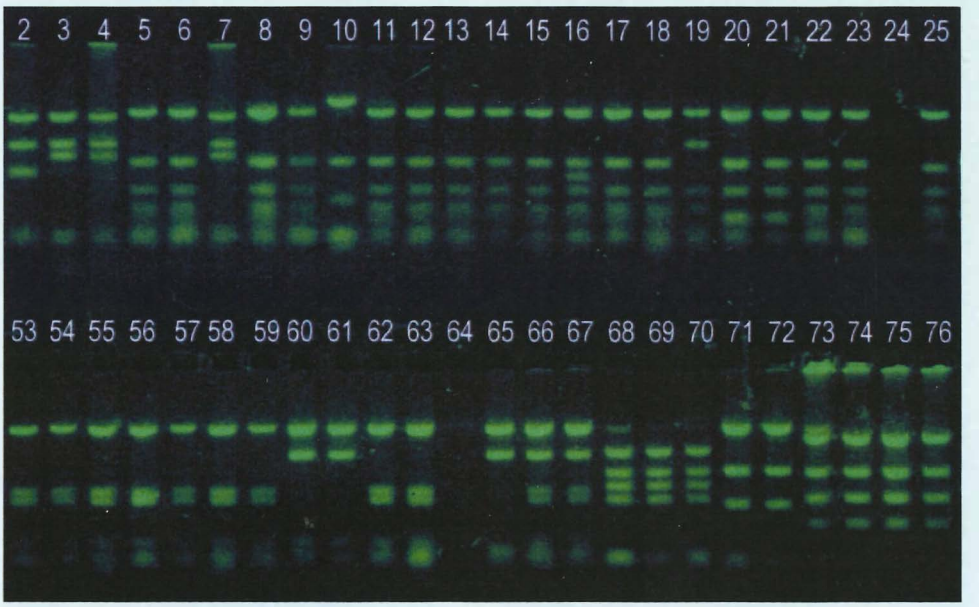
FISHERIES RESEARCH REPORT

No. 152, 2005

# Development of a DNA Database for Compliance and Management of Western Australian Sharks

## Final FRDC Report – Project 2003/067

R. McAuley, K. Ho, R. Thomas




Department of Fisheries

Australian Government
Fisheries Research and Development Corporation

CHEMISTRY CENTRE

Fish for the future

Western Australian Fisheries and Marine Research Laboratories

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**Fisheries Research in Western Australia**
The Fisheries Research Division of the Department of Fisheries is based at the Western Australian Fisheries and Marine Research Laboratories, PO Box 20, North Beach (Perth), Western Australia, 6920. The Fisheries and Marine Research Laboratories serve as the centre for fisheries research in the State of Western Australia.

Research programs conducted by the Fisheries Research Division and laboratories investigate basic fish biology, stock identity and levels, population dynamics, environmental factors, and other factors related to commercial fisheries, recreational fisheries and aquaculture. The Fisheries Research Division also maintains the State data base of catch and effort fisheries statistics.

The primary function of the Fisheries Research Division is to provide scientific advice to government in the formulation of management policies for developing and sustaining Western Australian fisheries.

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# Development of a DNA Database for Compliance and Management of Western Australian Sharks

## Final FRDC Report – Project 2003/067

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## Non Technical Summary

The capability to identify individual shark species from processed body parts is necessary for the WA Department of Fisheries to ensure the compliance of all WA fisheries with both existing protected species regulations and proposed new management measures for commercially important shark species. This project established a reference database of genetic profiles or 'fingerprints' for nine of Western Australia's protected and commercially important shark species. Fingerprints from a 10th species, the shortfin mako (*Isurus oxyrinchus*) could not be obtained due to probable primer sequence mismatching. The database, in conjunction with an associated inspection and testing regime, will act as a significant deterrent to the trade in body parts from protected shark species and also as a source of information on levels of bycatch of commercially important shark species in non-target fisheries. Genetic material from voucher specimens was collected by the principal investigator according to documented species identification and verification procedures. Samples were securely stored in tamper-proof containers until their transfer to the Chemistry Centre (WA) for genetic analysis and profiling. Additional fingerprints were derived from samples from the WA Department of Fisheries' shark DNA reference collection and included in the database, to ensure that as much genetic variation as possible was represented for each catalogued species. The collection, storage and transfer of each sample was documented and the integrity of each sample was verified on receipt by the project co-investigator prior to analysis. These continuity of evidence protocols were developed according to WA Police Service guidelines for forensic evidence collection to ensure the database's suitability as a provider of legally robust evidence.

## Objectives

1. Establish sampling protocols (ensuring methodology will be suitable for evidentiary purposes) and collect reference samples.
2. Establish 'legally robust' DNA processing protocols and process reference samples.
3. Establish a secure DNA 'fingerprint' database for WA shark species to act as both a provider of evidence and a deterrent to illegal fishing activity.

## Outcomes achieved to date

Continuity of evidence protocols and documentation were established for sample collection, storage, handling and security according to the standards adopted by the WA Police Department.

A total of 177 samples from 10 species have been collected and processed.

Mitochondrial DNA was successfully amplified from samples collected from nine species. The only samples (n=9) from which DNA could not be successfully amplified was the shortfin mako (*Isurus oxyrinchus*).

Genetic 'fingerprints' have therefore been produced for 168 samples from nine species using four different restriction enzymes.

The panel of 'fingerprints' produced by these enzymes for each species therefore constitutes the genetic database, against which 'test' samples can be compared.

This database is being maintained by the Department of Industry and Resources Chemistry Centre (WA), according to National Association of Testing Authorities accredited quality assurance procedures.

