Conservation of coral reef associated sharks in Australia and Indonesia

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TABLE OF CONTENTS

SUMMARY

In the past few decades, as a direct result of overfishing, shark numbers have declined dramatically across the world's oceans, which in some cases has resulted in trophic cascades affecting entire ecosystems. Genetic data, particularly when interpreted in conjunction with direct observations of animal movements and behaviour, can be applied to identify management units, an essential step in the establishment of effective management strategies. This thesis is structured into two main themes: first, I summarize the challenges and successes of shark conservation and management, both globally (chapter 2) and specifically for Australia and Indonesia (chapter 3), and second, I apply genetic methods to obtain conservation relevant information for coral reef associated sharks (chapters 4 to 10).

In the first part of my thesis I analyse trends in shark conservation research by evaluating 20 years of scientific output, with a focus on Australia and Indonesia. While scientific effort in shark conservation science has increased in the past two decades, it has done so with a strong geographic bias. Australia and the United States have made an overwhelming contribution to the research output, while countries like Indonesia, which is home to the largest shark fishery in the world, have historically contributed much less. This bias has important consequences in terms of shark conservation: countries which invested in shark research successfully established effective management policies, while nations which devoted less resources to shark conservation science failed to do so. Consequently, I suggest that poor management of shark fisheries might be remedied by increasing local research capacity, which has recently been driven by the establishment of international collaborations.

In the second part of my PhD I investigate how habitat and local selection influence patterns of genetic variation in the grey reef shark (*Carcharhinus amblyrhynchos*), an abundant coral reef predator which has undergone dramatic declines in recent years. I discover that genetic connectivity is affected by the spatial distribution of coral reef habitats, which act as stepping stones through which genetic connectivity can be maintained across large distance (>5000 km) by male dispersal. Furthermore, I identify signatures of local selection, suggesting that grey reef sharks in different regions of Australia and Indonesia may be locally adapted. I discuss the conservation implications of these discoveries in conjunction with recent studies on the movement ecology of grey reef sharks. I also describe the potential applications of integrating information from neutral genetic markers and markers under selection to identify conservation units and for monitoring the international shark fin trade.

DECLARATION

The work described in this thesis was conducted by myself, with the supervision of my supervisors: Associate Professor Adam Stow and Professor Robert Harcourt. The work herein described is original and is not being considered for a higher research degree in another institution.

All work involving animal handling was conducted in accordance with Macquarie University's Animal Ethics Committee, following the protocol number 2012/044-2. Samples were collected under permits from the Western Australia's Department of Environment and Conservation (permit number: SF008796) and the Great Barrier Reef Marine Park Authority (permit number: GI/13/35796.1). Research in Indonesia was conducted under research permit number 03B/TKPIPA/FRP/SM/III/2014 issued by the Indonesian State Ministry of Research and Technology (RISTEK) and in collaboration with the Indonesian Biodiversity Research Center and the Indonesian Institute of Science (Lembaga Ilmu Pengetahuan Indonesia).

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STATEMENT OF CONTRIBUTION

All data chapters (chapter 2-10) have multiple authors. My personal contribution to each of the data chapters is outlined in the table below

Chapter	**Design**	**Laboratory work**	**Data analyses**	**Writing**
Chapter 2	95%	NA	100%	80%
Chapter 3	95%	NA	100%	65%
Chapter 4	95%	90%	90%	95%
Chapter 5	95%	100%	90%	95%
Chapter 6	95%	100%*	100%	95%
Chapter 7	95%	100%*	100%	95%
Chapter 8	NA	NA	NA	95%
Chapter 9	50%	80%	80%	70%
Chapter 10	50%	80%	70%	30%

*with the exception of library preparation for next-generation sequencing, which was outsourced to Diversity Arrays Technology Pty. Ltd. (Canberra, Australia).

Chapter 1

General Introduction

Author:

Momigliano, Paolo

HUMAN IMPACT ON MARINE ECOSYSTEMS

Humans have impacted marine ecosystems for thousands of years, well before the rise of western industrial societies (Jackson *et al.* 2001). In the last century, however, the increase in global population in concert with the development of new technologies has led to the creation of industrial fishing fleets which have harvested marine resources on a scale unprecedented in human history (Jackson *et al.* 2001; Pauly *et al.* 2005). Overfishing has led to dramatic declines in marine predator biomass in many if not most regions of the world (Baum & Myers 2004; Baum *et al.* 2003; Myers & Worm 2003, 2005), with densities of some large marine predators reduced to the point in which they can no longer fulfill their ecological role (Casini *et al.* 2008; Daskalov 2002; Frank *et al.* 2005; Myers *et al.* 2007). Sharks, marine mammals, and other large marine predators play essential roles in shaping marine communities, often controlling both the abundance and behaviour of organisms in lower trophic groups (Heithaus *et al.* 2008). Predator removal may therefore have unpredictable and pervasive effects on marine food webs, resulting in trophic cascades and ecosystem phase shifts (Casini *et al.* 2008; Frank *et al.* 2005; Myers *et al.* 2007).

CAUSES AND EFFECTS OF THE GLOBAL DECLINE IN SHARK DENSITIES

Among the marine predators which have undergone dramatic declines in the past 100 years are sharks (Selachimorpha). Sharks are a diverse group of organisms, comprising more than 500 species, most of which are meso and high order predators inhabiting the continental shelves and slopes of tropical and temperate oceans (Compagno 2001). In the past few decades, large sharks have experienced dramatic declines across all oceans and ecosystems, from temperate seas (Baum & Myers 2004; Baum *et al.* 2003; Ferretti *et al.* 2008; Ferretti *et al.* 2010) to tropical coral reefs (Graham *et al.* 2010; Robbins *et al.* 2006; Ward-Paige *et al.* 2010). The main cause of the dramatic collapse of shark stocks is fishing mortality. Sharks are directly targeted for their fins and meat and caught as bycatch in fisheries targeting other species. Many species of sharks have conservative life-history strategies: they are slow growing, take a long time to reach sexual maturity and have low fecundity, and have therefore a low rebound potential compared with other marine fishes (Smith *et al.* 1998). These characteristics make them intrinsically vulnerable to even modest levels of fishing pressure (Field *et al.* 2009).

The decline of shark numbers is not a phenomenon isolated to a few regions where fishing pressure is extraordinarily intense, it has occurred in most coastal ecosystems around the globe. In the Mediterranean Sea, for example, the densities of large predatory sharks in the past century have declined between 96 and 99.9% (Ferretti *et al.* 2008). In the North-West Atlantic and Gulf

of Mexico similar trends have been recorded, with declines in biomass of large predatory sharks of between 87% and 99% (Baum & Myers 2004; Baum *et al.* 2003). In the tropics, alarming declines of reef shark populations have been recorded from coral reefs across the globe, from the Caribbean (Ward-Paige *et al.* 2010) to the Indian Ocean (Graham *et al.* 2010), and even within the comparatively well managed Great Barrier Reef Marine Park (GBR) (Ayling & Choat 2008; Robbins *et al.* 2006). Recently it has been shown that pristine coral reefs have top heavy food webs, where the biomass of apex predators overwhelms that of the fish assemblages, and reef-associated sharks make up the majority of apex predator biomass (Friedlander *et al.* 2014; Sandin *et al.* 2008; Stevenson *et al.* 2007). Reef shark density decreases over a gradient of increasing anthropogenic disturbance (Sandin *et al.* 2008), and in regions open to fisheries can be an order of magnitude lower than in pristine coral reefs .

