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STOCK STRUCTURE OF EXPLOITED SHARK SPECIES IN NORTH EASTERN AUSTRALIA

D J Welch
J Ovenden
C Simpfendorfer
A Tobin
J A T Morgan
R Street
J White
A Harry
R Schroeder
W G Macbeth

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JAMES COOK UNIVERSITY AUSTRALIA

NSW DEPARTMENT OF PRIMARY INDUSTRIES

THE UNIVERSITY OF QUEENSLAND AUSTRALIA

F&F

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D J Welch[1,2], J Ovenden[3], C Simpfendorfer[1], A Tobin[1], J A T Morgan[3], R Street[3], J White[1], A Harry[1], R Schroeder[1] and W G Macbeth[4]

[1] Fishing & Fisheries Research Centre, School of Earth and Environmental Sciences, James Cook University, Townsville, QLD 4810

[2] Queensland Primary Industries and Fisheries, Department of Employment, Economic Development and Innovation, PO Box 1085, Oonoonba, QLD 4810

[3] Molecular Fisheries Laboratory, Queensland Department of Employment, Economic Development and Innovation, The University of Queensland, Brisbane, QLD 4072

[4] New South Wales Department of Primary Industries, Cronulla Fisheries Research Centre of Excellence, PO Box 21, Cronulla, NSW 2230

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Corresponding author:

David Welch
Fishing & Fisheries Research Centre
School of Earth & Environmental Sciences
James Cook University
Townsville, QLD 4810
Phone: +61 7 4781 5114
Email: david.welch@jcu.edu.au

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Cover image (circled): Australian black tip shark, *Carcharhinus tilstoni*, identified as a hybrid crossed with a common black tip shark, *C. limbatus*. Photograph courtesy of Pascal Geraghty, NSW Department of Primary Industries.

Contents

Acronyms and Abbreviations

ABI .....Applied Biosystems Incorporated
AIC .....Akaike's Information Criteria
ANOVA .....Analysis of Variance
cm .....centimetre
DEEDI .....Department of Employment, Economic Development and Innovation
ECIFFF.....East Coast Inshore Fin Fish Fishery
FL.....Fork Length
FRDC .....Fisheries Research & Development Corporation
GBRMPA .....Great Barrier Reef Marine Park Authority
GBRWHA.....Great Barrier Reef World Heritage Area
HRM .....High-resolution-melt
ICPMS.....Inductively coupled plasma mass spectrometry
IUCN .....International Union for Conservation of Nature
JCU .....James Cook University
LA-ICPMS.....Laser ablation – inductively coupled plasma mass spectrometry
mm.....millimetre
MANOVA .....Multivariate Analysis of Variance
MTSRF.....Marine and Tropical Sciences Research Facility
NIST.....National Institute of Standards and Technology
NSW.....New South Wales
PCR.....Polymerase chain reaction
PCV.....Pre-caudal vertebral
QLD.....Queensland
QDPI&F.....Queensland Department of Primary Industries and Fisheries (now DEEDI)
QFIRAC.....Queensland Fishing Industry Research Advisory Committee
QFRAB.....Queensland Fisheries Research Advisory Board
QPIF.....Queensland Primary Industries and Fisheries (Department of Employment, Economic Development and Innovation)
SNP.....Single nucleotide polymorphism
STL .....Stretched total length
TACC .....Total Allowable Commercial Catch
TL.....Total Length
t.....Tonne

2007/035 Stock structure of exploited shark species in north-eastern Australia

Principal Investigator: David J. Welch

Address: C/- Fishing & Fisheries Research Centre
School of Earth & Environmental Sciences
James Cook University
Townsville, QLD 4810, AUSTRALIA
Telephone: +61 414 897 490

Objectives:

1. To determine the spatial and temporal stock structure of fished shark species along the Queensland east coast.
2. To use stock structure information to define appropriate management units for sustainable management of shark resources along the Queensland east cost.

Non-Technical Summary

OUTCOMES ACHIEVED TO DATE:

The project has provided management and other stakeholders with information necessary to make informed decisions about the management of four of the key exploited shark species caught in the Queensland inshore net fishery and northern New South Wales line fishery. The project has determined that spatial management of milk sharks within Queensland, and scalloped hammerhead, common black tip and Australian black tip sharks within Queensland and New South Wales is appropriate. The project has determined that both black tip shark species are likely to require co-operative management arrangements between Queensland and New South Wales. For scalloped hammerheads separate stocks between the two jurisdictions were identified from the fisheries-dependent samples, however genetic exchange across borders is likely to be facilitated by movement of adult females and perhaps larger males to a lesser extent. This information will greatly assist compliance with the Commonwealth *Environment Protection and Biodiversity Conservation Act (1999)* for shark fisheries in north-eastern Australia by providing the necessary basis for robust assessment of the status of stocks of the study species, thereby helping to deliver their sustainable harvest. It also helps to achive objectives of the Australian National Shark Plan.

The project provides the appropriate spatial framework for future montoring and assessment of the study species. This is at a time when shark fisheries are receiving close attention from all sectors and when monitoring programs are being implemented, aimed at better assessment of stock status.

This project has provided the crucial information for developing an appropriate monitoring design as well as the necessary basis for making statements about stock status.

The project has addressed research priorities identified by the Queensland Fisheries Research Advisory Board, Great Barrier Reef Marine Park Authority and Queensland Fisheries. Previously management has assumed a single stock for each species on the east coast of Queensland, and management of shark fisheries in New South Wales (NSW) and Queensland has been independent of one another.

The project has been able to enhance and develop links between research, management and industry. Strong positive relationships with commercial fishers were crucial in the collection of samples throughout the study area and fisheries managers were part of the project team throughout the study period. During the project the study area was extended to include both Queensland and NSW waters, creating mutualistic and positive links between the States' research and management agencies. Extension of project results included management representatives from NSW and Queensland, as well as the Northern Territory where similar shark fisheries operate and similar species are targeted.

The project was able to provide significant human capital development opportunities providing considerable value to the project outcomes. Use of vertebral microchemistry and life history characteristics as stock determination methods provided material for two PhD students based at James Cook University: Ron Schroeder, vertebral chemistry; and Alastair Harry, life history chacteristics.

The project has developed novel research methods that have great capacity for future application, including:

- Development of a simple and rapid genetic diagnostic tool (RT-HRM-PCR assay) for differentiating among the black tip shark species, for which no simple morphological identifier exists; and
- Development of laser ablation inductively coupled plasma mass spectrometry (LA-ICPMS) methods for analysing and interpreting microchemical composition of shark vertebrae.

The study has provided further confirmation of the effectiveness of using a holistic approach in stock structure studies and justifies investment into such studies.

The requirement for Australian fisheries jurisdictions to ensure the sustainable harvest of fish resources relies on robust information on the resource status. In northern Australia management of inshore fisheries that target shark is independent for each of these jurisdictions. However, the lack of information on the stock structure and biology of shark species in northern Australia means that the appropriate spatial scale of management is not known and assessment of the resource status is not possible. Establishing the stock structure of key shark species in commercial fisheries would immensely

improve the relevance of future resource assessments for fishery management of shark across northern Australia. In a time of widespread concern for shark fisheries everywhere there was an urgent need for stock structure information on targeted shark species. The project objectives therefore were to determine the stock structure of four key target shark species in northern Australian net fisheries and define appropriate management units for their sustainable management. The four shark species for this study were milk shark (*Rhizoprionodon acutus*), scalloped hammerhead (*Sphyrna lewini*), Australian black tip shark (*Carcharhinus tilstoni*), and the common black tip shark (*C. limbatus*). The project sampling regions covered the Queensland east coast however during the project collaborations were established with NSW Fisheries scientists and therefore extended the range of the study into northern NSW giving a more comprehensive study of stock structure for the east coast.

We used multiple techniques concurrently to determine the stock structure of each species. These techniques were: genetic analyses (mitochondrial DNA and microsatellites), vertebral microchemistry, and life history parameters. This holistic approach to stock identification gave the advantage of using techniques that were informative about the shark's life history at different spatial and temporal scales, increasing the likelihood of detecting different stocks where they existed and providing greater certainty in the signals given by the data. Genetics can inform about the evolutionary patterns as well as rates of mixing of sharks from adjacent areas, while vertebral microchemistry is directly influenced by the environment and so will inform about the patterns of movement during the sharks lifetime. Life history characteristics are influenced by both genetic and environmental factors.

During the project it became apparent that diagnostic tools used to distinguish between the two morphologically indistinct black tip shark species (mitochondrial DNA, vertebral counts, life history traits) produced many ambiguous identifcations. This meant that the ability to determine the stock structure for these two species was compromised and so vertebral microchemistry and life history characteristics for black tip sharks was not pursued further. This led to the development of a cost-effective and effective diagnostic molecular assay during this project for the identifications of black tip shark species. Further analyses revealed the remarkable discovery that *C. tilstoni* and *C. limbatus* are hybridising all along the north eastern Australian coast with crosses occurring both ways. Further, 1st and 2nd generation hybrids were detected indicating that at least some of the hybrids are reproductively viable. Genetic stock structure analayses were carried out for each black tip species with both species showing a distinct northern and southern stock with a boundary corresponding to southern limit of the Great Barrier Reef.

Both scalloped hammerhead and milk sharks were found to consist of single genetic stocks along the north eastern Australian coast, however vertebral microchemistry revealed that several separate stocks exist comprising largely of juveniles and adult males. For both species, and particularly scalloped hammerheads, it is hypothesisied that adult females extend into deeper waters farther offshore and are

largely unavailable to capture in the inshore fisheries as they currently operate. If so, then it is the females that are important in replenishing populations along the entire sampled range.

The implications of these results for management are that, where pragmatic, management agencies should adopt spatial management of these species according to the spatial scales and boundaries identified in this study. Future monitoring and assessment of the study species within their respective fisheries should also be conducted at a regional scale. We recommend that the evidence for differences in life history traits for scalloped hammerhead and milk sharks be further investigated through targeted sampling since this could have implications for the relative productivity of the respective stocks. We also recommend that the full extent of the black tip hybrid zone be determined by testing samples from Western Australia, the Northern Territory including the Gulf of Carpentaria, southern NSW and Indonesia. Investigation of hybrid fitness in light of future population viability is also needed. This information is urgently needed before mtDNA species identifications and haplotype frequencies can be used to infer restrictions to gene flow, which is essential base-line data for sustainable management.

KEYWORDS:

Scalloped hammerhead, *Sphyrna lewini*, Milk shark, *Rhizoprionodon acutus*, Australian black tip shark, *Carcharhinus tilstoni*, Common black tip shark, *Carcharhinus limbatus*, stock structure, spatial dynamics, population genetics, life history, microchemistry, fisheries, management

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Chapter 1: Introduction

1.1 Overview

Knowledge of the stock structure of marine species forms the basis for informed management whether for conservation or fisheries purposes. Whether species are comprised of a single mixing stock or many spatially independent stocks forms the basic unit for monitoring, assessment and management of that species. Although there exists a multitude of techniques that can be used to identify stock structure (e.g. tagging, parasite, biology), research on the stock structure of shark species globally are dominated by the use of genetic methods only. In this study we identify the stock structure of different shark species using multiple techniques applied concurrently and therefore represents the first use of a holistic approach (Begg and Waldman 1999) in determining shark stock structure.

The species in this study are all important target species for inshore net fisheries in northern Australia. They are the scalloped hammerhead, *Sphyrna lewini*, the milk shark, *Rhizoprionodon acutus*, the Australian black tip shark, *Carcharhinus tilstoni*, and the common black tip shark, *C. limbatus*. The region of focus for this study was north-eastern Australia comprising the Queensland east coast and northern New South Wales (NSW). The techniques used during the study to determine stock structure for each species were genetics (mitochondrial-DNA and microsatellites), vertebrae microchemistry (laser ablation – inductively coupled plasma mass spectrometry; LA-ICPMS), and life history parameters. The project therefore set out to identify the appropriate spatial scale for monitoring, assessing and managing these shark species in NSW and Queensland fisheries, and identify whether and where co-operative jurisdictional approaches were appropriate. Currently in both jurisdictions the status of shark stocks are unknown and there is very limited management.

In this report we present the results of the stock structure analyses for milk sharks and scalloped hammerheads separately for each method as individual chapters, followed by a chapter that integrates the results of all methods. Due to peculiarities in the data analyses for the two black tip species the results for these species are presented in a separate chapter and for stock structure analysis, using genetics only. In the current chapter (Chapter 1) we present background information included in the original project proposal, the project objectives and a general overview of the project methods. For scalloped hammerheads and milk sharks we present the results of the genetic stock structure analyses in Chapter 2, followed by the vertebral microchemistry and life history parameter analysis results in Chapters 3 and 4 respectively. In Chapter 5 the results of each technique are integrated to provide conclusions about management units for each species. Chapters 6, 7 and 8 present results for the analyses conducted on the two black tip shark species; diagnostic molecular assay development to distinguish between the two species, the discovery that common and Australian black tip sharks are

hybridising along the entire sampled north eastern coast, and genetic stock structure analyses respectively. In the final chapter we present outcomes, conclusions and recommendations arising from the project.

1.2 Background

Knowledge of the stock structure, spatial dynamics and fisheries biology of targeted species provides an essential framework for effective natural resource assessment and management. This information is urgently required for exploited shark species in northern Australia as there is significant concern about the sustainability of current shark harvests. Until this fundamental information is provided, assessments of the status of shark fisheries in tropical Australia, and the interventions that underpin their sustainable management, are uncertain. This project aimed to use a suite of complementary stock identification techniques to provide the necessary information on the spatial structure and effective management units of key exploited shark species in north-eastern Australia that is required for management of this highly valued resource. In doing so, this project directly addresses the Fisheries Research & Development Corporation (FRDC) strategic challenge to "maintain and improve the management and use of aquatic natural resources to ensure their sustainability". Further, determination of stock structure of targeted shark species in north-eastern Australia will inform the likely spatial scale of management units for these species across other regions of Australia where they are fished. That is, where there exist discrete or semi-discrete groups of particular shark species upon which fishing effort is imposed, then ensuring sustainable harvest requires the use of management interventions that allow harvest levels that are consistent with the biological attributes of that group of animals.

Global concern for the sustainability of shark stocks arises from their relatively low productivity, as sharks are typically long-lived, slow growing, late maturing, have low reproductive output and are often naturally in low abundance. These life history traits render sharks particularly vulnerable to fishing pressure, as they are unable to tolerate significant increases in mortality. On the Queensland east coast in 2008 approximately 1084 t of sharks were harvested in the East Coast Inshore Fin Fish Fishery (ECIFFF) (Anon 2010a), while in NSW shark catches increased dramatically from 2005, peaking at 457 t in 2006/07 (Macbeth *et al.* 2009). In 2009 an annual Total Allowable Commercial Catch (TACC) of 600 t was introduced for shark in the Queensland ECIFFF as a conservative measure to limit shark catch while uncertainty exists in their status. Similarly, in 2008, Industry and Investment NSW set a preliminary annual TACC of 160 t for a suite of large coastal and pelagic shark species to control the targeted demersal longline fishing for large coastal sharks in the NSW State-managed fisheries. While the fillets from sharks are of relatively low value on the domestic market, shark fins fetch a very high price on the Asian markets. Consequently, there is significant incentive for fishers to continue to target sharks in north-eastern Australia's inshore fisheries.

Despite the importance of these shark fisheries, very little was known about the stock structure and basic biology of exploited shark species in Queensland and NSW. Recent FRDC-funded projects (2001/077, 2002/064) have provided some preliminary biological information and examined the genetic stock structure of some key exploited tropical sharks (*Carcharhinus limbatus* and *C. tilstoni*) between northern Australia and Indonesia (see Ovenden *et al.* 2009, 2010). Further research is required to extend this knowledge to the east coast of Queensland and northern NSW so that priority management needs can be addressed, in particular the requirements for ecological sustainability under the Commonwealth's *Environment Protection and Biodiversity Conservation Act 1999* (EPBC Act). Information on the stock structure of exploited shark species is also important to the export accreditation process for the Queensland ECIFFF.

This project was developed in direct response to concerns raised by the Great Barrier Reef Marine Park Authority (GBRMPA) and Queensland Department of Employment, Economic Development and Innovation (DEEDI; formerly QDPI&F) about the sustainability of catches of sharks in Queensland's net fishery. This concern was substantiated by a risk assessment of Queensland sharks by Gribble *et al.* (2005) that identified a medium to high risk to the sustainability of a number of commercially harvested shark species. Accordingly, a description of the stock structure of target fished shark populations was identified as a high research priority by the Queensland Fishing Industry Research Advisory Committee (QFIRAC).

The project initially aimed to directly address this research priority by testing the hypothesis of broad scale spatial stock structure of three key harvested species from Queensland's inshore net fishery; Australian black tip shark (*Carcharhinus tilstoni*), the scalloped hammerhead shark (*Sphyrna lewini*) and the milk shark (*Rhizoprionodon acutus*). These three species comprise over fifty percent of the shark harvest on Queensland's east coast (Rose *et al.* 2003; Simpfendorfer *et al.* 2007) and span across two families and three different genera of sharks. During the early stages of the project it became obvious, due to the inability to morphologically distinguish between the Australian black tip shark (*C. tilstoni*) and the common black tip shark (*C. limbatus*), that sampling from catches would comprise both species. Also, since identifying between the two the species using genetic methods was required it made good sense to also include *C. limbatus* into analyses for stock structure determination. The FRDC agreed this was important and a further $14,250 in funding was granted to include *C. limbatus* into analyses meaning the project was focusing on four species. Also, during the project the opportunity to obtain samples of *S. lewini* and black tip sharks from NSW led to formal collaborative arrangements between states to examine stock structure across Queensland and northern NSW enabling a more comprehensive and appropriate study to take place.

Use of the techniques applied in other FRDC-funded projects that have examined stock structure for other finfish species (e.g. 1998/159 Northern Spanish mackerel, 2005/010 Grey mackerel, and

2007/032 Threadfin salmon) such as mt-DNA genetic analyses, microchemistry of internal hard parts and life history traits, provide a robust approach for investigating the stock structure of these shark species in Queensland. This multi-technique approach follows the dictum of the FRDC-funded workshop in July 1997, 'Taking Stock: Defining and Managing Shared Resources', which concluded that an analysis of stock structure is most effective if several techniques are used because of the different population scales addressed by each (Hancock 1998). For example, genetic analyses typically identify differences on large spatial and temporal scales, where gene flow is minimal. In contrast, microchemistry of internal hard parts (such as otoliths and vertebrae) reflects residence and movements of individuals in different ways, and may be used to resolve a genetically homogeneous population into discrete units of adult fish that may be more appropriate for management. The presence of spatial variation in life history traits indicates that populations may respond differently to fishing pressure and, therefore, may need to be managed as separate units even when other techniques suggest a more homogenous single stock.

The project was developed with the participation of management authorities in Queensland (DEEDI and GBRMPA), relevant researchers from other states with extensive experience in shark research, as well as industry groups along the Queensland east coast. As a consequence, this project gained strong support from all stakeholder groups and QFIRAC at the outset.

1.3 Need

The International Plan of Action for the Conservation and Management of Sharks ('IPOA-Sharks') was developed in 1999 in response to global concerns about the status of shark stocks. The Australian Government ratified the plan in 2004 and developed a National Shark Plan, with an overall objective to ensure the conservation and management of sharks and their long-term sustainable use in Australia.

Queensland fisheries legislation requires sustainable harvest of fish resources and their optimal use. Reliable and robust assessments of the status of fished resources are central to achieving such outcomes. Currently in Queensland, sharks are managed as a single stock with uniform management arrangements throughout the state. In NSW sharks are also managed under the assumption of single stocks and independently of Queensland. The lack of information on stock structure, however, means that the appropriate scale of management is not known and co-operative management among jurisdictions may be necessary. As well, fishers have no guidelines to encourage investment and long-term involvement in a fishery that supplies lucrative overseas markets. These management- and fisher-unfriendly circumstances must be viewed in the context of dramatic increases in catches of sharks on the Queensland east coast and NSW northern coast and the potentially high vulnerability of sharks to fishing pressure. Such a scenario highlights the urgent need for information on the stock structure of exploited shark species.

1.4 Objectives

1. To determine the spatial and temporal stock structure of fished shark species along the Queensland east coast.
2. To use stock structure information to definte appropriate management units for sustainable management of shark resources along the Queensland east coast.

1.5 Species background

1.5.1 Australian black tip shark (*Carcharhinus tilstoni*)

Distribution is restricted to the northern tropical and sub-tropical coastline of Australia (Last and Stevens 2009) however recently has been reported to occur as far south as Sydney on the east coast (Boomer *et al.* 2010). Research from the 1980s reported that they attain a size of 200 cm, are born as pups at approximately 60 cm, males mature at 110 cm and females at 115 cm. They are also reported to grow quickly reaching sexual maturity in three to four years (Davenport and Stevens 1988; Stevens and Wiley 1986). Tagging and genetic studies have suggested *C. tilstoni* individuals are capable of large-scale movements and that they comprise a single genetic stock across northern Australia (Ovenden *et al.* 2009). Recent observer surveys estimated that black tip sharks (*C. tilstoni* and *C. limbatus* combined) comprise approximately 28% (by number) of all sharks caught in the Queensland ECIFFF (Tobin, unpublished data) and approximately 7% (by number) of all targeted large sharks caught by demersal longline in northern NSW (Macbeth *et al.* 2009). Analysis of ECIFFF commercial logbook data estimates the harvest of “black tip sharks” to be approximately 59% by weight of the total shark harvest (Simpfendorfer *et al.* 2007).

1.5.2 Common black tip shark (*Carcharhinus limbatus*)

Distribution is cosmopolitan in tropical and warm temperate areas and includes much of the Australian coastline (Last and Stevens 2009). Their biology has been studied in South African, United States and Australian waters (Stevens and Wiley 1986; Castro 1996; Wintner and Cliff 1996). They are estimated to attain a size of 250 cm and are born between 40-70 cm. Size at maturity varies among regions with males reaching maturity between 135 and 180 cm while females mature between 120 and 190 cm. *C. limbatus* take longer to mature than *C tilstoni* with males maturing after five to six years while females take up to seven years. Morphologically the two species are identical and so distinguishing between the two species in Australian waters requires the use of either precaudal vertebral counts (Stevens and Wiley 1986) or genetic methods (Ovenden *et al.* 2010). Stevens and Wiley (1986) reported that precaudal vertebral counts for *C. limbatus* were in the range of 94-101 while for *C. tilstoni* they were in the range of 84-91. They are historically thought to be far less common in Australian waters than *C. tilstoni*. Studies on the global phylogeography of *C. limbatus* suggests the occurrence of

reproductive isolation in some regions, notably the western Atlantic compared to other regions, and in the Australian region the possibility of separate eastern and western Australian populations (Keeney and Heist 2006).

1.5.3 Scalloped hammerhead (*Sphyma lewini*)

Distribution is cosmopolitan in tropical and warm temperate seas and covers most of the northern Australian coastline (Last and Stevens 2009). Their biology has been studied in Australian, Indonesian and United States waters (Stevens and Lyle 1989; White *et al.* 2008; Piercy *et al.* 2007). They are reported to attain a length of 350 cm and are born at 45-50 cm. Males mature at 140-160 cm at between seven and ten years of age while females mature at 200-220 cm and at an age up to fifteen years. Movements are not well known although it appears that only males and juveniles occupy inshore shelf waters while adult females live in deeper water moving on to the shelf to mate and give birth. A study of the global phylogeography of *S. lewini* concluded that there is strong genetic differentiation among oceans and that there is high connectivity among adjacent coastal nursery populations although oceanic dispersal by females is rare (Duncan *et al.* 2006). Within the Australian region Ovenden and others (2009) found no genetic subdivision among *S. lewini* sampled from northern Australia and Indonesia. Recent observer surveys estimated that scalloped hammerheads comprise approximately eleven percent (by number) of all sharks caught in the Queensland ECIFFF (Tobin, unpublished data) and approximately three percent (by number) of all targeted large sharks caught by demersal longline in northern NSW (Macbeth *et al.* 2009). Analysis of ECIFFF commercial logbook data estimates the harvest of scalloped hammerheads to be approximately thirty percent by weight of the total shark harvest (Simpfendorfer *et al.* 2007).

1.5.4 Milk shark (*Rhizoprionodon acutus*)

Distribution is from tropical areas of the West African coastline throughout the tropical Indo-west Pacific including the tropical and sub-tropical coastline of Australia (Last and Stevens 2009). Milk sharks are a relatively small but fast growing shark attaining a size of approximately 100 cm, are born at 35-40 cm and most are mature by 75 cm, however these estimates can vary by geographical region (Harry *et al.* 2010; Henderson *et al.* 2006). Very little is known of the movements of *R. acutus* however they are known to occupy shallow coastal bays as juveniles and adult stages of their lives. Recent observer surveys estimated that milk sharks comprise approximately nine percent (by number) of all sharks caught in the Queensland ECIFFF (Tobin, unpublished data) however comprise less than one percent by weight of the total shark harvest in this fishery (Simpfendorfer *et al.* 2007). Milk sharks are almost certainly not caught in northern NSW waters (Macbeth *et al.* 2009).

1.6 Methods

This section provides an overview of the general approach taken during this study. The detailed methods specific to each of the techniques and the analyses undertaken are provided in the respective chapters. The general sampling approach taken during the project was driven by the overall objective of determining the stock structure of the selected shark species on the Queensland east coast to inform fisheries management (defining management units). The general approach of sampling was by using a phased approach whereby initial sampling was focused on locations at the extremes of the study area and evidence of a single stock at these spatial scales would prevent unnecessary sampling effort (Abaunza *et al.* 2008a). This type of approach has been used successfully now on several stock structure projects in northern Australia (Buckworth *et al.* 2007; Welch *et al.* 2009, 2010) and is described by three major elements:

ELEMENT 1 (Year 1): Broad spatial scale genetic and environmental differences in populations of Australian black tip shark, Common black tip shark, scalloped hammerhead shark, and milk shark between the far northern and far southern regions of Queensland's east coast to be assessed using a similar approach to other FRDC-funded projects examining stock structure (e.g. 2005/010 Grey mackerel, 2007/032 Threadfins). If the first year results do not support the notion of separate stocks in Queensland for any of the shark species, then the project will cease after Element 1 for that species.

ELEMENT 2 (Year 2): Finer spatial scale and short-term (inter-annual) temporal scale resolution of shark stocks, for those species where significant differences are identified in Element 1, is investigated at intermediate locations.

ELEMENT 3 (Year 3): Project results are finalised and the management units for each shark species defined for Queensland east coast waters. This information is presented to stakeholder groups and management agencies, with information transfer affected via established liaison and extension procedures of the collaborators' agencies.

The project used three basic techniques to examine shark stock structure: mt-DNA and microsatellite genetic analyses, vertebrae microchemistry, and life history parameters. In the first year of the project, these techniques were to be used to establish if broad spatial scale structural variation existed on the Queensland east coast through the collection of samples from the far northern and far southern regions of the Queensland east coast. Since the vast majority of catch of the selected shark species is by the commercial inshore net fishing sector, the locations of sample collection for each species were dictated by commercial fishing effort. This meant that the availability of samples of the project species was highly opportunistic and resulted in sampling locations that were not consistent across species. Samples were either provided directly from commercial fishers or by observers on board commercial net fishing vessels as part of a project funded by the Australian Government's Marine and Tropical Science Research Facility (MTSRF). The reliance on fisheries-dependent means of obtaining samples

also meant that samples collected differed in their characteristics from one location to the next. That is, for each species samples from the different locations were not similar in size classes, sexes, and/or stages of maturity and were due to factors such as the habitat type and depth that fishers operated in within each location. This meant that valid comparisons were not possible after the first year of sampling. At this stage of the project the FRDC approved the continuation so that samples could continue to be collected to supplement earlier collections and to provide collections from additional intermediate locations to describe fine spatial scale population structure. During the continuing sampling at further locations samples from NSW waters were sourced and included in analyses for black tip sharks and scalloped hammerheads.

For consistency with other research projects (e.g. MTSRF Inshore Biodiversity[1]) and State observer programs, regions on the north east coast were divided according to Figure 1.1. The sampling region for NSW was defined as 'Northern NSW' as defined by Macbeth *et al.* (2009). An overall summary of the sample collections for each species by region are provided in Table 1.1.

Table 1.1. Summary of all samples collected during the project for all four study species. For each region and species the number of samples collected of each gender and the sample mean size are provided. Data are only provided where gender was recorded.

Region	Males		Females		Total N
	N	Mean STL (mm)	N	Mean STL (mm)	
Milk shark, *Rhizoprionodon acutus*					
Far North	56	730	18	600	74
Cairns	14	636	8	588	22
Townsville	115	657	58	572	173
Mackay	23	861	1	955	24
Capricorn	1	890	2	721	3
Fraser Burnett	24	572	27	556	51
Brisbane	0	-	0	-	0
Northern NSW	0	-	0	-	0
Scalloped hammerhead, *Sphyrna lewini*[2]					
Far North	39	1225	25	949	64
Cairns	27	560	23	570	50
Townsville	186	1051	81	633	267
Mackay	20	930	10	622	30

[1] http://www.rrrc.org.au/mtsrf/theme_4/project_4_8_4.html

[2] Length measurements are fork length (mm).

Region	Males		Females		Total N
	N	Mean STL (mm)	N	Mean STL (mm)	
Capricorn	3	888	4	719	7
Fraser Burnett	7	702	8	743	15
Brisbane	14	1375	19	1474	33
Northern NSW	23	1678	20	1273	43
Australian black tip shark, *Carcharhinus tilstoni*					
Far North	32	1023	18	1044	50
Cairns	14	779	15	841	29
Townsville	103	889	126	989	229
Mackay	48	955	52	936	100
Capricorn	4	779	8	700	12
Fraser Burnett	0	-	0	-	0
Brisbane	12	711	7	761	19
Northern NSW	9	2109	6	2170	15
Common black tip shark, *Carcharhinus limbatus*					
Far North	2	968	2	1620	4
Cairns	6	837	9	800	15
Townsville	30	886	46	982	76
Mackay	1	741	7	921	8
Capricorn	8	816	4	801	12
Fraser Burnett	0	-	0	-	0
Brisbane	38	727	32	729	70
Northern NSW	38	2034	17	2081	55
Unidentified black tip shark					
Far North	13	577	7	466	20
Cairns	1	940	1	720	2
Townsville	129	884	123	981	252
Mackay	12	756	4	659	16
Capricorn	6	918	2	770	8
Fraser Burnett	15	1063	22	1035	37
Brisbane	0	-	0	-	0
Northern NSW	4	1450	1	2450	5

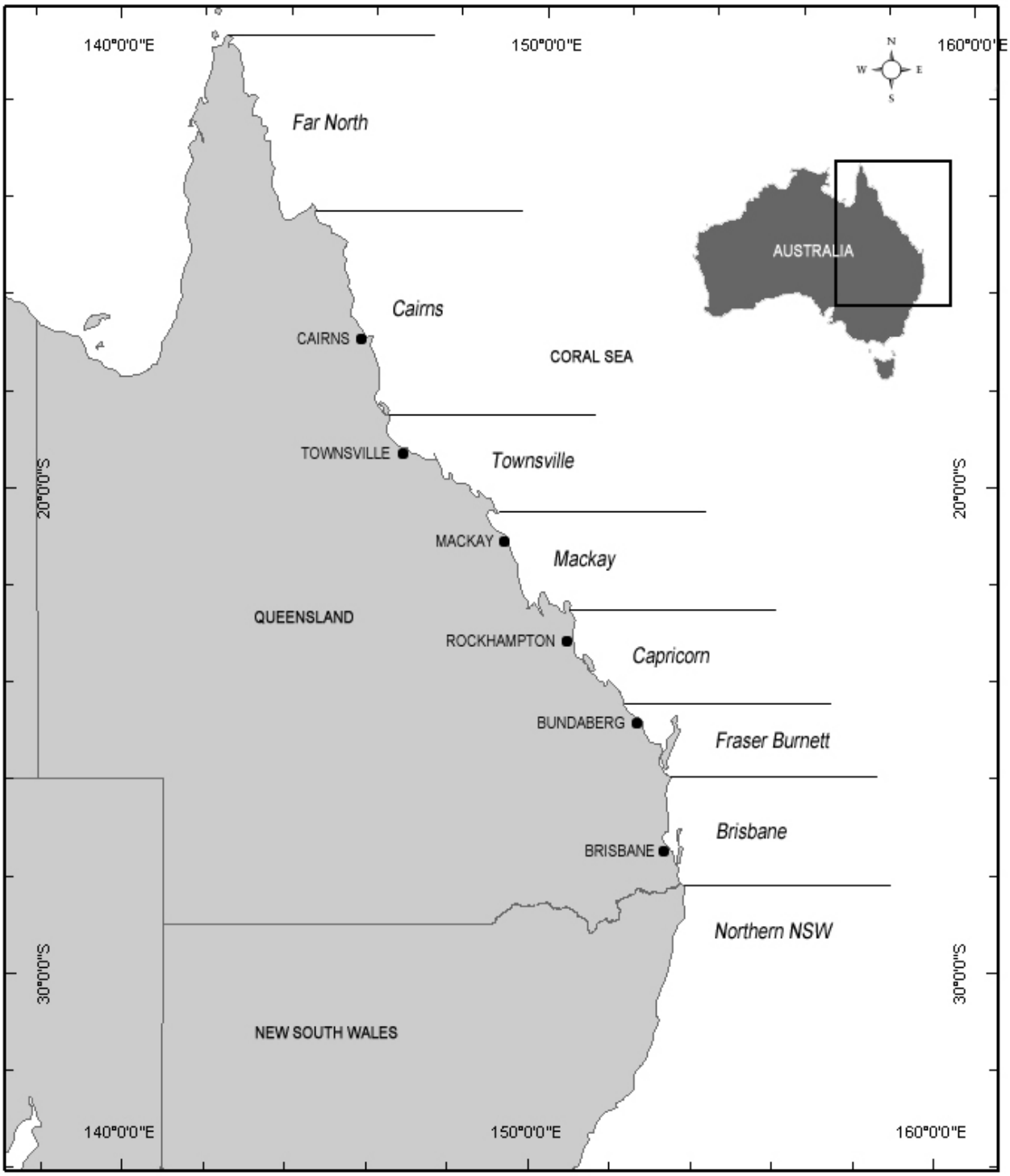

Figure 1.1. Regions from which samples were collected for all species along the northeast Australian coast.

Chapter 2: Genetic fisheries stock structure of two commercial shark species (*Rhizoprionodon acutus*, Rüppell, 1837 and *Sphyrna lewini*, Griffith and Smith, 1834) on the eastern coast of Australia

Jess AT Morgan, Jennifer R Ovenden, Raewyn Street, William G Macbeth, Andrew Tobin and David J Welch

2.1 Introduction

Australia is the custodian of a large amount of tropical marine biodiversity in the northern extent of its large exclusive economic zone. It is a significant slice of a marine biodiversity hotspot that has the richest marine fish fauna in the world (Randall 1998). Exploitation in the Australian zone is largely well managed and many species are protected. Nations to Australia's north have large population sizes and small amounts of land for agriculture, which increases their reliance of marine biodiversity for food. One group that is readily impacted by exploitation are elasmobranchs, but in tropical Australia these species are abundant. For example, in Queensland on the northeastern coastline, sharks represent about 20% (by weight) of the inshore commercial catch (Anon 2010a). The shark catch largely consists of five species, but numerous other elasmobranch species are also caught. The Queensland Government has a precautionary limit of 600 t per annum for the total allowable catch of elasmobranch species and has set additional alerts to overexploitation. These 'trigger points' are a reduction in the tonnage of landed catch by 30% over a three-year period and a significant change in the species composition of the landed catch. Preferably, management arrangements would be species-specific and based on a large body of species-specific demographic and biological information. In the absence of this, the question remains whether exploitation limits on elasmobranch species are conservative enough for nonspecific management to be effective.

