Application of DNA-based techniques for the identification of whaler sharks (*Carcharhinus* spp.) caught in protective beach meshing and by recreational fisheries off the coast of New South Wales

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The International Union for the Conservation of Nature's (IUCN) development of the Shark Specialist Group is indicative of the increasing environmental awareness of sharks' crucial ecological role as apex predators and that they are being threatened by human activities. Although the conservation status of certain carcharhinid species (*Carcharhinus limbatus, C. obscurus,* and *C. plumbeus*) are presently considered at low risk or near threatened according to the IUCN's threatened species categories,[1] species from the genus *Carcharhinus* are known to inhabit the waters of New South Wales (NSW), Australia (Stevens, 1984; Last and Stevens, 1994); however their conservation status has not been determined. Known as whaler or "requiem" sharks, they are also commonly caught off the coast of New South Wales in commercial fisheries (Stevens and Wayte[2]; Tanner and Liggins[3]), recreational fisheries (Pepperell, 1992; Gartside et al., 1999; Steffe et al.[4]) and by protective beach meshing (Reid and Krogh, 1992; Dudley, 1997).

Because of morphological similarities between a number of shark species in the genus *Carcharhinus* (Last and Stevens, 1994; Naylor and Marcus, 1994), taxonomic identification to species level has been difficult or inaccurate (or both)(Stevens and Wayte[2]). Historical catches of certain species of sharks in NSW commercial fisheries, recreational fisheries, and protective beach meshing have been recorded to genus level only (Pepperell, 1992; Reid and Krogh, 1992; Tanner and Liggins[3]). Formally trained Australian Fisheries Management Authority (AFMA) observers aboard commercial longlining vessels may experience difficulties in identification if distinguishing parts of a shark are discarded prior to confirmation of species (Stevens and Wayte[2]). Similarly, observers of protective beach meshing may find species identification difficult on severely decomposed sharks. Without proper identification, the exact number of individual species inhabiting NSW waters and the number of each species being landed cannot be determined (Chan, 2001).

The rise of molecular biological techniques in marine forensic science has facilitated the development of accurate taxonomic identification of shark species by sampling biological tissue (Martin, 1991; Lavery, 1992; Heist and Gold, 1999). DNA techniques require only muscle tissue, allowing biopsy tissue to be taken from specimens that can be released, rather than having to sacrifice the shark to obtain liver and heart tissue for allozyme analysis (Godfrey, 1997). Methods of taxonomic identification include PCR-based restriction fragment length poly-

[1] Musick, J., and S. Fowler. 2000. *Carcharhinus limbatus, C. obscurus* and *C. plumbeus* *In* IUCN 2002. 2002 IUCN red list of threatened species. http://www.iucn.org/redlist/2000index.html. [Accessed 1 October 2002.]

[2] Stevens, J. D., and S. E. Wayte. 1998. A review of Australia's pelagic sharks resources. Fisheries Research and Development Corporation project 98/107, 64 p. CSIRO Marine Research, GPO Box 1538, Hobart, Tasmania 7001 Australia.

[3] Tanner, M., and G. W. Liggins. 2000. New South Wales commercial fisheries statistics 1993/94 to 1996/98, 82 p. New South Wales Fisheries, PO Box 21, Cronulla, NSW 2230 Australia.

[4] Steffe, A. S., J. J. Murphy, D. J. Chapman, B. E. Tarlinton, G. N. G. Gordon, and A. Grinberg. 1996. An assessment of the impact of offshore recreational fishing in New South Wales waters on the management of commercial fisheries. Fisheries Research and Development Corporation Project 94/053, 139 p. New South Wales Fisheries, PO Box 21, Cronulla, NSW 2230 Australia.

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morphism (PCR-RFLP; Martin, 1991), DNA sequencing techniques (Heist and Gold, 1999), isoelectric focusing of muscle proteins (Renon et al., 2001; Smith and Benson, 2001) and direct multiplex PCR amplification (Shivji et al., 2002). These techniques use the differences in the sequences of nucleotide bases within a DNA strand among species. DNA techniques have high sensitivity, are easily reproduced, and allow the development of a unique "DNA fingerprint" for each species (Martin, 1991; Innes et al., 1998; Pepperell and Grewe, 1999). It was the aim of this project to initiate a shark DNA database for species identification of pelagic sharks (beginning with species from the genus *Carcharhinus*) in New South Wales by using PCR-RFLP techniques.

