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Shark jaws and teeth: an unexploited resource for population genetic studies

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Declines in shark populations worldwide are of particular concern to fishery managers and conservation biologists. Molecular-based technologies provide an efficient means to collect basic life-history information and estimates of dispersal that are critical to management. A novel method for obtaining high quality mtDNA from shark jaws and teeth for shark population genetic studies is described.

Key words: archived tissue samples; DNA extraction; historical samples; marine fish; mtDNA.

Sharks and other large marine predators have been subjected to intense exploitation both as by-catch in pelagic longline fisheries and as targets of direct fisheries in recent decades, and many shark populations now face serious declines (Baum *et al.*, 2003; Myers *et al.*, 2007). Sudden reductions in population size (*i.e.* population bottlenecks) can result in a loss of genetic variation and potentially can lead to inbreeding depression (Frankham *et al.*, 2002). More generally, lower levels of genetic variation can lessen the ability of the population to cope with environmental change, which also elevates the risk of extinction. Low levels of genetic diversity may arise from recent population bottlenecks induced, for example, by human activity, or may reflect historical processes such as founder effects (Menotti-Raymond & O'Brien, 1993). Several studies of marine fishes have shown direct connections between a reduction in population size because of overexploitation and the loss of genetic variation (Smith *et al.*, 1991; Hauser *et al.*, 2002; Hutchinson *et al.*, 2003).

Genetic analyses of extant populations allow researchers to infer historical population structure indirectly (Nystrom *et al.*, 2006). However, a more accurate assessment of population history can be obtained by measuring past levels of genetic diversity with historical samples and comparing them with current levels (Wisely *et al.*, 2002). To examine whether population declines of many shark species have led to the loss of genetic diversity, DNA needs to be extracted

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from samples collected prior to the population decline. As sharks are cartilaginous fish, bony skeletons are not available. In many cases, shark jaws and dried fins are the only potential source for historical samples as soft tissues degrade easily. Fortunately, shark jaws are often kept as trophies and can be readily sampled for many species. To assess historical levels of genetic variation in sharks, a method, modified from other historical DNA extraction protocols (Boom *et al.*, 1990; Matisoo-Smith *et al.*, 1997; Pichler *et al.*, 2001; Glenn *et al.*, 2006; Qiagen 2006), was developed to extract quality DNA from shark teeth and jaws.

The extraction of DNA from teeth and jaws was developed using 11 contemporary grey nurse shark *Carcharias taurus* (Rafinesque) teeth from Oceanworld Manly and Sydney Aquarium and from one contemporary ornate wobbegong shark *Orectolobus halei* (Whitley) jaw from Macquarie University. The method was also tested for older jaws and teeth. Together, 15 shark jaws [eight whaler shark (Family Carcharhinidae), four grey nurse shark, two tiger shark (*Galeocerdo cuvier* Péron and Lesueur in Lesueur) jaws and one school shark (*Galeorhinus galeus* L.)] and 19 shark teeth (18 grey nurse shark teeth and one whaler tooth) *c.* 20–40 years of age were used. Teeth or cartilage or both were sampled from each specimen.

Several safeguards were implemented to reduce contamination. Separate work areas for drilling and extraction were designated. Samples were drilled on an isolated bench, and DNA was extracted in a laminar flow cabinet. Work surfaces, hand drill, vice and clamps were washed with 70% ethanol and decontaminated with 50% bleach prior to use. Equipment was sterilized by autoclaving and decontaminated with bleach. Consumables used for the project were stored separately. Prepared reagents followed the Savannah River Ecology Laboratory DNA protocol (Glenn *et al.*, 2006).