To redress the lack of scientific information for management, this study focuses on the population structure of two commonly exploited species in Queensland; *Sphyrna lewini* (scalloped hammerhead) and *Rhizoprionodon acutus* (milk shark). Population structure is relevant for setting the scale of management arrangements, and once in place, for monitoring the effects of exploitation on a scale that matches the amount of demographic connectedness within the range of a species. In many respects *S. lewini* and *R. acutus* represent ends of a biological continuum. *Sphyrna lewini* is a coastal and

semioceanic shark found worldwide in the Atlantic, Pacific and Indian Oceans (Baum *et al.* 2007). *Rhizoprionodon acutus* is a continental shelf species with a more restricted distribution in the Indo-Pacific and western Africa. There is a considerable size difference between the species; *S. lewini* is large with a maximum size of around 340 cm (TL), and *R. acutus* is much smaller (100cm, Australia; 178cm Africa) (Last and Stevens 2009). Their resilience to exploitation is also different. *Sphyrna lewini* has low resilience because it is has a long generation time (around fifteen years), even though it has relatively high fecundity compared to other sharks (12-38 pups) (Baum *et al.* 2007). In contrast, *R. acutus* has smaller litters (1-8 pups), but its generation time is much shorter with rapid sexual maturity (2-3 years) and a maximum life span of eight years providing resilience to exploitation (Simpfendorfer 2003). Globally, *S. lewini* is listed as endangered by the IUCN. Australian populations are thought to be well-managed, but the increase in illegal, unregulated and unreported fishing in the north is of concern (Baum *et al.* 2007). *Rhizoprionodon acutus* is listed as Least Concern by the IUCN. The smaller body size of *R. acutus* implies lower vagility compared to *S. lewini* and combined with shorter generation times there is the expectation of a more pronounced population subdivision in *R. acutus.* These biological differences between the species suggest that population structure in *R. acutus* would be different to *S. lewini*.

There has been no previous stock structure research on *R. acutus.* However, stock structure of *S. lewini* has been investigated on a worldwide and regional scale. Duncan *et al.* (2006) and Quattro *et al.* (2006) reported pronounced genetic differences between *S. lewini* from major ocean basins (Atlantic, Pacific and Indian Oceans) in contrast to genetic connectivity between populations along continental margins. Genetic similarity among populations within the Indo-Pacific region was confirmed by Ovenden *et al.* (2009) between central Indonesia and northern Australia. Quattro *et al.* (2006) reported a cryptic lineage amongst Atlantic samples that had distinct vertebral counts. Regional scale genetic population structure has not been widely studied in the Carcharhinidae, with the majority of studies focussing on global phylogeography (Keeney and Heist 2006; Portnoy *et al.* 2010), except for studies focussing on natal philopatry (Keeney *et al.* 2003; Pardini *et al.* 2001). Genetic tools have been used to study the mating system in sharks, with some species having multiple paternity (Portnoy *et al.* 2007) and genetic tools are widely used for species identification (Mendonca *et al.* 2009; Ovenden *et al.* 2010; Wong *et al.* 2009).

In this study, two types of genetic markers were used to test for genetic population structure in *S. lewini* and *R. acutus* on a regional scale within Australia. Sequence polymorphism in a mitochondrial gene region (ND4) plus allele frequency variation at a range of microsatellite loci were deployed on each species. The markers represent genes that are neutral with respect to selection and are biparentally (microsatellite) or strictly maternally (mtDNA) inherited. Combined, they have the potential to provide a high degree of resolution at the intra-specific level per species. To ensure that population genetic subdivision would be detected if it were present, samples from a biogeographically distinct population

was included for each species. Samples were obtained from central Indonesia to provide this contrast within the dataset. Populations of *S. lewini* and *R. acutus* were sampled from similar locations along the coastline of Queensland and New South Wales to test the expectation that the scale of genetic subdivision would be finer in the species with lower potential vagility and shorter generation times (*R. acutus*) compared to the more benthic species with longer generation times (*S. lewini*).

2.2 Materials and methods

2.2.1 Sample Collection

Rhizoprionodon acutus and *S. lewini* were sampled from regions on the eastern coast of Queensland (Figure 2.1, Table 2.1). Four regions (*R. acutus* and *S. lewini*, Far North, Townsville, Mackay; *R. acutus*, Fraser Burnett; *S. lewini*, Brisbane) had sufficient sample sizes to be included in the genetic subdivision analysis. *Sphyrna lewini* was also sampled from the northern coast of New South Wales. Samples from one location in central Indonesia were included to test the power of the genetic loci to resolve population structure.

All samples were taken from the landed catch of the shark fishing sector. In Queensland, samples were provided by commercial fishers or collected by observers on commercial net boats as part of MTSRF Project 4.8.4 (GBRWHA Inshore fisheries). These collections were run in parallel with the Queensland Government fisheries observer program and staff from James Cook University (JCU) also assisted. In NSW, sharks were taken as part of the fisheries observer program. Sharks were sampled from artisanal markets in central Indonesia. For Australian samples, biological information was linked to samples taken for genetics, vertebrae microchemistry, and life history parameters on standardized datasheets.

Approximately 200 mg of muscle tissue was dissected and preserved in 1 ml of NaCl saturated solution with 20% dimethyl-sulphoxide. Sample vials were later air-freighted to the Molecular Fisheries Laboratory in Brisbane for DNA extraction and storage at -70°C.

2.2.2 Total genomic DNA extraction

Total genomic DNA was extracted from 10 - 25 mg of the tissue samples. DNA was extracted using a Qiagen DNeasy Blood & Tissue Kit (Qiagen, Doncaster Victoria) into a final elution volume of 200 µL.

2.2.3 Microsatellite loci

Genetic population structure was inferred from within and between population variation at microsatellite loci. Microsatellites developed for Carcharhinid sharks (Keeney and Heist 2003; Nance *et al.* 2009; Ovenden *et al.* 2006; Portnoy *et al.* 2006) were assessed for their utility in *R. acutus* and *S. lewini* (Table 2.2).

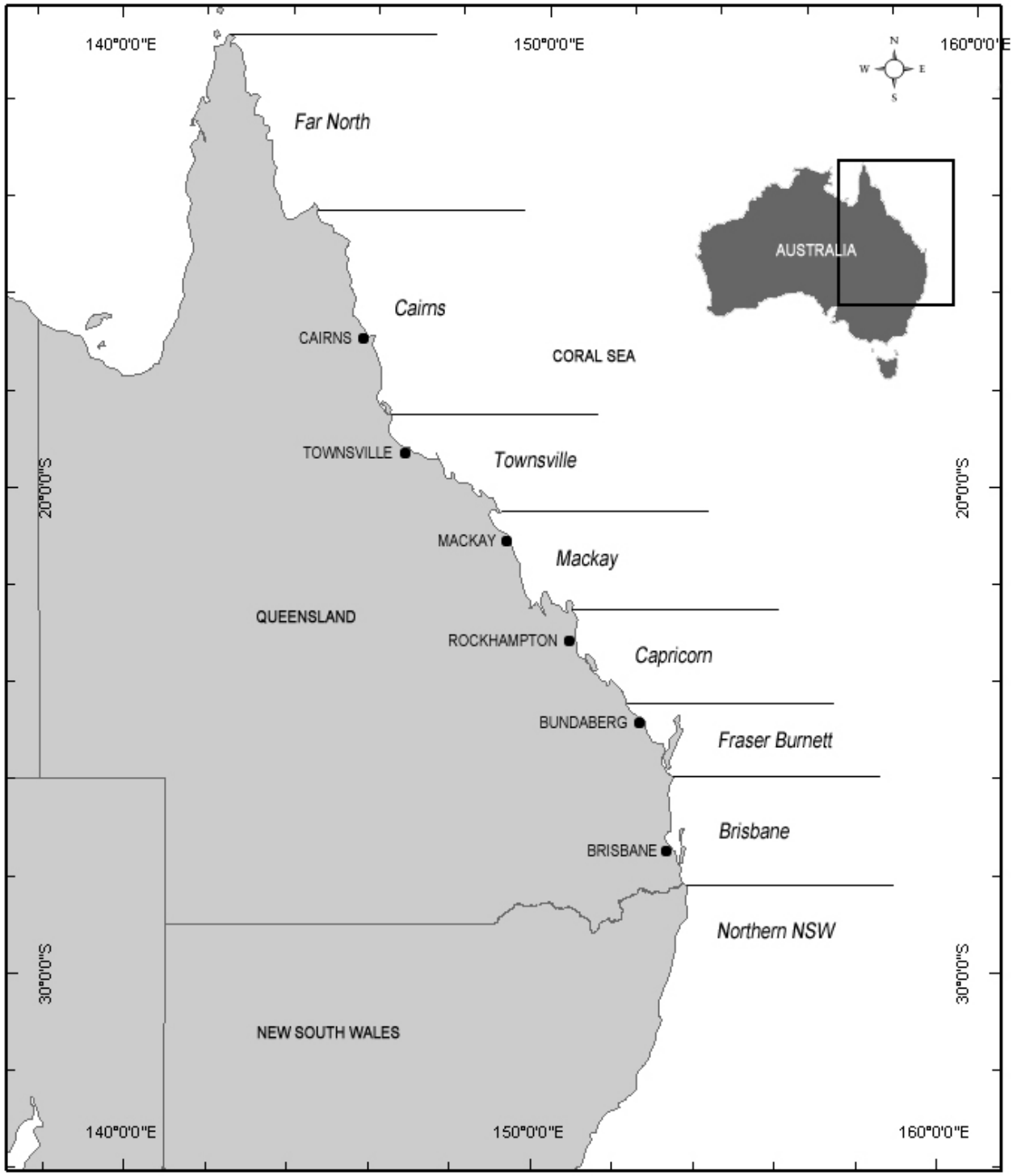

Figure 2.1. Sampling regions for *Rhizoprionodon acutus* and *Sphyrna lewini*. Sufficient genetics samples of *R. acutus* were obtained from regions Far North, Townsville, Mackay, and Fraser Burnett. Sufficient *S. lewini* were also obtained from the same regions but also included Cairns, Brisbane and northern NSW. Further samples of both species were obtained from central Indonesia (not indicated on map).

Microsatellite PCR amplifications were performed in 96-well plates using Perkin Elmer 9600 and 9700 series thermocyclers. PCR reactions using a Qiagen Multiplex PCR Kit (6 µL total volume) contained 3 µL of 2x Master Mix, 0.6 µL of 5x Q solution, 20 nM forward primer and 200 nM reverse primer, 200 nM FAM-labelled M13 primer and approximately 20 ng of genomic DNA template. Forward primers had an M13 extension (GAGCGGATAACAATTTCACACAGG) at the 5' end, enabling product amplification with the FAM-labelled M13 primer (Schuelke 2000). The DNA template and enzyme were denatured at 95°C for 15 minutes, followed by 35 cycles consisting of 94°C for 30 sec, 50°C for 45 sec and 72°C for 90 sec. A final extension at 72°C for 45 min was used to ensure complete addition of adenine to the PCR product, essential for consistent allele calling during genotyping. Products were separated on an ABI3130xl sequencer and genotypes were scored and binned using GeneMapper® v4.0 software (Applied Biosystems, Foster City, CA, USA).

Table 2.1. Sample size, gender distribution and mean stretch total length (STL) for *Rhizoprionodon acutus* and *Sphyrna lewini* samples from Eastern Australian regions, and one Indonesian region, for analysis with mitochondrial DNA (ND4 gene) and microsatellite loci.

Region[3]	Males		Females		Unknown gender or STL	Total N[4]
	N	Mean STL (mm)	N	Mean STL (mm)		
Rhizoprionodon acutus						
Kedonganan, Bali Indonesia	-	-	-	-	17 (15)	17 (15)
Far North	45	829	7	872	4	56
Cairns[5]	1	560	1	570	-	2
Townsville	50	757	14	634	-	64
Mackay	10	852	-	-	-	10
Fraser Burnett	24	560	27	556	9	60
Total	130		49		30	209
Sphyrna lewini						
Kedonganan, Bali Indonesia	-	-	-	-	33	33
Far North	21	1205	12	986	14	47
Cairns[3]	1	520	4	599	2	7
Townsville	29	840	20	676	-	49
Mackay	12	680	8	588	-	20
Fraser Burnett[3]	3	577	-	-	-	3
Brisbane	12	614, 704, 1760[6]	13	508, 679[3]	17	42
Northern NSW	23	2200	20	1650	-	43
Total	101		77		66	244

[3] See Figure 2.1 for sampling location.

[4] Microsatellite numbers bracketed (if different).

[5] Location excluded from population subdivision analysis due to low sample size.

[6] Subset of individuals measured. Individual STL presented.

2.2.4 Mitochondrial DNA

The extent of sequence variation in the NADH dehydrogenase subunit 4 (ND4), region of mtDNA was used to examine the extent of genetic population structure. The partial ND4 region (873 base pairs long) was amplified and sequenced using primers ND4 (CAC CTA TGA CTA CCA AAA GCT CAT GTA GAA GC) (Arévalo *et al.* 1994) and H12293-LEU (TTG CAC CAA GAG TTT TTG GTT CCT AAG ACC) (Inoue *et al.* 2001).

PCR amplification reactions were carried out in 50 µL volumes and contained 0.5 µM of each primer, combined with 10-100 ng of template DNA, 10x Taq buffer (containing 15 mM MgCl2), 0.8 mM dNTPs, and 0.6 units of Taq DNA polymerase (Qiagen, Valencia, CA, USA). Thermal cycling conditions consisted of an initial denaturation (94°C for 3 min) followed by 35 cycles of 94°C for 15 seconds, 60°C for 30 seconds and 72°C for 1 min, with a final extension step of 72°C for 10 minutes. Cycling was performed in either a PTC200 DNA Engine (MJ Research, USA) or PerkinElmer 9600 and 9700 series thermocyclers (PerkinElmer Australia, Melbourne, VIC). PCR products were viewed on a 1.5% agarose TAE gel stained with GelRed (Biotium, USA). PCR products were concentrated and desalted prior to sequencing using a DNA Clean & Concentrator-5 Kit (Zymo Research Corporation, Orange, CA, USA). Approximately 20ng of DNA was used in standard ABI Dye Terminator sequencing reactions using Big Dye v3.1 technology (Applied Biosystems, California) and were run on an Applied Biosystems 3130xl Genetic Analyser.

Table 2.2. Description of the microsatellite loci screened for *Rhizoprionodon acutus* and *Sphyrna lewini*.

Locus	Repeat	Reference	Species
Cli-100	$(TG)_4(GT)_{10}$	Keeney and Heist (2003)	*R. acutus* and *S. lewini*
Ct-06	$(CA)_{14}$	Ovenden *et al.* (2006)	*R. acutus* and *S. lewini*
Ct-07	$(GT)_{10}(GC)(GT)(GC)(GT)_2(GC)$	Ovenden *et al.* (2006)	*R. acutus* and *S. lewini*
Cpl-166	$(GT)_{17}$	Portnoy *et al.* (2006)	*R. acutus* and *S. lewini*
Cli-07	$(GT)_{20}$	Keeney and Heist (2003)	*R. acutus* only
Cli-107	$(GT)_{14}$	Keeney and Heist (2003)	*R. acutus* only
Cli-12	$(GT)_9$	Keeney and Heist (2003)	*S. lewini* only
PGL02	(XXX)n	Chapman *et al.* (2004)	*S. lewini* only
SLE018	(CA)5(TA)3(CA)3CG(CA)4TA(CA)4	Nance *et al.* (2009)	*S. lewini* only
SLE045	(CA)interrupted	Nance *et al.* (2009)	*S. lewini* only
SLE089	(GT)17(CT)2(GT)7	Nance *et al.* (2009)	*S. lewini* only

2.3 Data Analyses

2.3.1 Test of species identification

Mitochondrial DNA ND4 sequence was used to confirm the identity of all *R. acutus* and *S. lewini* samples by comparison to in-house ND4 reference sequences.

2.3.2 Equilibrium tests for nuclear genes

Microsatellite genotypes were tested for deviations from Hardy-Weinberg equilibrium and linkage disequilibrium using Genepop-on-the-web (Morgan 2000; Raymond and Rousset 1995). For the former, all locus *x* population combinations were tested, and for the latter all combinations of locus pairs was assessed for each population. A Bonferroni correction for simultaneous tests was applied at an α level of 0.05 to estimate critical levels of significance.

2.3.3 Null alleles at microsatellite loci

The software Microchecker v2.2.3 (Van Oosterhout *et al.* 2004) was used to explore cases of departure from Hardy Weinberg equilibrium. When null alleles were detected, their frequency was estimated using the maximum likelihood estimator of Dempster *et al.* (1977) for each locus and population. Calculations were performed in software FreeNA (Chapuis and Estoup 2007).

2.3.4 Genetic variation within and among populations

Population genetic structure was assessed by determining the proportion of total genetic variation that was found in pairwise comparisons between populations using an *F*-statistics approach. F_{ST} (actually theta) estimates were made following Weir and Cockerham (1984) based on allele frequency variance for microsatellites and based on Slatkin's linearized F_{ST} (Slatkin 1995) for mt-DNA sequence polymorphism. We corrected for the positive bias on F_{ST} estimates caused by the presence of null alleles at microsatellite loci using the ENA (excluding null alleles) approach of Chapuis and Estoup (2007). The corrected F_{ST} estimates were made using the software FreeNA (Chapuis and Estoup 2007). After estimating frequencies per population and per locus for null and non-null alleles, the software estimates F_{ST} using non-null alleles only. Null alleles are ignored in the computation meaning that the sums of allele and genotype frequencies are less than one, which is feasible according to Chapuis and Estoup (2007) based on Weir and Cockerham (1984). Missing data was assumed to be missing due to technical problems. F_{ST} estimates were made using FreeNA with and without the correction for null alleles for all loci or subsets of loci that excluded loci with null alleles.

2.3.5 Evolutionary history

Mitochondrial DNA sequence data was edited and aligned with Sequencher (v4.8 Gene Codes Corporation, Ann Arbor, MI, USA). Haplotype parsimony networks were estimated with the assistance of TCS (Clement *et al.* 2000). Neighbourhood joining phylogenetic trees were constructed from mt-DNA sequence data based on the number of polymorphic sites between haplotypes in PAUP* ver 4.0b10 (Swofford 2002). Arlequin v3.5 (Excoffier *et al.* 2005) was used to calculate a range of population genetic statistics relevant to sequence variation at the population level.

2.4 Results

2.4.1 Species identification

Genetic characterisation of shark tissue samples excluded twelve mis-identified animals; five great hammerheads (*Sphyrna mokarran*) and four smooth hammerheads (*Sphyrna zygaena*) that were confused for *S. lewini* and three *R. acutus* samples, genotyped to other genera, that were assumed to be mis-labelled. Overall there were 244 *S. lewini* and 209 *R. acutus* samples (Table 2.1).

2.4.2 Microsatellite Loci

Six microsatellite loci were polymorphic for *R. acutus*. Numbers of alleles per locus varied from three to four (Ct06) to 12 to 34 alleles per population (Cpl166, Table 2.3A). As expected for these di-nucleotide loci, the interval between alleles was two base pairs (or multiples of two base pairs). However, some loci had alleles separated by one base pair (Cli100, Cpl166, Ct06 and Cli107), which most likely reflected one base pair indels in flanking sequence or the repeat motif. There was no evidence of linkage disequilibrium between pairs of loci for any population of *R. acutus*.

Eight microsatellite loci were polymorphic for *S. lewini*. Three of eight microsatellite loci used to genotype *S. lewini* samples were developed for that species (SLE018, 045 and 089). Alleles separated by one base pair were detected at loci SLE018, SLE089, Cli12, Cli100, Ct07 and PGL02. The numbers of alleles per locus per population for *S. lewini* varied from two (Cpl166) to 16-24 (Cli12, Table 2.3B). Out of 168 combinations of locus pairs and population, there was evidence for linkage disequilibrium in the Townsville population for *S. lewini* at three pairs of loci (Cpl166/SLE018, Cli12/SLE045 and SLE018/SLE089). The linkage was judged to be slight because it was population specific and involved different pairs of loci.

Table 2.3. The population, sample size (N), number of alleles per microsatellite locus (Na), average observed heterozygosity (Ho) and expected (He) and unbiased (UHe) heterozygosity, *p*-value for test of Hardy-Weinberg equilibrium[7] (HWE) and null allele frequency[8] (Null) for each sampling location[9] for *Rhizoprionodon acutus* (Table 2.3A) and *Sphyrna lewini* (Table 2.3B).

Table 2.3A – *Rhizoprionodon acutus*

Sampling Location	Microsatellite Locus	N	Na	Ho	He	UHe	HWE	Null
Indonesia	*Cli07*	12	10	0.67	0.85	0.89	0.0200	0.31
	Cli100	15	12	0.73	0.89	0.92	0.0189	0.08
	Cpl166	11	14	0.45	0.92	0.96	0.0000	0.46
	Ct06	12	5	0.17	0.63	0.66	0.0000	0.48
	Ct07	10	7	0.60	0.79	0.83	0.4536	0.45
	Cli107	6	5	0.83	0.74	0.80	0.7784	0.63
Far North	*Cli07*	47	9	0.30	0.72	0.73	0.0000	0.40
	Cli100	56	5	0.41	0.39	0.39	0.6218	0.00
	Cpl166	56	32	0.64	0.91	0.92	0.0001	0.14
	Ct06	56	4	0.23	0.21	0.21	1.0000	0.00
	Ct07	56	7	0.55	0.54	0.54	0.8700	0.00
	Cli107	56	17	0.61	0.73	0.74	0.0223	0.07
Townsville	*Cli07*	55	10	0.29	0.77	0.77	0.0000	0.40
	Cli100	63	6	0.44	0.41	0.42	0.3532	0.05
	Cpl166	63	32	0.75	0.87	0.88	0.0012	0.08
	Ct06	64	3	0.23	0.26	0.26	0.2863	0.03
	Ct07	60	9	0.43	0.76	0.76	0.0000	0.26
	Cli107	54	19	0.54	0.66	0.67	0.0088	0.28
Mackay	*Cli07*	10	5	0.20	0.73	0.76	0.0000	0.31
	Cli100	10	2	0.20	0.18	0.19	1.0000	0.00
	Cpl166	10	12	0.70	0.89	0.93	0.0421	0.08
	Ct06	4	2	0.25	0.22	0.25	-	0.73
	Ct07	10	4	0.60	0.72	0.75	0.7127	0.06
	Cli107	10	8	0.70	0.74	0.78	0.4291	0.02
Fraser Burnett	*Cli07*	54	9	0.22	0.67	0.67	0.0000	0.38
	Cli100	60	5	0.42	0.46	0.46	0.3084	0.04
	Cpl166	60	34	0.77	0.94	0.95	0.0023	0.09
	Ct06	60	3	0.32	0.28	0.29	0.1991	0.00
	Ct07	60	7	0.67	0.59	0.60	0.5043	0.00
	Cli107	60	16	0.62	0.68	0.68	0.0032	0.04

[7] *P*-values that were significant after Bonferroni correction are underlined.

[8] Calculated according to Dempster *et al.* (1977).

[9] See Figure 2.1 for sampling locations.

Table 2.3B – *Sphyrna lewini*

Sampling Location	Microsatellite Locus	N	Na	Ne	Ho	He	UHe	HWE	Null
Indonesia	*Cli12*	33	22	13.78	0.58	0.93	0.94	0.0000	0.18
	Cli100	33	10	5.64	0.91	0.82	0.84	0.2391	0.00
	Cpl166	31	3	2.04	0.45	0.51	0.52	0.7161	0.03
	Ct07	33	9	4.14	0.73	0.76	0.77	0.1445	0.04
	PGL02	33	8	5.16	0.79	0.81	0.82	0.1386	0.03
	SLE018	32	5	3.28	0.50	0.69	0.71	0.0646	0.11
	SLE045	32	5	2.77	0.63	0.64	0.65	0.6703	0.00
	SLE089	33	14	8.47	0.85	0.88	0.90	0.3132	0.00
Far North	*Cli12*	47	19	13.94	0.68	0.93	0.94	0.0000	0.13
	Cli100	47	12	7.41	0.85	0.87	0.87	0.5089	0.00
	Cpl166	47	2	1.97	0.32	0.49	0.50	0.0181	0.12
	Ct07	47	7	3.07	0.72	0.67	0.68	0.9263	0.00
	PGL02	42	10	5.88	0.88	0.83	0.84	0.7071	0.00
	SLE018	45	7	3.80	0.58	0.74	0.74	0.0299	0.09
	SLE045	43	5	3.25	0.81	0.69	0.70	0.5407	0.00
	SLE089	44	14	8.29	0.91	0.88	0.89	0.7175	0.00
Townsville	*Cli12*	49	23	13.30	0.73	0.92	0.93	0.0024	0.10
	Cli100	49	11	7.51	0.80	0.87	0.88	0.0424	0.04
	Cpl166	49	2	1.99	0.43	0.50	0.50	0.3888	0.05
	Ct07	49	8	3.05	0.65	0.67	0.68	0.3527	0.04
	PGL02	49	10	5.44	0.73	0.82	0.82	0.0025	0.04
	SLE018	49	8	3.64	0.47	0.72	0.73	0.0000	0.15
	SLE045	49	5	2.83	0.61	0.65	0.65	0.3453	0.03
	SLE089	49	14	7.43	0.90	0.87	0.87	0.8876	0.00
Mackay	*Cli12*	20	16	11.76	0.70	0.92	0.94	0.0000	0.11
	Cli100	20	11	7.69	0.90	0.87	0.89	0.7732	0.00
	Cpl166	20	2	2.00	0.35	0.50	0.51	0.2006	0.10
	Ct07	20	5	3.25	0.80	0.69	0.71	0.1532	0.01
	PGL02	18	7	4.73	0.72	0.79	0.81	0.0287	0.06
	SLE018	20	4	3.36	0.40	0.70	0.72	0.0049	0.18
	SLE045	20	5	3.28	0.70	0.70	0.71	0.9324	0.01
	SLE089	20	8	6.06	0.90	0.84	0.86	0.9639	0.00
Brisbane	*Cli12*	42	24	11.96	0.83	0.92	0.93	0.0524	0.04
	Cli100	42	11	7.38	0.88	0.86	0.87	0.2135	0.00
	Cpl166	42	2	1.93	0.43	0.48	0.49	0.5267	0.04
	Ct07	42	7	3.17	0.76	0.68	0.69	0.8475	0.00
	PGL02	37	8	4.14	0.81	0.76	0.77	0.6403	0.00
	SLE018	42	6	3.20	0.48	0.69	0.70	0.0035	0.13
	SLE045	39	5	3.17	0.67	0.68	0.69	0.3385	0.00
	SLE089	42	12	7.44	0.88	0.87	0.88	0.1908	0.01

Table 2.3B – *Sphyrna lewini*

Sampling Location	Microsatellite Locus	N	Na	Ne	Ho	He	UHe	HWE	Null
Northern NSW	*Cli12*	41	20	12.23	0.71	0.92	0.93	0.0007	0.11
	Cli100	41	12	7.20	0.88	0.86	0.87	0.0741	0.00
	Cpl166	41	2	1.96	0.46	0.49	0.50	0.7513	0.02
	Ct07	41	6	2.72	0.61	0.63	0.64	0.1813	0.03
	PGL02	41	8	3.69	0.78	0.73	0.74	0.4456	0.00
	SLE018	39	6	2.80	0.44	0.64	0.65	0.0202	0.13
	SLE045	43	6	2.77	0.56	0.64	0.65	0.1119	0.02
	SLE089	41	12	9.89	0.90	0.90	0.91	0.1901	0.01

Several loci per population were out of Hardy-Weinberg equilibrium for both species. For *R. acutus*, nine of the thirty tests performed rejected the null hypothesis of Hardy-Weinberg equilibrium after Bonferroni correction. Instances of disequilibrium were spread across populations and commonly involved loci Cpl166, Cli07, Ct06 and Ct07 (Table 2.3A). Analysis with Microchecker software (Van Oosterhout *et al.* 2004) suggested that these were not due to scoring errors (large allele dropout or stuttering), but to an excess of homozygotes at the majority of allele size classes strongly suggesting the presence of null alleles. This was confirmed by the estimated frequency of null alleles at locus x population combinations in disequilibrium. Small sample sizes affected the reliability of the test for Hardy Weinberg equilibrium and estimation of null allele frequency. For instance, only ten *R. acutus* were genotyped for locus Ct07 and the *p*-value (0.4536) suggested Hardy Weinberg equilibrium, yet it was estimated that a null allele was present at a frequency of 0.45. For *S. lewini*, instances of Hardy Weinberg disequilibrium occurred commonly with loci Cli12 and SLE018. Microchecker software (Van Oosterhout *et al.* 2004) confirmed that this was not due to scoring errors, and at these loci *x* population combinations null alleles were estimated to be present (Table 2.3B). In a previous study of *S. lewini*, locus SLE018 showed evidence of null alleles in samples taken from the eastern Pacific Ocean (Nance *et al.* 2009).

F-statistic analyses from microsatellite loci were generally not informative about the genetic population structure of *R. acutus* or *S. lewini* on the eastern Australian coastline. However, there was strong evidence for separate populations of *R. acutus* in eastern Australia and Bali (Indonesia), and some evidence of separation on this scale for *S. lewini*. F_{ST} estimates (theta) for population pair-wise comparisons involving *R. acutus* from central Indonesia and eastern Australia were significantly larger than zero, and ranged from 0.1648 to 0.2682 (Table 2.4A). We were unable to reject the null hypothesis of panmixia for *R. acutus* on the eastern Australian coast as F_{ST} estimates between pairs of sampling locations were generally not significantly larger than zero. The two F_{ST} estimates that were significant for the eastern coast (regions Townsville and Fraser Burnett using all loci, and regions Mackay and

Fraser Burnett using a subset of loci) most likely occurred by chance. They were low (0.0184 and 0.0797) and not consistent among tests. There was no evidence of genetically separate populations for *S. lewini* between Indonesia and the eastern coast of Australia. However, two of the three significant population pairwise F_{ST} estimates for *S. lewini* were between the most spatially separate population pair (Table 2.4B).

The presence of null alleles was predicted to inflate F_{ST} estimates by depressing intra-population genetic diversity (Chapuis and Estoup 2007; Chapuis *et al.* 2008). This effect was most notable for comparisons between *R. acutus* from central Indonesia and eastern Australia, where F_{ST} estimates were around 0.2 (Table 2.4A). Other population pair-wise F_{ST} estimates (ie. among *R. acutus* sampling locations from eastern Australia and among *S. lewini* sampling locations) were close to zero, which masked the effect of null alleles on F_{ST} estimates.

Table 2.4. Pairwise F_{ST} (theta, below diagonal, with 95% confidence intervals above diagonal) using the ENA (excluding null alleles) approach of Chapuis and Estoup (2007) for (Table 2.4A) *Rhizoprionodon acutus* and (Table 2.4B) *Sphyrna lewini* from sampling locations in eastern Australia and central Indonesia[10].

Table 2.4A – *Rhizoprionodon acutus*						
Loci	Sampling Location	Indonesia	Far North	Townsville	Mackay	Fraser Burnett
Six (ie. all)						
Allele frequencies not adjusted for presence of null alleles						
	Indonesia	-	0.1068 to 0.3364	0.1002 to 0.3042	0.0520 to 0.2974	0.1025 to 0.2972
	Far North	0.2220	-	-0.0047 to 0.0253	-0.0137 to 0.0282	-0.0061 to 0.0018
	Townsville	0.1878	0.0073	-	-0.0218 to 0.0025	-0.0038 to 0.0190
	Mackay	0.1667	0.0024	-0.0087	-	-0.0097 to 0.0157
	Fraser Burnett	0.2039	-0.0015	0.0075	0.0034	-
Allele frequencies adjusted for presence of null alleles						
	Indonesia	-	0.1069 to 0.3461	0.0733 to 0.2825	0.0412 to 0.3047	0.1021 to 0.2934
	Far North	0.2227	-	-0.0019 to 0.0547	-0.0069 to 0.2291	-0.0016 to 0.0023
	Townsville	0.1648	0.0190	-	-0.0005 to 0.1855	0.0001 to 0.0423
	Mackay	0.1699	0.0703	0.0562	-	-0.0017 to 0.1861
	Fraser Burnett	0.1982	0.0003	0.0184	0.0585	-
Five (All except locus Cpl 166)						
Allele frequencies not adjusted for presence of null alleles						
	Indonesia	-	0.1598 to 0.3610	0.1210 to 0.3285	0.0740 to 0.3309	0.1680 to 0.3160
	Far North	0.2682	-	-0.0055 to 0.0293	-0.0152 to 0.0329	-0.0075 to 0.0005
	Townsville	0.2173	0.0087	-	-0.0270 to 0.0043	-0.0044 to 0.0206
	Mackay	0.2014	0.0059	-0.0109	-	-0.0042 to 0.0187
	Fraser Burnett	0.2501	-0.0031	0.0062	0.0097	-
Allele frequencies adjusted for presence of null alleles						
	Indonesia	-	0.1517 to 0.3851	0.0883 to 0.3090	0.0521 to 0.3378	0.1697 to 0.3167
	Far North	0.2671	-	-0.0032 to 0.0630	-0.0085 to 0.2816	-0.0020 to 0.0026
	Townsville	0.1943	0.0241	-	-0.0005 to 0.2146	-0.0039 to 0.0486
	Mackay	0.1995	0.0924	0.0731	-	0.0039 to 0.2263
	Fraser Burnett	0.2425	0.0002	0.0215	0.0797	-

[10] *F*-statistics that were statistically larger than zero are shown in bold. Sample sizes are presented in Table 2.1 and sampling regions are shown in Figure 2.1.