These declines have far-reaching effects on coastal marine communities. In the North-West Atlantic, for example, the ecological extinction of large predatory sharks drove an abrupt increase in elasmobranch meso-predators such as the cownose ray (Myers *et al.* 2007). Increased predation of cownose rays on scallops led to the collapse of a century-old fishery. Similarly, Ruppert *et al.* (2013) noted that in isolated coral reefs off Western Australia sharks were present in much larger numbers in unfished areas. The lower density of reef sharks in fished reefs was associated with higher density of smaller predatory fish and lower densities of herbivorous fish, an observation compatible with the hypothesis that a trophic cascade had occurred. Fished reefs showed lower recovery potential after catastrophic pulse-disturbances in the 1990s which led to a dramatic decline in coral cover (Ruppert *et al.* 2013), possibly because of the crucial role that herbivorous fish play in coral reef recovery (Hughes *et al.* 2007). The removal of predatory sharks may also have indirect effects on lower trophic groups. For example, tiger sharks can indirectly reduce grazing on seagrass meadows by dugongs and sea turtles through behavioral risk-effects, and it has recently been suggested that the recovery of turtle populations in concert with a decline in abundance of their main predator (the tiger shark) may have dramatic effects on seagrass habitats (Heithaus *et al.* 2014; Heithaus *et al.* 2008).

Given the global widespread decline in shark numbers, and its potential effects on marine ecosystems the need to implement effective conservation strategies for the recovery of shark populations has never been so crucial. Unfortunately, despite an increase in the number of studies on shark conservation and management in the past twenty years, there is still a paucity of data for most species in most regions of the world, hindering the development of effective management strategies for some of ocean's most important predators (see Chapter 2).

THE ROLE OF GENETICS IN THE CONSERVATION OF SHARKS

Dudgeon *et al.* (2012) recently reviewed some of the applications of molecular genetics for the conservation and management of elasmobranchs. Genetic tools can provide essential information for the management and conservation of sharks (Dudgeon *et al.* 2012). Demographic and evolutionary events, such as migration, local selection and speciation leave molecular signatures within individual genomes which can be used to reconstruct the underlying ecological and evolutionary processes. In the past quarter of a century, genetic techniques have been routinely employed to identify and delimit fishery stocks, describe phylogeographic patterns, investigate the extent of reproductive philopatry, describe mating systems, estimate effective population sizes and identify catch to the species level (Dudgeon *et al.* 2012). This information has played a crucial role in the development of effective management strategies for the conservation of sharks. For example, population genetic studies have been extensively used to delimit fishery stocks and conservation units of elasmobranchs (Dudgeon *et al.* 2009; Giles *et al.* 2014; Ovenden *et al.* 2009), thereby providing crucial information on the appropriate spatial scale of management. Determining effective population sizes can give hindsight into the viability of natural populations (Franklin & Frankham 1998), and studies revealing female natal philopatry can identify nursery areas which may warrant high conservation priority (Feldheim *et al.* 2014). Furthermore the use of DNA barcoding approaches has made it possible to determine the catch composition of poorly described fisheries (Sembiring *et al.* 2015), identify illegal catch to species level (Holmes *et al.* 2009) and monitor the international shark trade in order to detect the presence of protected and threatened species (Liu *et al.* 2013; Sebastian *et al.* 2008).

The advent of next generation sequencing technologies and the exponential increase in computing power has enormously reduced the cost and time of developing, screening and analysing thousands of genetic markers for large numbers of individuals. These large datasets, accessible today but unthinkable for non-model organisms only a decade ago, can be used to further address important questions in conservation, for example by identifying locally adapted populations which may warrant high conservation priority and detecting cryptic patterns of genetic structure (Allendorf *et al.* 2010; Funk *et al.* 2012).

THESIS OVERVIEW

This thesis is structured along two main themes: 1) summarizing the challenges and successes of shark conservation and management, both globally (chapter 2) and with a special focus on Australia and Indonesia (chapter 3) and 2) conservation genetics of coral reef associated sharks (chapters 4 to 10).

Chapter two and chapter three critically review the past literature on shark conservation and analyse publically available data on scientific effort and the status of shark conservation. These two chapters serve as a general introduction to the thesis. In Chapter two, I analyse trends in global shark conservation science and management in the past two decades. I review 20 years of scientific studies on shark conservation and management, identify taxonomic and geographic biases in the allocation of scientific effort, and discuss the consequences of scientific effort misplacement in terms of effective management. Furthermore, I use case studies to illustrate where the scientific community and policy makers have worked together to develop effective management policies and contrast these with where the feed-back process between science and management has broken down, or failed to start. Finally, I suggest a solution to this science-management disconnect through the implementation of the Adaptive Management Framework.

In chapter three I summarise the status of conservation of true sharks (Selachimorpha) in the Indonesian and Australasian regions, with a strong focus on Indonesia and Australia. I describe patterns of biodiversity and major threatening processes such as overfishing, habitat degradation and climate change. I use a number of case studies from Australia and Indonesia to illustrate some of the major challenges that shark management is facing and review current frameworks for shark management and conservation in the region, with a strong emphasis on the implementation of National Plans of Action (NPoA) for the management of sharks. Two sections of chapter three deal specifically with the conservation of coral reef associated sharks and the role of Marine Protected Areas (MPAs) in shark conservation, which are the main themes of the following chapters.

In chapter four I develop a number of genetic markers to study genetic connectivity in grey reef sharks (*Carcharhinus amblyrhynchos*). In chapter five I use these genetic markers to investigate genetic connectivity in the grey reef sharks in the Australian Great Barrier Reef (GBR) region. I use both empirical and simulated genetic data to determine the extent of connectivity between coral reefs in the GBR, and discuss the implications of these findings for the effectiveness of spatially discontinuous networks of MPAs as a tool for the protection of reef sharks.

In chapter six I use state-of-the-art genomics techniques and analyses to investigate cryptic genetic structure in grey reef sharks in Australia and Indonesia. I reveal that grey reef sharks are likely subject to spatially diversifying selection across the region and discuss the implications of these findings for the management of this species in Australia and Indonesia. Furthermore, I discuss the potential application of this approach for other shark species, and highlight the limitations of previous work aimed at identifying management stocks of elasmobranchs using exclusively neutral genetic markers.

I then move to investigate genetic connectivity in the grey reef shark at multiple spatial and temporal scales across a large section of its distribution range (chapter seven). I use the largest dataset ever used for any population genetic study on elasmobranchs, including more than 5400 genetic markers and samples collected from nine locations in the Indo-Pacific. I investigate the role of coral reef habitats in shaping genetic connectivity, and test the hypothesis that dispersal is sex biased. I discover that large scale and fine scale genetic connectivity is influenced by the spatial distribution of coral reef habitats, which act as stepping stones through which genetic connectivity is maintained across distances exceeding 5000 km by male dispersal. I discuss the implications of these findings for the effectiveness of MPAs for the protection of grey reef sharks, which is likely largely dependent on the extent of habitat fragmentation.