A new autoclaved drill bit (1·0–1·5 mm) and aluminium-foil autoclaved collection tray were used for each specimen. Prior to drilling, the outside of the tooth was wiped with 70% ethanol and sanded with autoclaved coarse sandpaper to remove contaminants. Slow rotation with a manual drill was used to minimize heat production during drilling. Each tooth was fixed horizontally with the vice to ensure that the tooth powder fell directly into the collection tray. Three to four holes were drilled inside the tooth root to obtain a sufficient amount of dentine powder (*c.* 0·02–0·06 g) for DNA extraction. Holes were *c.* 1·5 mm wide and 3·0 mm deep. Tooth powder was carefully poured into a 1·5 ml microfuge tube, and 500 μl of 0·5 M EDTA was added to prevent degradation or contamination of the downstream processing area with powder grains. The drill bit, collection tray, gloves and paper below the hand drill and the vice were discarded after each specimen was drilled, and working surfaces were decontaminated, as described above. An extraction blank for each batch of extractions was produced by swirling an autoclaved drill bit in a tube of 1 ml of 0·5 M EDTA and by carrying out the rest of the extraction as normal. Jaw samples were obtained either as pieces of cartilage or by drilling holes into jaws (as above) and collecting the powder.

DNA was extracted from the tooth powder using the silica-based method of Boom *et al.* (1990) as modified by Matisoo-Smith *et al.* (1997) (Protocol A). To test whether different methods of extraction play a major role in the yield of DNA and in the success of amplification by polymerase chain reaction

(PCR), another extraction method, Qiagen (Valencia, CA, U.S.A.) DNeasy tissue kit (Protocol B), was used. Dentine powder was transferred to a 1·5 ml microfuge tube and weighed, then 1 ml of 0·5 M EDTA was added to decalcify the sample. The sample was mixed thoroughly and incubated at 37° C overnight with agitation. The following day, the sample was centrifuged at 2000 *g* for 15 min and the supernatant was carefully discarded. The remaining pellet was washed with 1 ml of autoclaved milli-Q water to remove ions that had accumulated during the decalcification process. This procedure was repeated twice. Aliquots of 360 μl of buffer ATL (Quiagen) and 40 μl of proteinase K (20 mg ml^{-1}) were added to the final pellet, and the solution mixed with a vortex and incubated at 55° C until the pellet was completely lysed. The mixture was occasionally vortexed during the incubation. Lysis was usually completed in 2 h. A longer incubation time, 48 h, was also tried to increase DNA yield. The sample was centrifuged and the supernatant was transferred to a new 1·5 ml microfuge tube to avoid the transfer of dentine powder to downstream processes. A total of 400 μl of buffer AL (DNeasy tissue kit, Quiagen) was added to the supernatant, and the solution mixed thoroughly by vortexing and incubated at 70° C for 10 min. About 400 μl of 100% ethanol was added to the sample and agitated with a vortex. Up to 650 μl of the mixture was pipetted into a DNeasy spin column and centrifuged at 6000 *g* for 1 min. The flow-through discarded. This procedure was repeated until the entire sample was loaded into the spin column. The following steps were then carried out as described in the Animal Tissues Protocol in the DNeasy Blood & Tissue Handbook (Qiagen, 2006).

A *c.* 700 base pair (bp) fragment of the mtDNA control region was amplified with the PCR from tooth and jaw DNA extracts using the primers MtGNf (5′-AAY CTG RCA TCT GAT TAA TGC) and MtGNr (5′-CAT YTT AGC ATC TTC AGT GC) (Stow *et al.*, 2006). PCR amplification was conducted in 40 μl reactions containing genomic DNA (amount depended on extraction success), Taq buffer, 200 μM each deoxyribonucleotide triphosphate (dNTP), 2 mM $MgCl_2$, 1 μM each primer and 1 unit of *Taq*-DNA polymerase. PCR was performed with an MJ Research (Waltham, MA, U.S.A.) PTC100 thermocycler with initial denaturation for 3 min at 94° C, followed by six touchdown cycles of 94° C for 30 s, annealing temperatures at 65, 64, 63, 62, 61, 60, 59, 58, 57, 56 and 55° C for 30 s and an extension step of 72° C for 1 min. This was followed by 35 cycles at 55° C annealing temperature with a final 72° C for 5 min. Because of inhibitors in the sample, additional PCR protocols were tested: sample dilutions (1:1, 1:2, 1:10 and 1:100), PCR volume (10 and 40 μl) and addition of bovine serum albumin (BSA). Best results were achieved with 1:2 dilution, 40 μl PCR and 0·5 μg μl^{-1} BSA. Amplification success was tested by running samples on agarose gel. PCR products were purified using Promega (Madison, WI, U.S.A.) Wizard PCR Prep DNA Purification System. Forward primer was used for sequencing on an ABI 377 sequencer.