Table 2.4B – *Sphyrna lewini*							
Loci	Sampling Location	Indonesia	Far North	Townsville	Mackay	Brisbane	Northern NSW
Eight (ie. all)							
Allele frequencies not adjusted for presence of null alleles							
	Indonesia	-	-0.0070 to 0.0022	-0.0010 to 0.0082	-0.0101 to 0.0082	-0.0028 to 0.0098	-0.0020 to 0.0167
	Far North	-0.0029	-	-0.0056 to 0.0006	-0.0085 to 0.0009	-0.0005 to 0.0154	-0.0048 to 0.0116
	Townsville	-0.0011	-0.0027	-	-0.0125 to 0.0011	-0.0030 to 0.0032	-0.0038 to 0.0098
	Mackay	-0.0015	-0.0044	-0.0062	-	-0.0067 to 0.0094	-0.0015 to 0.0051
	Brisbane	0.0038	0.0067	0.0004	0.0017	-	-0.0045 to 0.0127
	Northern NSW	0.0069	0.0021	0.0026	0.0013	0.0031	-
Allele frequencies adjusted for presence of null alleles							
	Indonesia	-	-0.0057 to 0.00314	-0.0086 to 0.0084	-0.0093 to 0.0090	-0.0018 to 0.0099	-0.00122 to 0.0167
	Far North	-0.0019	-	-0.0051 to 0.0014	-0.0084 to 0.0013	-0.0009 to 0.0148	-0.0032 to 0.0113
	Townsville	-0.0006	-0.0022	-	-0.0114 to -0.0008	-0.0025 to 0.0042	-0.0029 to 0.0114
	Mackay	-0.0004	-0.0041	-0.0059	-	-0.0067 to 0.0096	-0.0012 to 0.0056
	Brisbane	0.0044	0.0064	0.0012	0.0017	-	-0.0037 to 0.0126
	Northern NSW	0.0074	0.0026	0.0037	0.0015	0.0035	-
Six (excluding loci Cli12 and SLE018)							
Allele frequencies not adjusted for presence of null alleles							
	Indonesia	-	-0.0037 to 0.0046	-0.0099 to 0.0114	-0.0099 to 0.0118	-0.0033 to 0.0111	0.0004 to 0.0219
	Far North	-0.0003	-	-0.0043 to 0.0022	-0.0081 to 0.0034	-0.0021 to 0.0174	-0.0032 to 0.0138
	Townsville	0.0004	-0.0013	-	-0.0140 to 0.0007	-0.0038 to 0.0037	-0.0029 to 0.0145
	Mackay	0.0004	-0.0033	-0.0071	-	-0.0103 to 0.0113	-0.0021 to 0.0067
	Brisbane	0.0048	0.0074	0.0004	0.0001	-	-0.0018 to 0.0186
	Northern NSW	0.0109	0.0030	0.0048	0.0015	0.0069	-
Allele frequencies adjusted for presence of null alleles							
	Indonesia	-	-0.0034 to 0.0054	-0.0038 to 0.0114	-0.0076 to 0.0127	-0.0002 to 0.0115	0.0018 to 0.0218
	Far North	0.0003	-	-0.0047 to 0.0028	-0.0150 to 0.0040	-0.0024 to 0.0173	-0.0022 to 0.0141

Table 2.4B – *Sphyrna lewini*							
Loci	Sampling Location	Indonesia	Far North	Townsville	Mackay	Brisbane	Northern NSW
	Townsville	0.0008	-0.0010	-	-0.0126 to 0.0003	-0.0034 to 0.0049	-0.0002 to 0.0159
	Mackay	0.0015	-0.0029	-0.0060	-	-0.0093 to 0.0123	-0.0017 to 0.0073
	Brisbane	0.0053	0.0074	0.0012	0.0010	-	0.0020 to 0.0179
	Northern NSW	0.0109	0.0036	0.0056	0.0021	0.0066	-

2.4.3 Mitochondrial DNA

ND4 sequencing

Nucleotide polymorphism in the 873 bp fragment of the mt-DNA ND4 gene was higher in *R. acutus* compared to *S. lewini.* For *R. acutus* 37 haplotypes were recorded (Table 2.5) compared to *S. lewini* samples with only 13 haplotypes (Table 2.6). A single *S. lewini* haplotype (SL01) dominated all populations, including Indonesia, with a mean abundance of 81%. The second most common *S. lewini* haplotye (SL06, 7% mean abundance) was also widely dispersed. Although often widely distributed, none of the remaining 11 *S. lewini* haplotypes were very abundant (<3%). The dominant *R. acutus* haplotype, RA01, was present in all populations along the Australian east coast, with a mean abundance of 44%. Four other common haplotypes (RA06 – 9.09%, RA11 – 7.18%, RA17 – 5.26% and RA18 – 5.26%) were also distributed across the full east coast sampling range (southern extent of distribution was Fraser Burnett). Unique Indonesian haplotypes were identified for both species; in fact no shared haplotypes of *R. acutus* were found between eastern Australia and Indonesia (Figures 2.2A, 2.3). Greater overlap of *S. lewini* haplotypes occurred between Australia and Indonesia, however, a genetically distant Indonesian haplotype was found (SL13). The unusual Indonesian haplotype SL13, differed from SL01 at 46 nucleotide positions but remained clustered with the *S. lewini* haplotypes in a neighbour joining tree containing sister species, *Sphyrna zygaena* and *Sphyrna mokarran* (Figures 2.2B, 2.3).

There was a large contrast between nucleotide diversity (the probability that two randomly chosen homologous nucleotides are different in a population) levels for the two shark species. Low nucleotide diversity (π) values for Australian east coast *S. lewini* (from 0.000372 to 0.001249) reflected the low number of polymorphic sites distinguishing the different haplotypes. In contrast, *R. acutus* populations had three- to ten-times higher nucleotide diversity values (0.003445 to 0.003895). Haplotype diversity (the probability that two randomly chosen haplotypes are different in the sample) was greater for *R. acutus* than *S. lewini*, as was the Watterson estimator (θ_s) suggesting a higher population mutation rate in *R. acutus* (Table 2.7).

Table 2.5. Mitochondrial DNA ND4 haplotypes[11] of *Rhizoprionodon acutus* from each sampling location (%) ranked by overall frequency. A dot (.) indicates sequence homology with the top reference sequence.

SNP Positioin	1	32	34	38	58	77	78	94	100	105	154	218	223	256	283	289	322	325	348	358	380	394	413	424	442	451	499	505	517	533	550	595	603	656	676	693	752	774	793	838	Region Overall %	Indoesia	Far North	Cairns	Townsville	Mackay	Fraser Burnett
ND4_RA01	T	G	T	C	A	A	T	T	A	C	T	T	A	T	T	T	T	C	G	T	G	C	C	T	T	C	C	A	T	T	T	T	T	A	C	T	G	T	G	G	37.32	0	37.50	50.00	43.75	50.00	38.33
ND4_RA06	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	9.09	0	12.50	0	12.50	0	6.67
ND4_RA11	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	C	.	.	.	.	.	A	.	.	.	7.18	0	5.36	0	7.81	0	11.67
ND4_RA17	.	.	.	.	G	.	.	.	.	.	.	.	.	C	C	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	C	.	.	.	.	.	A	.	.	.	5.26	0	10.71	0	1.56	10.00	5.00
ND4_RA18	.	.	.	.	G	.	.	.	.	.	.	.	.	C	C	.	.	T	.	.	.	.	.	.	.	.	T	.	.	.	C	.	.	.	.	.	A	.	.	.	5.26	0	3.57	50.00	9.38	0	3.33
ND4_RA02	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	3.83	0	0	0	4.69	0	8.33
ND4_RA07	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	.	.	.	.	.	3.83	0	5.36	0	1.56	0	6.67
ND4_RA12	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	T	T	.	.	.	C	.	.	.	.	.	A	.	.	.	2.87	0	1.79	0	1.56	10.00	5.00
ND4_RA19	.	.	.	.	.	.	.	.	.	C	.	.	C	C	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	C	.	.	.	.	.	A	.	.	.	2.87	0	5.36	0	1.56	10.00	1.67
ND4_RA20	.	.	.	.	.	.	.	.	.	.	.	.	.	C	C	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	C	.	.	.	.	.	A	.	.	.	1.91	0	3.57	0	1.56	10.00	0
ND4_RA31	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	T	.	.	.	T	.	.	.	.	C	.	T	.	.	.	.	.	.	1.91	23.53	0	0	0	0	0
ND4_RA33	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	T	.	.	.	.	C	.	T	.	.	.	.	.	.	1.44	17.65	0	0	0	0	0
ND4_RA35	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	T	G	.	.	.	C	.	T	.	.	.	.	.	.	1.44	17.65	0	0	0	0	0
ND4_RA05	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	0.96	0	1.79	0	1.56	0	0
ND4_RA08	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	0.96	0	0	0	1.56	0	1.67
ND4_RA09	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	0.96	0	0	0	1.56	0	1.67
ND4_RA13	.	.	C	.	.	.	.	.	.	.	.	.	.	C	.	.	C	.	.	.	.	.	.	.	.	.	T	.	.	.	C	.	.	.	.	.	A	.	.	.	0.96	0	0	0	0	0	3.33

[11] Genbank numbers HQ530174 to HQ530210 (for release on 18 April 2011).

SNP Positioin	1	32	34	38	58	77	78	94	100	105	154	218	223	256	283	289	322	325	348	358	380	394	413	424	442	451	499	505	517	533	550	595	603	656	676	693	752	774	793	838	Region Overall %	Indoesia	Far North	Cairns	Townsville	Mackay	Fraser Burnett
ND4_RA14	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	C	.	.	.	.	C	A	.	.	.	0.96	0	0	0	0	0	3.33
ND4_RA21	.	.	.	.	G	.	.	.	.	.	.	.	.	C	C	.	.	.	.	.	.	.	.	.	C	.	T	.	.	.	C	.	.	.	.	.	A	.	.	.	0.96	0	1.79	0	1.56	0	0
ND4_RA32	.	A	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C	.	.	T	.	.	.	T	.	.	.	.	C	.	T	.	.	.	.	.	.	0.96	11.76	0	0	0	0	0
ND4_RA34	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	T	.	.	.	T	.	.	.	.	C	.	T	.	.	.	.	.	.	0.96	11.76	0	0	0	0	0
ND4_RA36	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	T	.	.	.	T	.	.	.	.	C	.	T	.	.	.	.	.	.	0.96	11.76	0	0	0	0	0
ND4_RA03	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	0.48	0	0	0	0	0	1.67
ND4_RA04	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0.48	0	0	0	0	0	1.67
ND4_RA10	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	0.48	0	1.79	0	0	0	0
ND4_RA15	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	C	.	.	.	T	.	A	.	.	.	0.48	0	1.79	0	0	0	0
ND4_RA16	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	C	.	.	.	.	.	.	.	.	.	.	T	.	.	.	C	.	.	.	.	.	A	.	.	.	0.48	0	1.79	0	0	0	0
ND4_RA22	.	.	.	.	.	.	.	.	.	.	.	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	C	.	.	.	.	.	A	.	.	.	0.48	0	1.79	0	0	0	0
ND4_RA23	.	.	.	.	.	.	C	.	.	.	.	.	G	.	.	.	.	.	.	.	.	T	.	.	.	.	T	.	C	.	C	.	.	.	.	.	A	.	.	.	0.48	0	1.79	0	0	0	0
ND4_RA24	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	T	.	C	.	C	.	.	.	.	.	A	.	.	.	0.48	0	1.79	0	0	0	0
ND4_RA25	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	A	0.48	0	0	0	1.56	0	0
ND4_RA26	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	0.48	0	0	0	1.56	0	0
ND4_RA27	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0.48	0	0	0	0	10.00	0
ND4_RA28	.	.	.	.	.	.	C	.	.	T	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	T	.	C	.	C	.	.	.	.	.	A	.	.	.	0.48	0	0	0	1.56	0	0
ND4_RA29	.	.	.	.	G	.	.	.	.	.	.	.	.	C	C	.	.	.	C	.	.	.	.	.	.	.	T	.	.	.	C	.	.	.	.	.	A	.	.	.	0.48	0	0	0	1.56	0	0
ND4_RA30	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	.	.	.	.	.	.	.	.	.	T	.	.	.	C	.	.	.	.	.	A	.	.	.	0.48	0	0	0	1.56	0	0
ND4_RA37	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	T	.	.	.	.	C	.	T	.	.	.	C	.	.	0.48	5.88	0	0	0	0	0

The haplotype networks for *R. acutus* and *S. lewini* (Figure 2.3) show characteristic differences between the species. While both species have haplotypes that were reported in two or more Australian populations, many more rare *R. acutus* haplotypes were found. With the exception of the Indonesian population of *R. acutus*, the *R. acutus* and *S. lewini* networks show no clustering of haplotypes by sampling location. The Australian *R. acutus* haplotypes separate into three clusters with the five most common *R. acutus* haplotypes (63%) falling into two. There was no clustering pattern in the star-like *S. lewini* network; the single dominant haplotype (SL01) was centrally positioned.

As expected, Queensland populations of *R. acutus* and *S. lewini* were significantly different to the population sampled in Indonesia. Using mt-DNA ND4 sequence variation, all pairwise F_{ST} estimates between Queensland *R. acutus* sampling locations and the Indonesian location were significant (F_{ST} estimates 0.57 to 0.63, Table 2.8a). The Indonesian *S. lewini* population was also genetically different from Far North, Townsville and Northern NSW populations but couldn't be distinguished from Mackay or Brisbane populations (Table 2.8b). Australian east coast populations of *R. acutus* were not genetically different from each other using ND4 pairwise F_{ST} comparisons. For *S. lewini,* no pairwise comparisons between Australian east coast populations were significantly different, except for Townsville compared to Brisbane, but this significant value could have arisen by chance (*p*-value 0.03604). Plotting the distribution of dominant ND4 haplotypes onto a map for *R. acutus* (Figure 2.4a) and *S. lewini* (Figure 2.4b) highlights the similarities among east coast populations and the contrast with the Indonesian collection location, particularly for *R. acutus.*

2.4.4 Temporal stability of genetic population structure

Largely because this study relied on tissue samples from commercially caught sharks, there was little opportunity for repeated sampling at any location or species to confirm the stability of genetic population characteristics through time. Limited temporal sampling from Townsville from 2007 to 2009 was achieved for *R. acutus*. In 2007, fifteen animals were collected and nine haplotypes described. In 2008, 36 animals were collected representing 10 haplotypes of which six were new. In 2009, 13 animals were collected with nine haplotypes of which four were new. Sample sizes were insufficient to test for genetic population structure, however, haplotype RA01 dominated the catches over the three year time period (46%, 47%, and 31% respectively) and common haplotypes RA06, RA11 and RA18 were present at all time points. Overall, 19 haplotypes were described from 64 animals collected off Townsville. The high frequency of new haplotypes across temporal samples suggests that larger sample sizes would have been needed to fully characterise *R. acutus* haplotype diversity.

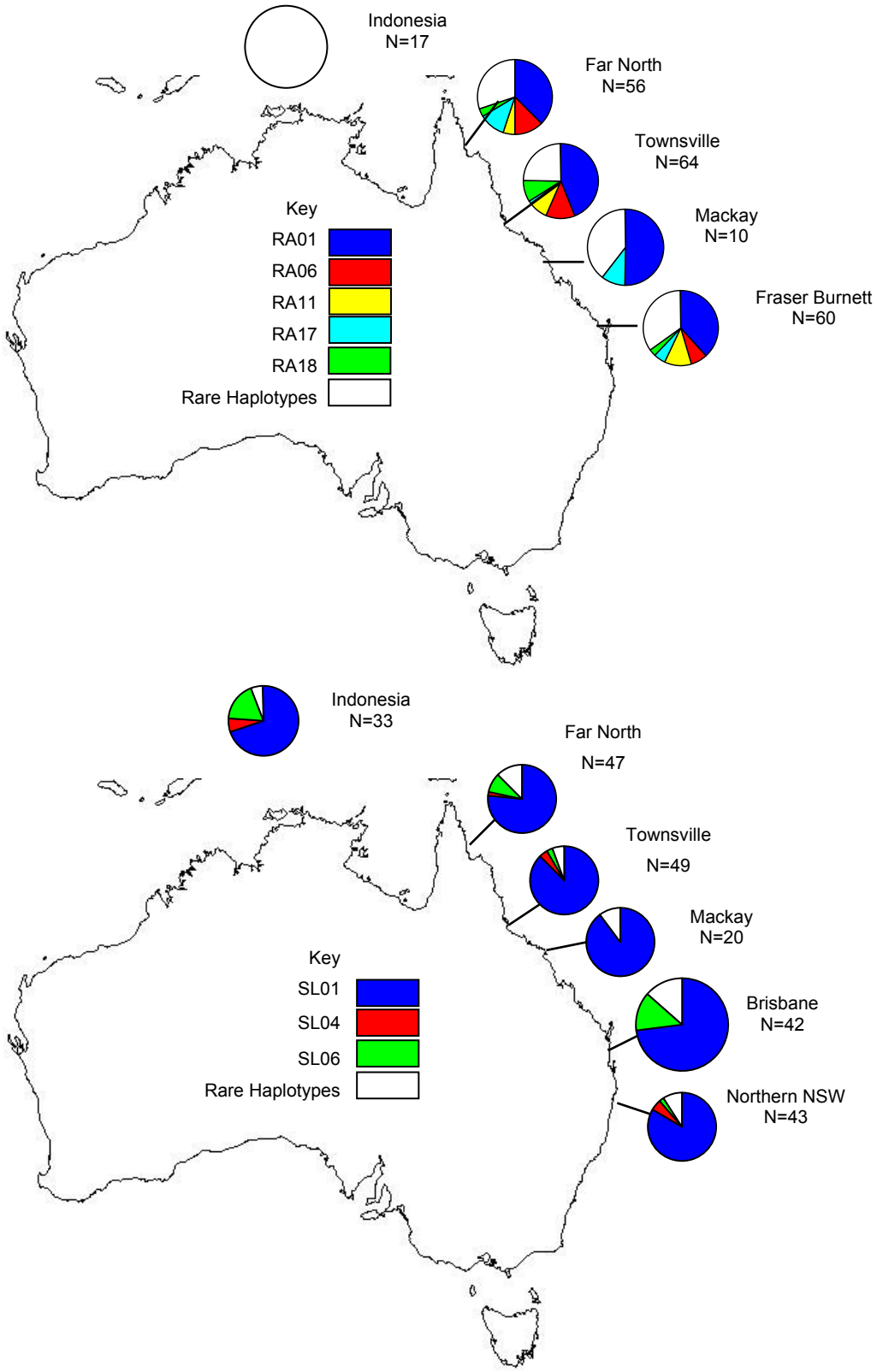

Figure 2.4. Maps profiling the distribution of the most common *R. acutus* (*top*) and *S. lewini* (*bottom*) mtDNA ND4 haplotypes from the major Australian east coast sampling sites and Indonesia.

Table 2.6. Mitochondrial DNA ND4 haplotypes[12] of *Sphyrna lewini* from each sampling location (%) ranked by overall frequency.

SNP position								1	1	1	1	2	2	2	2	2	2	3	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4	5	5	5	5
	0	1	2	3	4	5	7	0	3	3	6	0	1	3	3	4	8	2	2	3	4	8	8	0	1	1	2	3	4	4	5	7	8	9	2	3	4	5
	1	3	8	8	9	8	8	3	3	6	1	5	7	2	8	1	3	2	5	7	0	0	8	3	5	6	4	7	2	8	7	5	4	0	3	8	4	9
ND4_SL01	C	C	C	T	A	A	T	A	A	T	C	T	T	C	T	T	T	T	T	A	T	A	T	C	A	G	A	C	C	T	C	T	T	T	C	T	G	C
ND4_SL06	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
ND4_SL04	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ND4_SL07	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	C	.	.	.	.
ND4_SL03	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ND4_SL02	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ND4_SL05	.	.	.	.	G	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ND4_SL13	T	T	T	C	.	G	.	G	G	C	T	C	C	.	C	C	C	C	C	G	C	G	.	T	C	A	T	T	T	C	T	C	C	.	T	C	A	T
ND4_SL08	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ND4_SL09	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ND4_SL10	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ND4_SL11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ND4_SL12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

[12] Genbank numbers HQ530211 to HQ530223 (for release on 18 April 2011).

Table 2.6 (cont'd).

SNP position	5	5	5	5	6	6	6	6	6	6	7	7	7	8	Region Overall %	Indonesia	Far North	Cairns	Townsville	Mackay	Fraser Burnett	Brisbane	Northern NSW
	7	8	8	9	0	2	2	3	4	5	2	2	5	6									
	4	3	8	9	6	0	8	4	9	2	6	7	0	2									
ND4_SL01	C	A	C	C	T	C	C	T	C	C	T	C	A	C	81.15	69.70	76.60	100.00	87.76	90.00	100.00	76.19	83.72
ND4_SL06	.	.	.	.	.	.	.	.	.	T	.	.	.	.	6.97	18.18	8.51	0	2.04	0	0	11.90	2.33
ND4_SL04	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2.87	6.06	2.13	0	4.08	0	0	0	4.65
ND4_SL07	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2.05	0	2.13	0	0	5.00	0	4.76	2.33
ND4_SL03	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1.64	0	2.13	0	2.04	5.00	0	0	2.33
ND4_SL02	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1.23	0	4.26	0	2.04	0	0	0	0
ND4_SL05	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1.23	0	4.26	0	0	0	0	2.38	0
ND4_SL13	T	.	T	T	C	T	A	C	A	T	C	T	.	T	0.82	6.06	0	0	0	0	0	0	0
ND4_SL08	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0.41	0	0	0	0	0	0	2.38	0
ND4_SL09	.	.	.	.	.	.	.	.	.	.	.	.	G	.	0.41	0	0	0	0	0	0	2.38	0
ND4_SL10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0.41	0	0	0	2.04	0	0	0	0
ND4_SL11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0.41	0	0	0	0	0	0	0	2.33
ND4_SL12	.	G	.	.	.	.	.	.	.	.	.	.	.	.	0.41	0	0	0	0	0	0	0	2.33
Total Number															244	33	47	7	49	20	3	42	43

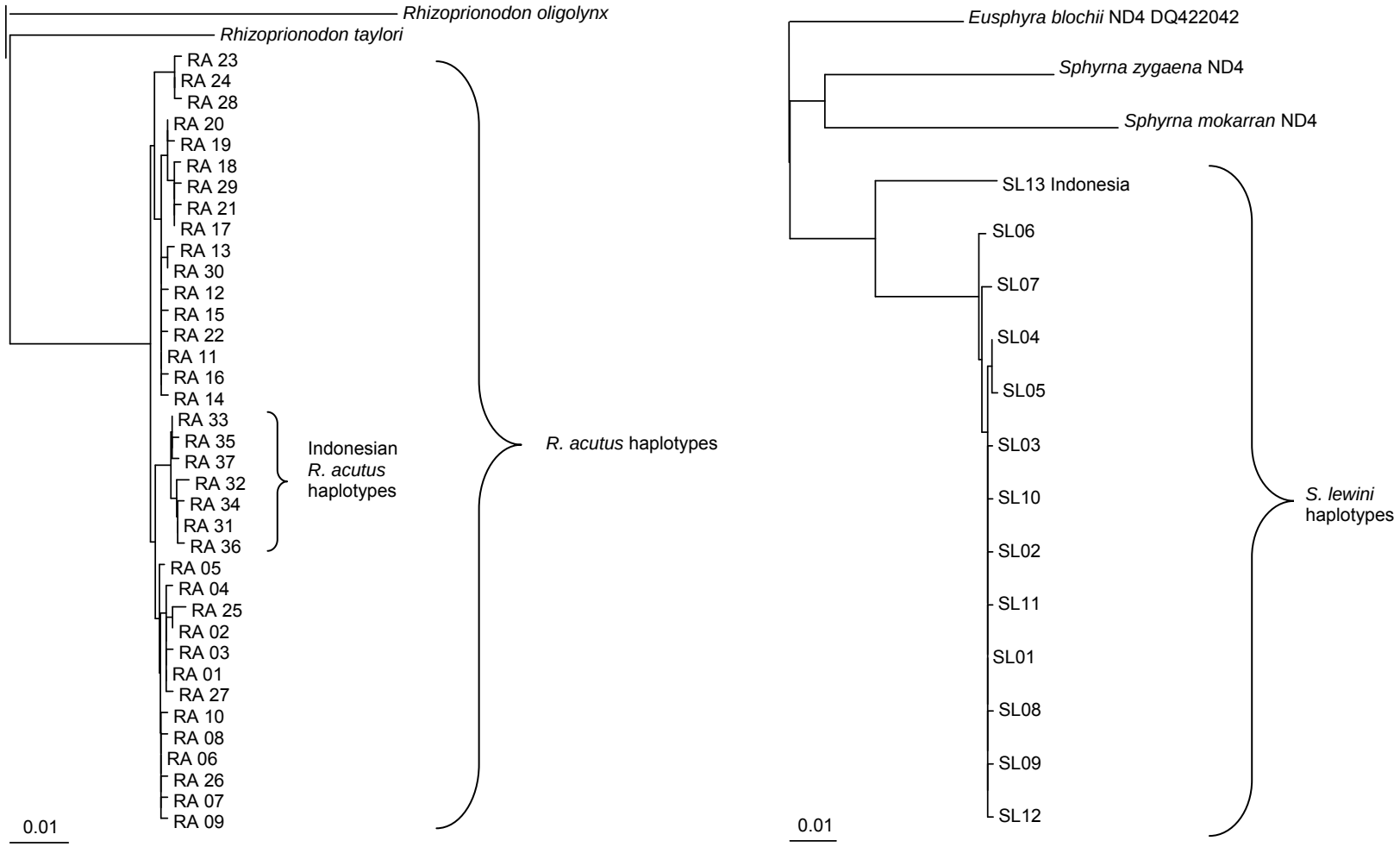

Figure 2.2. Phylogenetic neighbour joining tree of (a) *Rhizoprionodon acutus* and (b) Sphyrna *lewini* mtDNA ND4 haplotypes illustrating the position of Indonesian haplotypes.

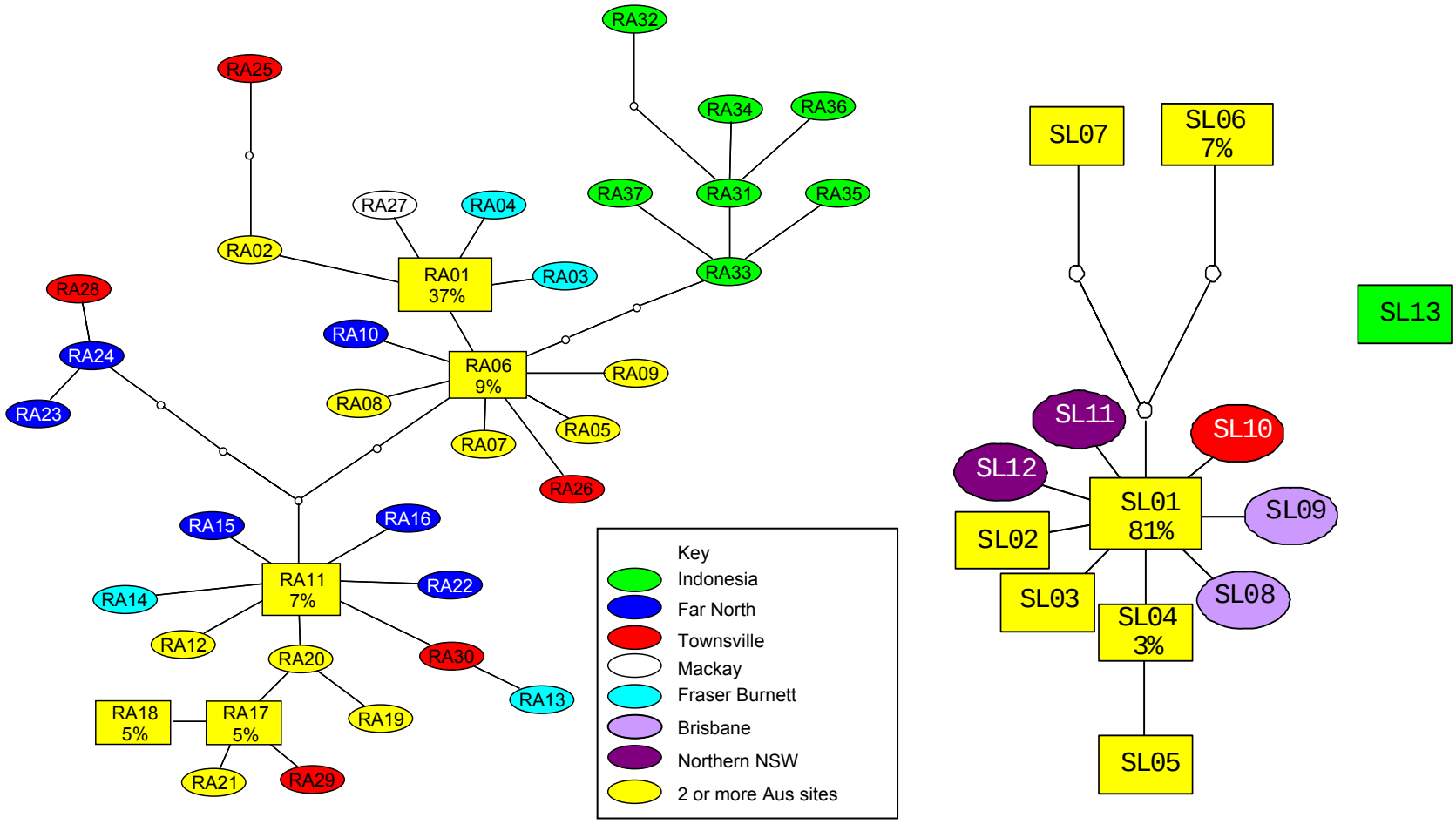

Figure 2.3. Mitochondrial DNA ND4 haplotype networks for (a) *Rhizoprionodon acutus* and (b) *Sphyrna lewini* indicating the sampling region and frequency of dominant haplotypes. Indonesian *Sphyrna lewini* haplotype ND4_SL13 is too divergent (46 steps) to be included in the network.

Table 2.7. Mitochondrial DNA ND4 molecular diversity indices for populations of *Rhizoprionodon acutus* and *Sphyrna lewini*. Statistics presented include the number of sharks sequenced (n), the number of unique haplotypes (u), the number of polymorphic sites (p), haplotype diversity (h, ± standard deviation), nucleotide diversity (π) and the Watterson estimator used to estimate population mutation ND4_RAte using segregating sites and non-recombining DNA (θ_S).

Region	n	u	p	h	π	θ_S
Rhizoprionodon acutus						
Indonesia	17	7	7	0.8897± 0.0398	0.002089±0.001414	2.070559±1.029486
Far North	56	17	20	0.8331± 0.0418	0.003860±0.002227	4.353872±1.495619
Cairns	2	2	7			
Townsville	64	19	23	0.7847± 0.0476	0.003536± 0.002065	4.864363±1.601907
Mackay	10	6	9	0.7778± 0.1374	0.003895±0.002460	3.181372±1.605053
Fraser Burnett	60	15	17	0.8277± 0.0414	0.003445± 0.002022	3.645562±1.285715
Total	209	37	40			
Sphyrna lewini						
Indonesia	33	4	46	0.4886±0.0921	0.007120±0.003859	11.33425±3.742889
Far North	47	7	9	0.4098±0.0885	0.001064±0.000829	2.037726±0.863100
Cairns	7	1	0			
Townsville	49	6	7	0.2313±0.0798	0.000372±0.000424	1.56993±0.715307
Mackay	20	3	4	0.1947±0.1145	0.000458±0.000495	1.127478±0.651884
Fraser Burnett	3	1	0			
Brisbane	42	6	9	0.4111±0.0908	0.001249±0.000930	2.091596±0.893926
Northern NSW	43	7	9	0.3012±0.0911	0.000581±0.000558	2.080087±0.887302
Total	244	13	52			

2.5 Discussion

This study represents a comprehensive, regional analysis of genetic population structure in two shark species (*S. lewini* and *R. acutus*), which are an important component of the elasmobranch commercial catch in north-eastern Australian fisheries. Two types of genetic markers (mt-DNA and microsatellites) were used per species, and large numbers of samples were studied from four to five regions per species on the eastern Australian coast. There was evidence of a single eastern coast genetic stock for *S. lewini* and a single eastern coast stock for *R. acutus*, each encompassing about two thousand kilometres of coastline. Results from both types of genetic markers for both species were concordant; comparisons between pairs of eastern Australian populations showed genetic similarity based on microsatellite allele frequencies and sequence polymorphisms in the mt-DNA ND4 gene region. Genetic homogeneity at this large spatial scale is not matched by environmental conditions. The regions sampled are environmentally heterogeneous; for example, they cross gradients of sea temperatures and extent of continental shelf, and they cross a major transition zone between sub-tropical rocky coast and the tropical Great Barrier Reef.

Table 2.8 Mitochondrial DNA ND4 population pairwise F_{ST} estimates (below diagonal) and *p*-value (above diagonal, α = 0.05, NS = not significant) for major sampling regions for *Rhizoprionodon acutus* (a) and *Sphyrna lewini* (b).

(a) *R. acutus*	Indonesia N=17	Far North N=56	Townsville N=64	Mackay N=10	Fraser Burnett N=60
Indonesia	-	0.00000	0.00000	0.00000	0.00000
Far North	0.56833	-	NS	NS	NS
Townsville	0.57691	0.00608	-	NS	NS
Mackay	0.63420	-0.03435	-0.02425	-	NS
Fraser Burnett	0.58784	0.00675	-0.00731	-0.02594	-

(b) *S. lewini*	Indonesia N=33	Far North N=47	Townsville N=49	Mackay N=20	Brisbane N=42	Northern NSW N=43
Indonesia	-	0.02703	0.01802	NS	NS	0.00000
Far North	0.03877	-	NS	NS	NS	NS
Townsville	0.07302	0.01114	-	NS	0.03604	NS
Mackay	0.03125	0.00032	-0.00611	-	NS	NS
Brisbane	0.02876	-0.01003	0.04730	0.01642	-	NS
Northern NSW	0.05965	0.00007	-0.01289	-0.02348	0.02345	-

Genetic population subdivision has been reported along this coastline for marine species. For example, Sumpton *et al.* (2008) reported a weak genetic disjunction on the north coast of New South Wales between populations of pink snapper (*Pagrus auratus*) and general isolation by distance on the eastern Australian coast. Their sampling locations overlapped with this study, particularly for *S. lewini*, which was sampled further south along the coastline (i.e. northern New South Wales coast) than *R. acutus.* At the northern extent of the coastline studied here, Ovenden *et al.* (Submitted) found genetic evidence for two populations of Spanish mackerel (*Scomberomorus commerson*). One population was centred in the Torres Strait region and the other encompassed regions from Cairns to Townsville southwards. For species studied within the Great Barrier Reef, genetic homogeneity has been the rule rather than the exception. For example, genetic homogeneity was reported for two popular recreational species, coral trout (*Plectropomus maculatus*) and stripey snapper (*Lutjanus carponatatus*) (Evans *et al.* 2010) and only limited genetic subdivision was found in a goby species (*Eviota queenslandica* and *E. albolineata*) that was specifically selected as a candidate for population genetic structure because of their cross-shelf distribution and short life-span (Farnsworth *et al.* 2010). Overall, the most likely explanation of lack of population genetic structure at large spatial scales for bony fish species is semi-passive dispersal of various life-history stages in long-shore currents as well as possible active dispersal of mature life-forms. This may not apply to sharks, however.

Sharks have a considerable potential for active dispersal, most likely as mature adults, although juveniles of some species (e.g. white shark, *Carcharodon carcharias*) also have the capacity to move long distances (Bruce 2008). While long-shore dispersal along the eastern Australian coast is the most likely explanation of the genetic homogeneity observed here for *S. lewini* and *R. acutus,* genetically distinct populations have been observed at this spatial scale in sharks. Ovenden *et al.* (2009) sampled the spot-tail shark (*Carcharhinus sorrah*) from several locations in northern Australia and from two locations in central Indonesia. The mean distance by sea between Australian and Indonesian samples would have been similar to the average pairwise distance between eastern Australian sampling locations in this study. Using a combination of microsatellites and mt-DNA sequence (control region), *F*-statistics for *C. sorrah* ranged from 0.751 to 0.903 (mt-DNA) to 0.038 to 0.047 (microsatellite loci). So, despite their apparent high vagility, barriers to dispersal in continuous marine environments can occur for sharks. It seems unlikely, however, that there are barriers to dispersal for *S. lewini* and *R. acutus* along the eastern Australian coastline. Genetic population analyses take an evolutionary viewpoint on the amount of dispersal that is needed to homogenise gene frequencies in populations. Other techniques for studying dispersal (e.g. tag-release-recapture) or similarity at a population level (e.g. otolith microchemistry, parasite abundance) provide information about the lifespan of individuals and provide an interesting contrast to genetic analyses. For example, for grey mackerel (*Scomberomorus semifasciatus*) on the Australian northern coastline more population subdivision was suggested by parasite (Charters *et al.* 2010) and otolith (Newman *et al.* 2010) analyses

than by population genetic studies (Broderick *et al.* Submitted). Information about dispersal barriers encountered during the lifespan of *S. lewini* and *R. acutus* is contained elsewhere in this report.