Materials and methods

Sharks of the genus *Carcharhinus* landed by recreational fisheries and caught in NSW beach meshing were identified to species level by using morphometric taxonomic guides and dentition identification (Cliff and Wilson, 1994; Last and Stevens, 1994; Naylor and Marcus, 1994). Positively identified sharks were retained as voucher specimens (see "Acknowledgments" section). A tissue biopsy (5–10 g) from the dorsal region on either side of all voucher specimens and unidentified sharks was taken and stored in 75% ethanol prior to DNA extraction. Mitochondrial DNA (mtDNA) of specimens from six species of the genus *Carcharhinus* (*C. brachyurus*, *C. brevipinna*, *C. falciformis*, *C. leucas*, *C. limbatus,* and *C. obscurus*; see Table 1 for sample sizes) was extracted by using a Fastprep DNA Extraction kit (BIO101, Integrated Sciences, Sydney, New South Wales) following the manufacturer's instructions. Approximately 200–300 mg of tissue was placed in a sterilized 1.5-mL eppendorf tube and after the addition of 1 mL Fastprep lysis buffer, incubated at 56°C for three hours prior to the extraction stage (Chan, 2001). Following the extraction procedure of the Fastprep protocol, quality and quantity of DNA was measured by using a GeneQuant DNA/RNA calculator (Amersham Biosciences, Sydney, New South Wales).

The polymerase chain reaction (PCR) was used to amplify the 1146 nucleotide base-pair (bp) cytochrome *b* (cyt *b*) region of the mtDNA (Martin and Palumbi, 1993; Kitamura et al., 1996). For each 50 µL PCR reaction, 100–200 ng of template mtDNA was used, 1.5 mM $MgCl_2$, 1X PCR buffer, 2 mM of dNTP, 5 mM of each external primer (5′–TGACTTGAARAACCAYCGTTG–3′ and 3′–CTCCAGTCTTCGRCTTACAAG–5′) and two units of DyNAzyme EXT DNA polymerase (Finnzymes, GeneWorks, Adelaide, South Australia) were added to a sterilized 200 µL PCR tube. The PCR was undertaken in a MJR MiniCycler (MJR, GeneWorks) with a heated bonnet on a cycle of 94°C for three minutes, followed by 35 cycles of 94°C denaturing for 45 seconds, 48°C annealing for 30 seconds, 72°C extension for 90 seconds, and a final 10-minute extension time at 72°C (Chan, 2001).

To determine if the cyt *b* region was successfully amplified, 10 µL of PCR product was added to 2 µL loading dye (25% bromophenol blue, 40% sucrose in distilled water) and loaded into wells of a 1.5% w/v agarose gel submerged in 0.5X TBE (Tris-borate-EDTA, pH 8) buffer, with 1 µg of a 100-bp DNA ladder (Sigma-Aldrich, Sydney, New South Wales) added to 5 µL distilled water added to each side-end well. The gel was subject to electrophoresis at 125 V for 45–60 minutes and then stained in ethidium bromide for 10 minutes, de-stained in fresh distilled water for 20 minutes prior to illumination under ultraviolet (UV) light to determine the success and yield of the amplification.

To obtain species-specific profiles, restriction fragment length polymorphism (RFLP) was used on the entire 1146 bp cyt *b* fragment (Martin, 1991; Chan, 2001). The successful amplified reaction products had the primers, taq polymerase, and buffer chemicals removed by using a BRESAspin PCR purification kit (GeneWorks). For each RFLP reaction, 30 µL of purified PCR-amplified cyt *b* mtDNA (300–1000 ng; 30 µL of distilled water was used for control reactions), 5 µL of 10X buffer, one unit of a restriction enzyme (*Alu* I, *Hae* III, *Pst* I, *Taq* I, and *Xho* I) and distilled water up to 50 µL volume was added to a sterilized 200 µL PCR reaction tube and incubated at 37°C for 2 hours in the MJR minicycler (with heated bonnet), with the exception of *Taq* I (incubated at 65°C for two hours in a water bath). After the allotted digestion time, 10 µL of loading dye was added to each tube prior to loading into a 1.5% w/v agarose gel submerged in 0.5X TBE buffer. In both end wells, 5 µL of distilled water + 1 µg of a 100 bp DNA ladder was added. The gel was subject to electrophoresis at 125 V for 60–90 minutes, stained in ethidium bromide for 10 minutes, and destained in fresh distilled water for 20 minutes prior to illumination under UV light. Enzyme-digested DNA fragments >100 bp were then "scored" to the nearest 25 bp based upon migration of the DNA fragment (the smaller the fragment, the faster the migration) and recorded for each enzyme and sample (Martin, 1991) by using the 100-bp DNA ladder as a standard measuring guide for size estimation.