**Keywords:** Sharks; compliance; protected species; mitochondrial DNA; PCR-amplification; DNA fingerprints

# 1.0 Background

There are four limited-entry 'shark' fisheries in Western Australia: the Joint Authority Southern Demersal Gillnet and Demersal Longline Fishery (JASDGDLF), the West Coast Demersal Gillnet and Demersal Longline Fishery (WCDGDLF), the Western Australian North Coast Shark Fishery (WANCSF) and the Joint Authority Northern Shark Fishery (JANSF). Additionally, sharks are subject to a number of sources of 'hidden' mortality in Western Australia, including fisheries, which take them as bycatch or byproduct (Harris and Ward, 1999; Stephenson and Chidlow, 2003; McAuley et al. 2005; Penn et al., 2005) and illegal domestic and foreign fishing (Rose and McLoughlin, 2001; Anderson and McCusker, 2005). The continued high value of shark fins on international markets is likely to have increased the incentive for operators to fin their shark bycatch at sea, despite State and Commonwealth legislation, aimed at prohibiting the practice (Rose and McLoughlin, 2001; Anderson and McCusker, 2005). This unquantified 'hidden' exploitation has the potential to compromise the continued viability of the target-shark fisheries as well as the successful conservation of vulnerable species.

Western Australian shark fisheries have undergone considerable fishing effort reduction over the last 15 years to ensure the sustainability of target stocks. While these measures have been successful in reducing overall effort, recent stock assessments of three commercially important species, the whiskery shark (*Furgaleus macki*) the dusky shark (*Carcharhinus obscurus*) and the sandbar shark (*Carcharhinus plumbeus*), have highlighted concern that these species are over-exploited (Gaughan and Chidlow, 2005; McAuley et al, 2005). The Western Australian Department of Fisheries is therefore considering additional species-specific management measures, such as commercial protection, size limits and bycatch limits, to reduce the mortality of these species. There is also serious concern at State, Commonwealth and international levels regarding the conservation status of several shark species. Currently, five species of sharks are protected in Western Australian waters: the white shark (*Carcharodon carcharias*) the grey nurse shark (*Carcharias taurus*) the speartooth shark (*Glyphis* sp. A., Last and Stevens, 1994), the northern river shark (Glyphis sp. C., Last and Stevens, 1994) and the whale shark (*Rhincodon typus*). Despite being protected under various State and Commonwealth regulations, there is thought to be a continuing trade in products from at least two of these: the white shark (jaws) and the grey nurse shark (flesh, fins and jaws).

Sharks are almost always processed at sea, which complicates the application and enforcement of conservation and management regulations. As processing usually involves evisceration, removal of the head and fins and 'bleeding' to prevent ammonia spoiling the flesh, the identification of individual species in catches is normally very difficult. However, DNA techniques, such as those proposed, have the potential for enabling identification of species even after they have been processed. When collected in accordance with appropriate evidentiary protocols, DNA evidence is also legally robust enough to be defensible in court (Lander, ES., 1989) and therefore can provide fishery and wildlife authorities with the capability to both enforce existing protected species regulations and potentially develop other species-specific management measures.

Due to the diversity of Western Australian shark fauna and the difficulties associated with the accurate identification of many species, much of the reported shark catch from non-target fisheries is either unidentified or misidentified. A reliable technique for the identification of shark species from processed body-parts also has the potential to be used for assessing the accuracy of reported catches and to determine species compositions of mis-reported catches.

Such data could be used to improve the accuracy of stock assessments (eg McAuley et al., 2005), risk assessments (eg FRDC project 2002/064, Northern Australian Sharks and Rays: the Sustainability of Target and Bycatch Fisheries, Phase II, FRDC project no. 2002/033 Rapid Assessment of Sustainability for Ecological Risk of Shark and Other Chondrichthyan Bycatch Species Taken in the SSF, SENTF, SETF and GABTF). More reliable bycatch data could also be used to provide additional information on exploitation levels of vulnerable and endangered species, such as the great white and grey nurse sharks, to assist in their recovery plans.

Although DNA fingerprinting has been used extensively in fin fish (Tagliavini, 1995), its potential for speciating cartilaginous fish has only been realised recently (Heist et al., 1995; Heist and Gold, 1999a; Shivji, et al., 2002). The proposed methods have previously been shown to be suitable for differentiating species of Australian sharks on the basis of genotype (Ho et al., 1998; Chan et al, 2003). The process involves isolating DNA from shark tissue, which is used in a biochemical reaction (PCR amplification) to produce billions of copies of a specific genetic sequence (Saiki et al., 1985; Kemp, 1989). PCR conditions will be strictly observed to prevent cross contamination (Kwok and Higuchi, 1989). The amplified DNA is converted by restriction endonucleases into smaller but discrete fragments which are separated according to size (Kelly and Smith, 1970; Roberts, 1983). The resulting banding pattern (restriction fragment length polymorphism) or DNA 'fingerprint' represents the genetic signature or blueprint for that species of shark.

This project aims to catalogue DNA fingerprints for up to 20 individuals of ten protected and commercially important Western Australian shark species to demonstrate reproducibility and to capture the extent of genetic variation. Following the implementation of this database, blind trials will be conducted against reference standards to show that species identification of unknowns is accurate and reproducible. The methodology will be accredited by validation and peer review through standard National Association of Testing Authorities (NATA) or equivalent accreditation processes.

With further verification and the addition of extra species, this project will constitute the basis for a national shark DNA database. The proponents also wish to examine the potential for developing a DNA-based 'field test-kit', which could be used by fisheries and wildlife management officers around the country to enforce compliance with the various protected species regulations, without the need for them to have a detailed understanding of shark species identification techniques or formal training in DNA techniques.

## 2.0 Need

The black-market trade in body parts from vulnerable and endangered sharks, has the potential to cause once common species to become, at least regionally, extinct. As sharks are almost always processed at the time of their capture, it is currently impractical for fisheries and wildlife officers to identify protected species with sufficient certainty of being able to mount a successful prosecution. There is therefore considerable potential (as well as financial incentive) for a continuing illegal trade in body parts from these species. A legally defensible method for identifying protected shark species is therefore urgently required to both ensure compliance with conservation regulations and to act as a deterrent to the illegal capture and trade of these species.

The sustainability of several commercially important shark species is also likely to be placed under increasing threat without better means of controlling the exploitation of individual species, such as commercial protection, species-specific size or bycatch limits. The development of such management measures is, however, dependent on a method for the reliable identification of individual species in processed catches. The proposed DNA database will provide the WA Department of Fisheries and other regulatory authorities with the tools necessary for enforcing both existing and future management and conservation regulations.