From genetic data alone, however, there are obvious limits to dispersal for *R. acutus*. While *S. lewini* may disperse widely between Australia and Indonesia, as shown here and by Ovenden *et al.* (2009), the population of *R. acutus* in central Indonesia was significantly different to the eastern Australian population of *R. acutus. F*-statistics between Indonesian and Australian *R. acutus* ranged from 0.58 to 0.63 (mtDNA ND4 region) and 0.16 to 0.27 (microsatellite loci) and no mtDNA haplotypes were shared between locations. This is strong indication that dispersal does not occur at this scale for *R. acutus.* Dispersal may also be restricted between northern Australian and central Indonesian populations, as suggested by the magnitude of population subdivision reported here. Future studies could address this by comparing *R. acutus* sampled from locations between the Gulf of Carpentaria and northwestern Australia to Indonesian locations. Compared to *S. lewini*, *R. acutus* showed higher levels of haplotype diversity (0.78 to 0.89 compared to 0.20 to 0.49) and a more extensive branching pattern in the haplotype network. In the network, Indonesian *R. acutus* haplotypes formed a well-resolved clade and there was evidence of two to three further clades among the Australian samples whose members were spatially overlapping. The network structure corresponds with category IV of Avise *et al.* (1987) that is characterised by extensive intraspecific gene flow not subdivided by barriers to dispersal. The *R. acutus* haplotype network also does not show the characteristic 'star-burst' pattern often linked with rapidly expanding populations (Posada and Crandall 2001).

This study has provided support for the application of a single set of management arrangements for two of the most commonly landed elasmobranch species in Queensland, *S. lewini* and *R. acutus.* Co-incidentally, and against expectations, there seems to be a single genetic stock for both species from northern New South Wales (*S. lewini*) or southern Queensland (*R. acutus*) to far north Queensland. The smaller body size, and hence presumed lower vagility of *R. acutus*, has not been responsible for genetic subdivisions within its range in Queensland. Despite passing through a heterogeneous environment encompassing temperate, sub-tropical and tropical ecosystems, this genetic study has found no obvious barriers to gene flow in either species within their range in Queensland. The distinction between the two species becomes apparent, however, when comparisons were made between the population on the eastern Australian coast and central Indonesia. As predicted, the smaller species (*R. acutus*) appears to be genetically subdivided, whereas the more oceanic larger species (*S. lewini*) is not affected. We urge fisheries authorities to support further stock structure and biological research on the remainder of the largely unstudied elasmobranch fauna in tropical Australia and to continually refine management strategies to ensure this unique part of Indo-Pacific biodiversity has a sustainable future in Australian waters.

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Chapter 3: Population structure of two inshore shark species (*Sphyrna lewini* and *Rhizoprionodon acutus*) using laser ablation inductively coupled plasma mass spectrometry (LA-ICPMS) along the east coast of Queensland, Australia

Ron Schroeder, Colin Simpfendorfer and David J Welch

3.1 Introduction

Sharks play an important role in the world's oceans as apex predators (Stevens *et al.* 2000a; Heithaus 2004); and recent declines in some populations have highlighted the need for improved management to ensure ongoing provision of ecosystem services and the maintenance of biodiversity (Heithaus *et al.* 2008). Sustainable management for sharks is especially important because many species have K-selected life history that result in low rates of population increase (Musick *et al.* 2000). The development of sustainable management systems depends on the availability of sound knowledge of several aspects of the biology and ecology of shark populations, including life history, population structure, changes in abundance and susceptibility to fisheries gear (Simpfendorfer and Donohue 1998). While the life history of sharks has been increasingly well studied (Carrier *et al.* 2004), stock structure has been poorly investigated even though knowledge of it dictates the spatial extent of management units. Improved knowledge of stock structure in shark populations will help improve the management of shark populations through identifying the appropriate spatial scales at which actions are applied. In this study we define 'stock' as a group of individuals that maintain spatial and temporal integrity by engaging in a distinct pattern of migration not shared by individuals of other contingents (Secor 1999).

The east coast inshore fin fish fishery (ECIFFF) operates along the Queensland eastern coastline. Of the 1800 t of fish commercially harvested in 2006, elasmobranchs (sharks and rays) constituted approximately thirty-five percent. Scalloped hammerhead sharks (*S. lewini*) and milk sharks (*R. acutus*) represent two important species to this composition. *R. acutus* have a continuous distribution from Indo-West Pacific region throughout the Indian Ocean, with isolated populations in the east Atlantic and Mediterranean Sea (Compagno *et al.* 2005). It is a coastal benthopelagic species that may range in size from 100 cm to 150 cm (Musick *et al.* 2004). They may occur in nursery areas, such as Cleveland

Bay in Australia, at all times of year, but may emigrate as they approach maturity (Simpfendorfer and Milward 1993). *S. lewini* are circumtropical ranging species, using nearshore locations as nursery areas areas (Branstetter 1990, Castro 1993, Simpfendorfer and Milward 1993). Living to approximately thirty years of age (Piercy *et al.* 2007), they are a benthopelagic species that can grow to over 300 cm (Musick *et al.* 2004). They will tend to reuse core areas while making occasional long distance excursions (Duncan and Holland 2006). However, transoceanic crossings for *S. lewini* appear uncommon (Kohler and Turner 2001; Duncan *et al.* 2006).

At present, management of the fishery is not based on quantitative population analysis, nor does it account for population connectivity of the various species. Management techniques include seasonal closures, catch limits and gear restrictions, and are principally aimed at teleost fish. Very little information is available for most of the relevant shark species, mandating the need for studies of stock structure and population connectivity. This in turn can be utilised to establish more informed management structure. A technique for examining stock structure that has become commonplace for use with teleost fishes is elemental analysis of their calcified structures using inductively coupled plasma mass spectrometry (ICPMS). Although usually applied to otoliths, several other structures of fish have been analysed using this technique, including scales, fin spines, eye lenses, etc. (Elsdon and Gillanders 2003). Most shark population structure analyses to date have been based on genetic markers (Keeney and Heist 2006; Ovenden *et al.* 2009), physical tagging (Stevens *et al.* 2000b), life history (Lenanton *et al.* 1990) or electronic tagging (Hunter *et al.* 2006). While effective within their scope, all techniques have limitations. Genetics can link fish to a population, but not to a specific geographic region (Ashford *et al.* 2005) because of the small amounts of migration between regions that can result in homology (Bentzen *et al.* 1996). Physical tagging on the other hand provides information for short time scales, but can be limited by the distribution of release and recapture effort and can be logistically challenging and costly (Ashford *et al.* 2005). Electronic tagging can provide data independent of recapture effort, but is expensive at the scale required to reliably identify stock structure (Sibert and Nielsen 2001).

The purpose of this study was to deploy the laser ablation inductively coupled plasma mass spectrometry (LA-ICPMS) method as described by Schroeder *et al.* (in review) to determine fine scale stock structure of *S. lewini* and *R. acutus* along the Queensland and northern NSW coast of Australia. Specifically, samples of both species were collected from six locations each several hundred kilometres apart for analysis of elemental composition and statistical comparison. This allowed inferences to be made regarding meta-population structure and possible migration habits.

3.2 Materials and Methods

3.2.1 Sample collection

All sharks were collected at inshore locations along the coast by observers on commercial fishing vessels between September 2007 and June 2009. Collections occurred at six locations: Far North (14.0° S, 144.0° E), Cairns (16.7° S, 145.9° E), Townsville (19.3° S, 147.2° E), Mackay (21.0° S, 150.0° E), Brisbane (27.0° S, 153.5° E), and Northern NSW (29.6° S, 153.5° E) (see Figure 1.1). Northern NSW consisted of only *S. lewini*. Sections of vertebral columns were cut from animals onboard vessels and frozen for future sample processing. Between 20 and 24 sub-samples of each species from each location were collected and attempts were made to minimise size variation of animals where possible, with *S. lewini* fork lengths ranging from 41 cm to 147 cm, and *R. acutus* ranging from 35 cm to 75 cm.

3.2.2 Sample preparation

Each vertebra centrum was defrosted and the neural arch and lateral processes removed prior to cleaning away as much organic tissue as possible. Individual vertebra were separated and soaked in 5% sodium hypochlorite solution for roughly thirty minutes. Samples were then dried for approximately eighteen hours in a drying oven at 50°C. A thin section of 500 microns was cut sagittally using a Buehler low speed Isomet diamond tipped rotary saw (Series 15HC wafering blade with tap water as coolant), then secured on a glass slide (25mm x 45mm) with clear polyester casting resin. The samples were lightly buffed for roughly five seconds with 3 micron lapping film while being rinsed in tap water. They were not polished due to potential for damaging the relatively soft sample material.

To analyse elemental composition of the vertebrae sections, a Coherent Geolas Pro 193 nm ArF Excimer laser unit was connected to a Varian 820-MS (Melbourne, Australia) Inductively Coupled Plasma Mass Spectrometer (ICPMS) via three metres of Tygon tubing (inner diameter 3.2mm). Vertebrae section slides were set on a chamber insert (55mm x 8mm height) and loaded into the circular sample chamber (55mm diameter x 15mm height). Helium was used as the carrier gas, flowing at 235 ml/min. The instrument was optimized to the maximum sensitivity (238U signal > 2 million cps for National Institute of Standards and Technology (NIST) 610) while keeping the oxide ratio low (ThO/Th ~0.3%) and 238U/232Th ~1. Other instrumental parameters were: RF Power 1300 W, sampling depth 5mm, plasma flow 16.5 l/min, auxiliary flow 1.65 l/min, carrier gas flow 0.97 l/min, and dwell time 20 ms. The laser repetition rate was fixed at 10 Hz on energy density of 6 J/cm2. A 31 µm laser beam was used and the scanning speed was set at 62 µm/s in a step repeat pattern. NIST 610 glass standard was processed at 60 to 120 minute intervals for purposes of correction of instrument drift during the data processing. Prior to measuring each transect, a cleaning run was made to remove surface contamination from the sample. The samples were processed in random order.

3.2.3 Sample data analysis

All data analysis was performed using R scripts. The output from LA-ICPMS was a time series for each element being analysed, with units of counts per second at the ICPMS detector. To process the raw data, several steps were undertaken. First, individual outlier points were identified as any point more than forty percent above or below the ten point simple central moving average, ignoring the current point. Outlier values were replaced with the calculated simple central moving average value. Second, the entire time series was then smoothed by a simple central moving average of width 11 points. Third, start and end points in the sample sequence were identified by visual inspection, determining at which point the signal moved substantially above the background signal or began dropping off the sample signal at the end. Fourth, the background signal inherent in LA-ICPMS was removed by subtracting the simple mean of points 5 through 20 (before the sequence start) from the entire time series. Fifth, the birth ring sequence location was calculated and based on typical measured diameters for each species (*S. lewini* = 4.76mm, *R. acutus* = 2.84mm), laser scan speed, ICPMS detection measurement period and the physical geometry of each individual sample. Sixth, the catch location influence section was defined as the outer 0.2mm lateral distance from the centrum edge, accounting for LA-ICPMS scan parameters and centrum geometry. Seventh, the whole life mean was calculated as the simple mean of the values between the birth ring and sequence end. Eighth, the catch location influence mean was the mean of the values between the catch location influence point and sequence end. Finally, each of the elements investigated was divided by the corresponding Ca level as an internal standard.

To account for the inherent drift in ICPMS sensitivity over time NIST 610 standards were used for external calibration (Jarvis *et al.* 1992) of whole life and catch location influence mean ratio values. Each LA-ICPMS processing day began and ended with a pair of NIST 610 standard transects, with periodic pairs of standards run throughout the day at 60-120 minute intervals. Data from each standard sample was processed by taking the mean of each element for the middle 60 points of the time series and calculating the ratio to corresponding Ca values. A linear interpolation model was created by using the mean of each pair of standard samples with run times as the independent variable, normalised to the first standard run of the first day of LA-ICPMS processing. An elemental ratio correction factor for each sample was calculated based on the actual sample run and date. Appropriate drift corrections for whole life mean and catch location influence mean for each sample were made by dividing the measured value by the corresponding correction factor.

All ratio data from the above preliminary processing was examined for normality and power transformed as appropriate. Principal components analysis (PCA) was used to provide visual exploratory analysis to determine obvious groupings of samples based on location. Due to scale differences in concentrations of various elements, PCAs were based on correlations matrix (Quinn and Keough 2002). Hypothesis testing began with MANOVA. Pillai's trace was used as the MANOVA test statistic for its robustness (Quinn and Keough 2002). Post hoc univarite ANOVAs were run after each

MANOVA to explore contribution of each response variable to overall variation between locations. Adjacent regions were analysed using Hotellings T2 test for pair-wise comparisons. Five location comparisons for *S. lewini* and four for *R. acutus* required Bonferroni adjustment of $p_{slewini}$=.0100 and $p_{racutus}$=.0125 to achieve overall p=.05.

3.3 Results

Principal components analysis (PCA) for *S. lewini* whole life comparison showed detectable groupings within the region and separation between each region, but with heavy overlap (Figure 3.1). The first two principal components explained 32% and 24% of overall variability. The largest variability corresponded to Mg, Mn, and Sr isotopes. Cairns had the most obvious grouping away from the overall means, associating heavily with Mg and Mn. Similar analysis for the catch location influence portion of the sequences indicated very similar patterns, and are not shown here.

PCA for *R. acutus* whole life comparison similarly showed separation between regions, with overlap (Figure 3.2). The first two principal components explained slightly less at 26% and 20%, with Ba and Mg appearing to have the heaviest influence. The Far North, Townsville, and Mackay regions have some association with Ba, while Cairns and Brisbane have some association with Mg. Again, PCA for catch location influence indicated similar patterns, so are not shown here.

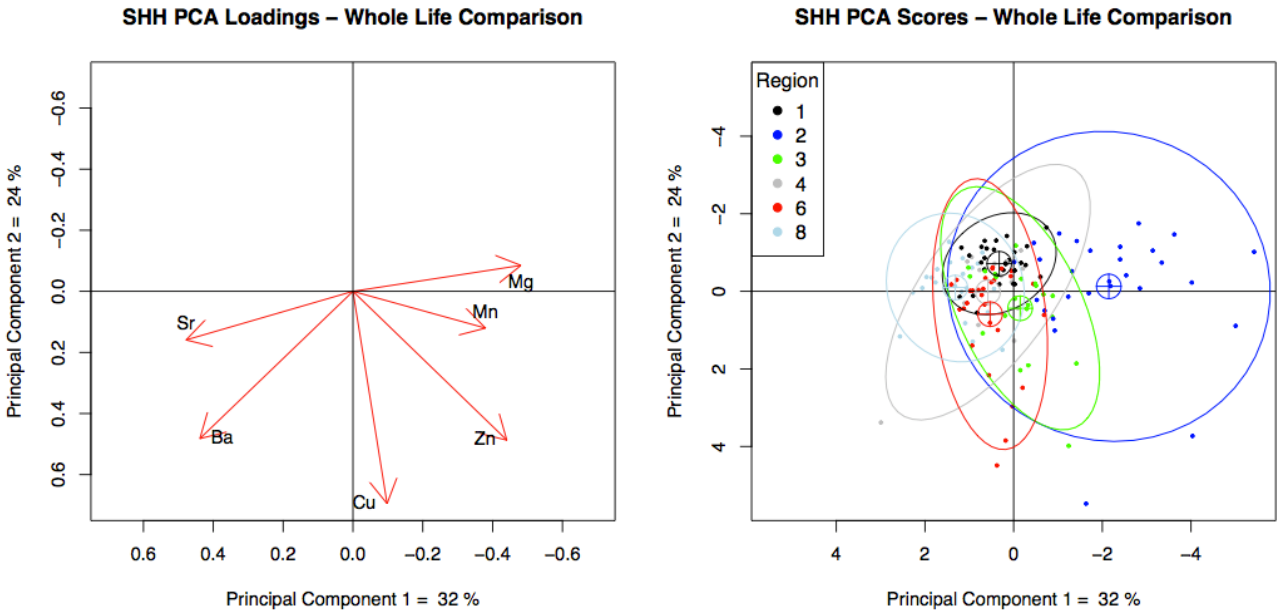


Figure 3.1. Principal components analysis for *S. lewini* "whole life" LA-ICPMS. Regions: 1 – Far North, 2 – Cairns, 3 – Townsville, 4 – Mackay, 6 – Brisbane, 8 – Northern NSW.

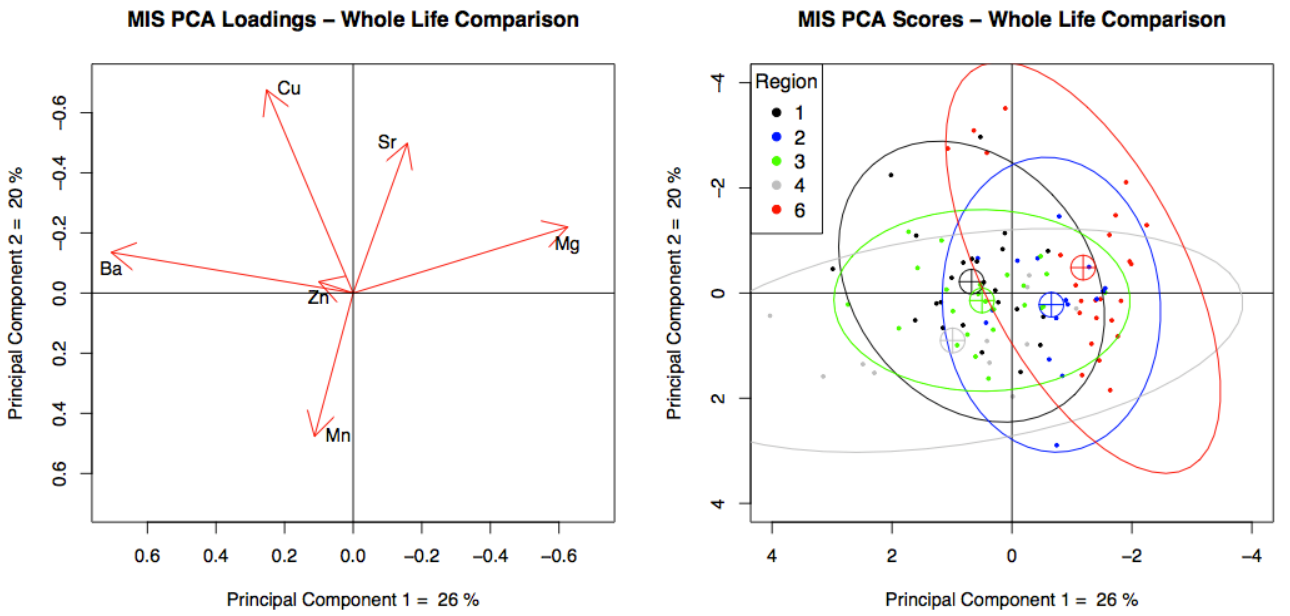


Figure 3.2. Principal components analysis for *R. acutus* "whole life" LA-ICPMS. Regions: 1 – Far North, 2 – Cairns, 3 – Townsville, 4 – Mackay, 6 – Brisbane.

The MANOVA hypothesis tests for both species and both analyses (whole life and catch location influence) showed high significance in testing for variation among groups (Table 3.1). Similarly the post-hoc ANOVAs for each element showed high significance among groups for most elements. One major exception is for Cu, which is likely due to contamination from the sectioning blade during preparation (Schroeder *et al.* in review). Zn showed high significance for both *S. lewini* post-hoc ANOVAs, but was not significant for either of the *R. acutus* tests. Sr was highly significant for all tests except for *R. acutus* whole life analysis, which showed no significance. It is logical that the catch location influence analyses would show greater PCA groupings and hypothesis testing effect size than the whole life analyses since the composition should reflect only the very end of the animal's life. This was not the case. While there were some differences in effect sizes between the analyses, there were no consistent increases in effect sizes from whole life to catch location influence sequence analyses.

While the above analyses appear to reject the simple hypothesis that there is no difference between sites, the pair-wise comparisons address adjacent sites. The Bonferroni adjusted Hotelling T^2 comparisons for both species' whole life sequences indicate highly significant separation between all adjacent locations except for Townsville-Mackay regions (Table 3.2).

Table 3.1. MANOVA and post-hoc ANOVA for both species and both analysis types (whole life or catch location influence).

Analysis	MANOVA	Mg	Mn	Cu	Zn	Sr	Ba
SHH Whole Life	Pillai = 1.65 *** F (5,138) = 11.3 p<.0001	F(5,138) = 43 *** 0. p<.0001	F(5,138) = 26 ** 1 * p<.0001	F(5,138) = 2.3 * 5 p = .044	F(5,138) = 7.1*** 3 p<.0001	F(5,138) = 20 *** 4 p<.0001	F(5,138) = 14 *** 6 p<.0001
MIS Whole Life	Pillai = 1.14 *** F(4,94) = 6.14 p<.001	F(4,94) = 26 *** 4 p<.0001	F(4,94) = 16.4 ** p<.0001	F(4,94) = 1.33 p = .266	F(4,94) = .297 p = .880	F(4,94) = 1.62 p = .176	F(4,94) = 10.7*** p<.0001
SHH Catch Location Influence	Pillai = 1.20 *** F(5,138) = 7.18 p<.0001	F(5,138) = 7 *** 68 p<.0001	F(5,138) = 17 ** 8 p<.0001	F(5,138) = 1.3 6 p = .242	F(5,138) = 4.5*** 5 p = .0007	F(5,138) = 6.1*** 2 p>.0001	F(5,138) = 13 *** 2 p>.001
MIS Catch Location Influence	Pillai = 1.28 *** F(4,94) = 7.21 p<.0001	F(4,94) = 63 *** 6 p>.0001	F(4,94) = 10.9 ** p>.0001 *	F(4,94) = .863 p = .489	F(4,94) = 2.45 p = .052	F(4,94) = 24.0*** p<.001	F(4,94) = 20.5*** p>.0001

Table 3.2. Hotellings T2 pair-wise comparisons. Bold entried are statistically significant.

Location Pair	*S. lewini*	*R. acutus*
Far North / Cairns	T^2 = 18.4, p>.0001	T^2 = 15.9, p>.0001
Cairns / Townsville	T^2 = 11.8, p>.0001	T^2 = 11.8, p>.0001
Townsville / Mackay	T^2 = 2.03, p>.09	T^2 = 1.22, p>.33
Mackay / Brisbane	T^2 = 27.8, p>.0001	T^2 = 27.2, p>.0001
Brisbane / Northern NSW	T^2 = 4.71, p>.0009	N/A

3.4 Discussion

In simply trying to infer stock separation between regions, it is not necessary to determine how environmental variables, such as temperature, ambient elemental concentrations, diet availability or salinity, influence the chemical composition of the animals involved. It is only necessary to compare the multivariate elemental fingerprints of animal groups from the different regions (Elsdon and Gillanders 2003). The fact that the exploratory PCA showed some grouping with heavy overlap between regions, and statistical hypothesis tests indicated significant separation between most regions may suggest these species display general site fidelity, but with limited regional migration. However, one must be cautious in interpreting results such as these. Little can be inferred from differences in elemental fingerprints, other than the stocks are different. If there is no difference in elemental fingerprints, such as between Townsville and Mackay in this study, one can not infer the stocks are the same since it is not known exactly what factors affect the trace elements in the vertebrae (Campana *et al.* 2000). It may be that the stocks freely migrate between the similar regions, or possibly that populations are isolated, but with similar influencing factors in each region.

For *S. lewini*, the above inferences of limited migration and structure are broadly aligned with expectations from other stock structure or migration determination methods. Using traditional tagging methods on the east coast of the United States, a total of 3,278 tagged *S. lewini* animals with a mean liberty time of 2.3 years (max. 9.6 years), the average distance travelled between tagging and recapture was less than 100km (max of 1,600km) (Kohler and Turner 2001). Another traditional tag-recapture study in northern Australia of many shark species, including *S. lewini*, demonstrated some animals moving considerable distances (>1,000 km in some cases), mainly along shore. However, most sharks appeared to move very little, often staying within fifty kilometres of initial tagging site (Stevens *et al.* 2000b). In an early ultrasonic telemetry study in Kaneohe Bay, Hawaii, juvenile *S. lewini* were found to have activity ranges between 0.46 km^2 and 3.52 km^2 within the bay over a twelve-day period (Holland *et al.* 1993). These studies suggest very limited home range for juvenile animals. Springer's (1967) general population model suggests that sharks come inshore for birthing where the pups stay near nursery areas until they move offshore as adults. This is consistent with expectations for *S. lewini* in that most samples found on the present inshore study were juveniles.

Traditional tagging or telemetry techniques are powerful in tracking individuals' general movements, but are not necessarily conducive to comparing populations between sites with statistical methods. Molecular methods have also been used to examine stock structure of *S. lewini*. Molecular methods are best used for determining structure over evolutionary time scales (Avise 2004). However, mixing rates need only be one percent or even less between stocks to prevent genetic differentiation (Bentzen *et al.* 1996). In a global *S. lewini* molecular based study, very little stock separation was found along coasts, while there was some level of structure detected across ocean basins (Duncan *et al.* 2006). In the present study the sampling sites are inshore and extend hundreds of kilometres along the coast

from each other. Coupling general site fidelity (or at least limited home range) with some animals occasionally travelling longer distances along the coast supports both the lack of coastal genetic structure and these microchemistry results. The microchemistry method addresses environmental influences on the animal from conception throughout life. It is logical that the technique will reveal shallower stock structure than molecular methods that reflect lineage up to the point of conception.

While substantial work has been performed on *S. lewini* stock structure and migration using other methods, very little has been done for *R. acutus*. However, some activity has occurred to understand movement of other species in the genus *Rhizoprionodon*. For example, *R. terranovae* were studied in Florida, United States, using acoustic telemetry tracking. It was found that juveniles had small home ranges, averaging 1.29 km^2. However, some animals may disappear for extended absences, returning to the tracking area at a later date of up to 1,352 days. Despite their small size, it appeared they use a series of coastal bays and estuaries as opposed to a discrete habitat. One individual was recaptured 169 km away from study site after 35 days at liberty (Carlson *et al.* 2008). A revised general model for smaller shark species suggests that despite limited site attachment, these animals that spend their entire life-cycle inshore are less reliant on a specific habitat (Knip *et al.* 2010). Although the home range size and distance travelled may be less than that of juvenile *S. lewini*, it appears the same general behaviour of showing some site fidelity with occasional longer distance movement may be occurring for *R. acutus*. Again, this is generally consistent with the results of the present study.

While the present study suggests that several metapopulations exist along the north-eastern Australian coast for both *S. lewini* and *R. acutus*, there are a number of unknown variables in this microchemistry method. Factors affecting elemental variation in the animals include water temperature, salinity, diet availability or ambient concentrations (Bergenius *et al.* 2005). The migration behaviour we wish to make inferences about may be directly affected by these same factors. Water temperature may have direct effect on distribution and migration of sharks (Grubbs *et al.* 2007). Diets may vary with geographic location (McElroy *et al.* 2006) and could be due to local availability of prey (Bethea *et al.* 2006). Young *C. leucas* will change location based on salinity levels in a river estuary (Heupel and Simpfendorfer 2008). Although neither of the species in the present study would be expected to inhabit rivers, the salinity decreases from river plumes during the wet season may affect animal movements.

As an inshore study with much of the area located in the tropics, it would be expected that run-off from the wet season will affect ambient concentrations of various elements. Detailed water analysis of elemental concentrations in various locations is beyond the scope of this study, as is the animals' physiological reaction related to these concentrations. Elemental concentration variation in sharks may be related simply to terrestrial runoff influencing ambient water concentrations and affecting the animals' uptake of related elements. As a simple example, if your compare the bedrock types underlying drainage basins for rivers near each of the study locations (Furnas 2003), an interesting

pattern may can be found corresponding to the Hotellings pair-wise comparisons in the present study (Table 3.3). Table 3.3 shows that there are large changes in bedrock type for rivers feeding each adjacent pair of locations, except for Townsville and Mackay. This may be a coincidental similarity or may be a causal factor. The purpose here is to suggest that there are too many possible variables affecting an animal's elemental fingerprint to explain any causal effects. The conclusion to be drawn from these results are simply that the stocks from the different locations are significantly different from a statistical standpoint, with the exception of Townsville/Mackay. This reinforces what is expected from more traditional movement studies and does not contradict what has been found in prior molecular studies.

Table 3.3. Bedrock types underlying river drainage basins and the corresponding regions from this study (data sourced from Furnas 2003).

Region	River	Igneous / Sedimentary percentage of bedrock
Far North	Normanby	10% / 90%
Cairns	Barron	38% / 62%
Townsville	Ross	100% / 0%
Mackay	Pioneer	98% / 2%
Brisbane	Mary	20% / 80%

Several additional areas can be addressed to increase the utility of this microchemistry method. In order to begin to understand what factors affect the animal's elemental profile, one must start with knowledge of where the animal has been. This may be from various tracking techniques, such as tag-recapture, acoustic telemetry, or preferably GPS tracking. Once the vertebrae and geographic history of many samples are acquired, it can be coupled with external environmental profiles. These would include items such as water chemical analysis, prey distribution models, or temperature profiles. Temporal effects must be considered to account for seasonal and annual variation in run-off or temperature. Analyses of this nature would be highly complex and require a very large sample size. It would also require much lower LA-ICPMS scan rate in order to substantially increase the resolution of transect data. However, if it is desired to infer more than 'the stocks are different', this step must be taken.

While there is significant inshore population structure in both *S. lewini* and *R. acutus* along the Queensland and northern New South Wales coast, there is some regional migration. This should be sufficient to provide reasonable connectivity between populations as well as some capability of replenishment of depleted neighbouring populations. However, this connectivity appears limited so it would be prudent to manage the fishery on a regional basis to limit localised stock depletion.

Chapter 4: Examination of the stock structure of milk shark, *Rhizoprionodon acutus*, and scalloped hammerhead, *Sphyrna lewini*, on the east coast of Australia using life history characteristics

Alastair Harry, Colin Simpfendorfer, Andrew Tobin and David J Welch

4.1 Introduction

Determination of the stock structure of fish species is a critical process in fisheries science as it provides the basis upon which monitoring, assessment and management of that species and their fisheries should be applied (Cadrin *et al.* 2005). One method of defining stock structure is by describing the life history characteristics unique to it e.g. growth rates, maturity, fecundity (Begg *et al.* 1999). The use of life history characteristics as a stock-identification tool in elasmobranchs specifically is not one that has been frequently employed, most likely due to historical lack of management of elasmobranch resources. However, a number of examples using commercially targeted species demonstrate that such methods can be used to successfully distinguish separate stocks. Yamaguchi *et al.* (2000) studied *Mustelus manazo* from five locations in Japan and Taiwan and found differences in most aspects of life history including maturity, fecundity and timing and frequency of reproduction. Because sharks from all areas except two showed variations, Yamaguchi *et al.* (2000) concluded that there was separation between some populations and possible mixing between the two biologically similar regions. *Mustelus antarcticus* populations off southern Australia also exhibit differences in various aspects of reproductive biology including timing and frequency of parturition (Walker 2007).

The milk shark, *Rhizoprionodon acutus*, Ruppell 1837 and the scalloped hammerhead, *Sphyrna lewini*, Griffith and Smith 1834, are two cosmopolitan species distributed in tropical and warm temperate seas circumglobally (Compagno *et al.* 2005). *R. acutus* is a small species that attains <1 m in size throughout most of its range and is restricted to coastal waters <200 m depth. *S. lewini* is a large coastal, semi-pelagic species that utilises coastal waters as nursery habitat and migrates offshore at larger sizes. Both species are exploited throughout their range in commercial and artisanal fisheries where they are targeted or taken as by-catch. Large population declines have been reported for *S. lewini* in many parts of the world and consequently this species is listed as endangered by the IUCN (Baum *et al.* 2007). In the waters off eastern Australia, both species are taken by a range of commercial fisheries. In the

inshore net component of the Queensland East Coast Inshore Finfish Fishery (ECIFF), *R. acutus* and *S. lewini* account for 8% and 11% of the total elasmobranch catch by number (Harry *et al.* in review-b). Both are also bycatch in trawl fisheries operating in Queensland. *S. lewini* is also captured by a longline fishery targeting shark in the waters off northern New South Wales (Macbeth *et al.* 2009). There is also an unquantified catch from the recreational sector. Prior to this study very little was known about the life history characteristics and movements of either species in Australian waters. As part of the current study aspects of the life history of *R. acutus* and *S. lewini* was investigated to make inferences about their stock structure in north-eastern Australia.

4.2 Methods

4.2.1 Sample collection

Biological samples were collected between December 2005 and May 2010 using fishery-dependent sources along the east coast of Australia from Princess Charlotte Bay, Queensland (13° 54' S) to the waters off Laurieton, New South Wales (31° 36' S) (see Figure 1.1). The majority of samples were obtained from a fishery observer program monitoring the commercial gillnet sector of the Queensland East Coast Inshore Finfish Fishery (ECIFF) (Harry *et al.* in review-b), in shallow waters up to 25 m depth. Additional fishery-dependent samples were sourced opportunistically from: the Queensland East Coast Otter Trawl Fishery (unknown depth); mid-shelf, demersal longline fisheries operating in both Queensland and New South Wales waters (30-110 m depth) (Macbeth *et al.* 2009); and the Queensland Shark Control Program.

4.2.2 Age determination and growth analysis

The age of individuals was established by counting growth band pairs deposited on vertebrae. Vertebrae samples were prepared, sectioned and aged using standard techniques for elasmobranchs (Cailliet and Goldman 2004). A full description of the methods used for age determination for each species can be found in Harry *et al.* (in review-a) and Harry *et al.* (2010). An information theoretic, multi-model-inference approach was undertaken to analyse growth (Katsanevakis and Maravelias 2008). Length-at-age data were fit to five commonly used growth models for elasmobranchs and the relative support for each model was evaluated using Akaike's Information Criteria (AIC) (Burnham and Anderson 2001). The best fit model was considered the one with the lowest value of AIC.

4.2.3 Reproductive biology and maturity analysis

Analysis of reproductive biology followed Walker (2005). Males were assigned a maturity stage based on the degree of clasper calcification. For females, maturity stage was based on macroscopic examination of the uterus. *In utero* embryos and ova were measured and counted to determine the timing and length of the gestation period and fecundity. The maximum diameter of the largest ovarian

follicle was also measured to determine the periodicity of reproduction. Length at birth was inferred from the largest embryos observed and from free-swimming individuals with open-umbilical scars indicating that they had recently been born. Population estimates of length and age at maturity were established by converting maturity stages to a binomial form (immature = 0, mature =1). Generalised linear models with a binomial error structure were used to model maturity stage (logit transformed) as a function of length or age.

4.2.4 Stock structure analysis

Statistical comparison of linear or generalised linear models (e.g. weight-at-length, length-at-maturity, fecundity-at-length) between regions was undertaken using analysis of covariance with region as a factor. Models were ranked based on computed AIC values and the one that minimised AIC was considered the best. Analysis of variance was used to test whether length at birth was statistically different between regions. Likelihood ratio tests and AIC were used for comparison of non-linear models (e.g. growth models) between regions (Kimura, 1980; Burnham and Anderson, 2001). Non-statistical regional comparisons involved examining differences in timing and frequency and seasonality of reproductive cycles. For some analyses, regions were pooled into tropical and temperate regions due to a lack of samples. The division between tropical and temperate regions was the Tropic of Capricorn, therefore the tropical region encompassed Far North to Capricorn, while the temperate region encompassed Fraser/Burnett to northern NSW (see Figure 1.1).

4.3 Results

4.3.1 Sample collection

A total of 300 *R. acutus* were obtained throughout the duration of sampling including 90 females (505-940 mm), 206 males (385-890 mm) (Table 4.1) and four individuals of unknown sex. Sample sizes were relatively low (n<60) in all regions except Townsville where 136 individuals were obtained. The samples predominantly comprised juveniles and adult males. Adult females were an infrequent component of the catch and only 24 were captured in total from only two regions: Far North and Townsville. Mean lengths and length compositions differed greatly between regions (Table 4.1, Figure 4.1). Females were largest in the Far North region and smallest in Fraser/Burnett. Males were largest in Mackay (and Capricorn where n = 1), and were smallest in Fraser/Burnett.