Chapter eight is a perspective paper in which I discuss the potential applications of Circuit Theory in the analysis of population genetics and acoustic telemetry data to create predictive models of dispersal. I suggests that these models may prove useful to identify potential shortcomings of the current model of discontinuous MPAs for the protection of organisms with high adult dispersal, such as sharks, and aid in the development of more effective networks of MPAs.

The following two chapters (chapter nine and chapter 10) report new distribution records of two threatened shark species, the grey nurse shark (*Carcharias taurus*) and the fossil shark (*Hemipristis elongata*) and reveal that these species are under fishing pressure from Indonesian fishers in areas where we did not even know they occurred. I report that grey nurse sharks, which are a fully protected species in Australia, are targeted by Indonesian fishers in an area of the Australian Exclusive Economic Zone known as the 1974 Memorandum of Understanding Box, where fishing by traditional Indonesian fishers is allowed. I discuss the implications of these findings for the conservation of grey nurse sharks, and highlight the challenges of regulating catches in remote areas where traditional foreign fishing is permitted. In the following chapter I reveal that the fossil shark, which was previously known to occur in

Indonesia only in the island of Java, has a much broader Indonesian distribution and is targeted by fishers throughout most of the country.

In the final chapter I discuss and synthetise the main findings of the thesis. Other manuscripts which I have authored and published during my candidature, but which are not directly relevant to the thesis, are presented in the appendices. In Appendix A I present a popular article I wrote describing the results from Chapter 2. Appendix B reports a manuscript I have co-authored on the mating system of captive grey nurse sharks. The papers presented in Appendix C and Appendix D deviate from the main topic of my thesis and deal with the biodiversity and ecology of harmful (Appendix C) and symbiotic (Appendix D) tropical microalgae in Australia.

This thesis follows Macquarie University's guidelines for "Thesis by Publications". All data chapters have either been already peer-reviewed and published (chapter 2, 3, 4, 5, 8, 9 and 10) or will be soon submitted for review in international scientific journals (chapter 6 and 7). The chapters have been reformatted to minimize differences in style.

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Chapter 2

Shark conservation, governance and management: the science-law disconnect.

Authors:

Momigliano, Paolo[1] and Harcourt, Robert[1]

Peer-Reviewed Book Chapter:

Momigliano P. and Harcourt R. (2014) Shark conservation, governance and management: the science-law disconnect. In Klein N. and Techera E. (eds) *Sharks: conservation, governance and management*. Abingdon, UK: Earthscan Series, Routledge, pp. 89–106.

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INTRODUCTION

Shark populations have experienced a dramatic global decline over the past few decades (Baum & Myers 2004; Baum *et al.* 2003; Ferretti *et al.* 2008). The collapse of shark populations is driven by two main factors. The first is the rise in demand for shark products (Lack *et al.* 2006; Rose 1996), which has led to a sharp increase in fishing effort and landings over the last 30 years. The second is the intrinsic vulnerability of sharks to fishing pressure, due to their conservative life history strategies (Field *et al.* 2009). In general sharks are k-selected, i.e. they are long lived, slow growing and take a long time to reach sexual maturity (Last & Stevens 2009). They invest considerable amounts of energy in the production of a few offspring that then have a high survival rate. These characteristics make them susceptible to even modest levels of fishing mortality. Unlike many bony fishes (teleosts), recruitment is tightly linked to stock size, meaning that any level of mortality in adult stocks will have immediate consequences in terms of recruitment in the following years (Smith *et al.* 1998). Recruitment overfishing, the level of harvesting at which the reproductive potential of a population is affected, starts early in the history of most shark fisheries. The rebound potential for shark populations is therefore lower than for most other harvested marine fishes. However, whereby this provides the rationale for sharks' intrinsically high risk of extinction, by the same reasoning lies therein a nugget of hope for successful management.

Conservative life history strategies lead to predictable demographies (Bradshaw *et al.* 2013). Recruitment levels in shark populations are usually stable compared to many commercially important teleosts, because the investment of substantial resources into offspring production means that juveniles are less susceptible to short-term environmental fluctuations. Moreover the rise in fishing effort has the potential to provide access to a large amount of shark life history information. Concomitant with this improved data, the long life expectancy of sharks makes them amenable to mark recapture analysis, from which survival and dispersal capabilities may be estimated (Bradshaw *et al.* 2013). These data can be used to produce viability analyses and predict future population trends, which can provide the scientific bases of future management policies aimed at the sustainable harvest of commercially important species and the recovery of threatened populations (Aires-da-Silva & Gallucci 2007; Bradshaw *et al.* 2007; Otway *et al.* 2004).

The development of effective policies for shark conservation is a complicated process that relies on a series of sequential steps involving scientific communities as well as the policy makers and other stakeholders (see below). Since sound scientific data are needed to inform decision-

makers, scientific effort needs to be directed towards areas relevant to the development of conservation policies. Knowledge gaps that prevent the development of efficient conservation strategies must be identified, and future research must be directed towards addressing scientific uncertainties. Conservation policies need to be evidence based, and subject to continuous scientific evaluation and review. The effectiveness of management policies also needs to be scientifically assessed based on clear performance indicators, and revised management strategies need to be developed based on such assessment. This process involves continuous feedback between scientific enquiry and policy development (see below). The recommendations of the United Nations International Plan of Action for the Conservation and Management of Sharks (IPoA) (FAO 1998) highlight the importance of evidence-based management and continuous feedback between the scientific community and policy makers.

Here we consider some of the main causes of potential mismatches in the science-policy feedback process. We review the last 20 years of scientific studies on shark conservation and management, and identify underlying issues that can lead to misplaced scientific effort. We also present examples where the scientific community and policy-makers have worked together in an organic way to develop effective management strategies, and compare these with where the process has broken down or even failed to start. Finally we suggest a possible way out of this morass through the effective adoption of the Adaptive Management Approach.

IS SCIENTIFIC EFFORT MISPLACED?

Research on areas other than conservation (for example alleviating poverty and providing alternative livelihoods for fishermen) may play an important role in ameliorating the threats of overfishing. However, scientific assessment remains a critical step in the development of effective management policies aimed at the conservation of sharks. If conservation science is to provide appropriate knowledge needed by policy-makers, then scientific effort needs to be directly relevant to management. Globally, if scientific effort is directed towards the development of management policies, we could predict that it would be concentrated in geographic areas where sharks are under high levels of threat or endangerment, and targeted at species which are of major conservation concern.

We analysed the global scientific output in shark management and conservation over the past 20 years (Jan 1, 1992-Dec 31, 2011) in order to evaluate whether scientific effort has been most effectively utilised. We undertook a Web of Science search and identified peer-reviewed articles reporting original research containing the words “Shark” and "Conservation" or "Management" either in their abstract or listed as keywords. For each paper, we recorded target

species (up to 5), and the location where the study was conducted. The final list included 479 publications.