Improved shark catch composition data, particularly from fisheries which take sharks as bycatch where they are often mis-identified, is also required to improve the accuracy of assessments of commercially-exploited stocks. The need for improved bycatch species composition data has been specifically recognised in the National Plan Of Action for the conservation and management of sharks (NPOA-sharks) and is therefore of national significance. The catalogue of DNA fingerprints established during this project will provide a basis for validating the accuracy of catch records data and determining the species composition of catches for which there are currently inadequate or no catch records (eg. unidentified shark bycatch or confiscated illegal catches).

# 3.0 Objectives

1. Establish sampling protocols (ensuring methodology will be suitable for evidentiary purposes) and collect reference samples.
2. Establish 'legally robust' DNA processing protocols and process reference samples.
3. Establish a secure DNA 'fingerprint' database for WA shark species to act as both a provider of evidence and a deterrent to illegal fishing activity.

# 4.0 Materials and methods

## 4.1 Sample collection, storage and transfer

A total of 177 samples were collected from two sources. The majority of samples (n=129) were collected by the principal investigator according to the methods developed for the current study (see below), during the course of field components of FRDC project 2000/134, *Biology and stock assessment of the thickskin (Sandbar) shark, Carcharhinus plumbeus, in Western Australia and further refinement of the dusky shark, Carcharhinus obscurus, stock assessment*. These voucher samples were collected between 18/3/03 and 17/8/03 from sharks caught by commercial fishing vessels during their regular fishing operations in the JASDGDLF and WCDGDLF and during fishery-independent surveys on-board the Department of Fisheries Research Vessel *Naturaliste*. The second source of samples was the WA Department of Fisheries' Shark Research Section genetic reference collection. Although not collected according to the same methods as voucher samples, these reference samples were necessarily included in the sample set to increase the sample sizes of those species which are only rarely caught by the WA target-shark fisheries. The majority of these reference samples (n = 40) were collected by Department of Fisheries' Shark Research Section staff on-board JASDGDLF, WCDGDLF and Southern and Western Tuna and Billfish Fisheries (SWTBF) vessels between 30/4/01 and 11/3/04. The remaining reference samples

(n=8) were collected by the principal investigator from JASDGDLF and WCDGDLF catches prior to commencement of the current project.

Sharks were identified using a modified form of the key to Australian sharks and rays given in *Sharks and Rays of Australia* (Last and Stevens, 1994), to ensure correct and consistent identification. The key was simplified to provide sufficient information for the identification of only those species that were being examined during the current project (Appendix I). A signed copy of the key, for each sample collected, was securely filed for future verification of each identification. Samples were collected from two protected species and eight of the most commercially important shark species caught in the Western Australian target-shark fisheries, as shown in Table 1.

**Table 1.** List of voucher and reference samples.

| Common Name | Scientific name | Acronym | No. samples | |
|---|---|---|---|---|
| | | | voucher | reference |
| Spinner Shark | *Carcharhinus brevipinna* | CB | 20 | |
| White Shark | *Carcharodon carcharias* | CC | | 2 |
| Dusky Shark | *Carcharhinus obscurus* | CO | 22 | |
| Sandbar Shark | *Carcharhinus plumbeus* | CP | 20 | |
| Grey Nurse Shark | *Carcharias taurus* | CT | 7 | 15 |
| Whiskery Shark | *Furgaleus macki* | FM | 20 | |
| Gummy Shark | *Mustelus antarticus* | MA | 20 | |
| Blue Shark | *Prionace glauca* | PG | | 22 |
| Smooth Hammerhead | *Sphyrna zygaena* | SZ | 20 | |
| Shortfin Mako | *Isurus oxyrinchus* | IO | | 9 |

Following species identification and verification, sharks were assigned a reference code and 5-10g of muscle and fin tissue excised from each specimen. Standard cleaning protocols were followed during and after tissue removal to avoid cross-contamination. Tissue samples (and subsamples) were placed in a sealed container, fixed in absolute ethanol to prevent DNA degradation and labeled with the specimen reference number. Samples were then secured in tamper-proof bags, which were stored for transportation in a secure lockable container.

Continuity of evidence protocols were established for sample collection, storage, handling and security according to the standards adopted by the WA Police Department. These protocols were developed in consultation with the WA Police forensics branch, with reference to their collection of forensic evidence guidelines. These protocols were refined throughout the project as experience was gained in the practicalities of collecting evidential material within commercial fishery environments. Continuity of sample/evidence sheets documented the collection of each sample and each time sample containers were opened, moved or transferred (see Appendix

II). These sheets will be used to document the future collection, storage, transport and transfer of further voucher samples and evidentiary material. Samples were ultimately placed in the custody of the Chemistry Centre (WA) for DNA fingerprinting analyses, where the integrity of each sample was verified by the project co-investigator. The security, handling, storage, containment and identity preservation of samples at the Chemistry Centre were conducted according to documented quality assurance procedures.

## 4.2 DNA processing and analysis

Genomic DNA was isolated from 10–200 mg tissue by incubation with 1.0mL of digestion buffer [75mM EDTA, 50mM Tris (pH 7.0), 1% SDS, 100µg proteinase K] for 4 hours at 60°C, according to the method of Sambrook et al., 1989. The digest was extracted with Tris-EDTA saturated (pH 7.0) phenol, followed by chloroform:isoamyl alcohol (24:1). The DNA in its aqueous phase was precipitated by adding 2.5 volumes of ice-cold absolute ethanol and stored at –20°C for one hour. DNA was recovered by centrifugation for 10 minutes at room temperature at maximum speed in a microcentrifuge. The pellet was washed with a 70% aqueous ethanol solution and resuspended in 100 µL of nuclease-free water.