A total of 512 *S. lewini* were obtained during sampling including 192 females (465-2,600 mm) and 320 males (465-2898 mm) (Table 4.2). The majority of samples were obtained from the Townsville region (n=268) and sample sizes from all other regions were comparatively low. The majority of samples were juveniles although a total of 70 adult males were captured. Only a single adult female was encountered during the study.

Table 4.1. Summary of *Rhizoprionodon acutus* samples collected within each sampling region, where *n* is total sample size, and *N* is the number of mature animals.

Region	Females				Males			
	n	*N*	Mean length (mm)	Range (mm)	*n*	*N*	Meanlength (mm)	Range (mm)
Far North	11	10	860	670-940	49		830	770-875
Cairns	8		588	564-615	14	11	636	500-830
Townsville	41	14	716	501-930	95	29	755	385-890
Mackay	1				23		861	770-890
Capricorn	2		721	547-895	1		890	
Fraser/Burnett	27		556	505-774	24	22	572	485-800
Brisbane	0				0			
Northern NSW	0				0			
Total	90				206			

Table 4.2. Summary of *Sphyrna lewini* samples collected within each sampling region, where *n* is total sample size, and *N* is the number of mature animals.

Region	Females				Males			
	n	N	Mean length (mm)	Range (mm)	n	N	Meanlength (mm)	Range (mm)
Far North	26		956	637-1,220	39	9	1,225	638-1,700
Cairns	23		570	494-665	27		560	505-685
Townsville	81		633	465-1,400	187	44	1,048	465-2,250
Mackay	10		622	482-840	20	3	930	466-1,970
Capricorn	4		719	665-770	3		888	690-1,205
Fraser/Burnett	15		1,084	502-1,221	10		1,068	702-950
Brisbane	12		1,474	1,000-1,820	11		1,375	545-2,300
Northern NSW	21	1	1,666	1,340-2,600	23	14	2,213	1,528-2,898
Total	192				320			

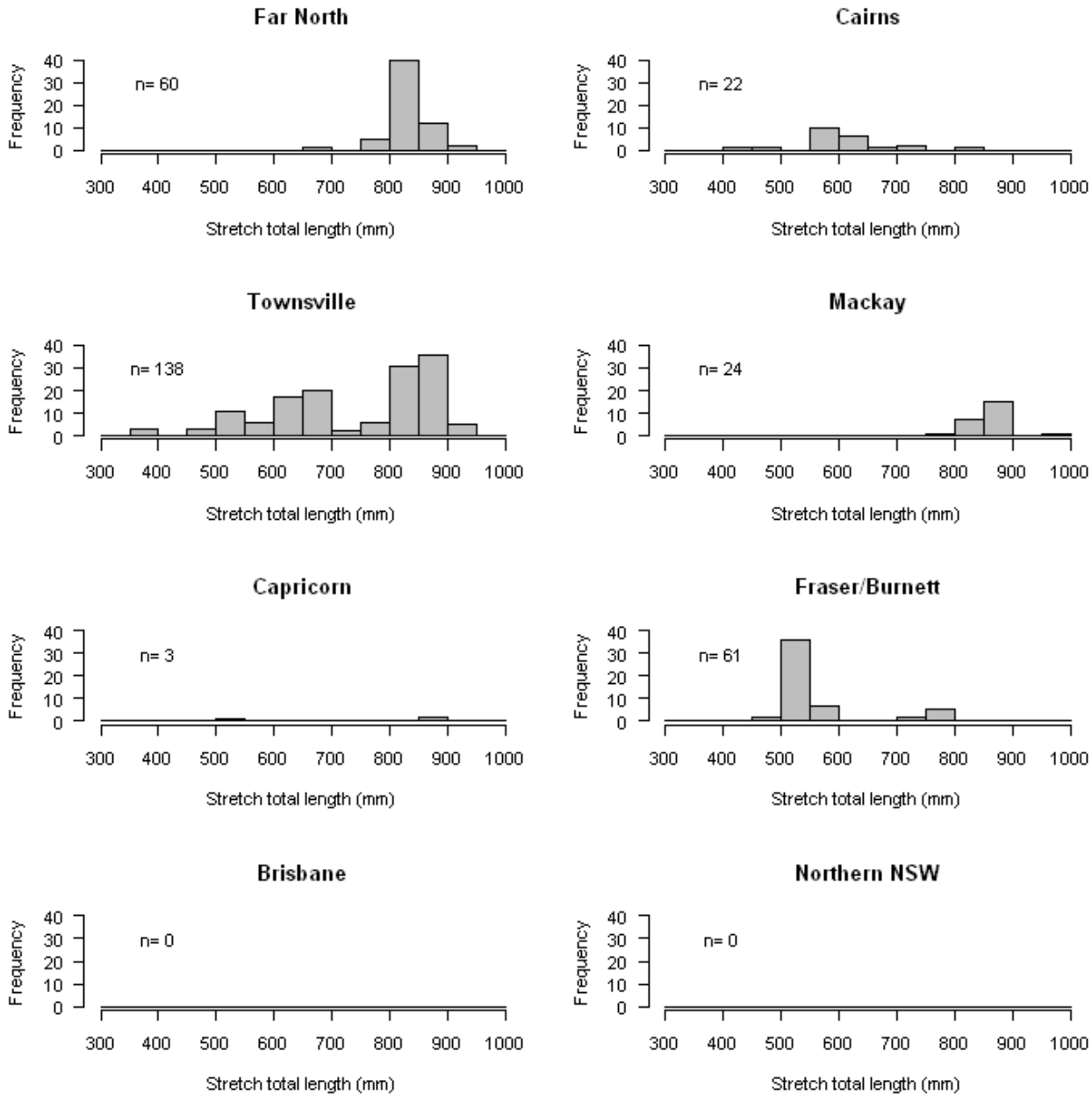


Figure 4.1. Length frequency of *R. acutus* samples separated by region.
The total number of samples in each region is denoted by n.

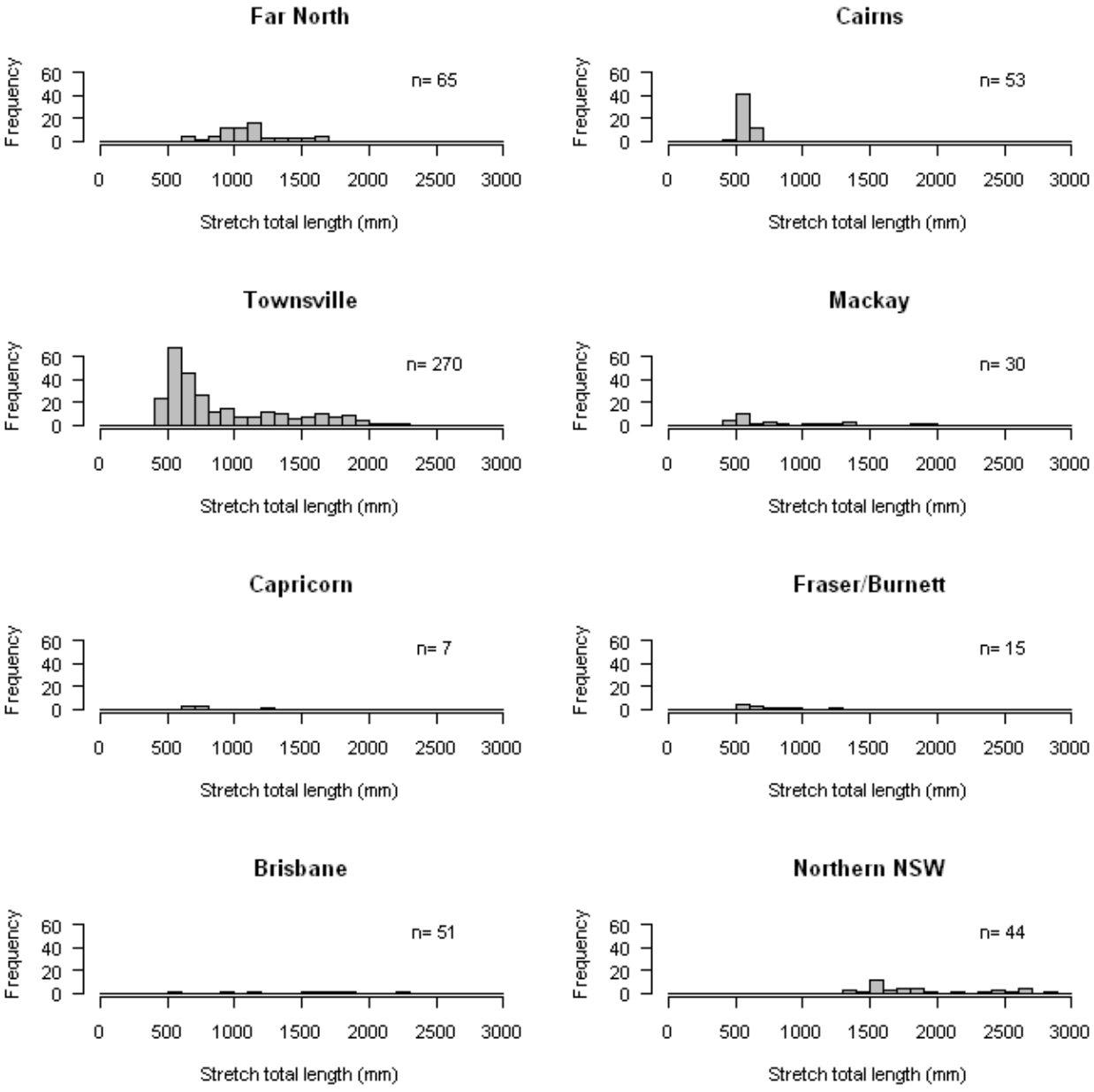


Figure 4.2. Length frequency of *S. lewini* samples separated by region. The total number of samples in each region is denoted by n. Lengths were not obtained for some animals in the Brisbane region.

4.3.2 Life history of *R. acutus*

The life history of *R. acutus*, inferred from samples collected in the present study, was described in detail by Harry *et al.* (2010). Off eastern Australia, 50% maturity occurred at 742 mm length and one year of age for males and 780 mm length and 1.8 years for females. Both sexes grew rapidly to an asymptotic size (821 mm for males, 859 mm for females) and reached maturity quickly, after which point minimal growth occurred. The oldest male and female were 4.5 and 8.1 years of age, while the largest male and female were 931 mm and 940 mm respectively. The mean fecundity from twenty pregnant females was 3.1 embryos (range 1-5) and there was a suggestion of increasing fecundity with increasing maternal length. Near-term pregnant females had large-yolky oocytes present indicating that mating probably occurs soon after birth and reproduction is annual though aseasonal (Harry et al, 2010). The largest embryo measured was 381 mm, while the two smallest free-swimming individuals were 385 mm, indicating a size-at-birth of approximately 350-400 mm.

4.3.3 Evidence of stock structuring in *R. acutus*

Insufficient sample sizes in all regions except Townsville, compounded by differences in size and sex structures of samples, prevented any inter-regional statistical comparison of life history traits. Pregnant females appeared to occur largely outside of the areas sampled and were only encountered in large numbers (>5) during two occasions: an observer trip in Princess Charlotte Bay (Far North) and an observer trip in the Whitsundays (Townsville). A non-statistical comparison of embryo lengths (Figure 4.3) provided some evidence of inter-regional differences. Embryo lengths from ten pregnant females sampled during September from Princess Charlotte Bay suggested an aseasonal cycle of reproduction (Figure 4.3). Females were in different stages of embryonic development, ranging from individuals recently pregnant with in utero eggs to individuals with pups close to full term. Conversely, embryo lengths from nine pregnant females sampled during August from the Whitsundays were suggestive of a seasonal cycle, as females were all at the same stage of embryonic development. Four other pregnant females measured from the waters off Townsville (Townsville region) between June and November all had embryo lengths > 300 mm, more indicative of an aseasonal cycle.

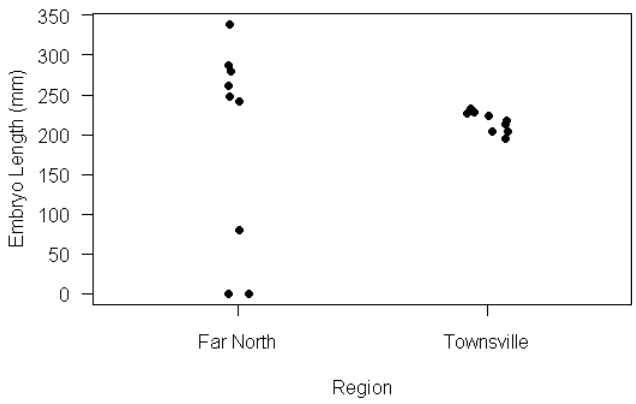


Figure 4.3. Mean embryo lengths of pregnant *R. acutus* collected during single sampling trips in the Far North (Princess Charlotte Bay) and Townsville (Whitsundays) regions.

4.3.4 Life history of *S. lewini*

The life history of *S. lewini* inferred from samples collected in the present study was described in detail by Harry *et al.* (in review-a). The absence of adult female samples meant only the reproductive biology of males could be observed directly. Off eastern Australia, fifty percent maturity occurred at 1,471 mm and 5.7 years in tropical waters (north of the Tropic of Capricorn and inclusive of regions Far North to Capricorn) and 2,043 mm and 8.9 years in temperate waters (south of the Tropic of Capricorn and inclusive of the regions Fraser-Burnett to northern NSW). Males attained a maximum age of 21 years and grew to at least 2,898 mm. The only adult female captured was 15 years old and 2,600 mm. Length at birth could be inferred from neonates with open-umbilical scars and was 465-563 mm. Neonates with open umbilical scars were captured throughout the year so the reproductive cycle appeared to be aseasonal but with a peak during summer.

4.3.5 Evidence for stock structuring of *S. lewini*

Sampling difficulties similar to those for *R. acutus* limited any fine-scale statistical comparison of inter-regional differences in life history. However, pooling samples into tropical and temperate regions allowed a broad-scale statistical comparison of male length- and age-at-maturity. Logistic regression models of length- and age-at maturity that included region (but not the interaction of region with length or age) were the most parsimonious given the data and showed that region had a strong effect on maturity (Figure 4.4, Table 4.3, Table 4.4 – adapted from Harry *et al.* (in review-a)). For male *S. lewini* in temperate waters, 50% maturity occurred at lengths 572 mm larger and ages 3.2 years older than those in the tropics.

Table 4.3. Summary of logistic regression analysis of length and age against maturity stage for male *Sphyrna lewini*. The effects of the factor region (tropical and temperate) on maturity stage was also examined, and the best model was chosen as the one that minimised the small-sample, bias-adjusted form of Akaike's Information Criteria (AICC). Akaike differences (Δ), akaike weights (w), residual deviance and residual degrees of freedom show the relative performance of competing models. K is the number of estimated regression parameters.

Potential process	Model	K	AIC_C	Δ	W	Residual deviance
Region affects length at maturity	Stage~L_{ST}+Region	3	52.72	0.00	0.5449	46.63
Region and the interaction of region with length affect length at maturity	Stage~L_{ST}+Region+ L_{ST}:Region	4	53.08	0.36	0.4551	44.93
Length at maturity is independent of region	Stage~L_{ST}	2	95.84	43.12	0.0000	91.80
Region affects age at maturity	Stage~Age+Region	3	45.13	0.00	0.7348	39.00
Region and the interaction of region with age affect age at maturity	Stage~Age+Region+Age:Region	4	47.18	2.04	0.2650	38.96
Age at maturity is independent of region	Stage~Age	2	61.22	16.08	0.0002	57.15

Table 4.4. Summary of four logistic regression models used to determine size and age at maturity of male *Sphyrna lewini*. Parameter values (with 95% CI) are given for the logistic regression model P(x) = 1/(1+exp(a+bx)), where P(x) is the proportion of individuals mature at a given size or age x, and a and b are fitted regression coefficients. L_{ST50} and A_{50} (with 95% CI) are population estimates of stretch total length (mm) and age (years) at 50% maturity, n is the number of mature animals, and N is the total number of animals.

Model	Region	a	b	L_{ST50} / A_{50}	n	N
Stage~L_{ST}+Region	Tropic	-25.29 (-38.72, -17.44)	0.017 (0.026, 0.119)	1471 (1423, 1519)	56	233
	Temperate	-35.12 (-53.83, -23.49)		2043 (1934, 2182)	14	31
Stage~Age+Region	Tropic	-8.90 (-13.94, -5.75)	1.575 (1.028, 2.368)	5.7 (5.1, 6.2)	25	160
	Temperate	-14.03 (-23.39, -7.75)		8.9 (7.5, 10.8)	13	27

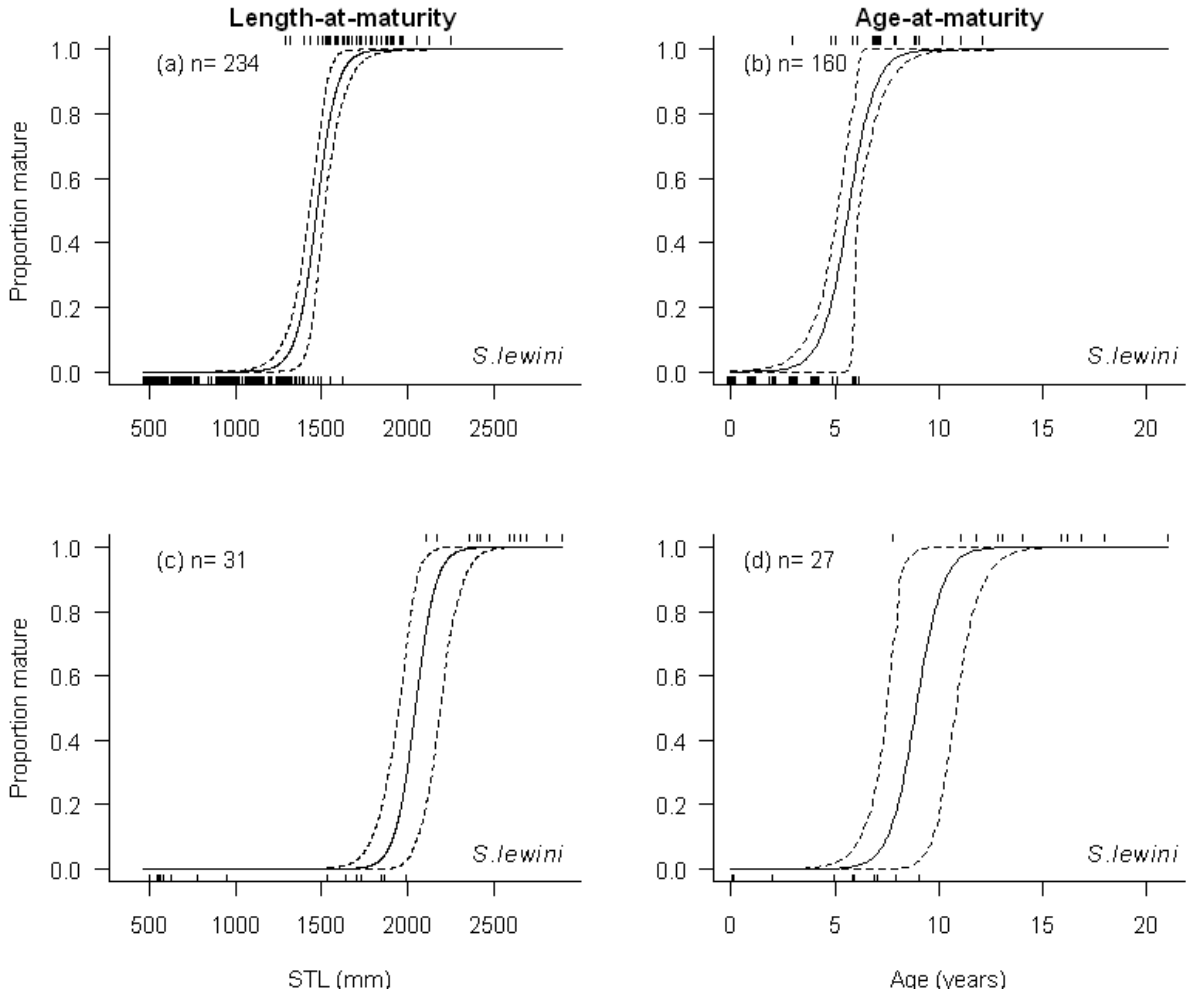


Figure 4.4. Length- and age-at-maturity ogives for *Sphyrna lewini* in tropical (a and b) and temperate (c and d) waters Solid lines are the expected proportion of population mature at a given length and dashed lines are 95% confidence intervals. Rug plots indicate observed data points.

Due to sampling restrictions and limitations in the data it was not possible to make valid comparisons of growth across all sizes. Samples from the tropics were predominantly juveniles and young adults aged 0-12 years while older adults (12-21 years) were only sampled in temperate waters (Figure 4.5).

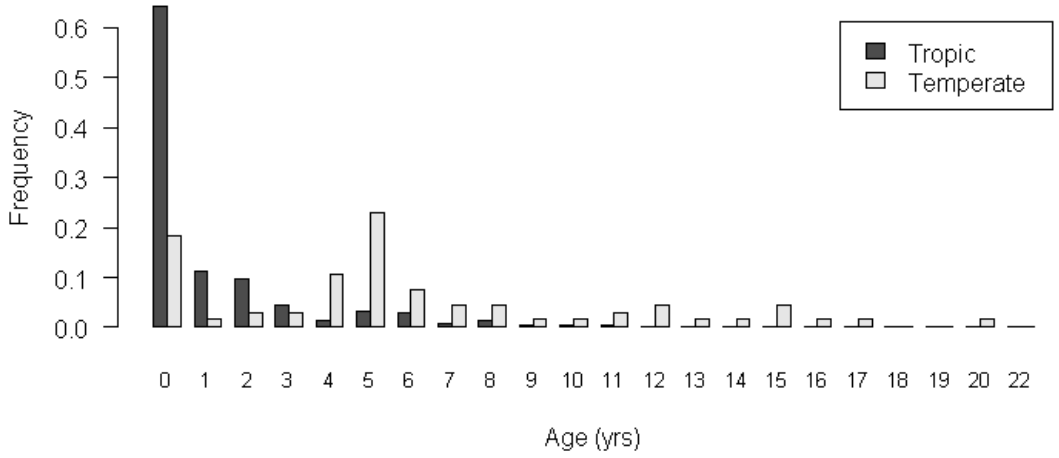


Figure 4.5. Comparison of ages of samples obtained from tropical and temperate regions for *S. lewini*. Frequency on the y-axis is expressed as a proportion of the total sample.

Examining the effect of region on growth curves of individuals aged 0-12 using likelihood ratio tests showed there was a strongly significant difference between regions across all parameters (χ^2 = 41.59, d.f. = 1, $p < 0.01$). This was mainly driven by a large difference in the parameter L_∞ between the regions, rather than differences in the parameters t_0 or *k*. Based on computed AIC values, the best model was one that had six fitted parameters: a unique L_∞ for each region; a unique value of *k* for each region; a single value of t_0 for both regions combine; plus a parameter for variance (Table 4.5).

Table 4.5. Summary of regional comparison of von Bertalanffy growth curves for ages 0-12 years. A description of each potential process modelled is given, along with the corresponding model formula. c is a dummy variable taking values of 0 or 1 and was used to incorporate region as a factor. n is the sample size, K is the number of estimated model parameters (plus one for variance), Δ is the Akaike difference, w is the Akaike weight, and RSE is the residual standard error.

Potential process	Model	n	K	AIC_C	Δ	w	RSE
All parameters are different between regions	STL ~ ($L_{\infty,0}$+$L_{\infty,1}$*c) * (1 - exp(-(k_0+k_1*c) * (Age - ($t_{0,0}$+$t_{1,0}$*c))))	380	7	3413.62	1.90	0.2117	88.3
k and t_0 differ, $L\infty$ does not	STL ~ (L_∞) * (1 - exp(-(k_0+k_1*c) * (Age - $t_{0,0}$+$t_{0,1}$*c))))	380	6	3418.48	6.76	0.0187	88.99
L_∞ and t_0 differ, *k* does not	STL ~ ($L_{\infty,0}$+$L_{\infty,1}$*c) * (1 - exp(-k * (Age-(t0,0+t0,1*c))))	380	6	3413.52	1.80	0.2224	88.41
L_∞ and *k* differ, t_0 does not	STL ~ ($L_{\infty,0}$+ $L_{\infty,1}$*c) * (1 - exp(-(k_0+k_1*c) * (Age-t_0)))	380	6	3411.72	0.00	0.5473	88.2
None of the parameters differ	STL ~ L_∞ * (1 - exp(-k * (Age-t_0))	380	4	3449.02	37.30	0	92.9

4.4 Discussion

4.4.1 Stock structure of *R. acutus*

The data obtained for *R. acutus* in the present study were unsuitable for most of the planned comparisons, both statistical and non-statistical. There was limited evidence to suggest that seasonality of reproduction may vary from aseasonal in the northern regions to seasonal in the southern regions. This in itself is evidence of separate stocks of *R. acutus* on the east Australian coast, though on its own is not compelling and would need to be substantiated through further targeted sample collection.

In northern Australia this species is known to reproduce aseasonally (Stevens and McLoughlin 1991) although elsewhere within its range it has a seasonal cycle (Capapé *et al.* 2006; Henderson *et al*. 2006). The observations of this study may indicate a transition between these modes of reproduction perhaps caused by stronger seasonal cues at higher latitudes. Alternatively, the observed phenomenon could be an issue of scale (e.g. samples from the Whitsundays may indicate seasonality at a fine-scale level, i.e. within individual schools). If the observations of the present study were representative of the wider population, it would suggest that females are not evenly mixing across wide-geographical areas (e.g. between Far North and Townsville). Evidence for both aseasonal and seasonal reproductive modes was found in the Townsville region, suggesting that the transition between these modes may occur within this region.

4.4.2 Stock structure of *S. lewini*

The large differences in length- and age-at maturity observed between tropical and temperate samples in the present study provide evidence of stock structuring at some level for *S. lewini*. These are further supported by the significant regional differences between growth models across ages 0-12, especially in the parameter L_∞. However, due to differences in fishing gear and sample characteristics among regions, other explanations cannot be conclusively ruled out. Most of the temperate samples came from a northern NSW mid-shelf longline fishery operating in depths 30-110 m, while the tropical samples were obtained from an inshore net fishery operating in depths of 0-25 m. Therefore the observed differences could be driven by depth, temperature, gear-selectivity, or other unknown variables. Off tropical northern Australia, Stevens and Lyle (1989) found that male *S. lewini* matured between 1,400-1,600 mm. In a sample of five *S. lewini* from NSW, Stevens (1984) found that a 2,190 mm male was immature and four males >2,350 mm were mature. These findings corroborate the evidence of the present study that there are large differences in maturity of *S. lewini* in Australian waters, and there may be some separation of stocks between tropical and temperate waters. If populations were evenly mixed along the coastline, we would expect to see some large immature males in the tropics and small mature males in temperate waters. Despite extensive sampling in the tropics around Townsville, no large (>1,800 mm) immature males were found. The clasper length data of Stevens and Lyle (1989) show a similar pattern with almost all males maturing in a very restricted size range.

4.4.3 Sampling issues and difficulties

The present study suffered from an obvious lack of consistency (size, sex, maturity) in samples collected among regions which limited the ability to make valid comparisons. The small-scale nature of the ECIFFF, where the majority of samples were sourced from, combined with its large geographical area meant that the numbers of samples required for this study could not be obtained. Furthermore, because sharks were not landed whole, and sampling was via onboard vessel observer programs, it was rarely possible to obtain anything other than lengths, sex and vertebrae for most individuals. In the absence of reproductive data and weights, limited analyses could be undertaken. Many of the idiosyncrasies of a shark's life history further complicated this study and were not anticipated in the original design. For example, the large size and comparatively low abundance of *S. lewini*, meant that even if targeted sampling had been possible for all regions, statistical comparisons may not have been possible. The large size also meant purchasing samples from remote areas was a costly or simply unfeasible solution. Another challenge for studies such as this is the complex population structure inherent in many sharks which included strong segregation by size and sex, and often large-scale migrations. Both *R. acutus* and *S. lewini* were predominantly caught as juveniles or adult males, with adult females largely absent from the catch. For *S. lewini*, which utilise tropical inshore habitats as nurseries and migrate offshore at larger sizes, some inter-regional comparisons would have been meaningless, as different components of the population were found in different regions (e.g. adults, juveniles).

4.4.4 Conclusions

We have demonstrated the potential for the use of life history characteristics for stock identification of *S. lewini* and *R. acutus*; however we have also demonstrated the potential challenges in the sample collections required for life history parameter estimation of elasmobranchs. These challenges are due to separation of life history stages, habitat preferences and variability in small-scale fisheries characteristics over large areas. These issues are likely to be exacerbated in elasmobranchs because they are often found in low abundance, and are frequently by-product or by-catch species rather than the target species in fisheries. Based on life history data we found some evidence to support stock structuring for both *R. acutus* and *S. lewini* off eastern Australia. This is strongest for *S. lewini* where large differences in length- and age-at-maturity and growth in ages 0-12 were found between the tropics and temperate waters. This pattern has been observed in previous studies of this species in Australian waters. The pattern of stock structuring in both species appears to be based on separation at large spatial scales however these results should be considered preliminary. Therefore further work is required to substantiate these findings before making any management recommendations.

Chapter 5: Management units for scalloped hammerhead, *Sphyrna lewini*, and milk shark, *Rhizoprionodon taylori*, on the Australian east coast: integrated analysis

David J. Welch, Jenny Ovenden, Jess A.T. Morgan, Raewyn Street, Colin Simpfendorfer, Andrew Tobin, Alastair Harry, Ron Schroeder, Jimmy White, William Macbeth, Pascal T Geraghty and Ashley J. Williams

5.1 Introduction

Determination of the stock structure of exploited fish populations is the basic element upon which appropriate fisheries management is determined. Despite this, research into fish spatial dynamics in a fisheries context has been lacking until recently and most fisheries in Australia assume a single stock for management due to a lack of relevant knowledge. Such an assumption is akin to management by guesswork and recent research has documented that some fish species exhibit highly localised stock structure (Welch *et al.* 2010), which can have significant implications for management of their fisheries under a single stock assumption. The identification of discrete stocks within a fishery therefore determines the appropriate spatial scale at which management should be applied, or in other words, identifies the management units for the particular fishery. Further, these spatial units also inform the basis for monitoring, assessment and research on the fisheries in question (Secor 2005).

Studies on the stock structure of exploited species have historically relied on the use of a single technique to discriminate different stocks. These techniques can vary and include the use of molecular approaches (Ovenden and Street 2003), otolith characteristics (e.g. chemical composition, shape; Newman *et al.* 2010), parasite incidence (Charters *et al.* 2010), life history characters (Begg 2005), or mark-recapture studies (Zischke *et al.* 2009). The choice of technique used in stock structure studies should depend on the research questions since each technique will address different aspects of the fish population, spatially and temporally (Begg and Waldman 1999). Because of this, the use of different techniques concurrently in a holistic approach to stock identification provides a very powerful and robust approach (Begg and Waldman 1999), The use of a holistic approach to stock structure research has proved very successful in recent studies (Abaunza et al 2008b; Buckworth *et al.* 2007; Welch *et al.* 2009; Welch *et al.* 2010). A holistic approach, when used on the same samples concurrently, provides greater power in the detection of different stocks than by single methods alone. Using this 'weight of evidence' approach greater certainty can be had where stock separation is detected (or not). One of the

limitations of single-technique studies is that in comparing fish from different locations the lack of evidence for stock differentiation cannot with any certainty conclude that they are not different stocks. The result may simply be a reflection of the discriminating power of the particular method for that species or may reflect similar environmental conditions at the locations from which fish were sampled. With the use of a holistic approach a consistent result among techniques of no difference among different regions, though not constituting 'proof', provides greater confidence that the fish from the regions in question are actually part of a single stock. The use of life history characteristics as part of a multi-technique approach to stock identification also provides the added benefit of informing about the productivity of individual stocks detected and therefore helps assess the appropriateness of different management strategies.

In this study we used a holistic approach to determine the stock structure of two species of elasmobranchs commonly taken in the inshore net fisheries of north-eastern Australia. The two species examined were the scalloped hammerhead, *Sphyrna lewini*, and the milk shark, *Rhizoprionodon acutus*. The null hypothesis being tested for each species was that along the north-eastern Australian coast they were comprised as single unit stocks. Since we set out to define the management units for scalloped hammerhead and milk shark, and because of the known characteristics of the inshore net fisheries operating on the north-eastern Australian coast, we defined a 'stock' as a semi-discrete group of animals that maintain spatial and temporal integrity by showing distinct movement patterns that are essentially not shared by individuals of other groups. The techniques used during this study were genetic analyses using mitochondrial DNA and microsatellites, vertebral micro-chemical analyses, and life history parameter analyses. These techniques were used on the same samples for each species to provide for a holistic and integrated interpretation of all results (Begg and Waldman 1999). In this chapter we integrate the results from the respective techniques using a framework that allows for accurate interpretation and identification of the appropriate spatial management units for scalloped and milk sharks in north-eastern Australia.

5.2 Methods

Detailed descriptions of the methods used for the respective techniques are provided in the relevant data chapters. Included are details of the data analyses and the results of these analyses for each of the individual techniques. Since the data types generated from each of the techniques used vary, the integration of all data into a single analysis is not possible. Further, the differences in the spatial and temporal scales at which each of the techniques are informative would also make interpretation of combined data challenging.

In this study, the basis for data analyses to determine stock structure for each technique relied on pair-wise comparisons of each of the locations sampled. To integrate the results of the respective analyses we used a framework developed by Welch *et al.* (2010) which relies on a matrix of the results of pair-

wise comparisons which readily facilitates interpretation of the overall results. The matrix gave an overview of the respective results clearly demonstrating where different stocks were detected and which methods discriminated these stocks. This also provided a basis for interpreting the mechanisms upon which stock structure was influenced. For example, in conjunction with sample data it identified whether stock isolation was influenced by juvenile movement, adult movement, or a combination of the two. The ability to readily assess the likely mechanisms influencing stock structure patterns is important in identifying appropriate management responses.

5.3 Results

5.3.1 Milk shark

Results from both microsatellite and mitochondrial DNA analyses indicated that *R. acutus* sufficiently mix among locations along the north-eastern Australian coastline to form a single genetic stock. They were found to be genetically separate to *R. acutus* from Indonesia perhaps providing some indication of the spatial scales that may limit mixing; however the north-east Australian coast and Indonesia are separated not only by distance but by an open expanse of deep water in the Arafura and Timor Seas which may also represent a barrier to dispersal. The most telling results, however, came from the vertebral microchemistry analyses which did indicate differences among most north-eastern Australian regions except for Mackay and Townsville. Although limited to a single pairwise comparison of regions (Far North and Townsville) and based on limited data, the life history data concurred with the microchemistry indicating a difference in the nature and timing of reproduction.

Table 5.1. Results matrix for *Rhizoprionodon acutus* of the pairwise regional comparisons among the four different techniques. Significant results for each pairwise comparison are indicated by bold upper case letters and non-significant results by lower case letters using the following lettering: M – microsatellites, D – mitochondrial DNA, V – vertebral microchemistry*, L – life history characteristics. Where the analysis was not carried out is indicated by '-'.