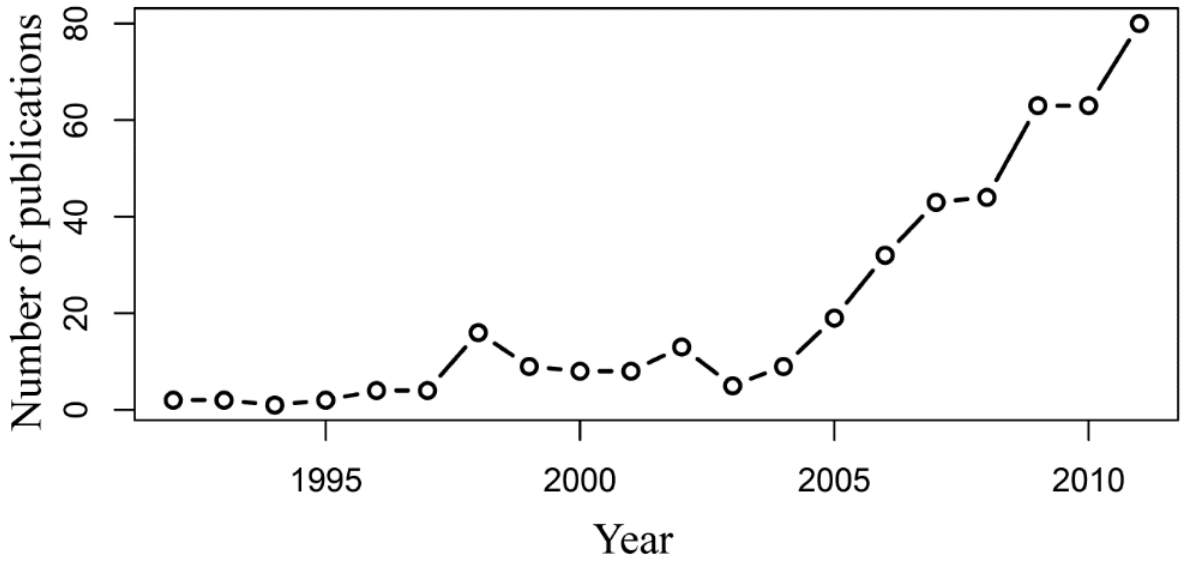

Figure 1: *Global trend of scientific effort (number of publications) in the period 1992-2011 including in their title, abstract or keywords the words "Shark" and "Conservation" or "Management".*

A sharp increase in research effort has occurred in the last seven years (figure 1). Empirical studies on shark conservation and management were very rare in the 1990s (in 1992 only two studies were published), but in the 2000s shark conservation and management studies became increasingly more common, and in 2011, 80 studies on topics relevant to shark conservation were published worldwide. This trend suggests that considerable effort has been placed in research relevant to the conservation and management of shark species at the global scale. However, if we deconstruct the data and look at how scientific effort has been distributed across nations and across species, some clear trends arise.

Geographic bias in scientific and fishing effort

Since fishing mortality is the highest threat to shark populations (Field *et al.* 2009), if scientific effort was directed towards areas of highest need we would expect scientific output to be correlated with fishing effort on a geographical basis. Nations responsible for the majority of shark landings should place considerably more effort into research relevant to fishery management. However if we plot nation by nation scientific effort against shark landings, a very different picture emerges (figure 2).

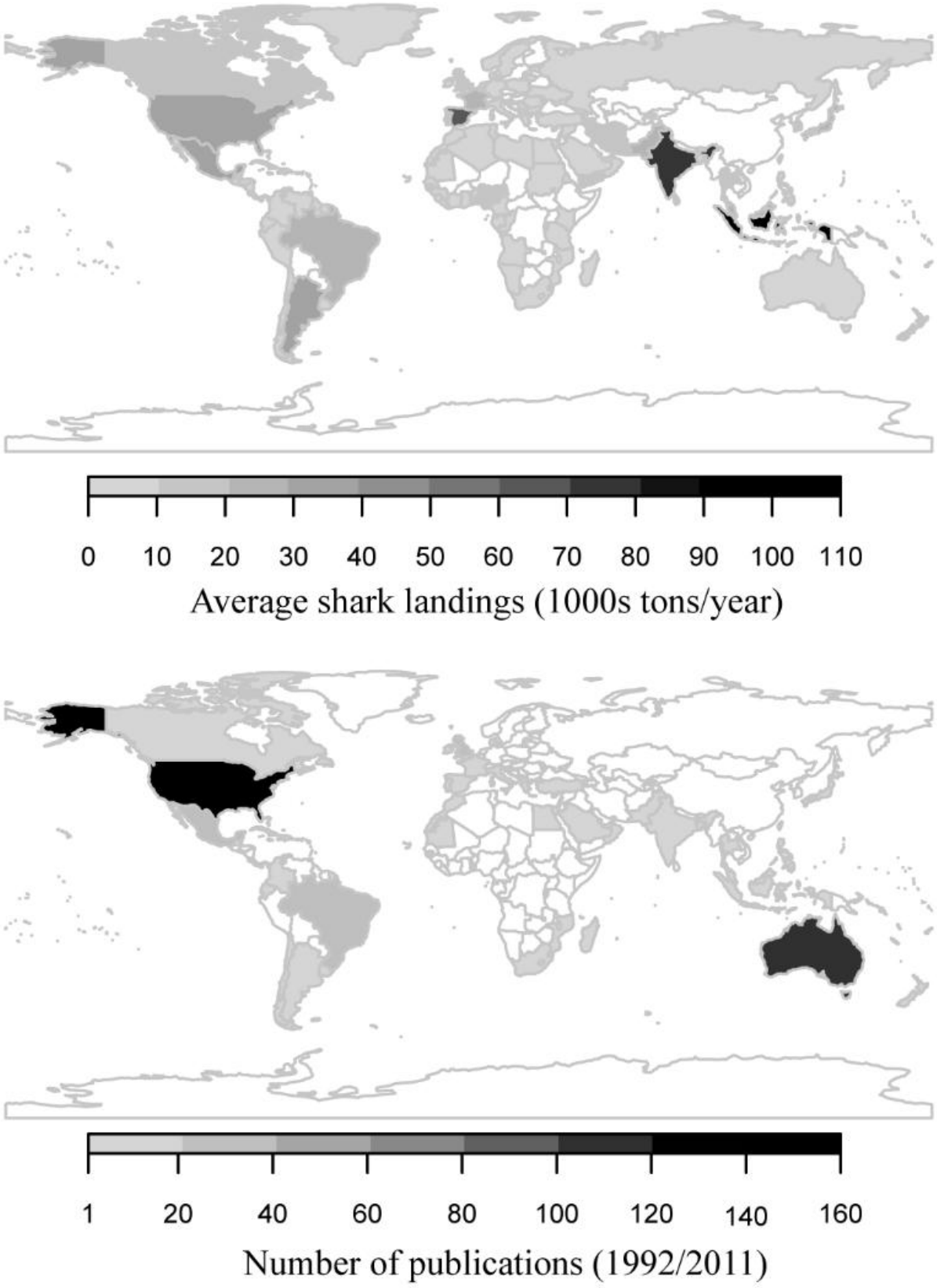

Figure 2: *National contributions to shark landings (above) and scientific output (below). Shark landing data were obtained from the Food and Agriculture Organization (FAO)*

