicate, however, that all of the species found in our survey are known from Madagascar with the exception of the genus *Mustelus*. The total number of species occurring in Antongil Bay is at least 19 (Table I). As the fisheries surveyed in the present study operate mostly within or just on the edge of the continental shelf, this is probably not a total account of the shark biodiversity of the area given that deeper waters were not sampled. Additional species in the area include species of ragged tooth shark (Family Odontaspididae), which was observed in Antongil Bay in 2001 (P. Doukakis, 2001), as well as species of dogfish (Family Squalidae), nurse (Family Ginglymosomatidae), and thresher (Family Alopiidae) sharks as well as silky (*C. falciformes*) and blacktip reef (*Carcharhinus melanopterus*) sharks (Smale 1998). Our survey does, however, represent, the species targeted by the fisheries.

The only other published accounts of shark fisheries species diversity in Madagascar come from southern and northwest areas. In the south, 13 species were documented in the fishery, including species found in Antongil Bay as well as species of Isurus and Alopias and *C. falciformes* (McVean et al. 2006). The species most represented in the northwest fishery were those also encountered in Antongil Bay (*C. amblyrhinchos*, *C. albimarginatus*, *C. sorrah*, *S. lewini*, *L. macrorhinus*) as well as *C. melanopterus* and *Triaenodon obesus* (du Feu 1998).

The scalloped hammerhead was the most commonly encountered species identified in the Antongil Bay shark fishery. This species is considered to be endangered by the IUCN (Table I) and is increasingly threatened by fishing and trade, as recently illustrated by the 2010 petition to list the species and four congeners under Appendix II of the Convention on International Trade in endangered species. The species is declining across its circum-global range (see http://www.cites.org/eng/cop/15/prop/E-15-prop-15.pdf and references therein). Here, we found adults as well as juveniles in the fishery and acquired additional field data indicating that pregnant *S. lewini* females are harvested in the target, large-mesh artisanal gill-net fishery (P. Doukakis, unpublished data). Given this evidence, Antongil Bay appears to be a breeding ground for *S. lewini*. This species obtains a premium on the marketplace due to its fin characteristics and so pressure on the species will probably continue without some conservation intervention

Table II. Comparison of local names assigned at time of collection with DNA-based species identification.

Local name	DNA-based species ID	*n*
Amboarano	*Stegostoma fasciatum*	1
Antendromaso	*C. brevipinna* (1), *C. sorrah* (2), *P. glauca* (1), *S. lewini* (19)	23
Antsingora	*C. brevipinna* (9), *C. sorrah* (2), *L. macrorhinus* (8), *R. acutus* (1), *S. lewini* (3)	23
Antsingora ambanivava	*R. acutus*	1
Antsingora biloha	*C. albimarginatus*	1
Antsingora bosy	*G. cuvier* (3)	3
Antsingora dofodoha	*C. amboinensis*	1
Antsingora fasika	*R. acutus*	1
Antsingora fotsy	*C. amboinensis* (1), *C. obscurus* (2), *C. plumbeus* (1), *C. sorrah* (1), *L. macrorhinus* (1), *R. acutus* (2)	8
Antsingora lava tsiko	*L. macrorhinus* (3)	3
Antsingora mainty	*C. obscurus*	1
Antsingora tapakafo	*C. brevipinna*	1
Antsingora tasika	*R. acutus*	1
Antsingora vato	*R. acutus* (2)	2
Sorkay	*Rhinobatos* sp.	1

Data in parentheses are the number of individuals within each assignment and category.

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(Abercrombie et al. 2005). *Sphyrna* species also made up a significant portion of the catch in southern Madagascar fishery (McVean et al. 2006).

Of the other species comprising the bulk of the fishery, the spottail and spinner are considered near threatened, while *R. acutus* is considered to be of least concern due to its wide distribution and relatively productive life history (Table I). For *R. acutus*, both juveniles and pregnant females occur in the fishery, indicating that the fishery could have a potential impact on the species locally. Given that the population structure of this species remains largely unknown, it is difficult to predict the potential for local extirpation. One-third of the spinner sharks examined in the present study were young juveniles with total lengths below 1 m and so the Bay may be an important nursery for juvenile spinner sharks. At least four additional species occurring in the fishery are considered vulnerable by IUCN (Table I), so special attention may be warranted regarding the continued harvest of these species. The endangered great hammerhead appears to be only occasionally harvested, with only one record here over 2 years of monitoring. Smale (1998) also collected a juvenile of this species from the main market near Antongil Bay (Maroantsetra; for details on location, see Doukakis et al. 2007).