Results and discussion

PCR-RFLP profiles were successfully developed for six species of the genus *Carcharhinus*; distinct and discrete patterns were observed for each species with five restriction enzymes (Table 1, and Chan, 2001). The only intraspecific polymorphism observed was for two specimens of *C. brevipinna* with the *Xho* I restriction enzyme. Increasing the sample size of all species may identify more intraspecific polymorphisms. Because of the relatively small sample sizes, no statistical analyses were undertaken. Other restriction enzymes were tested (Chan, 2001), and with the possible inclusion of other species from the genus *Carcharhinus* into this database in the future, these restriction enzymes may be required in order to discern the additional species. Because some of the fragment sizes were rounded to the nearest 25 bp, the sum of the fragments for a restriction enzyme of a species may be more than 1146 bp, the size of the cyt *b* uncleaved region for sharks (Martin and Palumbi, 1993). Fragments <100 bp were not recorded because the DNA ladder had a lower limit of 100 bp.

Table 1

Summary of PCR-RFLP banding patterns for the cytochrome *b* (cyt *b*) region in *Carcharhinus* spp. Fragment sizes are given in number of base pairs (bp) and have been rounded to the nearest 25 bp. Where the enzyme appeared not to have cleaved the cyt *b* region, it was scored "1146." *n* = denotes sample size. ✓ = denotes fragment size present.

		Carcharhinus species						
Enzyme	Fragment size	*C. limbatus* (*n*=9)	*C. brachyurus* (*n*=12)	*C. leucas* (*n*=3)	*C. obscurus* (*n*=29)	*C. falciformis* (*n*=12)	*C. brevipinna* Haplotype 1 (*n*=6)	*C. brevipinna* Haplotype 2 (*n*=2)
Alu I	1000				✓			
	700		✓					
	600						✓	✓
	500					✓		
	450	✓						
	350	✓		✓			✓	✓
	300		✓			✓		
	200				✓			
Hae III	1100	✓		✓				
	975		✓			✓	✓	✓
	750				✓			
	225				✓			
	175		✓		✓	✓	✓	✓
Pst I	1146		✓		✓	✓	✓	✓
	975	✓		✓				
	175	✓						
Taq I	1146	✓					✓	✓
	1100			✓	✓			
	850		✓					
	650					✓		
	325		✓					
	300					✓		
Xho I	1146	✓		✓	✓		✓	
	850		✓			✓		✓
	325		✓			✓		

These techniques can be used to complement morphometric identification (Cliff and Wilson, 1994; Last and Stevens, 1994; Naylor and Marcus, 1994) or can be used to identify "cryptic" species when morphological identification cannot be done. Other "cryptic" species caught in beach meshing and by recreational fisheries can be added to the DNA database, such as hammerhead sharks (*Sphyrna* spp.) which are commonly caught and are recorded in catch records to genus level only (Pepperell, 1992; Reid and Krogh, 1992; Chan, 2001). Although this project positively identified six species from the genus *Carcharhinus*, other species of this genus are known to inhabit the NSW coastline (Stevens, 1984; Last and Stevens, 1994). During the warmer months, when the northern currents extend farther south to the Sydney region, transient tropical *Carcharhinus* spp. may appear off the coast. In the northern regions of NSW, there have been recorded catches of the blacktip reef shark (*C. melanopterus*) by shore-based anglers (Gartside et al., 1999). Although transient tropical whaler sharks may not have permanent stocks in NSW waters, it is important to discern them from resident *Carcharhinus* spp. prior to any species-specific stock assessment. Given the number of shark species and difference in life histories (Last and Stevens, 1994; Smith et al., 1998), identification to species level is crucial.

The use of genetic techniques allows, for the first time, accurate identification of species of whaler sharks that were landed by recreational fisheries and caught in protective beach meshing in NSW and that have been historically recorded to genus level. Continual sampling and formal identification are required for comparison of catches between species of *Carcharhinus*. Genetic techniques have the potential to be used for all other shark species and fisheries within the Australian Fishing Zone (AFZ). The use of genetic techniques has been employed in the field of law enforcement to prevent the selling of protected fish species at local fish markets where the majority of the carcass is not retained (Ward et al., 1999). This use could

be extended to ensure that protected shark species such as the grey nurse shark (*Carcharias taurus*), white shark (*Carcharodon carcharias*), and the smalltooth sand tiger shark (*Odontaspis ferox*) are not sold.

This project is the first time that *Carcharhinus* spp. have been formally identified to species level in the 60-year history of NSW protective beach meshing and only the second time in NSW recreational fisheries after Stevens (1984). The depositing of voucher specimens and all DNA biopsies at the Australian Museum ensures that these valuable and irreplaceable biological samples can be used in future research. It is evident that DNA techniques can be used to taxonomically identify "cryptic" specimens, especially *Carcharhinus* spp., and *Sphyrna* spp. to species level that were once recorded to genus level only in many fisheries based in NSW (Pepperell, 1992; Reid and Krogh, 1992; Chan, 2001; Tanner and Liggins[3]). It is important that sharks that are caught be recorded to the lowest taxonomic level for management and conservation strategies. Long-term routine sampling and recording to species level will provide useful data on which conservation management strategies can be developed as part of the Australian national plan of action for the conservation and management of sharks.

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