Region	Far North	Cairns	Townsville	Mackay	Fraser Burnett	Brisbane
Far North						
Cairns	- - V -					
Townsville	m d V L	- - V -				
Mackay	m d V -	- - V -	m d v -			
Fraser Burnett	m d - -	- - - -	m d - -	m d - -		
Brisbane	- - V -	- - V -	- - V -	- - V -	- - - -	

5.3.2 Scalloped hammerhead

Similar to *R. acutus* the results from both the microsatellite and mitochondrial DNA analyses indicated a single genetic stock of *S. lewini* on the north-eastern Australian coast. Comparisons with samples taken from Indonesia were also similar indicating the exchange of genetic material across a very large spatial scale. Vertebral microchemistry analyses indicated differences among all adjacent regions except for Townsville and Mackay, similar to what was found for *R. acutus*. From this result it was inferred that all non-adjacent regions were different, despite not being explicitly tested. Results of life history parameter analyses were consistent with the vertebral microchemistry results despite having to pool data into tropical (Far North, Cairns, Townsville, Mackay) and sub-tropical (Brisbane, Northern NSW) regions for a meaningful comparison among regions. The evidence for a tropical/sub-tropical separation in *S. lewini* populations was strengthened by analyses detecting differences in both size/age at maturity estimates and also in growth parameter estimates, particularly L_∞.

Table 5.2. Results matrix for *Sphyrna lewini* of the pairwise regional comparisons among the four different techniques. Significant results for each pairwise comparison are indicated by bold upper case letters and non-significant results by lower case letters using the following lettering: M – microsatellites, D – mitochondrial DNA, V – vertebral microchemistry*, L – life history characteristics. Where the analysis was not carried out is indicated by '-'. Results of life history analyses are provided in the top half of the matrix since a comparison necessitated the pooling of tropical (Far North, Cairns, Townsville, Mackay) and sub-tropical (Brisbane, Northern NSW) regions.

Region	Far North	Cairns	Townsville	Mackay	Brisbane	Northern NSW
Far North					L	
Cairns	- - V					
Townsville	m d V	- - V				
Mackay	m d V	- - V	m d v			
Brisbane	m d V	- - V	m d V	m d V		
Northern NSW	m d V	- - V	m d V	m d V	- - -	

*Significant differences among non-adjacent regions are inferred based on adjacent regional comparisons.

5.4 Discussion

This study has been able to determine the likely spatial scale of stock structuring in *R. acutus* and *S. lewini* that is appropriate for fisheries management by integrating the results of different techniques used for determining stock structure. Using this holistic approach, and drawing on previous research, the project has identified the likely spatial dynamic behaviours of each species on the north-eastern Australian coastline and the relevance of these movements to current fisheries practices.

In making these interpretations it has been important to consider the different techniques and the nature of the information each technique provides (Table 5.3). Genetic methods are less likely to identify differences than many other techniques simply because it may only require a few individual animals to mix between one area and another for a homogeneous stock to be determined. Although this would be determined as a single genetic stock, if the genetic exchange is low enough between regions occupied by that stock then it is still possible that fishing impacts may vary spatially for that stock, potentially leading to localised depletions. Vertebral chemistry in contrast is more likely to detect differences in the species spatial dynamics within a genetic stock by averaging the chemical signal over the course of the lifetime of animals from different regions. In this study the lifetimes being examined are up to eight years for *R. acutus* and up to 21 years for *S. lewini*. Similarly life history characteristics reflect isolation in time and space and can manifest over generational time scales or longer and like vertebral chemistry are influenced by environmental factors, along with other possible factors (Table 5.3).

Table 5.3. Intrinsic time scales of the different stock identification techniques used in this study (adapted from Buckworth *et al.* 2007 and Welch *et al.* 2009).

Method	Intrinsic time scale	Origin of information
Genetic spatial analyses	10,000-500,000 years	Rate of evolution of genetic markers
Genetic temporal analyses	5-50 years	Comparison of genetic markers over time
Vertebral microchemistry	5-10 years	Average ambient chemical environment over sharks life span
Life history parameters	5-10+ years	Sharks life span and longer. Mediated by the environment, genetic influences, generation times and density-dependent mechanisms

5.4.1 Milk shark

On the north-eastern coast of Australia, *R. acutus* is comprised of a single genetic stock however within this stock they are comprised of several sub-stocks based on our ‘stock’ definition: a semi-discrete group of animals that maintain spatial and temporal integrity by showing distinct movement patterns

that are essentially not shared by individuals of other groups. This was inferred directly from the vertebral microchemistry results primarily but was also supported by the life history parameter analyses. This is not that surprising given this species is relatively small with a maximum size of approximately one metre and therefore an assumed low vagility. One of the major difficulties during this study was in obtaining adequate numbers of samples from each of the study regions that were comparable in size, sex composition and stages of maturity. For example, only 24 adult females from a total sample of 300 animals were obtained and only from two of the regions: Far North and Townsville. Further, twice as many males as females were obtained from the overall sample. Given the low sample sizes in some regions it was not possible to conclude unequivocally that fewer female milk sharks overall are taken in the Queensland east coast inshore net fishery; an inference possible from these data. However it does appear that fewer adult females are taken. This may be evidence of some segregation of sexes and life history stages based on habitat preferences with adult females more likely to inhabit areas not fished often by the net fishery (e.g. deeper water). This possible higher rate of offshore movement by adult females may also result in greater longshore movement and be the mechanism of genetic exchange along the east coast, with sub-adults and males showing a higher tendency for site fidelity. Evidence from genetic studies on some other shark species (e.g. white sharks, Pardini et al 2001; black tip sharks in the Atlantic, Kenney et al 2003) have indicated that it is the males that show a lower site fidelity than the females, which is contrary to the results indicated by our study.

Previous research on movements of *R. acutus* are lacking in the literature. One movement study on a similar species, the Atlantic sharpnose shark (*R. terraenovae*), conducted in Florida, USA, used conventional tag-recapture and acoustic tracking methods (Carlson *et al.* 2008). They found that in general individual sharks had small home ranges however were capable of large-sale movements with one shark moving 169 km after only 35 days, while the longest movement reported was 399 km. This is consistent with results of the present study for *R. acutus* with our sampling regions separated by 240-550 km.

5.4.2 Scalloped hammerhead

Similar to the milk shark, *S. lewini* represents a single genetic stock along the north-eastern Australian coastline, however within this region is comprised of several sub-stocks as indicated by both vertebral microchemistry and life history parameter analyses. This was a surprising result given that they are known to be a large and often oceanic shark that is capable of very large movements. The north-eastern Australian animals were also found to be genetically similar with those from central Indonesia located approximately four thousand kilometres away. As discussed above and elsewhere in this report, the tendency for many shark species to disaggregate themselves according to habitats, sex, maturity stage, and a combination of these factors, make the collection of comparable samples challenging. Despite this, and notwithstanding size, for *S. lewini* the characteristics of the sampled catch among regions was generally comparable in that regardless of the region they consisted entirely of juveniles

(male and female) and adult males. In fact of a total of 512 *S. lewini* sampled only one adult female was caught. These results suggest that stock structuring evident is due to *S. lewini* juveniles and males showing site fidelity at least within the spatial extent of our study regions. Movement between these sub-stocks, and facilitating genetic exchange, appears to be driven primarily by adult females and possibly to a lesser extent by large adult males. These larger animals are likely to prefer deeper waters farther offshore than where current fishery practices occur, at least within Queensland. In northern NSW where the continental shelf is a lot closer to the coastline, shark fisheries tend to operate in deeper waters and the animals sampled from this region were the largest regardless of sex. It was also the region from where the only adult female was collected.

The observations in this study are consistent with previous studies on the movements of *S. lewini* in other parts of the world. Duncan *et al.* (2006) conducted a global genetic phylogeographic study of *S. lewini* and found strong genetic subdivision among ocean basins and weak or non-existent separation along continental margins. Other studies have found that juvenile *S. lewini* occupy distinct inshore nursery areas and tend to remain relatively localised for early periods of their development, mainly for predator avoidance (Duncan and Holland 2006).

From a fisheries perspective on the north-eastern Australian coastline, the characteristics of the catches from waters of the Great Barrier Reef Marine Park compared to that farther south tend to show larger animals on average being taken in the southern regions. This may be a reflection of the nearness of the continental shelf to where fishing takes place and possible depths fished. It may also reflect different habitat types. Regardless, the vertebral chemistry results indicate that, up until the time of capture,
S. lewini display limited movement on average within the spatial scales of our sampling regions. That is, the *S. lewini* individuals available to capture by the inshore fisheries operating along the north-eastern Australian coastline form discrete sub-stocks for fishery purposes. Perhaps one of the most surprising lines of evidence to support this is the significant differences detected in size/age at maturity and in growth between tropical and sub-tropical regions. Although the size structures of the samples taken from the respective pooled regions were different and could have affected the validity in the growth comparison, the maturity ogives generated included reasonable sample sizes with good overlap in sizes of animals among the regions. These tropical and temperate regions represent distinctly different habitat types typified by shallow, soft-bottomed turbid Great Barrier Reef lagoon waters in the tropics and deeper, clearer, sandy-bottomed waters adjacent to the continental shelf in the sub-tropics. These vastly different environmental conditions are very plausible explanations for the differences in life history characteristics observed. A more thorough analysis of this observation would require sample collections adequate enough to conduct comparisons of maturity among the different regions used here, particularly those adjacent to the distinct change in habitat discussed above (Capricorn vs. Fraser Burnett).

5.4.3 Management implications

For the purposes of fisheries management on the north-eastern Australian coastline *R. acutus* and *S. lewini* are comprised of multiple stocks separated by spatial scales of several hundreds of kilometres. As such the preferred management approach should be at a regional scale and based on the stock boundaries identified in this study. This also means that monitoring of these species within their respective fisheries, and their assessment, should also be conducted at a regional scale. It should also be noted in both species that the lack of difference detected between samples collected in the Townsville and Mackay regions may be due to there being a single stock, however it may also be due to similar chemical signatures in those regions reflected in the vertebrae and possible due to similar environmental conditions experienced by the animals from those regions. A move towards regional scale management in coastal fisheries may be viewed as increased complexity of fisheries management, however it may also be viewed as a way forward given the likely increase in the future of resource allocation issues among sectors and the increasing interest among local communities in co-management approaches. Either way, there is increasing evidence for localised stock structure among different coastal species in north-eastern Australia. Further, future fisheries monitoring should at the very least incorporate this into future programs.

Chapter 6: Diagnostic molecular assay for Australian whaler sharks, *Carcharhinus tilstoni*, *C. limbatus* and *C. amblyrhynchoides* using real-time PCR and high-resolution melt analysis

JAT Morgan, R Street, D Broderick, A Harry, C Simpfendorfer and JR Ovenden

6.1 Introduction

Whaler sharks are a widespread and diverse lineage of sharks belonging to the genus *Carcharhinus*. The genus contains over 30 commercially important species (Compagno *et al.* 2005) with 21 species occurring in Australian waters, predominantly in the tropical north (Last and Stevens 2009). The Australian black tip shark *C. tilstoni*, the common black tip shark *C. limbatus*, and the graceful shark *C. amblyrhynchoides* are three very closely related species within the genus. The two black tip species are morphologically indistinguishable externally (Compagno *et al.* 2005). In Queensland, 267 tonnes of *C. tilstoni* and *C. limbatus* was landed in 2008, which was a 20% increase over 2007 (Anon 2010a). In the Northern Territory, 371 tonnes was landed in 2009, which was a 15% increase over 2008 (Anon. 2010b).

Morphological species identification of *C. limbatus* and *C. tilstoni* is currently based on pre-caudal vertebral (PCV) counts. Counts for *C. limbatus* range from 94 to 101 and counts for *C. tilstoni* range from 84 to 91 (Last and Stevens 2009), although Ovenden *et al.* (2010) reported slightly lower counts (80 to 85) for *C. tilstoni*. The two black tip species can be distinguished genetically with diagnostic mutations found in the mitochondrial DNA COI barcode gene (Ward *et al.* 2008), control region and ND4 gene (Ovenden *et al.* 2010). Of the three mitochondrial genes studied, the ND4 gene has the greatest number of diagnostic mutations (nine) separating *C. limbatus* from *C. tilstoni* (Ovenden *et al.* 2010). In contrast, the control region could not unambiguously separate *C. limbatus* from *C. amblyrhynchoides* and the two black tip species differ by only two diagnostic mutations in the CO1 gene (Ovenden *et al.* 2010).

Reliable catch data is essential for effective fisheries management. Fishery observer programs operate in all Australian States but the difficulty of distinguishing *C. limbatus* from *C. tilstoni* means that they are currently counted together as unidentified black tip whalers. Once validated, DNA sequencing is a

reliable and sensitive technique for identifying species at any age. It can also be applied to tissue samples such as fin clips or fillets where identity is in doubt and full frames are not available. The importance of distinguishing the two black tip species for fisheries management was highlighted by Ovenden *et al.* (2010) who found that the relative proportion of *C. tilstoni* to *C. limbatus* off northern Australia was closer to 50:50 and not 300:1 as reported by Stevens and Wiley (1986). Accurate species identification is vital for determining stock structure and population subdivision, as well as ensuring sustainable harvest of each species.

Sequencing DNA requires experienced personnel and is relatively costly and time-consuming, which can be prohibitive for large numbers of samples. More rapid PCR (polymerase chain reaction) based diagnostic assays are increasingly being used for shark identification (Chapman *et al.* 2003; Pank *et al.* 2001). Real-time PCR offers more sensitive and specific detection and is faster than standard PCR (Heid *et al.* 1996). Real-time high-resolution-melt (HRM) PCR, developed by Wittwer *et al.* (2003), has advantages over other genotyping technologies. It is more cost effective than sequencing or TaqMan®-probe based real-time PCR, it is fast, powerful and accurate, and it is simple to run provided the laboratory has access to an HRM capable real-time PCR machine (Reed *et al.* 2007).

A real-time high-resolution-melt PCR species-diagnostic assay (RT-HRM-PCR) was developed based on the ND4 gene for rapid and inexpensive species identification of *C. tilstoni* and *C. limbatus*. *Carcharhinus amblyrhynchoides* was included in the RT-HRM-PCR assay because this species falls between the two black tip species in a phylogenetic tree (Ovenden *et al.* 2010).

The RT-HRM-PCR assay was tested against the 96 black tip sharks with known PCV counts and ND4 sequences (63 *C. tilstoni* and 33 *C. limbatus*) and five graceful sharks (*C. amblyrhynchoides*). The assay was then tested against a further 160 black tip sharks for which identity was later confirmed by ND4 sequencing. DNA from 9 non-target *Carcharhinus* species; *C. sorrah, C. obscurus, C. falciformis, C. plumbeus, C. altimus, C. amboinensis, C. dussemieri, C. fitzroyensis* and *C. brevipinna* was tested to assess the level of cross-species reactivity of the assay. These species are frequently caught with, and occasionally misidentified as, black tip sharks in the field (particularly as juveniles).

6.2 Methods

6.2.1 Tissue collection, DNA extraction amplification and sequencing

Shark tissue samples from target, and non-target species were collected via observer surveys, research projects and fishery monitoring programs on commercial net boats. Reference tissue samples were those reported in Ovenden *et al*. (2010). PCV counts were taken on additional *C. tilstoni* and *C. limbatus* samples by dissection. In addition to Australian samples, *Carcharhinus amblyrhynchoides*

and *C. limbatus* samples were collected from Indonesian markets. The provenance of these samples was determined to be within 300 km based on interviews with vendors.

Genomic DNA was extracted from 50 mg of tissue using a DNeasy Tissue Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The mitochondrial DNA ND4 region was amplified and sequenced using primers ND4 (CAC CTA TGA CTA CCA AAA GCT CAT GTA GAA GC) (Arévalo *et al.* 1994) and H12293-LEU (TTG CAC CAA GAG TTT TTG GTT CCT AAG ACC) (Inoue *et al.* 2001). PCR amplification and sequencing reactions were carried out following Ovenden *et al*. (2010). Sequence data was edited and aligned with Sequencher (v4.8 Gene Codes Corporation, Ann Arbor, MI, USA).

The RT-HRM-PCR assay was designed against a subset of samples consisting of 63 *C. tilstoni*, 33 *C. limbatus* and five *C. amblyrhynchoides.* Initial attempts to develop the assay using one pair of generic primers surrounding species-specific mutations were unsuccessful due to overlapping melt profiles. Instead, three pairs of species-specific primers were designed within the mitochondrial DNA ND4 region. The 3-prime nucleotide of each primer was anchored on a species-specific single nucleotide polymorphism (SNP). The primers were designed to be multiplexed in the same reaction tube (i.e. had similar annealing temperature). Design parameters were for small products under 300 bp, primers 18-27 bp long with melt temperature 57-63°C and GC content 20-80%.

Real-time PCR was conducted in a Rotor-Gene 6000 (Corbett Research, Mortlake, NSW, Australia) using a Type-it HRM PCR Kit (Qiagen, Valencia, CA, USA). Reactions (15 µL) included 7.5 µL of 2x HRM PCR Master Mix (containing HotStarTaq Plus DNA Polymerase, EvaGreen dye, optimised concentration of Q-solution, dNTPs, and MgCl2; Qiagen, Valencia, CA, USA), the six oligonucleotide primers each at a final concentration of 0.3 µM, and 10-50 ng of extracted DNA. Temperature cycling conditions were 5 min at 95°C, followed by 40 cycles of 10 sec at 95°C and 30 sec at 61°C, acquiring SYBR fluorescence on the green channel at the end of every extension step. The high-resolution melt was run in 0.1 degree increments between 73 and 82°C. At the completion of the run, the dynamic tube was turned on and the data was slope-corrected. After preliminary testing, the threshold line was set to 0.01 for all assays. Cycle threshold (Ct) scores, corresponding to the cycle number at which the amplification curve crosses the threshold line, were recorded for each sample. A negative (no template) control was included in each PCR run. Positive controls of each species representing the known haplotypes in the amplification region (four *C. limbatus*, four *C. tilstoni* and two *C. amblyrhynchoides*) were included to span the known diversity within each species.

6.3 Results

Tissue samples were collected predominantly from the east coast of Queensland but also included samples from New South Wales, the Northern Territory (including the Gulf of Carpentaria), Western Australia, and Indonesia (Table 6.1). PCV counts of the black tip sharks ranged from 83 to 89 for

C. tilstoni and 96 to 102 for *C. limbatus.* The mtDNA ND4 sequences separated the sharks into the same two clusters (Figure 6.1). Including the mtDNA ND4 haplotypes described by Ovenden *et al*. (2010) and Boomer *et al*. (2010), an alignment was made for each species.

Table 6.1. Origin of shark samples sequenced for mtDNA ND4 for use in development and testing of RT-HRM-PCR assay.

Species	Sample Origin					
	Indo[13]	WA	NT + GOC	QLD	NSW	Total
Carcharhinus tilstoni	-	-	1	133	-	134
C. limbatus	2	1	1	89	29	122
C. amblyrhynchoides	2	-	3	-	-	5
C. altimus	-	-	-	-	1	1
C. amboinensis	-	-	-	1	-	1
C. brevipinna	-	-	-	13	14	27
C. dussumieri	-	-	-	1	-	1
C. falciformis	-	-	-	-	1	1
C. fitzroyensis	-	-	-	1	-	1
C. obscurus	-	-	-	-	5	5
C. plumbeus	-	-	-	-	1	1
C. sorrah	-	-	-	2	-	2

Based on these alignments, species-specific primers were designed to target *C. tilstoni*, *C. limbatus* and *C. amblyrhynchoides* (Table 6.2). Ideally, species-specific primers would avoid amplifying regions containing SNP that were variable within species, but this was unavoidable here due to the limited number of diagnostic sites available to anchor primers and the nucleotide diversity of the ND4 region. The 17 *C. tilstoni* ND4 haplotypes (from 63 animals) amplified four RT-HRM-PCR products, the eight *C. limbatus* ND4 haplotypes (from 33 animals) amplified four RT-HRM-PCR products and the three *C. amblyrhynchoides* ND4 haplotypes (from five animals) amplified two RT-HRM-PCR products (Table 6.3). The quantitation curve, melt curve and normalised high resolution melt curve demonstrate good amplification for the different species and clear melt profiles despite the presence of several RT-HRM-PCR haplotypes per species (Figure 6.2a, b and c respectively). Peak melt temperatures for each species showed no overlap and differed by at least 1°C (Table 6.4).

[13] Indo = Indonesia; WA = Western Australia; NT + GOC = Northern Territory plus Gulf of Carpentaria; QLD = East Coast of Queensland; NSW = New South Wales.

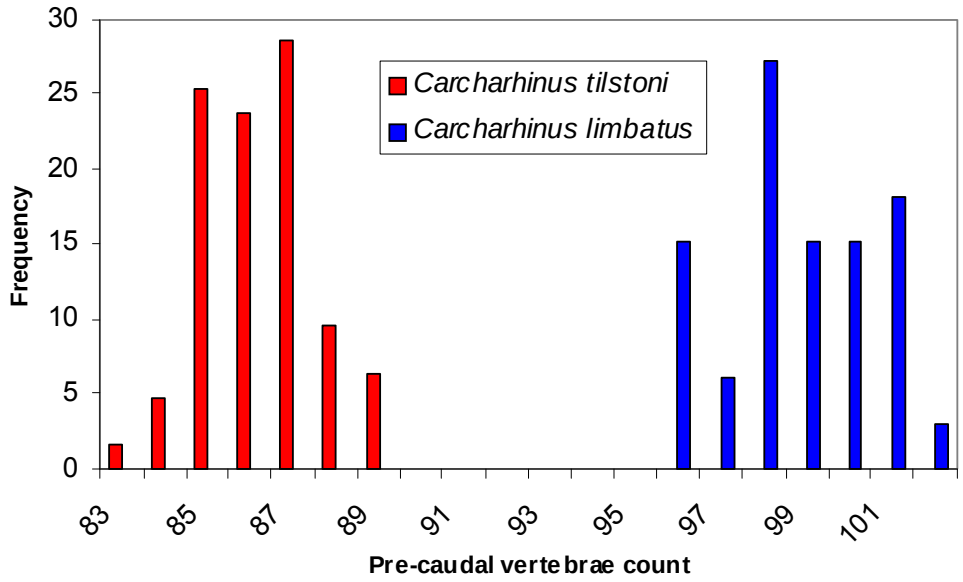


Figure 6.1. Frequency histogram (%) of pre-caudal vertebrae counts of sharks typed as *C. tilstoni* (red, 63 animals) and *C. limbatus* (blue, 33 animals) based on mtDNA ND4 sequences.

Table 6.2. Species-specific primer pairs for the real-time PCR assay. Underlined nucleotides are homologous to a diagnostic SNP in the target species. The position of the five prime nucleotide of the primer in Genbank accession GQ227268 is given.

PrimerName	Target species	Primer sequence (3' to 5')	Site
CT-F	*C. tilstoni*	CCCAYGGTTTAATYTCATCAGCCT	285
CT-R	*C. tilstoni*	GGAGAATGATTTGGATTCCTCGG	389
CL-F	*C. limbatus*	ACCAAAGAAATAGCCTACCCATTC	92
CL-R	*C. limbatus*	TTTGGATTCCTCGAGCTAGAAGG	380
CA-F	*C. amblyrhynchoides*	TGTCCTACTAAAACTAGGAGGTTAT	31
CA-R	*C. amblyrhynchoides*	AACAAATAGAGCTAGTTATTACGATG	161

Primer dimers resulted in low level amplification in the no-template negative control samples (mean cycle threshold score = 28.0), but these products did not produce a melt curve in the range of the assay so were readily identifiable. Weak non-specific amplification was also observed for most of the non-target species. The false-positive samples had high cycle threshold scores, on average ten cycles higher than the target species (indicating late DNA amplification) which flagged these samples as suspicious, despite their having weak melt curves similar to the target black tips (Table 6.5). Of the non-target species, C. *fitzroyensis* amplified a strong enough product and melt profile to be confused with

C. tilstoni and occasionally a strongly amplified *C. brevipinna* was confused for a weakly amplified *C. limbatus*. Figure 6.3 demonstrates a profile of a mixed catch of sharks with unknown black tips in pink and non-target species (*C. brevipinna* and *C. obscurus*) in black. The non-target species are distinguished by their late amplification on the quantitation curve (Figure 6.3a) and the black tips clearly fall into either the *C. tilstoni* (red) or *C. limbatus* (blue) melt profiles (Figure 6.3b and 6.3c).

Table 6.3. Intraspecific RT-HRM-PCR products for characterised *C. tilstoni*, *C. limbatus* and *C. amblyrhynchoides* ND4 haplotypes. RT-HRM-PCR amplification products were produced using the species-specific primers from Table 6.2.

Species ND4 haplotype	Genbank accession[214]	Variable position in RT-HRM-PCR amplified product			
C. tilstoni		5	14	104	
ND4_CT01, ND4_CT05, ND4_CT07, ND4_CT09, ND4_CT13, ND4_CT14, ND4_CT15	GQ227268, HM231105, HQ530167, HM231106, HQ530169, HQ530170, HQ530171	T	C	C	
ND4_CT02, ND4_CT04, ND4_CT06, ND4_CT08, ND4_CT_10, ND4_CT11, ND4_CT12, ND4_CT16	GQ227269, GQ227271, HQ530164, HQ530163, HQ530165, HQ530168, HQ530166, HQ530172	T	T	C	
ND4_CT03	GQ227270	C	T	C	
ND4_CT17	HQ530173	T	C	T	
C. limbatus		214	231	234	267
ND4_CL02, ND4_CL04, ND4_CL05, ND4_CL07, ND4_CL08	GQ227273, GQ227275, HM231104, HQ530161, HQ530162	G	T	C	C
ND4_CL01	GQ227272	G	T	T	C
ND4_CL03	GQ227274	G	C	C	T
ND4_CL06	HQ530160	A	T	C	C
C. amblyrhynchoides		56			
ND4_CA01, ND4_CA02	GQ227276, GQ227277	T			
ND4_CA03	GQ227278	C			

[14] Genbank accessions GQ 227268 to 78 from Ovenden *et al.* (2010) and HM 231104 to 06 from Boomer *et al.* (2010). ND4_CT05 and ND4_CT09 are referred to differently by Boomer *et al. (*2010). Genbank HQ to be released on 18 April 2011.

The RT-HRM-PCR assay was tested on 160 unknown black tip samples for validation. The assay identified 71 *C. tilstoni* and 89 *C. limbatus*. The identity of the samples was confirmed by sequencing the mtDNA ND4 region (amplified with primers ND4 and H12293-LEU) and all were correctly typed using the RT-HRM-PCR assay.

Table 6.4. PCR amplification product size and melt temperatures for each species.

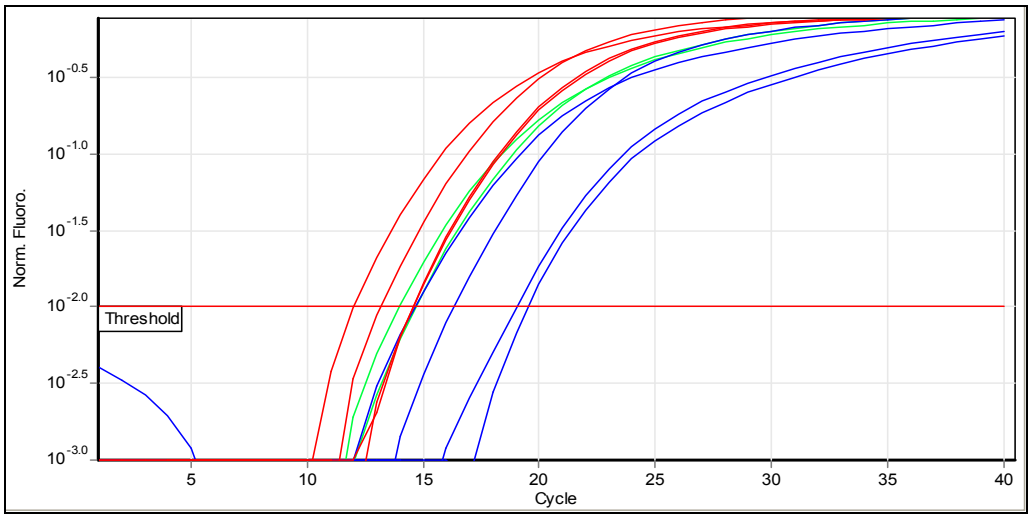
Species	Product size (bp)	Melt temp range and mean °C
C. amblyrhynchoides	131	74.5 – 75.0 – 75.5
C. tilstoni	105	76.5 – 77.0 – 77.5
C. limbatus	289	79.5 – 80.3– 81.0

Table 6.5. Average cycle threshold scores, standard deviation (SD) and sample size (N) for target and non-target species in RT-HRM-PCR assay and description of melt profiles compared to targets.

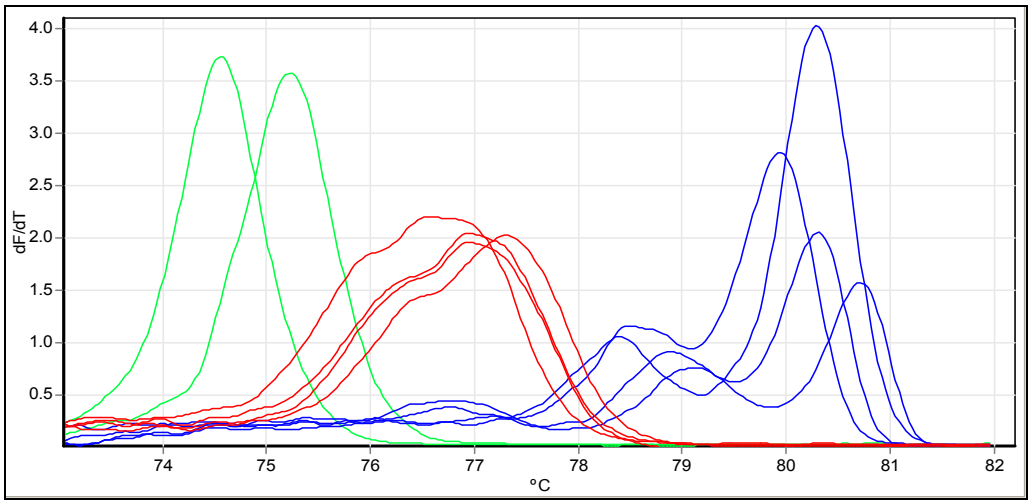
Species	N	Average Cycle Threshold score	SD	Melt Profile[15]
Target				
C. tilstoni	134	15.38	1.91	CT
C. limbatus	122	16.70	2.26	CL
C. amblyrhynchoides	5	16.78	4.00	CA
Non-target				
C. altimus	1	27.64	-	weak CL
C. amboinensis	1	31.46	-	weak CT
C. brevipinna	27	25.79	2.97	weak CL
C. dussumieri	1	28.18	-	weak CT
C. falciformis	1	27.95	-	weak CT
C. fitzroyensis	1	19.97	-	CT
C. obscurus	5	26.87	0.68	weak CT
C. plumbeus	1	27.19	-	no melt
C. sorrah	2	26.00	4.04	weak CT
No Template Control	6	28.00	3.59	no melt

[15] CL = *C. limbatus*; CT = *C. tilstoni;* CA = *C. amblyrhynchoides.*

(a) Quantitation Curve



(b) Melt Curve



(c) Normalised High Resolution Melt Curve

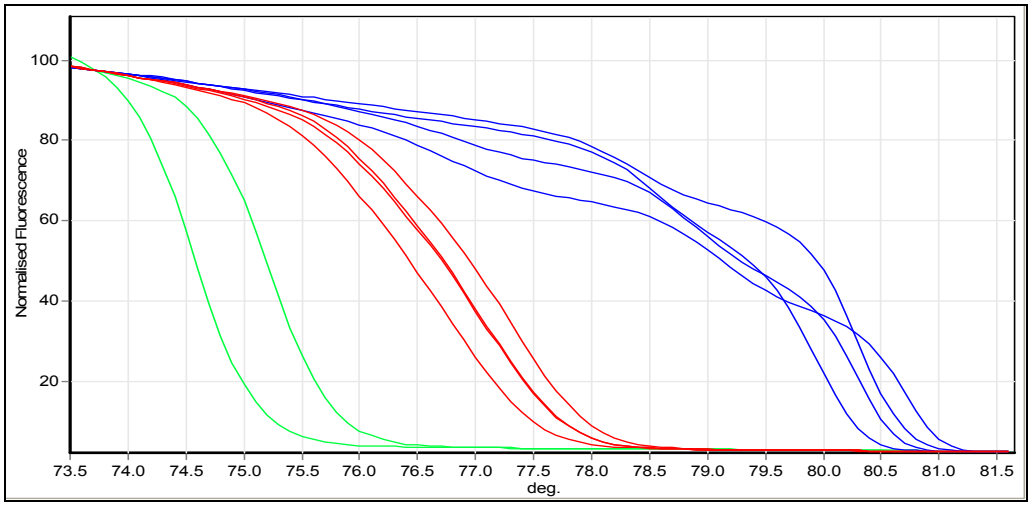
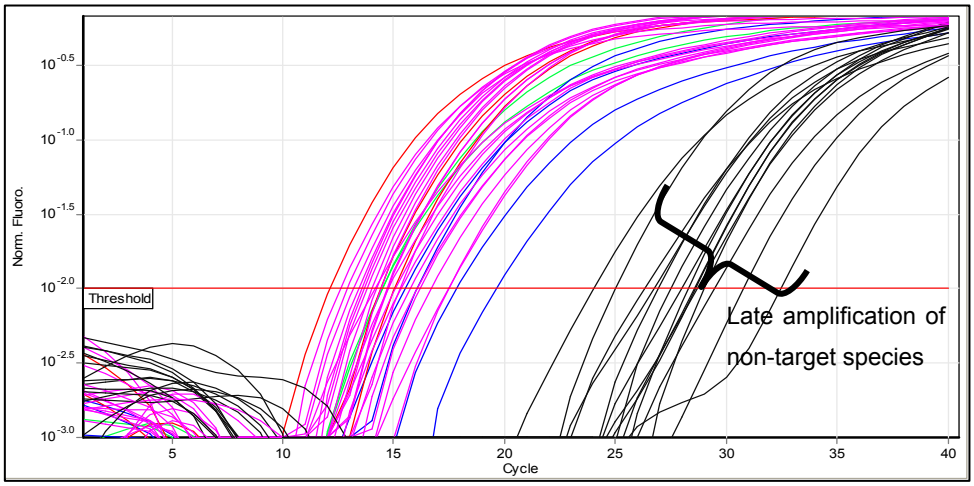
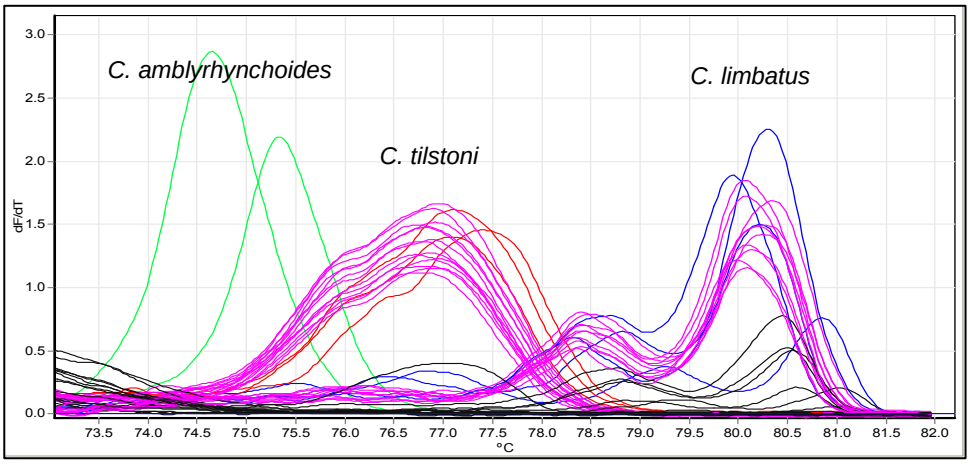


Figure 6.2. Real-time PCR analysis for each of the characterised RT-HRM-PCR haplotypes for the three whaler shark species, green *C. amblyrhynchoides,* red *C. tilstoni* and blue *C. limbatus* showing a. quantitation curve, b. melt curve, and c. normalised high resolution melt curve.