PCR amplifications (Saiki et.al., 1985) were performed in 20 µL reactions containing 10mM Tris-HCl, 1.5mM $MgCl_2$, 50mM KCl (pH 8.3), 0.25µM of primers, 250µM dNTPs and 0.75 units of Taq DNA polymerase. DNA was denatured at 94°C for 2 minutes, followed by 35 amplification cycles (melting: 94°C for 15 seconds; annealing 45°C, for 30 seconds; extension: 72°C for 1 minute) and a final extension at 72°C for 10 minutes. As contingency against sequence mismatching, which can lower the efficiency of the PCR amplification process, two pairs of primers complementary to conserved sequences on the vertebrate mitochondrial D-loop were evaluated. The first pair, designated cyt1/cyt2 (5'-CCATCCAACATCTCAGCATGATG-AAA-3' and 5'-GCCCCTCAGAATGATAT TTGTCC-TCA-3'; Kocher et al., 1989; Meyer et al., 1995) flanked a 360bp region on the cytochrome *b* gene. The second pair designated c12R1/c12R2 (5'-CATATTAAACCCGAATGATATTT-3' and 5'-ATAATAGGGTATCT-AATCCTAGTTT-3'; Martin et al., 1992; Tabata et al. 1997) spanned a 2080bp region comprising the 12S rRNA gene and a portion of the cytochrome *b* gene.

Up to 10µL of amplified DNA were digested with 5 units of restriction enzyme for 4 hours at 37°C. A panel of restriction endonucleases (*Hae III, Rsa I, Hinf I, Hpa II, Pst I and Cfo I*) was tested to assess the relative effectiveness of each enzyme and to provide multiple references for species in which any single restriction enzyme produced non-specific profiles. Digests were analysed by horizontal gel electrophoresis on 3.5% agarose (Agarose MS, Roche Diagnostics) against a DNA molecular weight marker. Ethidium bromide stained DNA were photographed under UV transillumination and scanned using a Bio-Rad model GS 650 densitometer. Photographs of DNA profiles (fingerprints) were converted into bitmaps and transferred to a computer as Tagged Image File Formats (TIFF). Gel track images were normalised against a DNA size marker using the Bio-Rad Molecular Analyst Fingerprinting software. Molecular weight was estimated against the DNA Molecular Weight Marker VIII using the Multi-Analyst/PC (BIO-RAD) software. Restriction fragments (>70bp) were sized to the nearest 5 base pairs. Where multiple intraspecific haplotypes were found, they were labelled in numerical order.

## 5.0 Results

Although 360bp fragments were successfully PCR amplified to conserved sequences on the **cytochrome (cyt *b*) gene** using the first primer pair (cyt1/cyt2), extraordinary bands were co-amplified from several species (eg. *S. zygaena* and *C. plumbeus*). Since multiplication of amplified bands is a potentially significant source of artefacts, investigations with the cyt1/cyt2 primer pair were therefore discontinued. The second primer pair (c12R1/c12R2), which flanked portions of the cyt *b* gene and the 12S rRNA gene on the **mt D-loop region**, successfully amplified 2.1kb fragments from samples of the four species of carcharinids (*Carcharhinus brevipinna, C. obscurus, C. plumbeus* and *Prionace glauca*), the two triakid species (*Mustelus antarcticus* and *Furgaleus macki*), two of the three lamnid species (*Carcharias taurus* and *Carcharodon carcharias*) and the one species of hammerhead (*Sphyrna zygaena*). However, only two of the nine samples from the shortfin mako (*I. Oxyrhincus*) tested positive for the 2.1kb amplicon and only weak signals were obtained from the two *C. carcharias* samples.

Amplified 2.1kb DNA fragments from nine species (excluding *I. oxyrinchus*) were successfully digested by four restriction endonucleases, *Hae III*, *Rsa* I, *Hinf I* and *Hpa II*. However, preliminary trials revealed that samples from nearly half of the species tested, resisted digestion by *Cfo I* and *Pst I* and these enzymes failed to generate any restriction fragments in samples from *C. brevipinna*, *C. plumbeus*, *F. macki* and *S. zygaena*. The use of *Cfo I* and *Pst I* was therefore discontinued.

Digestion with ***Hae III*** produced species-specific haplotypes in *C. brevipinna, C. carcharias, C. obscurus, F. macki, M. antarticus, P. glauca* and *S. zygaena* (Table 2, Appendix III). Three haplotypes were produced in *C. plumbeus* (CP.1, 2 & 3) and four in *C. taurus* (CT.1, 2, 3 & 4). Interestingly, the fingerprints of a *C. plumbeus* haplotype (CP.1) and a *C. taurus* haplotype (CT.2) appeared to be homologous. A similar pattern emerged for both *C. carcharias* and *C. obscurus* except for the presence of a 225bp fragment in *C. obscurus*. The most number of *Hae III* restriction sites (7) were found in *C. brevipinna* whose restriction fragments were 700, 480, 370, 290, 225, 145, 105 and 75bp long. The least (3) was found in several sharks including *S. zygaena* (755, 625, 530 and 280bp in length). Thirteen unique polymorphisms were generated for this library from a total of fourteen haplotypes.

Digestion with ***Rsa I*** produced species specific haplotypes in *C. carcharias, F. macki, M. antarticus, P. glauca* and *S. zygaena* (Table 2, Appendix III). *C. obscurus* was the most polymorphic (3 haplotypes), followed by *C. taurus, C. plumbeus* and *C. brevipinna* each with two haplotypes. An interesting but desirable feature was the absence of interspecies homology. The same pattern emerged for both *C. plumbeus* (CP.2) and *C. taurus* (CT.2) except for the presence of the 430bp and 365bp fragments in the *C. taurus*. The most number of *Rsa I* restriction sites (8) were found in *C. carcharias* whose restriction fragments were 1050, 795, 600, 475, 370, 100 and 75bp long. The least (2) was found in *S. zygaena* (1080, 590 and 130bp in length). Fourteen unique haplotypes were generated for this library from a total of fourteen haplotypes.

Digestion with ***Hinf I*** produced single species specific haplotypes in all the sharks except for *C. taurus* and *M. antarcticus*, where two intraspecies haplotypes were detected (Table 2, Appendix III). Interspecies homology was detected between *C. plumbeus* haplotype CP.1 and *C. taurus* haplotype CT.2. The same pattern emerged for both *C. carcharias* and *C. obscurus* except for the presence of an extra 225bp fragment in *C. obscurus*. The most number of *Hinf I* restriction sites (6) were found in *C. carcharias* whose restriction fragments were 955, 840,

700, 520, 290, 100 and 70bp long. The least (2) was found in *S. zygaena* (1115, 530 and 190bp in length). Six unique polymorphisms were generated for this library from a total of 11 haplotypes.