Based on the present study, monitoring fisheries based on local names will not be an accurate means of tracking species-level exploitation in the fishery (Table II). The possible exceptions are the names used to identify tiger and zebra sharks (Table II). Surprisingly, even hammerheads were not identified by a single local name. Given that most of the hammerheads harvested in the Bay belong to a single species (*S. lewini*), it may be fairly easy to train fishers to record a hammerhead catch and gain a fairly good picture of the level of exploitation of *S. lewini*.

More than 50% of the species identified occurred in both artisanal and traditional fisheries, including the most commonly encountered species and all of the vulnerable and endangered species, with two exceptions. The vulnerable *C. obscurus* was found only in the artisanal fishery, while the one specimen of the endangered S. *mokarran* was found only in the marketplace. Since *C. obscurus* inhabits nearshore and continental shelf environments, it may well also occur within the traditional fishery. Conservation measures for these vulnerable and endangered species must therefore focus on both fisheries, with the possible exception of a targeted effort to reduce the capture of *C. obscurus* in the artisanal fishery. Since only five *C. obscurus* were harvested in our study, the fishery probably poses little threat to the species. It should be noted that the species was only observed in January and February and thus there may be some seasonality to its infrequent occurrence.

While the guitarfish, *Rhinobatos* sp., was only observed here in the traditional fishery, field observations indicate that adults are harvested in the artisanal fishery (P. Doukakis, unpublished data). This species is protected by a local "fady" or traditional taboo that forbids that harvest of the species for folkloric cultural reasons. Artisanal fishers ignore this fady because prices for fins are extremely high due to their high quality. Traditional fishers, however, observe the taboo and throw back any guitarfish that they take in their nets. Field observations indicate that many more juvenile *Rhinobatos* occur in the traditional fishery than have been recorded here, probably because of this taboo.

A useful next step will be to determine whether there are temporal and/or spatial patterns characterizing the breeding activity of those sharks utilizing the Bay as a breeding area. This will help guide potential area and seasonal fishing restrictions. Although the sample size is small, our data indicate that breeding of *R. acutus* may take place between December and April given that this is when pregnant females and juveniles were detected in our survey. Additionally, since the data used here were collected in 2001/02, it would be useful to again survey the shark fauna harvested in the Bay to see whether the species composition has shifted. Ultimately, understanding the impact of the shark harvest and the best methods for restricting the fishery will come from detailed studies of the ecosystem dynamics of the Bay and the socioeconomic characteristics of the fishery. For now, any restriction on the large-mesh gillnets used in the artisanal fishery will benefit the shark fauna as well as other marine resources in the area.

Barcoding and species-specific PCR are both useful techniques for shark species identification. When access to sequencing is limited, the PCR assays can be used for identifying some very common species. A complementary barcoding approach would, however, generate the most species identifications.

Conclusions

The use of genetic methods, including barcoding and species-specific ITS2 PCR, has proven useful in characterizing the species composition of the fishery in Antongil Bay, Madagascar. At least 19 species occur in the fishery, and Antongil Bay appears to be a breeding area for scalloped hammerhead, milk, and possibly spinner and bull sharks. While the BOLD database performed well in identifying the present species, there is still a great need for voucher sequences for many shark species. Despite the logistical challenges associated with the preservation and archival of large-bodied animals like sharks, these sequences should be accompanied by voucher specimens deposited in museums or, at the very least, voucher photographs. Creation of a vouchered tissue collection will also benefit development of additional ITS2 species-specific PCR assays. Conservation efforts should focus on additional studies of the current harvest

in the fishery in terms of species diversity, temporal and spatial variability and socioeconomics, as well as potential restrictions of the large-mesh gillnet fishery.

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