(a) Quantitation Curve



(b) Melt Curve



(c) Normalised High Resolution Melt Curve

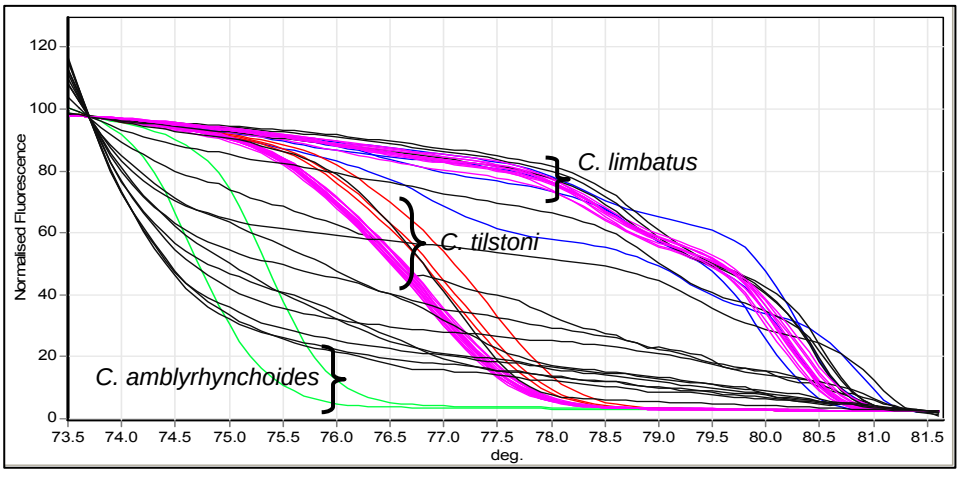


Figure 6.3. Example of a real-time PCR analysis of unknown samples (identified by observer/fisherman as black tips) in pink including non-target species *C. brevipinna* and *C. obscurus* in black compared to targets (green *C. amblyrhynchoides,* red *C. tilstoni* and blue *C. limbatus*) showing a. quantitation curve, b. melt curve, and c. normalised high resolution melt curve.

6.4 Discussion

A real-time high-resolution-melt PCR diagnostic assay has been developed to distinguish between the Australian black tip *C. tilstoni*, the common black tip *C. limbatus* and the graceful *C. amblyrhynchoides* shark species. The assay targets species diagnostic mutations in the mtDNA ND4 gene. Individuals identified as *C. tilsoni* or *C. limbatus* using the assay had pre-caudal vertebrae (PCV) counts in the expected range. PCV counts from 66 *C. tilstoni* ranged from 83 to 89, while counts for 33 *C. limbatus* ranged from 96 to 102 vertebrae. Both these ranges overlapped with those reported by Stevens and Wiley (1986) and Ovenden *et al.* (2010). This confirms the reliability of the mtDNA ND4 gene, and the RT-HRM-PCR assay reported here, for identification of the three shark species in this closely related clade (Ovenden *et al.* 2010).

The diagnostic RT-HRM-PCR assay multiplexes three pairs of species-specific primers anchored in the ND4 gene. Although a generic-primed assay would have been preferable all attempts to create one failed due to overlapping melt profiles. This overlap in melt profiles was possibly due to strong secondary structures in the flanking sequence surrounding the diagnostic mutations. Greater melt profile differentiation was achieved by amplifying different sized fragments (105, 131 and 289 bp) using species-specific primers. Within species variability could not be avoided but despite this variability the range of melt temperatures for the different species differed by at least 1°C. A large number of ND4 sequences from the three target species, collected from the Northern Territory fishery (Field *et al.* In preparation), have since been included in the assay alignment and the RT-HRM-PCR primers remain species-specific suggesting that the assay should reliably identify *C. tilstoni*, *C. limbatus* and *C. amblyrhynchoides* despite the occurrence of ND4 haplotypes not reported here. However, the accuracy of species identification using the RT-HRM-PCR assay should be confirmed by sequencing, particularly if applied to samples collected outside Australian waters. None of the black tip sharks screened from the 2007-2009 Queensland east coast shark observer programs typed as *C. amblyrhynchoides* indicating that this species is either readily distinguishable from *C. limbatus* and *C. tilstoni* in the field or, more likely, that it is not a significant component of the Queensland east coast shark fishery catch.

In our experience, juvenile *Carcharhinus* species are most likely to be misidentified as target black tip shark species. *Carcharhinus brevipinna* is also frequently confused with the black tip shark target species, especially when *C. brevipinna* is abundant. The RT-HRM-PCR assay cross-reacted with a false-positive signal for most of the tested non-target *Carcharhinus* species, including *C. brevipinna,* but amplification and melt profiles were weak compared to the target species flagging them as suspicious samples. Of the non-target species, C. *fitzroyensis* was the most likely to produce a false-positive *C. tilstoni* in the assay. Black tip shark surveys conducted in waters where C. *fitzroyensis* is abundant should be wary of using the RT-HRM-PCR assay as their only diagnostic tool.

The RT-HRM-PCR assay reported here provides a rapid, relatively inexpensive method for identifying three closely related and morphologically similar black tip shark species (*C. tilstoni, C. limbatus* and *C. amblyrhynchoides*) from a small tissue sample. The assay was 100% accurate; 160 black tip shark tissue samples were correctly identified by the assay based on subsequent full sequencing of the species-diagnostic mt-DNA ND4 region (Ovenden *et al.* 2010). The assay requires DNA extraction and PCR amplification on a specialised HRM thermocycler, but species identification is instantaneous. It is less expensive and more rapid than full sequencing as gel electrophoresis, PCR clean-up, sequencing reactions, chromatogram editing, sequence alignment and analyses are unnecessary. The RT-HRM-PCR assay will be a useful diagnostic tool if large numbers of samples need to be identified and when it is not practical to conduct pre-caudal vertebrae counts in the field. This will be useful in accurately determining the species-specific composition of black tip shark catches in northern Australian commercial net fisheries.

Chapter 7: Black tip sharks, *Carcharhinus tilstoni* and *C. limbatus* are hybridising along the east coast of Australia

JAT Morgan, R Street, A Harry, C Simpfendorfer, DJ Welch, J White, PT Geraghty, WG Macbeth and JR Ovenden

7.1 Introduction

The Australian black tip shark, *Carcharhinus tilstoni* (Whitley), and the common black tip shark, *C. limbatus* (Müller & Henle) are very closely related whalers with overlapping distributions in northern Australian waters (Last and Stevens 2009). Although the two species are morphologically cryptic externally (Compagno *et al.* 2005), they do differ in several respects. The only known fixed morphometric character separating the species is the number of pre-caudal vertebrae (PCV), which ranges from 80-91 for *C. tilstoni* and 94-110 for *C. limbatus* (Lavery and Shaklee 1991; Ovenden *et al.* 2010; Stevens and Wiley 1986). The common black tip *C. limbatus* grows larger (males to 2,300 mm, females to 2,700 mm) than the tropical Australian endemic, *C. tilstoni* (males to 1,450 mm, females to 1,800 mm) (Stevens and Wiley 1986). The Australian endemic also matures at a smaller size and typically gives birth to fewer pups of a smaller size (Stevens and Wiley 1986). The two species can also be distinguished genetically with diagnostic mutations found in the mitochondrial DNA CO1 barcode gene (Ward *et al.* 2008), control region and ND4 gene (Ovenden *et al.* 2010). Phylogenetic analysis of the two species groups them closely with a third species, the graceful shark *C. amblyrhynchoides* (Lavery 1992; Ovenden *et al.* 2010; Ward *et al.* 2008).

The relative abundance of the two black tip species along the Australian coastline has recently come into question. In 1986, Stevens and Wiley reported a ratio of 300:1 *C. tilstoni* to *C. limbatus* off northern Australia based on an allozyme genetic study. The relative importance of *C. limbatus* to the commercial harvest in northern Australia was still considered to be minor in 2009 (Last and Stevens 2009). However, in 2010 Ovenden *et al*. (2010) reported roughly equal frequencies of *C. tilstoni* and *C. limbatus* off northern Australia based on mitochondrial DNA sequencing. The magnitude of the change in the relative frequency of the two species was thought to be more likely a sampling and methodology artifact than a true indication of changing catch composition.

Black tips are a significant component of the Queensland east coast shark fishery. Little is currently known about the relative frequencies of the two species or whether stocks are genetically structured

along the Australian east coast. During the course of investigating stock structure and abundance, several animals were identified with clear mismatches between genetic and morphometric diagnostic characters. One hypothesis was that mismatches were due to hybridisation, although hybridisation in elasmobranchs has not been previously reported. A nuclear genetic marker was identified and applied to the mismatched animals. The significance of widespread hybridisation along the Australian east coast is discussed. The possible mechanisms that might be involved in maintaining the black tip sharks as separate species are considered including whether the hybridization event has a recent versus ancient origin.

7.2 Methods

7.2.1 Sample collection and DNA extraction

Carcharhinus tilstoni and *C. limbatus* were sampled along the eastern coast of Australia, predominantly from far north Queensland, Townsville, Mackay, Brisbane and northern New South Wales (see Figure 1.1). All samples were taken from the landed catch of the commercial net fishing sector. In Queensland, sampling was via commercial fishers directly or by observers on commercial net boats being conducted as part of MTSRF Project 4.8.4 (GBRWHA Inshore fishery). These collections were run in parallel with the Queensland Government fisheries observer program and staff from James Cook University (JCU) also assisted. In New South Wales, sharks were taken as part of the fisheries observer program. Biological information was linked to samples taken for genetics on standardised datasheets. Approximately 200 mg of muscle tissue was dissected and preserved in 1 ml of NaCl saturated solution with 20% dimethyl-sulphoxide. Total genomic DNA was extracted from 10-50 mg of muscle tissue using a Qiagen DNeasy Blood & Tissue Kit (Qiagen, Doncaster Victoria) into a final elution volume of 200 µL.

7.2.2 Mitochondrial DNA ND4

Species identifications were confirmed by a real-time high-resolution-melt PCR assay (Morgan *et al.* in prep-a). The assay targets species diagnostic mutations in the mtDNA NADH dehydrogenase subunit 4 (ND4) gene. Non-target species were removed.

7.2.3 Nuclear DNA marker CT06 development and screening

Diagnostic mutations in the flanking sequence of microsatellite locus CT06 (Ovenden *et al.* 2006) were identified by direct sequencing four homozygous individuals (based on genescan analysis of the locus) of each species. Once identified, the diagnostic polymorphisms were validated by sequencing twelve more individuals per species with known PCV counts and matching ND4 genotype. Amplification was between primers CT06F (CTGGCTGTCTCACTGAATGG) and CT06R (GGAAGGCCATATTCCAATCG) (Ovenden *et al.* 2006).

PCR amplification reactions were carried out in 10 µL volumes and contained 0.5 µM of each primer, combined with 5-50 ng of template DNA, 10x Taq buffer (containing 15 mM MgCl2), 0.8 mM dNTPs, and 0.6 units of Taq DNA polymerase (Qiagen, Valencia, CA, USA). Thermal cycling conditions consisted of an initial denaturation (94°C for 1 min 30 secs) followed by 35 cycles of 94°C for 25 seconds, 55°C for 25 seconds and 72°C for 1 minute, with a final extension step of 72°C for 10 minutes. Cycling was performed in a PTC200 DNA Engine (MJ Research, USA). PCR products were viewed on a 1.5% agarose TAE gel stained with GelRed (Biotium, USA). PCR products were desalted prior to sequencing using Exosap-it® (USB Corporation distributed by GE Healthcare Bio-Sciences, Rydalmere NSW, Australia).

Sequencing reactions used 20 ng of DNA and ABI Big Dye v1.1 technology designed for smaller products where sequence is needed close to the primer (Applied Biosystems, California) and were run on an Applied Biosystems 3130xl Genetic Analyser. With the exception of the preliminary screen of the six homozygous animals, only the forward primer (CT06F) was used as a sequencing primer due to frame shifts caused by heterozygous microsatellite alleles in the reverse primer reads. Sequence data was edited and aligned with Sequencher (v4.8 Gene Codes Corporation, Ann Arbor, MI, USA). Subsequent screening of samples followed the same PCR amplification and one-directional sequencing protocol.

7.3 Results

7.3.1 Sample mismatch between morphology and mt-DNA

A set of 42 animals were found with discordant morphology and ND4 mt-DNA species identifications. Pre-caudal vertebrae counts for 24 animals ranged from 98 to 105, typing them as *C. limbatus* but their mt-DNA matched *C. tilstoni*. Similarly three animals had PCV counts 83 to 88 indicating *C. tilstoni* morphology but possessing a *C. limbatus* mt-DNA ND4 haplotype. An additional three animals had intermediate PCV counts of 93 with one genotyping as *C. limbatus* and the other two as *C. tilstoni*. A further eleven animals from northern NSW were genotyped as *C. tilstoni* but had stretch total lengths well above (STL > 2,000 mm, but no PCV counts) the range recorded for this species. Finally, a neonate caught off Townsville was identified as a *C. limbatus* using ND4 mt-DNA, but its length (STL = 651 mm) was well below that observed for *C. limbatus*. Neither morphological plasticity nor genotyping errors could readily explain the mismatches, thus a hybrid hypothesis was formulated that required a nuclear genetic marker for verification.

7.3.2 Nuclear marker

In the search for species-diagnostic mutations several nuclear markers were screened including ribosomal DNA ITS1 and ITS2 (primers designed by authors and available on request), RAG2 (Cooper *et al.* 2009) and flanking sequence for microsatellite loci CLI-12 (Keeney and Heist 2003), CT-07 and CS-02 (Ovenden *et al.* 2006) (data not shown).

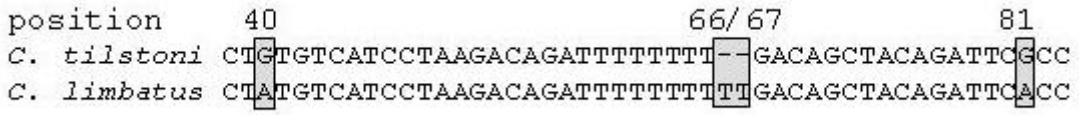


Figure 7.1. Species diagnostic sites in five prime flanking sequence of nuclear DNA marker CT06 (Ovenden *et al.* 2006)

Table 7.1. 'Pure' black tip sharks (*C. limbatus* and *C. tilstoni*) used for nDNA CT06 assay validation (shaded). Specimens had concordant nDNA CT06, mt-DNA ND4 and morphological characteristics (where available). (na = data not available).

Collection Location	N	Sex	STL range (mm)	PCV range	mt-DNA	nDNA
C. limbatus						
Far North	7	na	na	na	*C. limbatus*	*C. limbatus*
Cairns	5	5F	na	na	*C. limbatus*	*C. limbatus*
Townsville	7	5F, 2M	676-1380	93-101	*C. limbatus*	*C. limbatus*
Mackay	5	4F, 1M	741-1490	100	*C. limbatus*	*C. limbatus*
Brisbane	11	4F, 6M, 1na	665-749	96-110	*C. limbatus*	*C. limbatus*
Northern NSW	11	6F, 5M	740-2670	na	*C. limbatus*	*C. limbatus*
total	46	24F, 14M	665-2670	93-110		
C. tilstoni						
Far North	4	3F, 1M	678-1500	na	*C. tilstoni*	*C. tilstoni*
Townsville	8	3F, 5M	611-856	83-93	*C. tilstoni*	*C. tilstoni*
Mackay	10	4F, 6M	742-1660	84-88	*C. tilstoni*	*C. tilstoni*
Brisbane	1	F	785	87	*C. tilstoni*	*C. tilstoni*
total	23	11F, 12M	611-1660	83-93		

Species-diagnostic mutations were found in the five prime flanking sequence of nuclear microsatellite locus CT-06 (Figure 7.1). Further sequencing of twelve individuals per species validated three of the four diagnostic mutations (positions 40, 66 and 81), but identified an allelic difference within *C. limbatus*

at position 67 with either a T or a gap at this site. Heterozygote *C. limbatus* at position 67 were more difficult to score downstream (3 prime) due to the sequence frame-shift, however, the identity of position 81 was generally resolvable by eye as a G or an A as the immediate flanking sequence both upstream and downstream was C. Forty-six 'pure' *C. limbatus* (STL ranging from 665-2,670 mm and PCV range 93-110) and 23 'pure' *C. tilstoni* (STL ranging from 611-1,660 mm and PCV 83-93) were genotyped with the CT06 marker (Table 7.1). Pure animals were those with congruent ND4 and STL and/or PCV counts.

Screening of the 42 mismatched animals confirmed the hybrid hypothesis with forty animals returning a hybrid genotype (Table 7.2). The two mismatched animals with pure (i.e. non-hybrid) genotypes had PCV scores of 93, one genotyped as pure *C. tilstoni* and the other as pure *C. limbatus*. The third animal with PCV count of 93 typed as a backcrossed B1 hybrid.

Table 7.2. Hybrid black tip sharks (*C. limbatus* and *C. tilstoni*) (first generation F1 or backcrossed B1) and their collection location. Shading indicates samples with an observed mismatch between morphology and mt-DNA (na = data not available).

Collection Location	N	Sex	STL range (mm)	PCV range	mt-DNA	nDNA
F1 hybrids						
Far North	1	na	na	na	*C. limbatus*	Heterozygote
Townsville	4	4F	651-1000	98-101	*C. limbatus*	Heterozygote
Mackay	1	F	765	101	*C. tilstoni*	Heterozygote
Brisbane	1	M	700	107	*C. limbatus*	Heterozygote
Brisbane	4	3M, 1na	682-730	97-101	*C. tilstoni*	Heterozygote
Northern NSW	1	F	730	na	*C. limbatus*	Heterozygote
Northern NSW	5	3M, 2F	1580-2570	na	*C. tilstoni*	Heterozygote
total	17	7M, 8F	651-2570	97-107		
B1 Hybrids						
Far North	6	3M, 2F, 1na	840-1620	na	*C. limbatus*	*C. tilstoni*
Townsville	1	M	678	101	*C. limbatus*	*C. tilstoni*
Townsville	3	1M, 2F	1130-1415	93-99	*C. tilstoni*	*C. limbatus*
Mackay	3	3F	631-996	83-88	*C. limbatus*	*C. tilstoni*
Brisbane	17	9M, 6F, 2na	650-904	96-105	*C. tilstoni*	*C. limbatus*
Northern NSW	10	6M, 4F	1540-2560	na	*C. tilstoni*	*C. limbatus*
total	40	20M, 17F	631-2560	83-105		

Two classes of hybrids were identified; F1 and B1. F1 hybrid individuals were heterozygous for the CT06 diagnostic SNPs and had either *C. tilstoni* mt-DNA or *C. limbatus* mt-DNA. Both types of maternal mt-DNA were found among F1 hybrids indicating that hybridisation occurs in both directions (e.g. female *C. tilstoni* with male *C. limbatus* and visa versa). B1 hybrids were homozygous for the CT06 diagnostic SNPs and had conflicting nDNA and mt-DNA species identifications. For example, a hybrid with the ND4 mt-DNA of *C. limbatus*, but the CT06 genotype of *C. tilstoni* was classified as a B1. The presence of B1 suggests that F1 are capable of producing offspring, most likely as back-crossed matings with a parental species. Note, that matings between male and female F1 may lead to the B1 type and that matings between F1 and B1 may also be possible.

Screening of additional samples identified a further eight F1 and nine B1 hybrids. The hybrid animals were collected from every sampled population from the Far North of Queensland to northern NSW (Figure 7.2, Table 7.2). The hybrids did not display any sex bias and both F1 and B1 animals were caught with STL over 2500 mm suggesting they are not restricted in size.

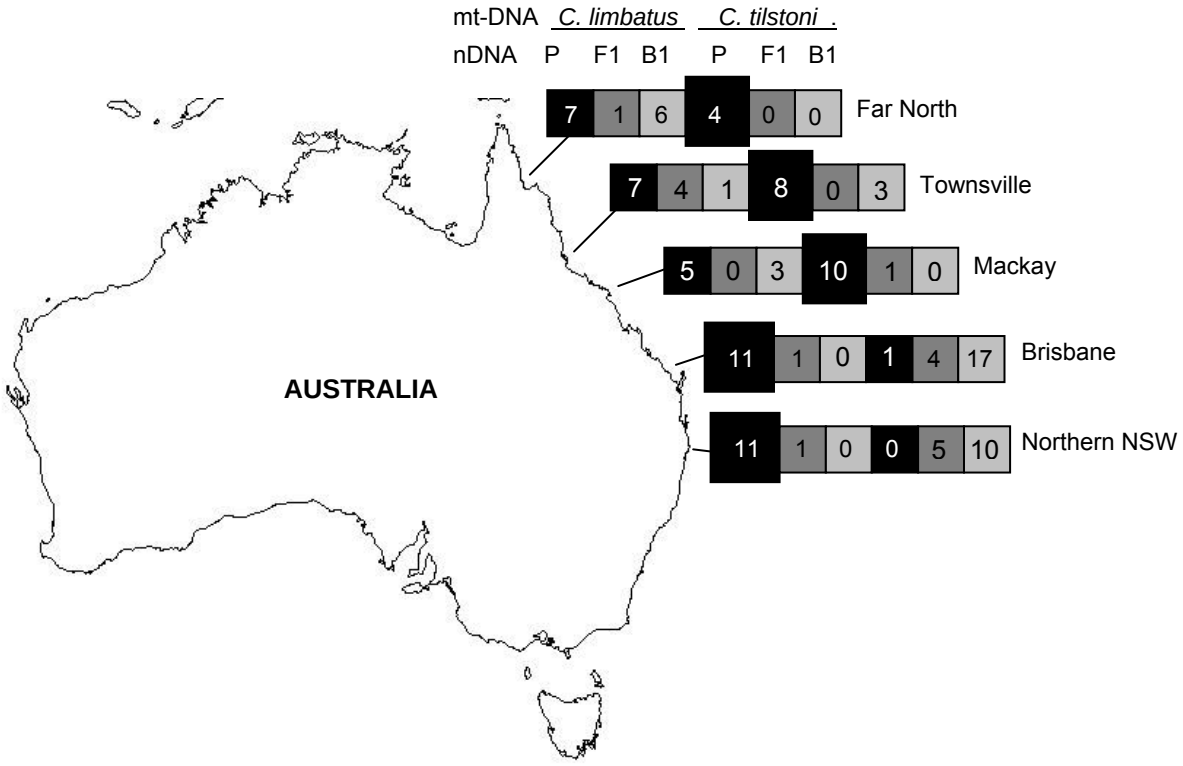


Figure 7.2. Map showing the distribution of 'pure' (P, Table 7.1) and different hybrid types (*C. limbatus* or *C. tilstoni*, F1 first generation hybrid or B1 backcross hybrid, Table 7.2) along the east coast of Australia. Enlarged boxes indicate most abundant 'pure' species based on ND4 mt-DNA haplotype. 'Pure' individuals had concordant nDNA, mt-DNA ND4 and morphological characteristics (where available).

7.4 Discussion

Nuclear and mitochondrial DNA screening of black tip sharks *C. limbatus* and *C. tilstoni* collected along the east coast of Australia has identified 57 hybrid animals. The hybrid zone spans over 2,000 km ranging from far north Queensland to northern NSW. Although the maximum size and size at maturity are quite different between the two species (Stevens and Wiley 1986), the genetic characteristics of the hybrids suggest they are freely interbreeding with no bias towards one type of cross-mating (ie. reciprocal crosses).

Only one pure *C. tilstoni* was caught off Brisbane or northern NSW, whereas F1 and B1 hybrids with *C. tilstoni* mt-DNA haplotypes were common at these locations. It is possible that the larger body size of hybrids may confer selective advantage in cooler temperate waters of the southern part of the coastline thus allowing introgression of *C. tilstoni* mitochondrial and nuclear genes into the *C. limbatus* population. *Carcharhinus tilstoni* is generally regarded as a more tropical-adapted species, while *C. limbatus* may be more adapted to temperate waters. Large body size is thought to confer higher fitness in cooler waters (Gunter 1950), but this is at odds with the occurrence of the larger bodied *C. limbatus* in tropical Indonesian waters to the exclusion of *C. tilstoni*.

The hybrid zone is likely to be more extensive than reported here. *Carcharhinus tilstoni* were identified in waters adjacent to Sydney on the mid-NSW coast (Boomer *et al.* 2010) based on mt-DNA ND4 sequence. However, these animals may be hybrids as some had large STL measures (Boomer, pers. comm.). If so, the hybrid zone extends a further 1,000 km south than reported here, spanning almost the entire east coast of Australia.

The F1 hybrids appear to be reproductively viable, as 40 of the 57 hybrids were likely the result of F1 hybrid matings, either with other F1s or pure animals. No evidence of reduced hybrid fitness was observed; although only a few characters linked to fitness (e.g. size and gender) were recorded. Further investigations into hybrid fitness are recommended as it may vary along the hybrid zone. The zone extends through a large number of environmental gradients, such as water temperature, turbidity and biotic communities. The presence of hybrids along several thousand kilometres of coastline suggests hybrid fitness is robust to environmental variation.

Nuclear DNA, being inherited from both parents, can readily identify F1 hybrids (heterozygous nuclear alleles) and second generation hybrids (homozygous nuclear alleles but in conflict with the mt-DNA identity, i.e. backcross or B1 here). A single nuclear marker however, cannot distinguish later generations of hybrids and, depending on the cross and the assortment of alleles, it may miss backcrossed B1 hybrids. Thus, estimates of hybrid abundance using these markers alone are likely to underestimate their true incidence. Increasing the number of nuclear diagnostic markers would assist in the identification of ‘pure’ (i.e. non-hybrid) individuals and would assist in understanding the degree of

genome mixing or introgression occurring between the two species. Unless hybrid fitness is significantly below parental fitness, all animals in the population are likely to have a hybrid ancestry (Allendorf *et al.* 2001).

Determining the mechanism and timescale leading to the first hybridization event is difficult because most animal hybridization models are based on two closely related allopatric species coming into contact (Barton and Hewitt 1985). The region of overlap becomes a hybrid zone and hybrid success is determined by fitness and the ability to compete with the parental species. In this instance, the two species live sympatrically and the hybrid zone spans the entire species range along the east coast of Australia. Understanding the original black tip speciation event may assist in interpreting the current hybridization event.

Given the global distribution of *C. limbatus,* it is likely that an ancestral population of this species was cut off (possibly by a land bridge across the Torres Strait separating northern Australia from eastern Australia). Evolution in isolation led to the formation of *C. tilstoni*. With the opening of the Torres Strait, the two species then expanded their ranges and became sympatric. Along the east coast *Carcharhinus tilstoni* appears to be better adapted to the Australian tropics where it generally outnumbers *C. limbatus.* The supposed absence of *C. tilstoni* from south-east Asia may be associated with this species having less affinity for crossing deep water. Deep water appears to reduce gene flow in a closely related whaler species, *C. sorrah* (Ovenden *et al.* 2009).

The extensive hybrid zone and the high abundance of hybrid animals suggest that the timing of the initial hybridization event was not recent. Yet the two species appear to be maintaining their species diagnostic morphometric and life history differences (i.e. there appears to be little phenotypic introgression). Of the 57 hybrids identified only 3 animals had intermediate PCV counts of 93. Interestingly, one was a pure *C. tilstoni*, one was a pure *C. limbatus* and the third was a backcrossed hybrid. The remaining animals had either less then 90 or more than 95 vertebrae. The mode of inheritance of the number of PCV is not known. The character appears to be continuous within species, but the lack of intermediate phenotypes among hybrids suggests that it is a discrete character. Other life history measures supporting the independence of the species were size at reproductive maturity, pup size, litter size and timing of birth (*C. tilstoni* being on average smaller and earlier than *C. limbatus* for all of these measures) (Alastair Harry, unpublished data).

Reproductive isolating mechanisms can be either pre-zygotic or post-zygotic. The most common isolating mechanism is non-overlapping distributions. Shark catches along the Australian east coast regularly contain both species so they do not appear to be niche partitioning on a local scale. The species have a slightly asynchronous pupping period with *C. tilstoni* pups appearing first, a month earlier than *C. limbatus* (Alastair Harry, unpublished data). Gestation time is shorter for *C. tilstoni* (ten

months) (Stevens and Wiley 1986) compared to *C. limbatus* (10-12 months) (Castro 1996; Compagno 1984) although not measured in Australian populations) so the species could differ in their time and place of mating. Neonates (generally less than four weeks old and showing unhealed umbilical scars (Castro 1996) of both species were caught together suggesting that the species have overlapping pupping grounds. Physical size differences can also isolate species preventing them from cross-mating successfully. Although adult black-tips differ considerably in size this is not preventing the species from reciprocal mating (mating success may be size limited). It seems unlikely that pre-zygotic isolating mechanisms could have broken down over the full extent of the hybrid zone.

The post-zygotic isolating mechanism of hybrid infertility is not occurring, since both F1 and second generation hybrids have been found. However, reduced hybrid fertility could be a post-zygotic isolating mechanism. Hybrid F2 breakdown or outbreeding depression occurs when unrelated genes interact via epistasis suppressing or masking expression (Whitlock *et al.* 1995). Haldane's rule states that epistasis is much more likely to affect genes on the sex chromosomes and the heterogametic sex (probably males in this case; Maddock and Schwartz 1996) will suffer the greatest impact being absent, rare or sterile (Haldane 1922). Although no evidence is currently available to suggest reduced hybrid fitness, this scenario best explains the apparent absence of intermediate phenotypes (resulting from genetic introgression) and the maintenance of species characters. Based on results to date, the incidence of hybrids ranges from 3% of the catch in the north, to 21% in the south. If the two black tip species have been hybridising since they came back into contact with each other, but if the hybrid offspring are less viable and are an evolutionary dead-end, then their mixed genes won't contribute to subsequent generations and the species effectively stay pure. The reproductive biology of wild black tip sharks may be even further complicated. Chapman *et al.* (2008) reported genetic evidence of asexual development in an aquarium reared Atlantic *C. limbatus*, although it may not occur in the wild. If Australian black tips are capable of parthenogensis then it could be a mechanism for less-fit hybrids to reproduce without having to mate.

The hybrid swarm theory (Seehausen 2004) predicts that when closely related species which don't normally interact come into contact, hybridisation and adaptive radiation will be promoted. This scenario may explain the high incidence of *C. tilstoni* hybrids in the temperate south of the range. Hybrid *C. tilstoni* may gain an ecological advantage over pure *C. tilstoni* enabling them to radiate into cooler waters. Ocean warming may also be facilitating this process. A similar scenario could be postulated for the northern edge of the *C. tilstoni* range. The discrepancy identified in the reported relative frequencies of the two species off northern Australia from 1986 (Stevens and Wiley 1986) to 2010 (Ovenden *et al.* 2010) could be a reflection of a hybrid swarm. Hybrid animals may be better adapted to expand into Asian seas although no reports of *C. tilstoni* have been documented from Asia.

Genetic introgression can cause the fusion of species, the genetic swamping of one species by another, or the creation of new species (Seehausen 2004). East coast black tip shark populations show considerable genetic structure, *C. tilstoni* dominates catches in the north and *C. limbatus* in the south. The habitat parameters vary enormously across the distribution from tropical to temperate environments. Close monitoring of shark stocks and hybrid frequency is needed along the entire species range to assess if environment-dependant hybrid fitness exists among populations.

The consequence of finding hybrids for black tip fishery management is serious. If hybrids have reduced fitness and are genetic dead ends then estimates of population productivity, ignoring the presence of hybrids, will be inflated and harvesting could result in overfishing. In contrast, if hybrids suffer no fitness cost or display increased fitness, then over time the two species will merge into a single species and biodiversity will be lost. If fishing techniques are unwittingly targeting one species over the other then fishing pressure may be facilitating greater hybridization in some populations. Also of concern is the status of the third species in this cryptic lineage the graceful shark, *C. amblyrhynchoides*. This species falls as an intermediate between *C. tilstoni* and *C. limbatus* on a phylogenetic tree. The graceful shark is not commonly caught on the east coast but may be interacting, and possibly hybridising, with the black tips off northern Australia. If *C. tilstoni* and *C. limbatus* were to be synonymised then the species status of *C. amblyrhynchoides* would be in doubt.

Several unknowns need answers to better manage black tip sharks along the Australian east coast. The full extent of the hybrid zone needs to be determined by testing samples from Western Australia, the Northern Territory, southern NSW and Indonesia. Hybrid fitness desperately needs to be investigated using a range of genetic and non-genetic characters. More diagnostic nuclear markers, and morphological characters, are needed to assess the level of introgression in the two genomes. Now that hybrids can be identified obtaining life history measurements to assess their capacity to reproduce, their susceptibility to parasites and general health compared to the parental species will assist in understanding whether the two species will be maintained or combined into one hybrid species in the future.

Chapter 8: Genetic stock structure exists along the east coast of Australia for black tip sharks, *Carcharhinus limbatus* and *C. tilstoni* based on mitochondrial DNA

Jess Morgan, Jenny Ovenden, Raewyn Street, Pascal T Geraghty and David J Welch

8.1 Introduction

The Australian black tip shark, *Carcharhinus tilstoni,* and the common black tip, *C. limbatus,* are externally indistinguishable species (Compagno *et al.* 2005) with overlapping distributions in northern Australian waters (Last and Stevens 2009). The two species differ in their number of pre-caudal vertebrae (Last and Stevens 2009) and they can also be distinguished genetically with diagnostic mutations found in the mitochondrial DNA CO1 barcode gene (Ward *et al.* 2008), control region and ND4 gene (Ovenden *et al.* 2010).

Until recently, the distribution of *C. tilstoni* was thought to be restricted to tropical northern waters, while *C. limbatus* had a more extended distribution reaching as far south as Sydney (Last and Stevens 2009). In 2010 Boomer *et al.* (2010) reported *C. tilstoni* off Sydney, based on mt-DNA identification. A nuclear genetic marker developed by Morgan *et al.* (Chapter 6) was used to confirm that the two black tip species are hybridising extensively along the east coast of Australia. They found hybrids to be as prevalent as 21% in catches from Brisbane and northern NSW.

Combined, the two black tip whalers constitute a significant proportion of Queensland's east coast shark fishery (Anon 2010a). Little is known about the relative frequencies of the two species or whether stocks are genetically structured along the Australian east coast. Hybrid fitness is unknown although the F1 generation is reproductively viable (Chapter 7). The extent of the hybrid zone is enormous, over 2,000 km, suggesting that genetic mixing between the two species may be high.

A basic understanding of the species composition and stock structure of black tip sharks along the east coast of Australia is desperately needed. Genetic subdivision in the two species of black tip has been investigated by sequencing the mt-DNA ND4 gene of samples collected by east coast shark fisheries. Although hybrids cannot be determined using this genetic marker it provides information about maternal movements and historical barriers to gene flow. The population-level information provided by comparing

the distribution of different mitochondrial DNA haplotypes will assist in better understanding gene flow and the role that migration may have played in spreading hybrid animals.