A handful of states are responsible for the vast majority of shark landings. Indonesia (>100,000 tons/year), India (>70,000 tons/year), Spain (>60,000 tons/year), Taiwan (>40,000 tons/year) Mexico, Argentina and the USA (>30,000 tons/year) make up approximately half of the total landings. Nevertheless, with the exception of the United States and Mexico, these nations have placed very little research effort in shark conservation and management (figure 2). Indonesia and India are the leading harvesters of shark products in the world, but these countries rank very low in terms of scientific output. Only nine studies were conducted in Indonesia during the past 20 years, most of which were basic accounts of species composition and life history traits from major landing sites (Blaber *et al.* 2009; Varkey *et al.* 2010; White 2007a, 2010; White *et al.* 2008; White & Kyne 2010; White 2007b). While these studies may constitute a basis for future scientific assessment of national fisheries, they are inadequate with respect to providing necessary information for the development of evidence-based management policies. The situation is very similar in India, Spain, Taiwan and Argentina. More than half of the studies published in the past 20 years have been carried out in the USA and Australia (144 and 117 respectively). While the USA is an important player in shark fisheries at the global scale, Australia, with landings totalling less than 10,000 tons per year, contributes only 1% towards total global catches. The fact that the USA and Australia are leading the way in shark research is not surprising. The USA leads scientific research globally producing as many papers across science annually as the rest of the world combined, and Australia is one of the top 10 nations worldwide in terms of scientific impact (May 1997). Furthermore, the USA has economically important shark fisheries, and Australia harbours the highest diversity of shark fauna on Earth (White & Kyne 2010) .

The most likely reason for the disconnect between the production of quality, relevant science and catch size is that scientific effort is highly correlated with economic wealth (May 1997), and the nations that are responsible for most of the shark catches are comparatively poor. Research is expensive, and often funded directly by governments particularly in areas specifically relevant to natural resource management. Developing countries such as Indonesia, and even comparatively more wealthy countries such as India and Mexico, simply do not have the research capacity and the financial resources to carry out extensive research surveys relevant to natural resource management on the same scale as the USA or Australia.

This geographic bias has important consequences in terms of management, and had major repercussions on the implementation of Nation Plan of Actions for the Conservation and Management of Sharks (NPoA) following the United Nation's (UN) International Plan of Action's (IPoA) guidelines. The two countries that contributed the most to global scientific

effort in shark management and conservation were among the first to implement NPoAs (in 2001 and 2004 respectively)(NPoA-Australia 2004; NPoA-USA 2001). Australia is the only country in which the NPoA strictly followed the IPoA guidelines (Davis & Worm 2012), and is the only nation that has carried out extensive reviews of the first NPoA, conducted a new shark assessment report and released a second NPoA in 2012 (NPoA-Australia 2012). By comparison, countries that are major players in the shark fishing industry but did not invest in shark conservation research, such as India and Indonesia, have still failed to produce NPoAs following the IPoA guidelines. While India has not implemented a NPoA, the Indonesian government has committed to manage its shark resources through the formulation of a NPoA. However, the Indonesian NPoA released in 2010 did not propose any specific management strategy, rather it simply highlighted the need to obtain more scientific data to inform management without specifying a framework for increasing research effort. If advances are to be made through mechanisms such as the IPoA then the most significant shark fishing nations must be encouraged and assisted to develop and implement effective NPoAs. Enhanced collaboration among institutions in developing countries and countries leading in conservation research will likely be a key process for increasing local research capacity.

Shark conservation science and the issue of taxonomic chauvinism

The goal of conservation biology is to provide the scientific tools needed for the preservation of species that are, either directly or indirectly, threatened by anthropogenic processes (Soulé 1985). If conservation research is to be informative in terms of management of threatened species, it must give preference to species that are subject to the highest risks and depend for their survival upon the prompt implementation of conservation policies. This leads to a quandary, as species that are threatened are usually less common and /or abundant than species facing little threat and are therefore often more difficult to study. Accordingly, we wondered whether research effort in shark conservation and management does in fact reflect conservation priority, or whether other factors, such as economic importance or the "charisma" of the study organism are at play. We define economically important species as those species that are directly targeted by important fisheries (data from IUCN, http://www.iucnredlist.org), and charismatic species as sharks that are well-known by the general public and are often used either as flagships for environmental organizations or as eco-tourism attractions (primarily the great white shark, whale shark, basking shark, mako shark, tiger shark, oceanic whitetip shark, hammerhead sharks, grey reef shark and grey nurse/sand tiger/ragged tooth shark).

We found that while research effort is very high for Vulnerable and Near Threatened species, only a small fraction of the scientific output is directed towards species that are Endangered and Critically Endangered (figure 3A). Furthermore, of the 20 most studied shark species only one is Endangered, but most are either charismatic or of economic importance or both (figure 3B). Species that received the most scientific interest are often not the ones facing the highest threats. Research effort is biased towards charismatic species and species of economic importance, while comparatively little research effort is placed on species that are facing immediate risk of extinction or on species for which insufficient data are available for assessment. This is despite the fact that this scientific effort is specifically linked to conservation and / or management.

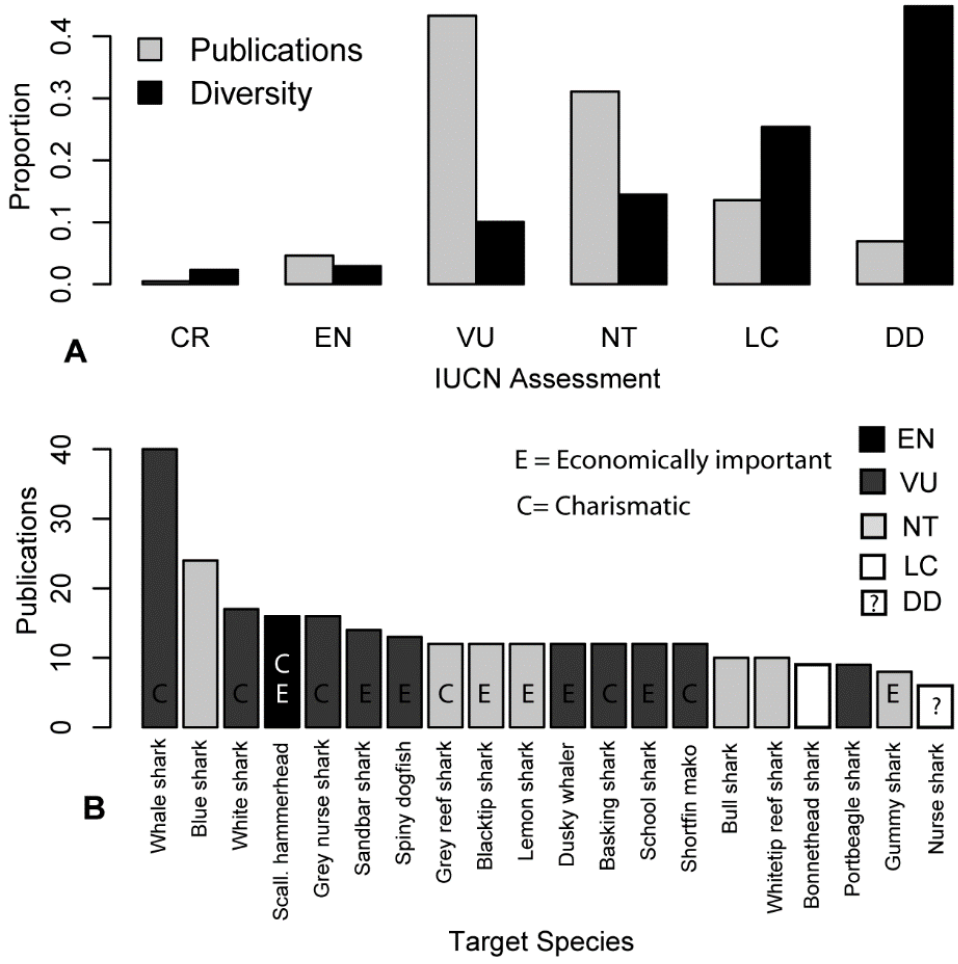

Figure 3: *Proportion of the scientific output and taxonomic diversity for each IUCN assessment class (A), and number of publications for the most well studied 20 species of sharks grouped by IUCN assessment (B). (EN=endangered, VU = Vulnerable, NT= Near Threatened, LC = Least Concern, DD = Data Deficient)*

Under-representation of Endangered and Critically Endangered species.