Digestion with ***Hpa II*** produced single species specific haplotypes in *C. brevipinna, C. carcharias, C. obscurus, F. macki, M. antarticus, P. glauca* and *S. zygaena.* Two haplotypes were produced in *C. plumbeus* and also in *C. taurus* (Table 2, Appendix III). Interspecies homology was detected in haplotypes CB1, CO1, CP1, CT2 and MA1. Another close match was recorded between *F. macki* (FM.1) and *P. glauca* (PG.1) but for the presence of an extra 75 bp fragment in *P. glauca*. The most number of *Hpa II* restriction sites (9) were found in *C. carcharias*, whose restriction fragments were 970, 580, 335, 160, 95 and 75bp long. Only two were found in *F. macki* (990, 860 and 175bp in length). A unique feature was the 550bp and 600bp doublet in *S. zygaena* which only became apparent after extended gel electrophoresis. Seven unique polymorphisms were generated for this library from a total of 10 haplotypes.

**Table 2.** Number of haplotypes from each restriction endonuclease that collectively comprise the database of DNA fingerprints for Western Australian sharks.

| Acronym | Species | No. Samples | DATABASE No. Haplotypes | | | |
|---|---|---|---|---|---|---|
| | | | ***Hae III*** | ***Rsa I*** | ***Hinf I*** | ***Hpa II*** |
| CB | *Carcharhinus brevipinna* | 20 | 1 | 2 | 1 | 1 |
| CC | *Carcharodon carcharias* | 2 | 1 | 1 | 1 | 1 |
| CO | *Carcharhinus obscurus* | 22 | 1 | 3 | 1 | 1 |
| CP | *Carcharhinus plumbeus* | 20 | 3 | 2 | 1 | 1 |
| CT | *Carcharias taurus* | 22 | 4 | 2 | 2 | 2 |
| FM | *Furgaleus macki* | 20 | 1 | 1 | 1 | 1 |
| MA | *Mustelus antarticus* | 20 | 1 | 1 | 2 | 1 |
| PG | *Prionace glauca* | 22 | 1 | 1 | 1 | 1 |
| SZ | *Sphyrna zygaena* | 20 | 1 | 1 | 1 | 1 |
| | *Total* | 168 | 14 | 14 | 11 | 10 |
| | *Unique Haplotypes* | | 13 | 14 | 6 | 7 |

The level of inter-specific discrimination of profiles generated by each enzyme, was directly associated with the number of unique haplotypes that each produced (Table 2). Of the four restriction enzymes used to generate fingerprints for inclusion in the database, *Rsa I* produced discriminatory profiles in all nine species that tested positive for the 2.1kb amplicon (Table 3). Digestion with *Hae III*, *Hinf I* and *Hpa II* produced discriminatory profiles for seven, five and three species, respectively.

**Table 3.** Summary of restriction endonuclease panel results.

| Acronym | Species | *Hae III* | *Rsa I* | *Hinf I* | *Hpa II* |
|---|---|---|---|---|---|
| CB | *Carcharhinus brevipinna* | ● | ● | ❍ | ❍ |
| CC | *Carcharodon carcharias* | ● | ● | ● | ● |
| CO | *Carcharhinus obscurus* | ● | ● | ● | ❍ |
| CP | *Carcharhinus plumbeus* | ❍ | ● | ❍ | ❍ |
| CT | *Carcharhinus taurus* | ❍ | ● | ❍ | ● |
| FM | *Furgaleus macki* | ● | ● | ● | ❍ |
| MA | *Mustelus antarticus* | ● | ● | ❍ | ❍ |
| PG | *Prionace glauca* | ● | ● | ● | ❍ |
| SZ | *Sphyrna zygaena* | ● | ● | ● | ● |

● = Highly discrimitory for that species; ❍ = Less discrimitory for that species

## 6.0 Discussion

The PCR amplification technique has already proven successful in speciating tissue samples from several shark species (Ho et al., 1998; Heist and Gold, 1999a; Chan et al., 2003), and preliminary results from tests of dried shark fins (Ho, unpublished data) have demonstrated its applicability to speciating sharks from degraded DNA samples. The simpler and cheaper RAPD (random polymorphic DNA) PCR (Bardakci and Skibinski, 1994) is not suitable for the potentially low quality DNA presented from evidentiary exhibits. Other more sophisticated techniques such as microsatellite analysis (Heist and Gold, 1999b; Feldheim et al., 2001; Schrey and Heist, 2003), which is highly discriminatory, require extensive method development. In the context of fishery management and elasmobranch conservation objectives, gene cloning, screening and DNA sequencing, are both time and cost prohibitive. However, further improvements in these technologies, combined with improved genetic analysis infrastructure and resourcing may accelerate the adoption of more effective and economically-scaled methods for DNA fingerprinting.

The mass of samples ranged from 10mg to 2.5g (as received), although most were approximately 120mg. Overall, the use of absolute ethanol for preserving samples that were generally below 500mg, appeared suitable. Since the quality and quantity of extractable DNA decreases over time and depends on storage conditions after collection (Kirby, LT, 1992), it was unsurprising that, in a small number of samples, there was evidence of decomposition from the presence of volatiles (n=2) and discolouration (n=2). The cartilaginous matrix in a few samples (n=2) was also not conducive to thin sectioning and may have led to a lower than expected yield of DNA. Whilst genomic DNA isolated from the samples showed varying degrees of degradation, they were generally suitable for PCR amplification. However, the yield of DNA from 10mg samples was at the lower limit of that required for replicate analyses.