8.2 Methods

8.2.1 Sample collection, DNA extraction, amplification and sequencing

Carcharhinus tilstoni and *C. limbatus* were sampled along the east coast of Australia via fisheries observer programs from the landed catch of the shark fishing sector (see Figure 1.1). Where possible the gender and stretched total length (STL) was recorded. Total genomic DNA was extracted from ten to 50 mg of muscle tissue using a Qiagen DNeasy Blood & Tissue Kit (Qiagen, Doncaster Victoria). To determine relative species abundances at collection locations along the Queensland coast, sharks were identified as *C. tilstoni* or *C. limbatus* using the RT-HRM-PCR assay developed by Morgan *et al.* (in prep-a). Hybrids cannot be distinguished using mitochondrial DNA; they were typed as the maternal species. The majority of samples were sequenced for the ND4 region to determine haplotype frequencies per species among collection locations. To ensure both species were represented at each location, all samples from the less abundant species were included when the abundance bias was pronounced. An 873 bp region of the mtDNA ND4 gene was amplified and sequenced using primers ND4 (CAC CTA TGA CTA CCA AAA GCT CAT GTA GAA GC) (Arévalo *et al.* 1994) and H12293-LEU (TTG CAC CAA GAG TTT TTG GTT CCT AAG ACC) (Inoue *et al.* 2001) following Ovenden *et al.* (2010). Sequence data was edited and aligned with Sequencher (v4.8 Gene Codes Corporation, Ann Arbor, MI, USA). A haplotype parsimony network was estimated using TCS (Clement *et al.* 2000). Statistical testing for stock structure in the two black tip species was not conducted as the assumptions of Wright's Island model (Wright 1943) were most likely contravened in the presence of hybrids.

8.3 Results

In total 752 unidentified black tips were typed to mitochondrial species; 477 *C. tilstoni* and 275 *C. limbatus* (Table 8.1). Hybrids were typed to their maternal species. Relative species abundance varied among seven regions along the east coast from far north Queensland to northern NSW. *Carcharhinus tilstoni* was most common in the north and *C. limbatus* was most common in the south. However, abundance was not directly related to latitude; *C. tilstoni* was relatively the most common at the central coast site of Mackay. Comparison of STL among locations showed that the Queensland fishery targets considerably smaller animals (mean black tip STL= 904 mm) than the NSW fishery (mean black tip STL = 1,920 mm). There was no marked gender bias in the sampling.

Table 8.1. Mitochondrial species identity using the RT-HRM-PCR assay from unidentified black tip shark tissue samples from seven locations on the eastern Australian coastline (see Figure 1.1). Gender and size (STL, stretched total length) is given, where known.

Region	Far North	Cairns	Townsville	Mackay	Capricorn	Brisbane	Northern NSW
C. tilstoni							
Male	32	15	103	50	4	12	9
Male STL mm	700-1490	615-1453	403*-1560	721-1485	675-870	650-779	1580-2520
Female	18	14	127	52	8	7	6
Female STL mm	678-1660	670-1110	390*-1930	632-1660	651-725	705-904	1540-2570
unknown sex/STL	13	1	3	0	0	3	0
Total *C. tilstoni*	63	30	233	102	12	22	15
C. limbatus							
Male	3	6	30	1	8	38	43
Male STL mm	840-985	742-1030	620-1380	741	715-985	665-835	740-2630
Female	2	14	47	7	4	31	21
Female STL mm	1620#	706-875	651-1400	631-1490	700-885	681-841	730-2670
unknown sex/STL	9	0	4	0	0	7	0
Total *C. limbatus*	14	20	81	8	12	76	64
Ratio per region *C. tilstoni* : *C. limbatus*	4.5 : 1	1.5 : 1	2.88 : 1	12.75 : 1	1 : 1	1 : 3.45	1 : 4.27

* Four (4) embryos screened with STL<600.

\# Only one STL available.

DNA sequences (ND4) were obtained for 509 subsampled animals, representing 283 *C. tilstoni* and 226 *C. limbatus* mitochondria (hybrids type to maternal species) (Table 8.2). From the 509 animals, 16 *C. tilstoni* and six *C. limbatus* mitochondrial haplotypes were identified (Tables 8.3a and 8.3b). Three of the *C. tilstoni* haplotypes (ND4_CT01, ND4_CT02 and ND4_CT04) and two *C. limbatus* haplotypes (ND4_CL02 and ND4_CL04) had been described by Ovenden *et al.* (2010). Three haplotypes described by Boomer *et al.* (2010) were found. They were allocated names ND4_CT05, ND4_CT09 and ND4_CL05 here, but were referred to differently by Boomer *et al.* (2010). A further eleven *C. tilstoni* and three *C. limbatus* mitochondrial haplotypes were new to this study (Tables 8.3a and b). Two common haplotypes dominated all populations for both species; ND4_CT05 and ND4_CT04 for *C. tilstoni* and ND4_CL05 and ND4_CL02 for *C. limbatus*.

Table 8.2. Breakdown of *Carcharhinus tilstoni and C. limbatus* subsamples for which mt-DNA ND4 sequences were obtained.

C. tilstoni	Males		Females		Unknown	ND4
Location	N	STL range (mm)	N	Mean STL (mm)	gender / STL	Total N
Far North	32	700-1490	18	678-1660	13	63
Cairns	7	615-1075	10	680-1110	1	18
Townsville	46	613-1560	50	593-1930	0	96
Mackay	25	721-1480	33	632-1660	0	58
Capricorn	3	675-870	8	651-725	0	11
Brisbane	12	650-779	7	705-904	3	22
Northern NSW	9	1580-2520	6	1540-2570	0	15
Total	134		119		30	283
C. limbatus		Males		Females	Unknown	ND4
Region	N	Mean STL (mm)	N	Mean STL (mm)	gender / STL	Total N
Far North	3	840-985	2	1620, na	9	14
Cairns	6	742-1030	10	706-875	0	16
Townsville	26	620-1380	41	651-1400	3	70
Mackay	1	741	7	631-1490	0	8
Capricorn	8	715-985	4	700-885	0	12
Brisbane	38	665-835	31	681-841	7	76
Northern NSW	19	1680-2630	11	830 -2670	0	30
Total	101		106		19	226

The ND4 haplotype network (Figure 8.1) clearly separated the two species. For both species, all of the unique haplotypes (found in only one locality) were from the north with only common haplotypes found south of Townsville. Haplotypes did not cluster by geographic origin within the network. The two most common haplotypes for each species (ND4_CT05 and ND4_CT04 for *C. tilstoni* and ND4_CL05 and ND4_CL02 for *C. limbatus*) fell at internal nodes within the network although ND4_CT04 may be derived from ND4_CT02.

A map displaying the distribution of common haplotypes along the east coast (Figure 8.2) shows a transition from dominant haplotype CT05 in the north to CT04 in the south for *C. tilstoni*, and from dominant haplotype CL02 in the north to CL05 in the south for *C. limbatus*. The transition occurs between Capricorn and Brisbane for *C. tilstoni* and between Mackay and Capricorn for *C. limbatus*. The Capricorn region marks the change from *C. tilstoni* dominated populations in the north to *C. limbatus* dominated populations in the south (Figure 8.2). Greatest haplotype diversity for both species was seen from Townsville north. Only three *C. tilstoni* and three *C. limbatus* haplotypes were detected in northern NSW.

Table 8.3a. Relative frequency (%) of mitochondrial DNA ND4 haplotypes of *Carcharhinus tilstoni* from each sampling region. Highlighted columns correspond to species diagnostic mutations separating *C. tilstoni* from *C. limbatus*. Dots indicate the same nucleotide as the first reference line. Regions are 1 = Far North Queensland, 2 = Cairns, 3 = Townsville, 4 = Mackay, 5 = Capricorn, 7 = Brisbane, 8 = Northern NSW.

C. tilstoni	Genbank Accession[16]				1	1	2	2	2	2	2	2	2	3	3	3	4	5	5	5	5	5	6	7	7	% per Region							Total number
			7	8	1	3	1	1	3	5	8	9	9	0	6	8	1	2	3	3	5	5	7	7	9								
		1	6	5	5	9	1	7	2	9	3	3	8	8	7	8	6	3	0	1	3	6	1	5	0	1	2	3	4	5	7	8	
Ref ND4_CT01	GQ227268	C	C	T	T	C	C	C	C	T	T	T	C	T	C	C	G	C	G	T	T	C	T	T	C	11	-	-	-	-	-	-	7
(ND4_CT05)	HM231105	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	27	61	72	91	82	9	33	166
ND4_CT04	GQ227271	.	T	.	.	.	.	.	.	.	.	.	T	.	.	.	.	T	.	.	.	.	.	.	.	37	6	8	3	9	91	60	64
ND4_CT02	GQ227269	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	T	.	.	.	.	.	.	.	16	-	2	-	-	-	-	12
ND4_CT10	HQ530165	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	T	.	.	C	.	.	.	.	-	11	5	-	-	-	-	7
ND4_CT06	HQ530164	.	.	.	.	T	.	.	.	.	.	.	T	.	.	.	.	T	.	.	.	.	.	.	.	3	-	2	3	-	-	-	6
ND4_CT15	HQ530171	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	-	-	4	2	-	-	-	5
(ND4_CT09)	HM231106	.	.	.	.	.	.	T	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2	6	1	-	-	-	-	3
ND4_CT11	HQ530168	.	T	.	.	.	.	.	.	.	.	.	T	.	.	.	.	T	.	C	.	.	.	.	.	2	-	2	-	-	-	-	3
ND4_CT17	HQ530173	.	.	.	.	.	.	T	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	-	-	1	-	9	-	7	3
ND4_CT07	HQ530167	.	.	.	.	.	.	T	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	11	-	-	-	-	-	2
ND4_CT08	HQ530163	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	A	T	.	.	.	.	.	.	.	2	-	-	-	-	-	-	1
ND4_CT12	HQ530166	.	T	.	.	.	.	.	.	.	.	.	T	.	.	.	.	T	A	.	.	.	.	.	.	2	-	-	-	-	-	-	1
ND4_CT13	HQ530169	T	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	6	-	-	-	-	-	1
ND4_CT14	HQ530170	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	-	-	1	-	-	-	-	1
ND4_CT16	HQ530172	.	.	.	.	T	.	.	.	.	.	.	T	.	.	.	.	T	.	.	.	T	.	.	.	-	-	1	-	-	-	-	1
																				Total number					63	18	96	58	11	22	15	283

[16] Genbank accessions GQ 227268, 69 and 71 from Ovenden *et al.* (2010) and HM 231105, 06 from Boomer *et al.* (2010). ND4_CT05 and ND4_CT09 are referred to differently by Boomer *et al.* (2010). Genbank HQ to be released on 18 April 2011.

Table 8.3b. Relative frequency (%) of mitochondrial DNA ND4 haplotypes of *Carcharhinus limbatus* from each sampling region. Highlighted columns correspond to species diagnostic mutations separating *C. tilstoni* from *C. limbatus*. Dots indicate the same nucleotide as the first reference line. Regions are 1 = Far North Queensland, 2 = Cairns, 3 = Townsville, 4 = Mackay, 5 = Capricorn, 7 = Brisbane, 8 = Northern NSW.

C. limbatus	Genbank Accession[17]		1	2	2	2	3	3	3	4	5	5	6	7	8	8	% per Region							Total number
		8	1	1	5	9	0	0	6	6	2	3	7	7	3	4								
		5	5	1	9	3	5	8	7	3	1	0	1	5	1	6	1	2	3	4	5	7	8	
ref ND4_CL01	GQ227272	C	C	T	C	C	G	C	T	A	G	G	C	C	G	G	-	-	-	-	-	-	-	0
(ND4_CL05)	HM231104	.	.	.	.	.	.	.	.	G	.	A	.	.	.	.	21	31	10	25	67	97	87	125
ND4_CL02	GQ227273	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	57	63	79	75	33	3	7	87
ND4_CL04	GQ227275	.	.	.	.	.	.	.	.	G	.	.	.	.	A	.	7	6	4	0	-	-	7	7
ND4_CL08	HQ530162	.	.	.	.	.	.	.	.	G	A	A	.	.	.	.	-	-	6	-	-	-	-	4
ND4_CL06	HQ530160	.	.	.	.	.	A	.	.	G	.	.	.	.	.	.	7	-	1	-	-	-	-	2
ND4_CL07	HQ530161	.	.	.	.	.	.	.	.	G	.	.	.	.	.	A	7	-	-	-	-	-	-	1
												Total number					14	16	70	8	12	76	30	226

[17] Genbank accessions GQ 227272, 73 and 75 from Ovenden *et al.* (2010) and HM 231104 from Boomer *et al*. (2010). ND4_CL05 is referred to differently by Boomer *et al.* (2010). Genbank HQ to be released on 18 April 2011. Note ND4_CL01 not found in this study.

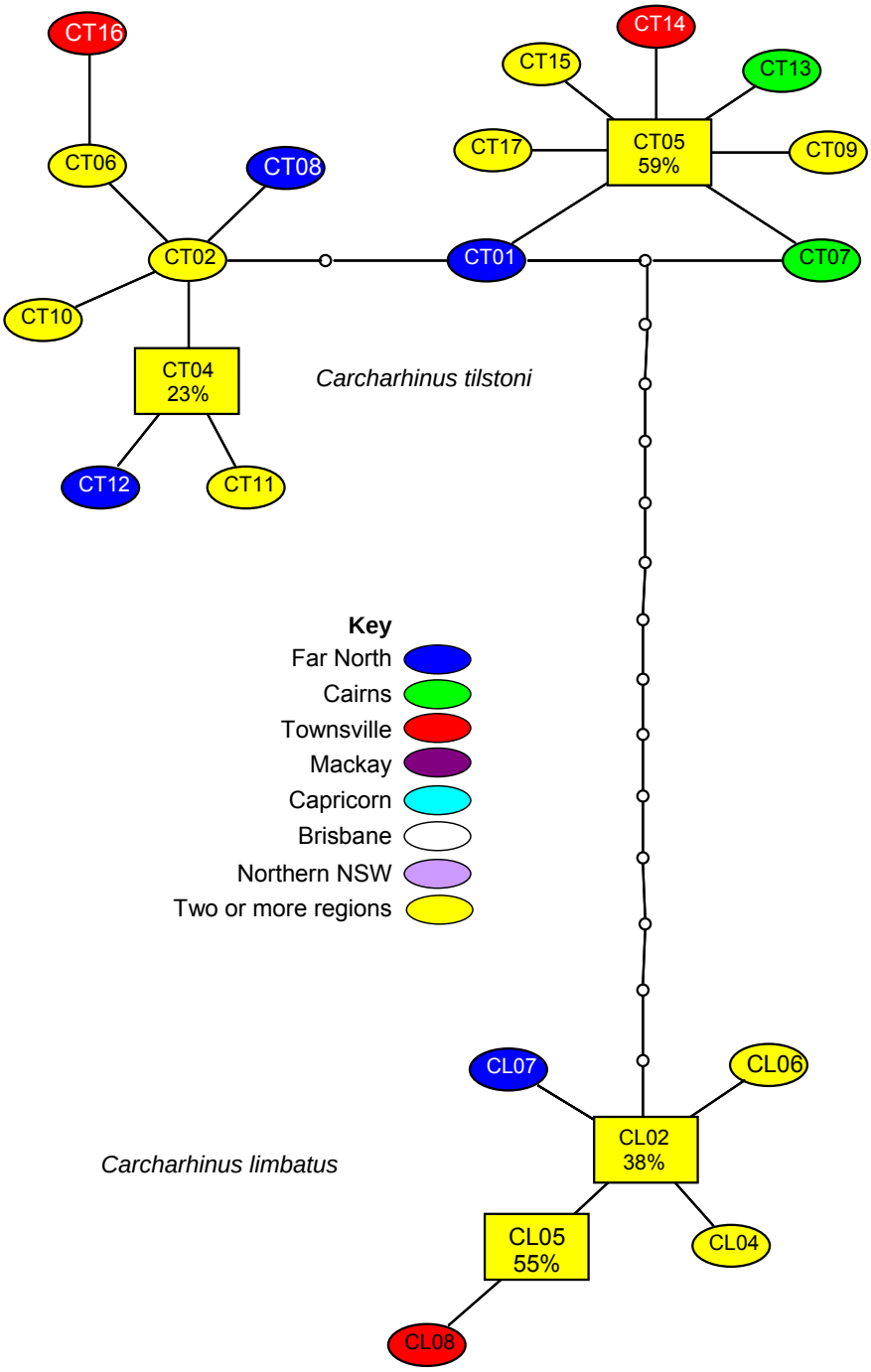


Figure 8.1. *Carcharhinus tilstoni and C. limbatus* mtDNA ND4 network showing the frequency of two most common haplotypes for each species in boxes and geographic origin in colours following key.

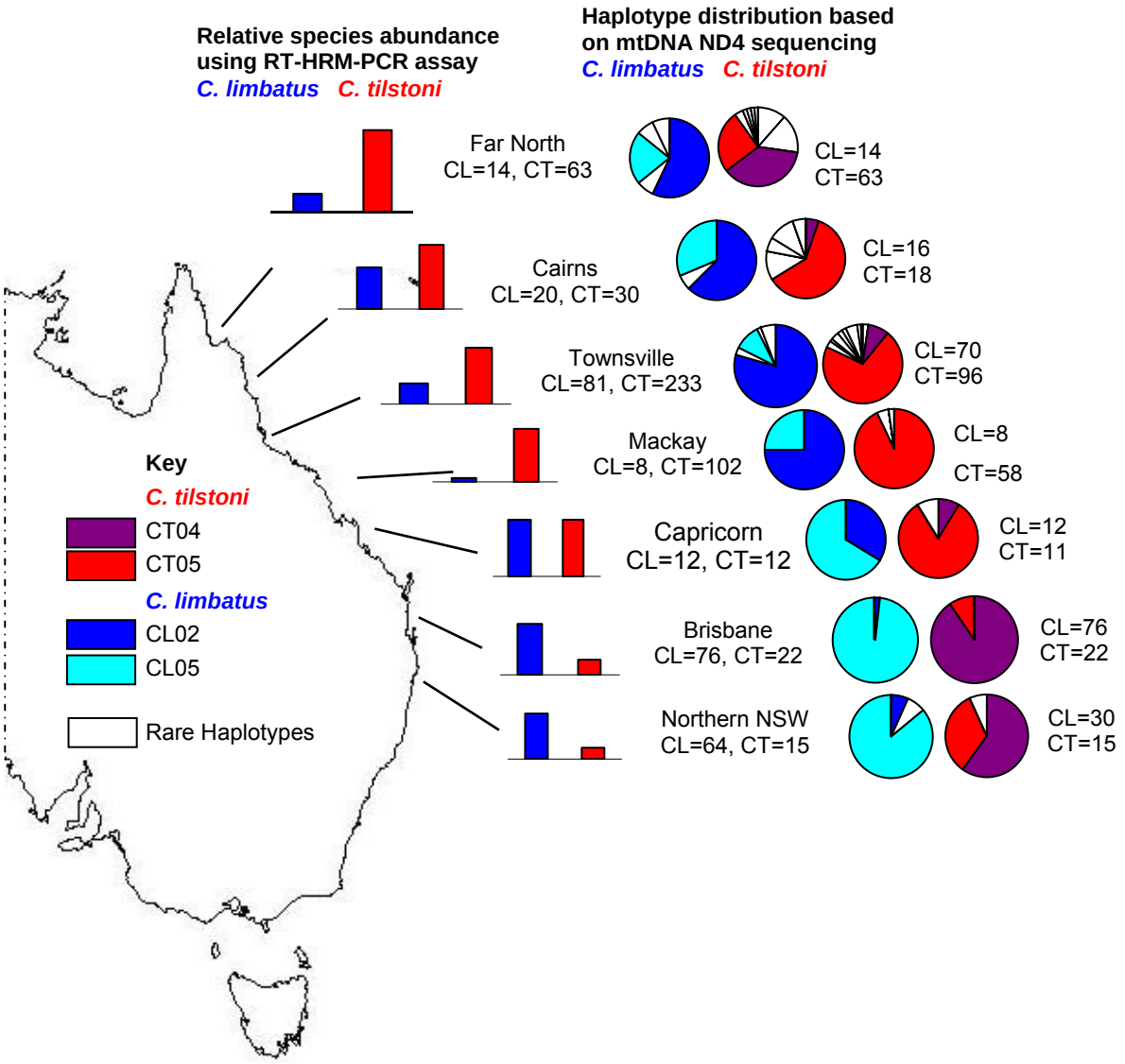


Figure 8.2. Relative mt-DNA species abundance and distribution of ND4 haplotypes along the east coast for *C. tilstoni* (red) and *C. limbatus* (blue). The dominant two alleles for each species are coloured following the key.

8.4 Discussion

The relative abundance of black tip whalers along the east coast changes from *C. tilstoni* dominated populations north of the Capricorn region to *C. limbatus* dominated populations south of the Capricorn region. The Tropic of Capricorn lies at 23.5° latitude and corresponds to the southern edge of the Great Barrier Reef. Ocean temperature and depth may be important parameters affecting the distribution of the two black tip shark species. A negative relationship exists between body size and ocean temperature; it is better to grow bigger in cold water (Gunter 1950). The maximum length and length at maturity of *C. limbatus* changes, depending on the geographic location of the population. For example, in the Gulf of Mexico *C. limbatus* females mature at 1,360 mm and reach a maximum length of 1,830 mm (Carlson, Sulikowski *et al.* 2006), while off eastern Australia maturity is reached at approximately 2,050 mm and maximum length is 2,670 mm (Macbeth *et al.* 2009). In contrast female *C. tilstoni* from the Arafura Sea mature at 1,150 mm and reach a maximum length of 1,600 mm (Stevens and Wiley 1986). It appears that *C. tilstoni* has adapted to living in a tropical environment by evolving a smaller body size.

Along the east coast of Australia *C. tilstoni* displays much greater diversity than *C. limbatus* (16 haplotypes compared to 6). A worldwide phylogeographic study of *C. limbatus* based on mitochondrial DNA control region sequences (Keeney and Heist 2006) found 37 haplotypes and major population subdivision across the Atlantic Ocean, but not the Pacific Ocean. Two *C. tilstoni* haplotypes included in their phylogeny were paraphyletic, falling within the eastern Atlantic and Indo-Pacific *C. limbatus* clade (Keeney and Heist 2006). Being globally distributed the overall genetic diversity of *C. limbatus* is likely to be large compared to *C. tilstoni*. In northern Australia Ovenden *et al.* (2010) found roughly equal numbers of *C. tilstoni* and *C. limbatus* haplotypes for both the mitochondrial control region and ND4. The reduced diversity of *C. limbatus* compared to *C. tilstoni* along the Queensland east coast supports the hypothesis that the origin of *C. tilstoni* may have been the north Queensland coast. Movement of animals south appears to be limited for both species with decreased haplotype diversity for both *C. tilstoni* and *C. limbatus* in the south of the range. Once in temperate waters, however, *C. limbatus* takes over as the most abundant species.

The Tropic of Capricorn not only appears to be a barrier to species movements but this region also marks a change in dominant haplotypes within both species. This marked change in stock structure highlights the importance of the barrier in restricting gene flow between the north and south. The existence of the barrier suggests that hybridization was probably not the result of a single recent event that then radiated over the entire range. The presence of three *C. tilstoni* mitochondrial haplotypes off northern NSW is also significant. All fifteen of the animals typed as *C. tilstoni* from northern NSW were hybrids. These animals must be the result of at least three different hybrid matings. Hybridisation is not rare, nor is it restricted to specific mitochondrial haplotypes.

The relative lack of *C. limbatus* haplotypes compared to *C. tilstoni*, the decrease in haplotype diversity in both species towards the south and the relative abundance per location of species could be linked to known hybridisation between the species (Morgan *et al.,* Chapter 7). These effects could be the product of a fitness differential between hybrids and non-hybrids, between male and female hybrids, as well as across environmental gradients (e.g. reef to rocky shore, tropical to temperate, inshore to offshore). If female hybrid offspring of particular crosses (e.g. female *C. tilstoni* with male *C. limbatus*) had lowered fitness, then the frequency of haplotypes of the maternal species would be affected under certain circumstances (e.g. when hybridisation was common, a particular cross dominated and female hybrid fitness was low). Information on stock structure of these vulnerable shark species is essential for sustainable management given exploitation is permitted. More information on the nature of hybridisation between these species is urgently needed before mt-DNA species identifications and haplotype frequencies can be used to infer restrictions to gene flow, which is essential base-line data for sustainable management.

Both species of black tip sharks are displaying genetic stock structure along the east coast of Australia. The central position of *C. tilstoni* haplotype ND4_CT02 in the haplotype network (Figure 8.1) suggests it may be an ancestral haplotype. Although based on limited samples, ND4_CT02 appears to be a more significant component of shark stocks from the Northern Territory and Western Australia (Ovenden *et al.* 2010). This result suggests that *C. tilstoni* may show significant east to west genetic structure based on ND4 sequences although Ovenden *et al.* (2010) did not detect patterns of population subdivision between Western Australia, the Northern Territory or Queensland samples of *C. tilstoni* using mitochondrial DNA control region sequences or microsatellites.

Mitochondrial DNA can clearly separate *C. limbatus* from *C. tilstoni*. Unfortunately the marker cannot identify hybrids. Despite this short-coming mt-DNA information adds value to hybrid studies by providing the direction of the cross, and the ability to identify back-crossed hybrids (Morgan *et al.* In prep-b). Investigating mt-DNA haplotype diversity and distributions can also provide information about stock structure. Irrespective of hybrids, black tip sharks display marked stock structure along the east coast of Australia. A genetic barrier exists around the Capricorn region that marks a change in the dominant species and also a change within both species of the dominant haplotype. The barrier corresponds to the Tropic of Capricorn which marks the southern end of the Great Barrier Reef and the transition from tropical to temperate waters. Fisheries managers should consider black tip shark stocks on either side of this barrier as independent. Stocks of both species in the south display limited genetic diversity and high levels of hybridisation indicating that they may be more sensitive to fishing pressure.

Chapter 9: Conclusions

9.1 Benefits and Adoption

The results from this study have provided important knowledge for the future management of some of the most commonly taken shark species in inshore fisheries of north-eastern Australia. The project has provided important knowledge on the spatial dynamics of scalloped hammerhead shark, *Sphyrna lewini*, milk shark, *Rhizoprionodon acutus*, common black tip shark, *Carcharhinus limbatus*, and the Australian black tip shark, *C. tilstoni*. The project also developed reliable genetic techniques for the identification of the morphologically indistinct black tip species, and in doing so, made a remarkable discovery in confirming the existence of hybridisation occurring between the two black tips, *C. limbatus* and *C. tilstoni*. Shark fisheries in north-eastern Australia have received much attention in recent times with growing concern from conservation groups in particular of their sustainability. This project has identified the spatial scales that are appropriate for management and will directly assist resource managers in NSW and Queensland. This information will also benefit resource managers in the Northern Territory and Western Australia. The results of the study have been communicated directly to managers from the different jurisdictions across northern Australia so that informed and timely uptake of results can be incorporated into local fisheries management, monitoring and assessment. Within Queensland the information this study provides is very timely given that new monitoring and research programs on sharks are currently being implemented. Further, co-investigators of the project team (Welch, Tobin and Simpfendorfer (Queensland); Macbeth (NSW)) provide advice directly to key stakeholder advisory groups and have updated these groups on the project results, further facilitating future adoption of results where necessary.

9.2 Further Development

Recommendations for further research and development activities for scalloped hammerheads, milk sharks, and black tip sharks include the following:

- Where pragmatic, management agencies should adopt spatial management of these species according to the spatial scales and boundaries identified in this study.
- Future monitoring and assessment of the study species within their respective fisheries should also be conducted at a regional scale. Estimation of mortality rates for the study species should be applied based on the spatial scales and boundaries identified.
- Research on the movement of adult female scalloped hammerhead is required to confirm the spatial dynamics hypothesisied here. Also research into the reproductive output of females and an assessment of take in other fisheries of adult females may be prudent.

- There is some uncertainty as to the portion of scalloped hammerhead stocks that are female and samples taken in this study suggest a male bias. This observation may be assessed indirectly through fisheries monitoring programs by monitoring the proportions of female young of the year in catches.
- Further research to establish the stock structure of milk sharks in the Gulf of Carpentaria and their connectedness to the east coast.
- To confirm the differences in scalloped hammerhead maturity and growth schedules further research should collect comprehensive and comparable samples and conduct comparisons among the different regions used here, particularly for Capricorn vs. Fraser Burnett, which represents the divide between tropical and sub-tropical/temperate regions and vast differences in habitat types and environmental conditions.
- Regional differences in milk shark reproductive seasonality should be further assessed and confirmed with targeted sample collections as this could influence risk to fishing.
- Where monitoring/research programs require accurate determination of black tip species composition in northern Australian fisheries catches, the RT-HRM-PCR assay is a useful diagnostic tool if large numbers of samples need to be identified and when it is not practical to conduct pre-caudal vertebrae counts in the field.
- The full extent of the black tip hybrid zone needs to be determined by testing samples from Western Australia, the Northern Territory including the Gulf of Carpentaria, southern NSW and Indonesia. Monitoring of shark stocks and hybrid frequency is also needed along the entire species range to assess if environment-dependant hybrid fitness exists among populations. This information is urgently needed before mtDNA species identifications and haplotype frequencies can be used to infer restrictions to gene flow, which is essential base-line data for sustainable management.
- Black tip hybrid fitness urgently needs to be investigated using a range of genetic and non-genetic characters. Life history measurements that assess reproductive capacity and their susceptibility to parasites and general health compared to the parental species will assist in understanding whether the two species will be maintained or combined into one hybrid species in the future.
- More diagnostic nuclear markers, and morphological characters, are needed to assist in the identification of ‘pure’ (i.e. non-hybrid) individual black tip sharks and would assist in understanding the degree of genome mixing or introgression occurring between the two species.
- Research into the original black tip speciation event may assist in interpreting the current hybridisation event.
- Further stock structure and biological research on the remainder of the largely unstudied elasmobranch fauna in tropical and sub-tropical Australia and to continually refine management strategies to ensure a sustainable future in Australian waters.

- That future such sampling and monitoring programs consider fisheries-independent approaches to minimise the challenges due to separation of life history stages, gender, habitat preferences and variability in small-scale fisheries characteristics over large areas that are typical in elasmobranch fisheries.

9.3 Planned Outcomes

The planned outcomes that this project has achieved include:

The project has provided management and other stakeholders with information necessary to make informed decisions about the management of four of the key exploited shark species caught in the Queensland inshore net fishery and the northern NSW line fishery. The project has determined that spatial management of milk sharks within Queensland, and scalloped hammerhead, common black tip and Australian black tip sharks within Queensland and NSW is appropriate. The project has determined that both black tip shark species are likely to require co-operative management arrangements between Queensland and NSW, while for scalloped hammerheads separate stocks between the two jurisdictions were identified from the fisheries-dependent samples, however genetic exchange across borders is likely to be facilitated by movement of adult females and perhaps larger males to a lesser extent. This information will greatly assist compliance with the Commonwealth *Environment Protection and Biodiversity Conservation Act (1999)* for shark fisheries in north-eastern Australia by providing the necessary basis for robust assessment of the status of stocks for the study species, thereby helping to deliver their sustainable harvest. It also helps to achieve objectives of the Australian National Shark Plan.

The project provides the appropriate spatial framework for future montoring and assessment of the study species. This is at a time when shark fisheries are receiving close attention from all sectors and when monitoring programs are being implemented with the aim to better assess stock status. This project has provided the crucial information for developing an appropriate monitoring design as well as the necessary basis for making statements about stock status.

The project has addressed research priorities identified by the Queensland Fisheries Research Advisory Board, Great Barrier Reef Marine Park Authority and Queensland Fisheries. Previously management has assumed a single stock for each species on the east coast of Queensland, and management of shark fisheries in NSW and Queensland has been independent of one another.

The project has been able to enhance and develop links between research, management and industry. Strong positive relationships with commercial fishers were crucial in the collection of samples throughout the study area and fisheries managers were part of the project team throughout the study

period. During the project the study area was extended to NSW waters creating mutualistic and positive links between NSW and Queensland research and management agencies. Extension of project results included management representatives from NSW and Queensland as well as the Northern Territory where similar shark fisheries operate and similar species are targeted.

The project was able to provide significant human capital development opportunities providing considerable value to the project outcomes. Use of vertebral microchemistry and life history characteristics as stock determination methods provided material for two PhD students based at James Cook University: Ron Schroeder, vertebral chemistry; and Alastair Harry, life history chacteristics.

The project has developed novel research methods that have great capacity for future application. These are:

- Development of a simple and rapid genetic diagnostic tool (RT-HRM-PCR assay) for differentiating among the black tip shark species, for which no simple morphological identifier exists; and
- Development of laser ablation inductively coupled plasma mass spectrometry (LA-ICPMS) methods for analysing and interpreting microchemical composition of shark vertebrae.

The study has provided further confirmation of the effectiveness of using a holistic approach in stock structure studies and justifies investment into such studies.

9.4 Conclusions

The conclusions from the project are:

- Fisheries for scalloped hammerhead sharks on the north east Australian coast are comprised of multiple stocks comprised of localised juveniles and adult males.
- Fisheries for milk sharks on the Queensland east coast are comprised of multiple stocks comprised primarily of localised juveniles and adult males.
- Fisheries for common black tip and Australian black tip sharks on the north-east Australian coast are comprised of two stocks with a boundary corresponding approximately to the southern limit of the Great Barrier Reef.
- On the north eastern Australian coast all four study species are comprised of single genetic stocks with genetic exchange facilitated by adult movement. For scalloped hammerhead and milk sharks this genetic exchange is likely to be primarily driven by longshore movements of adult females.
- On the north eastern Australian coast line hybridisation between the common and Australian black tip sharks is occurring. This may have been going on for some time and the prevalence of hybrids is

unusually high across the entire study area. The fitness and viability of hybrids are potentially of serious concern to the future of black tip populations.

- Fisheries-dependent methods of sampling elasmobranch species presents challenges in overcoming the tendency of many species to spatially distribute themselves based on combinations of life history stage and gender and propose the use of fisheries-independent methods of sampling, exclusively or in parallel with fisheries-dependent methods, for future stock structure and life history studies.
- The use of the different techniques in identifying stocks proved extremely useful in delineating spatially discrete stocks from genetic stocks and in identifying possible mechanisms influencing the observed stock structure in each species.

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Appendices

Appendix 1: Intellectual Property

No patentable or marketable products or processes have arisen from this research. All results will be published in scientific and non-technical literature. The raw data from compulsory fishing logbooks remains the intellectual property of Fisheries Queensland (DEEDI). Raw catch data provided by individual fishers to project staff remains the intellectual property of the fishers. Intellectual property accruing from the analysis and interpretation of raw data vests jointly with James Cook University, Fisheries Queensland, Fisheries Western Australia, Northern Territory Department of Primary Industries, Fisheries and Mines, The University of Queensland and the Principle Investigator.

Appendix 2: Project Personnel

Fishing & Fisheries Research Centre, James Cook University

David J. Welch (QPIF, DEEDI)..... Principal Investigator
Colin Simpfendorfer..... Co-investigator
Andrew Tobin ..... Co-investigator
Olivier Bittar..... Research assistant
Jimmy White..... Research assistant
Alastair Harry..... Student and Research assistant
Ron Schroeder ..... Student
Ann Penny..... Liaison Officer
Katia Bazaka ..... Administrative Officer

Fisheries Queensland, Department of Employment, Economic Development and Innovation (DEEDI)

Jenny Ovenden ..... Co-Investigator
Jessica Morgan ..... Fisheries geneticist
Raewyn Street..... Fisheries technician
Mark Lightowler..... Fisheries manager

Great Barrier Reef Marine Park Authority

Rachel Pears..... Manager

NSW Department of Primary Industries

Will Macbeth..... Research scientist
Pascal Geraghty..... Fisheries technician

Contributing Fishers

Greg Radley
Russell Marriage
Trevor Falzon
Bill Gilliland
Dane King
Joe Greenhalgh
Trevor Draper
Ben Gilliland
Steve Howe

Appendix 3: Genetic raw data (under construction)

Data available at http://www2.dpi.qld.gov.au/extra/era/index.html