DNA extractions and PCR amplifications for contemporary teeth and jaw consistently produced similar DNA yields and quality with each of the extraction methods tested. A single individual was randomly selected from these PCR products and sequenced (608 bp) and proved unambiguously to be grey nurse shark. DNA extraction and PCR amplification were also successful for 15 (44%) of 34 historical samples. Nine of these 15 samples were sequenced

and each yielded full length and high quality sequences. No differences were observed between the two extraction methods, and the longer proteinase K incubation time seemed to increase the yield of DNA.

The results demonstrate that high quality mtDNA can be extracted from shark teeth and jaws and PCR amplified and sequenced. The success rates of DNA extraction and PCR amplification were much higher for contemporary tooth and jaw samples (100%) than for historical tooth and jaw samples (44%). This drop off in success rate is expected because nucleic acids gradually degrade over time because of accumulation of hydrolytic and oxidative damage (Lindahl, 1993). Thus, low concentrations of DNA and degenerated DNA are one of the main problems in using historical samples for population genetic studies. Ancient and historical samples usually yield small DNA fragments (rarely >300 bp) (Höss & Pääbo, 1993). The primers used in this study were designed to amplify a long *c.* 700 bp fragment, and this may, in part, be why amplifications were successful in only 15 historical tooth and jaw samples. The next step is to design specific primers for different shark species to amplify shorter fragments of the mtDNA control region (*i.e.* 200–300 bp). Another option would be to design specific primers around polymorphic sites in the control region to genotype degraded DNA.

Several factors may explain the failure of PCR amplification in some samples. Inhibitors in the DNA extract may have blocked DNA polymerase activity and thus inhibited PCR amplification. Both DNA extracts and some reagents may contain inhibitors. For example, both DNA extraction methods used here were silica based, and even small amounts of silica can inhibit PCR (Höss & Pääbo, 1993). The addition of BSA, which chelates inhibitory proteins, to a PCR mix increased amplification success in contemporary samples, demonstrating that DNA extracts contained some inhibitors. In future, DNA extraction methods should be optimized to minimize inhibitory effects during the PCR.

The methods reported here can be used to detect illegal trade in shark products. While the morphology of shark jaws, sometimes teeth, can be used for identifying species, genetic analysis of these samples has the added potential of revealing the population of origin. Surprising levels of genetic partitioning are becoming evident for some sharks (Pardini *et al.*, 2001; Stow *et al.*, 2006), and this attribute lends itself to identify the stock source of shark products. This method has important implications for identifying the source of shark material, especially where species are protected throughout only parts of their distribution.

This is the first report of the extraction of DNA from shark teeth and jaws. The successful amplification of historical samples demonstrates that shark teeth are potentially useful to provide insights into historic changes in population genetic diversity in shark. Moreover, because shark jaws are collected by game fishermen throughout the world, historical and contemporary samples are easily collected. Given the rapid rate of depletion of shark populations (Baum *et al.*, 2003; Myers *et al.*, 2007), with the possibility of localized extinctions (Baum *et al.*, 2003), there is a degree of urgency in evaluating genetic diversity. In the first instance, the methods reported here will be used to evaluate genetic variation in the critically endangered grey nurse shark in eastern Australia.

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