During the planning phase of this project, it was thought that first primer pair (cyt1/cyt2), which flanked a small (360 bp) region on the cytochrome *b* gene, would prove to be more suitable for analysing the potentially low quality DNA that might be presented as evidentiary material (eg. dried or frozen tissue). Unfortunately, as the co-amplification of extraordinary bands resulted in unreplicable profiles for several species (eg. *S. zygaena* and *C. plumbeus*), these priming sequences utimately proved to be unsuitable for our purposes and its investigation was therefore discontinued. However, the second primer pair (c12R1/c12R2), which has proven to be effective in Red sea Bream (*Pagrus major*, Tabata et.al., 1997), produced replicable 2.1 kb fragments from samples of nine of the study species. However, only two of the nine samples from the shortfin mako (*I. Oxyrhincus*) tested positive for the 2.1 kb amplicon and only weak signals were obtained from the two samples from *C. carcharias.* This unexpected result for *I. Oxyrhincus* was consistent with mismatches between the priming sequence and not because of a lack of DNA. Therefore, the use of alternative primers requires further investigation if this species is to eventually be included in the database. Overall, the c12R1/c12R2 primer-pair was sufficiently 'generic' to enable DNA to be amplified in adequate quantities for restriction enzyme analysis. The success of the PCR amplification process supports our decision to use mitochondrial (mt) DNA in preference to nuclear DNA because of its high copy number in the cell (Magoulas, 2005) and its resistance to cellular degradation.

Amplified DNA was readily digested by *Hae III, Rsa I, Hinf I* and *Hpa II.* The individual libraries of genetic profiles resulting from digests by these four enzymes, collectively form the database. Whilst these four restriction endonucleases produced distinct and discrete fingerprints, which allowed discrimination of samples to species level, interspecific similarities were also detected in some digests. Furthermore, all four restriction endonucleases showed intraspecific variations. Of the six enzymes evaluated, *Rsa I* was the most discrimitory followed by *Hae III*. Although the level of interspecies polymorphism for *Hinf I* and *Hpa II* sites was low, their use in conjunction with other restriction enzymes reduces the potential for misidentification of the study species. The database is therefore comprised of matrices of multiple DNA profiles for each of the nine study species for which fingerprints were successfully produced. As a host of other potentially informative enzymes are also available, it is desirable that as many of these as possible be evaluated in the future to assess their suitability for speciating sharks' DNA.

The DNA database and associated evidentiary sampling protocols that were developed during this project provide four immediate benefits to the agencies responsible for management and conservation of sharks in Western Australia. Firstly, they constitute a legally defensible basis for ensuring compliance with existing protected species regulations. Two catalogued species, the white shark (*Carcharodon carcharias*) and grey nurse shark (*Carcharias taurus*), are protected under the Environment Protection and Biodiversity Conservation Act (1999), the WA Wildlife Conservation Act (1950) and, in the case of *C. carcharias*, also under the WA Fish Resources Management Act (1994). As sharks are processed (headed, gutted and fins removed) shortly after their capture, the identification of body parts from these species during at-sea inspections and in landed catches, has previously been too uncertain to enable prosecution. This database now enables unambiguous identification of these species from only small samples of tissue, even when evidentiary material has been frozen or dried. However, further samples from *C. carcharias* will be required to ensure that as many of this species' haplotypes as possible are represented in the database. These samples will be collected opportunistically (according to the established evidentiary protocols) and incorporated into the database as they become available.

Secondly, the outputs from the project provide a basis for development of species-specific management responses, similar that which is currently being implemented for the dusky shark, *C. obscurus*. Stock assessments for this species (Simpfendorfer, 1999; McAuley et al. 2005) have indicated that in conjunction with catches by the target demersal gillnet fishery (JASDGDLF and WCDGDLF), small catches of dusky sharks older than six years of age are likely to cause this population to decline. The WA Department of Fisheries is therefore in the process of listing dusky sharks with an interdorsal length greater than 70cm (*ca*.150cm FL) as commercially protected, to ensure the sustainability of this stock and the ongoing viability of the temperate demersal gillnet fishery. This database will be essential for ensuring compliance with this new regulation and for developing similar measures for other commercially important species, should they become necessary at some point in the future.

Thirdly, the relevant management agencies' improved ability to detect catches of protected species will act as a significant deterrent to the illegal trade in their body parts. To ensure the database has this desired effect, the Western Australian commercial and recreational fishing sectors will be notified of the implementation of the database and associated testing regime through relevant publications. Any successful prosecutions resulting from the genetic identification of protected species, will be publicised in, e.g. commercial fishing magazines, newspapers and the FRDC R&D news magazine to reinforce the database's deterrent effect.

Finally, this catalogue of reference DNA samples also provides the Department of Fisheries with the ability to assess the contribution of catalogued species to previously unidentifiable shark catches by non-target fisheries and from illegal sources. With suitable levels of testing, these currently hidden sources of fishing mortality can potentially be quantified and thereby used to improve the stock assessments of commercially important shark species.

The protocols and documentation for collecting, transporting and storing genetic samples that were developed during this project will be incorporated into the Western Australian Department of Fisheries operational policy through its Fisheries Officer Instructions. An operational strategy for inspecting and testing samples from shark catches will then be developed by the Department's Regional Services Branch (RSB). Methods for collating data on the number of inspections, number of tests conducted and numbers of protected species identified will also be developed during this process in order to measure the longer-term success of the project. Following the development of these strategies, the project investigators will make themselves available to attend RSB annual regional meetings in order to workshop any unresolved issues relating to the implementation of inspection and testing procedures.

## 7.0 Benefits

The primary beneficiaries of this project will be the Western Australian commercial shark fisheries, Department of Fisheries and the broader community. The adoption of legally robust methods for identifying individual species in shark catches will help to ensure the conservation of at-risk species and the sustainability of target species of the WA commercial shark fisheries. Not only is the economic viability of these approximately $7 million per year fisheries dependent on their target species' biological sustainability but also on obtaining approval from the Commonwealth Department of Environment and Heritage (DEH) under their Ecologically Sustainable Management of Fisheries guidelines. This approval is likely to be dependent on the fisheries being able to demonstrate that their continued operation is not threatening the

sustainability of either protected or commercially exploited species. The genetic database and associated inspection/testing regime should make it easier to demonstrate that the WA target-shark fisheries are not causing excessive mortality of protected species and that the bycatch of commercial species in non-target fisheries is not threatening their sustainability. This project therefore also delivers a tangible benefit to DEH in providing a more robust basis for their assessment of the WA target-shark fsheries, as well as other fisheries which take incidental catches of sharks.