Scientific output (standardized by species diversity) is not homogeneous across species listed in different IUCN categories (χ^2= 736.8, df=5, p<0.0001). The proportion of the scientific output on NT, VU and EN species is higher than the proportion of taxonomic diversity that is accounted for by these categories, while for LC species it is lower. This trend indicates that scientists are focusing more on species of conservation concern than on species facing no threat. However, while NT and VU species received much attention from the scientific community, of the 479 scientific articles reviewed, only 22 (<5%) had Endangered and Critically Endangered species as their target. Of these, 16 (>70%) were focused on a single species of economic importance: the scalloped hammerhead (*Sphyrna lewini*). Alarmingly, nearly 50% of shark species are DD, having received virtually no attention by the scientific community. The lack of data for these taxa hampers any attempt at effective management.

There are objective difficulties in conducting informative research on Endangered and Critically Endangered species. Common, widespread and abundant species do not usually go extinct in the blink of an eye: they first become rare. Therefore, species that are facing immediate threats of extinction are often rare to begin with and/or have limited distribution ranges, making collecting data a challenging task. It must be stressed that species that are rare or have low densities at present are not necessarily ecologically unimportant, and may have been abundant and relatively common in the past (Pauly 1995). For example, the angelshark (*Squatina squatina*) was once a common catch in the Mediterranean, but is now extremely rare and has been extirpated throughout most of its range (Cacchi & Notarbartolo di Sciara 2000; Morey *et al.* 2006). Similarly the Harrison's dogfish was a common catch in south-eastern Australia deep water trawling industry, but became rare as a result of overfishing (Graham *et al.* 2001). Adding to the difficulty of obtaining scientific data, Critically Endangered and Endangered species are often endemic to countries that have limited capacity to undertake research. Of the seven species of angelsharks listed as Critically Endangered and Endangered, five occur in countries with a poor record of research in shark management (Taiwan, Argentina, Ecuador) (data from IUCN, http://www.iucnredlist.org/). Another example is the Endangered whitefin topeshark (*Hemitriakis leucoperiptera*), endemic to degraded coastal waters of the Philippines, a country where only a single peer-reviewed study related to shark management and conservation has been conducted in the past 2 decades. The underrepresentation of Endangered and Critically Endangered species in the literature is, in part, related to the strong geographic bias in research effort we reported earlier.

Are shark researchers taxonomic chauvinists?

Sharks are an extremely diverse group, numbering in excess of 500 species. However, more than 60% of the published literature has focused on a limited group of 20 species and the ten most studied species contribute >40% to the scientific output over the last two decades.

Among the 20 most studied taxa, only one is listed globally by the IUCN as Endangered. All but five are either well-known charismatic species, or species that are directly targeted by economically important fisheries. There are of course obvious reasons for concentrating research effort towards species of economic importance. First, to ensure the economic and ecological sustainability of target fisheries. Furthermore, many fisheries are managed on a single species cost-recovery basis and therefore it is easier to obtain public funding for species of economic importance. This is combined with the management requirement to collect data on major target species meaning that this data is often readily available in the form of fisher's logbooks and other records. On the other hand, species-level data for bycatch species of little economic importance are often scarce.

While directed fisheries have contributed to the collapse of many sharks stocks around the globe, the highest threat to sharks is probably represented by mixed fisheries in which species with a low rebound potential are caught as bycatch in an otherwise sustainable industry (Musick *et al.* 2000). Therefore the lack of reliable data and the low level of scientific attention that bycatch species receive may have the effect of delaying management action. One example of the effect of fisheries on bycatch shark species is the collapse of deepwater dogfish (Squalidae) and angelshark (*Squatina* spp.) stocks in south-east Australia. These slow-growing deepwater sharks were caught as bycatch in the lucrative gemfish (*Rexea solandri*) fishery. Since no information on bycatch trends were available for the first three decades of the fishery, by the time the first scientific data on bycatch became available their stocks had already declined by nearly 99% (Graham *et al.* 2001).

Our review also reveals that shark research specifically relating to conservation and management is skewed towards charismatic species. This bias towards charismatic species is not a prerogative of shark researchers. Taxonomic chauvinism is a well-known issue in ecology and conservation science (Bonnet *et al.* 2002; Clark & May 2002). Researchers may choose 'popular' organisms as their target species because of their personal preference, or because it is easier to leverage funding. Furthermore, publishing studies in high impact scientific journals is often easier if a popular species is chosen, reflecting a taxonomic bias in the peer-review process (Bonnet *et al.* 2002). For example, a reasonably trivial discovery, such as the occurrence of sex-

bias dispersal, may earn the authors' a *Nature* paper if the species involved is the great white shark (Pardini *et al.* 2001). Focusing on charismatic species is not necessarily a bad strategy in aiming at conservation for the greater good of marine life in general and flagship species have proven their worth in other fields of conservation. Charismatic sharks may act as "flagships", creating public awareness and stimulating conservation action. However, due to their generally wide ranging habits, and given the absence of informative science for the majority of other taxa, creating public awareness by focusing on flagship species is unlikely to provide major benefits to the majority of shark species.

IS MANAGEMENT EVIDENCE-BASED?

The implementation of management policies is a complex process. While scientific evidence may suggest which actions need to be undertaken to ensure the conservation of a species, other factors play a major role in policy development. Efficient evidence-based management strategies may seem unfeasible because of their economic cost, low chances of success, or because they may not be welcomed by stake-holders and the wider community that perceive them as a limitation of their freedom to use natural resources for economic and leisure activities. Due to the complexity of marine ecosystems as a whole, science may point to management actions that are counter- intuitive and therefore unlikely to win stakeholder support. The result may be failure to initiate conservation action or worse, implementation of management policies that may be achievable, but lack a biological basis and therefore are likely to be ineffective (Svancara *et al.* 2005). Here we review a case in which scientific data informed a successful evidence-based management strategy, and two cases in which no management actions followed scientific assessment.

The management of Sandbar Sharks in the United States and the Mediterranean Sea

The sandbar shark (*Carcharhinus plumbeus*) is a coastal species that supports large commercial fisheries around the globe. Sandbar sharks are large and long lived, reaching sexual maturity at 15-16 years, and have low reproductive output (Sminkey & Musick 1995). Because of their life history strategies they are very susceptible to fishery overexploitation, and have been listed by the IUCN as Vulnerable (Musick *et al.* 2009). While fishery stocks have been overexploited in many different countries, management of sandbar sharks in the United States is an example of appropriate management based on sound scientific data. Here we review the fundamental steps in scientific assessment and policy development of sandbar shark fisheries in the United States, and contrast these with the situation in the Mediterranean Sea.