Should threats to the sustainability of these study species be identified in the future, the genetic reference collection also enables the development of species-specific management responses. This will further benefit the WA shark fisheries in that management measures (e.g. commercial protection, size limits, bycatch/bag limits, etc.) can be fine-tuned to respond to specific threats, thereby negating the need for more restrictive broad-scale responses, such as effort reduction. This type of approach is currently being undertaken in response to the risk of low levels of larger dusky shark mortality, through the commercial protection of dusky sharks with interdorsal lengths greater than 70cm (~150cm Fork Length).

## 8.0 Acknowledgements

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# 10.0 Appendices

## Appendix I. Species Identification Key

Department of Fisheries
Government of Western Australia

**SHARK DNA DATABASE PROJECT**

**Species identification key**

| Sample ID No: | Date: | Time: | Vessel: |
|---|---|---|---|
| Latitude: | Longitude: | Skipper: | Crew: |

**Circle appropriate responses. If response = Y proceed to next question unless otherwise directed.**

**KEY TO FAMILY**

1. 5 GILLSLITS ON EITHER SIDE OF HEAD (NOT VENTRAL) **Y N**

2. MID-BASE OF 1st DORSAL-FIN ANTERIOR TO PELVIC-FIN ORIGIN **Y N**
3. SNOUT ELONGATE AND FLATTENED (BLADE-LIKE) **(if N go to 4)** **Y N**
4. ANAL FIN PRESENT **Y N**
5. HAMMER-SHAPED HEAD **(if Y = Sphyrnidae; if N go to 6)** **Y N**
6. DORSAL-FIN SPINES ABSENT **Y N**
7. CONICAL SNOUT **(if N go to 11)** **Y N**
8. CAUDAL KEELS PRESENT **(if N = Odontaspididae)** **Y N**
9. LUNATE CAUDAL FIN **Y N**
10. MINUTE TEETH & GILLSLITS EXTEND ONTO VENTRAL SURFACE **Y N**
    **(if N = Lamnidae)**
11. UPPER CAUDAL FIN LOBE <30% OF TOTAL LENGTH **Y N**
    *upper caudal fin length* ☐ cm; *total length* ☐ cm
12. CAUDAL FIN WITH SUBTERMINAL NOTCH **Y N**
13. NICTITATING MEMBRANE PRESENT **Y N**
14. SPIRACLES PRESENT **(if N = Carcharhinidae)** **Y N**
15. NO PRECAUDAL PITS **(if Y = Triakidae)** **Y N**

**KEY TO SPECIES**

**Family Sphyrnidae:**

1. HEAD WIDTH <40% OF TL *head width* ☐ cm; *total length* ☐ cm **Y N**
2. ANTERIOR MARGIN OF HEAD LACKING MEDIAN INDENTATION **Y N**
   **(if 1 & 2 = Y, *Sphyrna zygaena*)**

**Family Odontaspididae:**

1. 1st DORSAL FIN EQUAL IN SIZE TO 2nd DORSAL & ANAL FINS **Y N**
   *1st dorsal fin height* ☐ cm; *2nd dorsal fin height* ☐ cm; *Anal fin height* ☐ cm
2. 1st DORSAL FIN ORIGIN BEHIND PECTORAL FIN FREE REAR TIPS **Y N**
   (if 1 & 2 = Y, *Carcharias taurus*)

Department of Fisheries
Government of Western Australia

# SHARK DNA DATABASE PROJECT

## Species identification key

**Family Lamnidae:**

1. FLAT, BROADLY TRIANGULAR UPPER TEETH WITH SERRATED EDGES **Y N**
   **(if 1=Y, *Carcharodon carcharias;* if N go to 2)**
2. (a). SINGLE CAUDAL KEEL **Y N**
   (b). MONOCUSPID TEETH **Y N**
   (c). PECTORAL FIN LENGTH < HEAD LENGTH *head length* ☐ cm **Y N**
   **(if 2 (a), (b) & (c) = Y, *Isurus oxyrinchus*)** *TL* ☐ cm

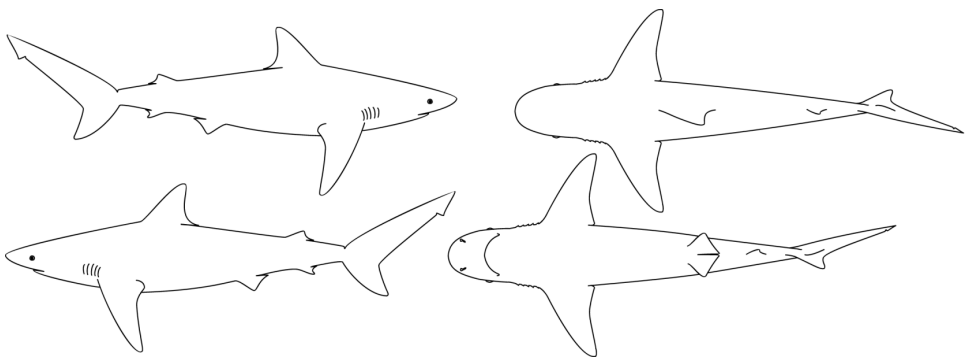
**Family Triakidae:**

1. NASAL BARBELS PRESENT **(if 1 = Y, *Furgaleus macki;* if N go to 2)** **Y N**
2. WHITE SPOTS ON BODY **Y N**
3. >120 cm TL *TL* ☐ cm **(if 2 & 3 = Y, *Mustelus antarcticus*)** **Y N**

**Family Carcharhinidae:**

1. LIVE COLOURATION VIVID BLUE **(if Y = *Prionace glauca;* if N go to 2)** **Y N**
2. FINS WITH DISTINCTIVE MARKINGS **(if N go to 4)** **Y N**
3. DISTINCT BLACK TIPS TO ALL FINS EXCEPT PELVIC **(if Y= *C. brevipinna*)** **Y N**
4. UPPER TEETH BROADLY TRIANGULAR, SERRATED **Y N**
5. INTERDORSAL RIDGE PRESENT **Y N**
6. 1st DORSAL FIN HEIGHT <12.5% OF TOTAL LENGTH
   *1st dorsal height* ☐ cm; *total length* ☐ cm **Y N**
   **(if N = *Carcharhinus plumbeus;* if Y go to 7)**
7. 2nd DORSAL FIN INNER MARGIN <1.6 TIMES 2nd DORSAL FIN HEIGHT **Y N**
   *2nd dorsal fin height* ☐ cm; *2nd dorsal fin inner margin length* ☐ cm
8. PREORAL LENGTH <9.5% OF TOTAL LENGTH **Y N**
   *preoral length* ☐ cm; *total length* ☐ cm **(if 7 = Y, *Carcharhinus obscurus*)**

Signed _____ INDICATE SAMPLE LOCATION:



## Appendix II. Continuity of sample/evidence form

Department of Fisheries
Government of Western Australia

**SHARK DNA DATABASE PROJECT**

**Continuity of sample/evidence**

Collection/Seizure

| | |
|---|---|
| Date & time sample taken | ……/……/…….. ; …… : …… hours |
| Location | |
| Is sample a voucher sample or unidentified sample? | voucher / unidentified |
| Sample number (or seizure receipt number if appropriate) | |
| Officer who collected sample | |
| Division/section/office | |
| Assisting Officer/s | |
| Sample caught or consigned by / seized from | |
| Boat Name | FBL # |
| Skipper informed of sample taken | yes / no How |
| Date & time sample placed into storage | ……/……/…….. ; …… : …… hours |
| Sample stored at (office) | |

Maintenance

| Alcohol levels checked and / or topped up (date & initial) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | |
| | | | | | | | | | |

Transportation

| | |
|---|---|
| Date & time sample removed from storage | ……/……/…….. …… : …… hours |
| Transported by (print & sign) | |
| Delivery location | |
| Received by (print & sign upon receipt) | |
| Date & time sample received | ……/……/…….. …… : …… hours |
| Collected by (print & sign upon receipt) | |
| Delivery location | |
| Received by (print & sign upon receipt) | |
| Date & time sample received | ……/……/…….. …… : …… hours |
| Collected by (print & sign upon receipt) | |
| Delivery location | |
| Received by (print & sign upon receipt) | |
| Date & time sample received | ……/……/…….. …… : …… hours |
| Collected by (print & sign upon receipt) | |
| Delivery location | |
| Received by (print & sign upon receipt) | |
| Date & time sample received | ……/……/…….. …… : …… hours |

Department of Fisheries
Government of Western Australia

# SHARK DNA DATABASE PROJECT

## Continuity of sample / evidence

### Analysis

**Fingerprinting**

Date DNA profile recorded ……/……/…..…....

Unidentified sample identified as (species)..... .....

### Evidence

Will sample be required as evidence .....yes / no (circle)

Received letter for professional opinion.....yes / no (circle) date ……/……./……..

**Post analysis storage**

Location sample stored following analysis .....

Date sample arrived for storage ……../……../……

Person who placed sample into storage.....

Reference number .....

### Disposal

Disposal method .....

Approval to dispose ..... yes / no (circle) signature of SFO.....

Date sample disposed ……/……/…..…..

## Appendix III. Shark Restriction Fragment Length Polymorphism and Restriction Enzyme Matrix

| Species | *Hae III* | *Rsa I* | *Hinf I* | *Hpa II* |
|---|---|---|---|---|
| *Carcharhinus brevipinna* | 700, 480, 370, 290, 225, 145, 105, 75 | 705, 580, 465, 370, 95, 75<br><br>695, 580, 455, 365, 135, 100, 75 | 895, 520, 450, 260, 100, 75 | 970, 580, 335, 160, 95, 75 |
| *Carcharodon carcharias* | 1060, 490, 100, 75 | 1050, 795, 600, 475, 370, 100, 75 | 955, 840, 700, 520, 290, 100, 70 | 1210, 1030, 775, 590, 470, 100, 75 |
| *Carcharhinus obscurus* | 1060, 490, 225, 80 | 1165, 785, 585, 470, 365, 80<br><br>750, 560, 455, 360, 80<br><br>660, 560, 450, 360, 175, 90, 75<br><br>550, 445, 350, 75 | 865, 470, 260, 70 | 965, 580, 335, 160, 75<br><br>920, 735, 560, 330, 160, 75 |
| *Carcharhinus plumbeus* | 955, 490, 290, 190, 150, 100, 75<br><br>980, 490, 380, 290, 185, 75<br><br>970, 650, 280, 190, 100, 75 | 1020, 430, 360, 135, 100, 75<br><br>1030, 770, 100, 75 | 950, 535, 460, 265 | 995, 590, 330, 160, 75 |
| *Carcharhinus taurus* | 900, 630, 530, 75<br><br>970, 485, 285, 190, 140, 75<br><br>915, 645, 410, 100<br><br>935, 625, 495, 145 | 1080, 610, 330<br><br>1010, 760, 430, 365, 100, 80 | 920, 400, 260, 135<br><br>905, 525, 445, 260, 75 | 700, 415, 200, 160, 70<br><br>960, 580, 330, 160, 75 |
| *Furgaleus macki* | 995, 410, 315, 190, 135 | 900, 580, 365, 210 | 1135, 275, 195, 140, 70 | 990, 860, 175 |
| *Mustelus antarticus* | 1120, 485, 145, 75 | 915, 765, 595, 380, 135, 105 | 920, 525, 450, 260, 100, 75<br><br>935, 770, 450 | 975, 570, 330, 165, 75 |
| *Prionace glauca* | 955, 650, 280, 225 | 1030, 600, 360, 80 | 930, 505, 450, 260, 75 | 990, 855, 160, 75 |
| *Sphyrna zygaena* | 755, 625, 530, 280 | 1080, 590, 130, | 1115, 530, 190 | 560, 325, 160 |

# List of Fisheries Research Reports

Not all have been listed here, a complete list is available online at http://www.fish.wa.gov.au/res

83 The Western Rock Lobster fishery 1985/86. Brown, R.S. and Barker, E.H. (1990).

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