In the United States, sandbar shark landings started to increase dramatically in the 1980s, following a rise in demand of shark products. Landings peaked in 1989 and started to decline thereafter. By the early 2000s, spawning stocks had declined by approximately 70% (SEDAR 2011). In 1993, the Fishery Management Plan for Sharks of the Atlantic Ocean (1993 FMP) established a framework to determine fishery quotas and bag size limits based on Maximum Sustainable Yield (MSY). In the 1990s and 2000s significant effort had been placed in identifying fishing stocks using genetic and mark-recapture data and collecting life-history parameters to estimate demographic responses to fishery mortality and rebuilding strategies. Most of these efforts led to the publication of high quality peer-reviewed articles that were the basis of stock assessment reports (Grubbs *et al.* 2007; Heist & Gold 1999; Heist *et al.* 1995; McCandless & Frazier 2010; Merson & Pratt Jr 2001; Rechisky & Wetherbee 2003). The National Marine Fisheries Service (NMFS) of the National Oceanic and Atmospheric Administration (NOAA) convened fishery stock assessments for the sandbar sharks in 1996, 2002, 2005/2006 and again in 2011. Following each of the stock assessments, new management strategies were implemented (SEDAR 2011). These included reduced fishery quotas and recreational bag limits (1999), the establishment of time/area fishery closures, prohibition of shark finning (2006), the establishment of a rebuilding strategy (2008) and of a research fishery for the collection of life history traits of sandbar sharks (2008). Since 2008, it has been illegal to land any sandbar sharks without a scientific permit. While stocks are still depleted and recovery may take decades, this is an example of how scientific effort can inform appropriate adaptive management.

Unlike the United States stocks, sandbar sharks are not subject to management in the Mediterranean Sea. Once commonly caught, and often seen at fish markets in the Levantine and Sicily, sandbar shark numbers have experienced a dramatic decline and virtually disappeared from landings in most of the Mediterranean (Ferretti *et al.* 2005) with the exception of the Gulf of Gabès in southern Tunisia (Saïumldi *et al.* 2005). They are still sporadically caught as bycatch in tuna and swordfish longline fisheries (Megalofonou 2005). Following a local assessment, the IUCN listed sandbar shark in the Mediterranean as Endangered (Musick *et al.* 2009); however, no rebuilding strategy has been implemented in the Mediterranean Sea and stocks remains unmanaged. Important nursery grounds have been identified off the coast of Tunisia and Turkey, but no spatial closures have been implemented to protect juveniles. In 2003, an Action Plan for the Conservation of Cartilaginous Fishes in the Mediterranean Sea was released (RAC/SPA 2003), and a new shark assessment report was presented in 2009.

While in these documents the sandbar shark is listed as a commercial species, no stock assessment and no management actions are planned for the Mediterranean Sea.

The Shark Meshing (bather protection) program in New South Wales.

The Shark Meshing (Bather Protection) Program (SMP) was established in NSW in 1937 and has operated continuously since. The SMP was introduced following a spate of shark attacks at Sydney beaches and it is designed to reduce shark attacks and interactions between sharks and swimmers. To achieve these goals, the New South Wales (NSW) government established a system of large mesh gill nets deployed immediately proximate to 51 popular beaches along the coast of NSW. The SMP is not a physical barrier to prevent sharks from reaching popular beaches, but rather aims at reducing interactions with large sharks by culling local populations. The intent of the SMP is to reduce densities of large dangerous sharks (e.g. white sharks, tiger sharks and bull sharks), but these species have always constituted a relatively small proportion of the catch.

In the first decades of the SMP, grey nurse sharks (*Carcharias taurus*) were caught in large numbers (Reid *et al.* 2011), particularly in the Newcastle region circa 200 km north of Sydney [1] where the SMP appears to have been a contributing factor in the dramatic decline of this species (Green *et al.* 2009). The eastern Australian grey nurse shark population is now listed as Critically Endangered under the Environment Protection and Biodiversity Conservation Act (1999)[2] and as an isolated population of <2000 individuals (Ahonen *et al.* 2009; Stow *et al.* 2006) is facing extinction if threatening processes are not mitigated (Otway *et al.* 2004). Few grey nurse sharks are now caught in beach nets in NSW, probably due to the reduction in numbers yet the SMP still accounts for approximately 5-10% of anthropogenic mortality (Reid *et al.* 2011). Accordingly, the SMP remains one of the key threatening factors for the eastern Australian population. Grey nurse sharks are not the only protected non-target species that is caught as bycatch in the SMP. Hammerheads have made up more than 50% of the catch in the SMP over the past two decades (Green *et al.* 2009). Three species of hammerheads are caught in the SMP: the smooth hammerhead (*Sphyrna zygaena*), which likely makes up the vast majority of the catch, the great hammerhead (*S. mokorran*) and the scalloped hammerhead (*S. lewini*). The smooth hammerhead is listed by the IUCN as Vulnerable and the great

[1] The Newcastle region is the area surrounding Newcastle, a coastal city located in the NSW Hunter Region approximately 160 km north of Sydney.

[2] The Environment Protection and Biodiversity Conservation Act (1999) is the most significant federal environmental legislation in Australia, providing the main legislative framework for the protection of the Australian environment, including its biodiversity.

hammerhead (*Sphyrna mokarran*) and the scalloped hammerhead (*Sphyrna lewini*), are fully protected species in NSW and listed respectively as Vulnerable and Endangered. In March 2009, a report to the NSW Department of Primary Industries reviewed the existing NSW SMP and using all the available scientific evidence made explicit recommendations about alternatives to shark nets in order to reduce bycatch in areas where grey nurse sharks are caught (Green *et al.* 2009). While the SMP appears to have been effective in reducing shark fatalities since its establishment, alternative bather protection programs exist, that are more selective with respect to target species. One alternative strategy is to replace part of the nets with baited drumlines, which consists of baited hooks secured above the bottom (Dudley *et al.* 1998; Sumpton *et al.* 2011). A long-term study in Queensland showed that baited drumlines are efficient in catching dangerous sharks, while greatly reducing bycatch of endangered shark species including hammerheads and grey nurse sharks as well as increasing bycatch survival rates (Sumpton *et al.* 2011). The partial replacement of mesh nets with baited drumlines has been shown to be effective in avoiding an increase in shark attack in South Africa (Cliff & Dudley 2011), where the replacement program is now being expanded.

Despite evidence that the SMP is classified under legislation as a Key Threatening Process[3] to more than one protected, threatened species in NSW; that alternatives exists and that all the science points to the effectiveness of alternatives both to mitigate conservation issues and maintain protection, management decisions do not concur. The NSW government has continued the SMP in its current form despite recommendations solicited directly from its own Department of Primary Industries (Green *et al.* 2009). In fact, the SMP has been championed by the NSW government for decades as an effective strategy for bather protection, and it is likely that an extensive review of the SMP will attract a heated public debate.

THE ADAPTIVE MANAGEMENT APPROACH- RESOLVING THE SCIENCE MANAGEMENT DISCONNECT?

In summary, we have seen that science can effectively inform appropriate management policy but that critical to effective management is that the stakeholders, including science practitioners, are all brought on board in the process. How can this be done most effectively? We propose that one method that may help reduce the apparent disconnect is to adopt the adaptive

[3]A Key Threatening Process is a process that threatens or may threaten the survival, abundance or evolutionary development of a native species or ecological community. The SMP is listed as a Key Threatening Process under the NSW Fisheries Management Act 1994 and the Threatened Species Conservation Act 1995 because of its adverse effects on great white sharks (*Carcharodon carcharias*), the critically endangered grey nurse shark (*Carcharias taurus*), hammerheads (*Sphyrna* spp.) and other non-target sharks, as well as vulnerable and endangered marine mammals (dugons, fur seals etc..) and turtles.

management approach based on the Adaptive Environmental Assessment and Management (AEAM) process originally proposed by Holling (1978). Research suggests that this approach provides an effective tool in the long-term management of complex and uncertain environments (Walters 1986; Walters 2007; Walters & Green 1997).

The adaptive management approach provides a useful framework because the objective is to formulate a workable management plan while also trying to gain a better understanding of the underlying ecology through experimentation. AEAM probes the dynamic responses of a system in order to improve management and is particularly important when counterintuitive responses arise. For example, scientists or managers may attempt to base predictions on simple, common sense arguments (e.g. ‘‘reducing mortality rate of sharks should cause their abundance to increase’’), when in fact the complexity of ecological systems means that responses may depend on indirect and multiple causal pathways whereby reduction in mortality in one life history phase may actually decrease production. Management problems typically differ from pure scientific problems in a number of ways:

1) management questions commonly imply a much broader perspective on a system than technical experts adopt;

2) management questions are about distinguishing between alternative policies, not about precision in prediction, as such; and

3) the breadth of interests implied in management questions requires confronting ignorance and uncertainty.

The adaptive management approach confronts these issues explicitly. Its core methodologies include:

1) Bringing multiple perspectives into dialogue to develop a broad view of the problems.

2) Embracing uncertainty by focusing on exploring its shape, rather than by trying to eliminate it across the board.

We suggest that an appropriate approach to resolve the disconnect is for research to be driven by a focus on achieving workable management outcomes informed as much as possible by objective scientific investigation. A powerful method to enable this approach consists of conducting workshops involving key stakeholders (fisheries management agencies, scientists, shark fishing / tourism industry etc), the objectives of which are to:

1) identify the various perspectives on the problem;

2) seek to reach consensus on the nature and scope of the problem being addressed;

3) achieve a better understanding of shark/ fisheries interactions and to gain an understanding of all information currently available;

4) identify gaps and shortfalls in existing information;

5) prioritise data gathering efforts to focus on that information required to direct management or to test specific hypotheses; and

6) identify potential management objectives

Walters (2007) has outlined both the strength of this approach and also identified the risks of failure. He points out that while AEAM workshops frequently can provide consensus about the need for an experimental management program, that without a champion (usually from within a regulatory agency, the '*compleat emmanuensis*' *sensu* Holling 1978), AEAM programs fail to materialise.

Given the rate at which shark numbers are declining, and the relatively limited scientific effort apportioned to understanding their demise, the time is ripe for a renewed attempt to enact management with a common purpose and with the highest chance of success. We have learnt only too well that management cannot succeed without stakeholder support. The most powerful management incorporates science as a means of informing best practice. AEAM has struggled not to gain consensus in terms of desired outcomes, which is relatively straightforward, but to follow through with effective experimental approaches due to the complexity of marine ecosystems combined with the expense of monitoring the process.

We suggest that with the new opportunities that arise from technological advances, we might be on the verge of a new and effective era in management. With new technology comes the ability to create novel and innovative monitoring systems that use the experience and expertise of the industry partners themselves (eg electronic tagging, geo-referenced GPS, electronic logbooks) to economically collect the data. This is what is required for large scale AEAM experimentation and is now feasible even in relatively poor nations. This combined with the ability to economically communicate widely and swiftly to all stakeholders and for real time

monitoring of catches, combined with the ability to model ecosystems with a much higher degree of finesse, bodes well for future success.

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Chapter 3

Predators in danger: shark conservation and management in Australia, New Zealand, and their neighbours.

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SUMMARY

In this chapter we examine the biodiversity and the status of conservation and management of shark species in Australasia and Indonesia. Almost 17% of shark species in the region are listed by the International Union for the Conservation of Nature (IUCN) as threatened, and approximately 40% are of conservation concern, their future being dependent on the implementation of appropriate management strategies.

Overfishing is a major threat to sharks, as their life history strategies make them susceptible to even modest levels of fishing mortality. In Australia and New Zealand many shark stocks experienced dramatic declines as a consequence of overfishing , including species such as Harrison's dogfish (*Centrophorus harrisoni*), school sharks (*Galeorhinus galeus*) and sandbar sharks (*Carcharhinus plumbeus*) ; however in the past decades substantial improvements in the management of shark fisheries have taken place. On the other hand, shark fishing in Indonesia is largely unreported and unregulated and fishing by Indonesian vessels is likely to have consequences that go beyond the depletion of local populations, affecting shark populations in neighbouring countries such as Australia.

We illustrate examples of overfishing in the region, discuss the potential effects of habitat degradation and climate change in the future and examine current management frameworks for the conservation of shark species in the region with an emphasis on the implementation of Nation Plans of Action for the Conservation and Management of Sharks (NPoAs).

SHARK BIODIVERSITY IN THE INDO-AUSTRALASIAN REGION

The history of shark evolution has been one of enduring success. Since their first appearance in the late Silurian more than 400 MYA (Last & Stevens, 2009), sharks have colonized most of the marine realm, from the clear tropical waters of shallow coral reefs to the freezing depths of the Arctic Circle (Benz *et al*., 2004). With over 500 extant species representing eight orders and more than 30 families, sharks are an extremely diverse group (Compagno, 2001). A few shark species (approximately 5%) are oceanic species with a circumglobal distribution while the vast majority of taxa are comprised of demersal species, which inhabit the tropical and temperate continental shelves and slopes (Tittensor *et al*., 2010).

At the global scale, patterns of shark species richness are governed by a small set of environmental variables, with sea surface temperature (SST), coastline length, and primary productivity being the main drivers of diversity (Lucifora *et al.*, 2011, Tittensor *et al*., 2010). Shark species richness is highest at mid latitudes (20º- 40º) in coastal areas with high

productivity (Lucifora *et al*., 2011, Tittensor *et al*., 2010). Indo-Australasia, the geographic region including Indonesia, Australia, New Guinea, New Zealand and New Caledonia supports an extraordinary diversity of shark species. Approximately 240 species are found in the continental shelves and slopes of this region, accounting for nearly 50% of global diversity. Within the region, diversity is highest in Australia (182 species), which alone accounts for 36% of global biodiversity (Last & Stevens, 2009). Indonesia, with approximately 100 species is second, and the remaining subregions (New Zealand, New Guinea and New Caledonia) follow with approximately 60 species (White & Kyne, 2010). The highest species richness is found on the temperate continental shelf of New South Wales, the tropical waters of the Great Barrier Reef on the eastern Australian coast, and the coral reefs of Western Australia (figure 1A). A large proportion of the shark species that inhabit the region are found nowhere else in the world, and Australia has the highest proportion of endemic species (39%).

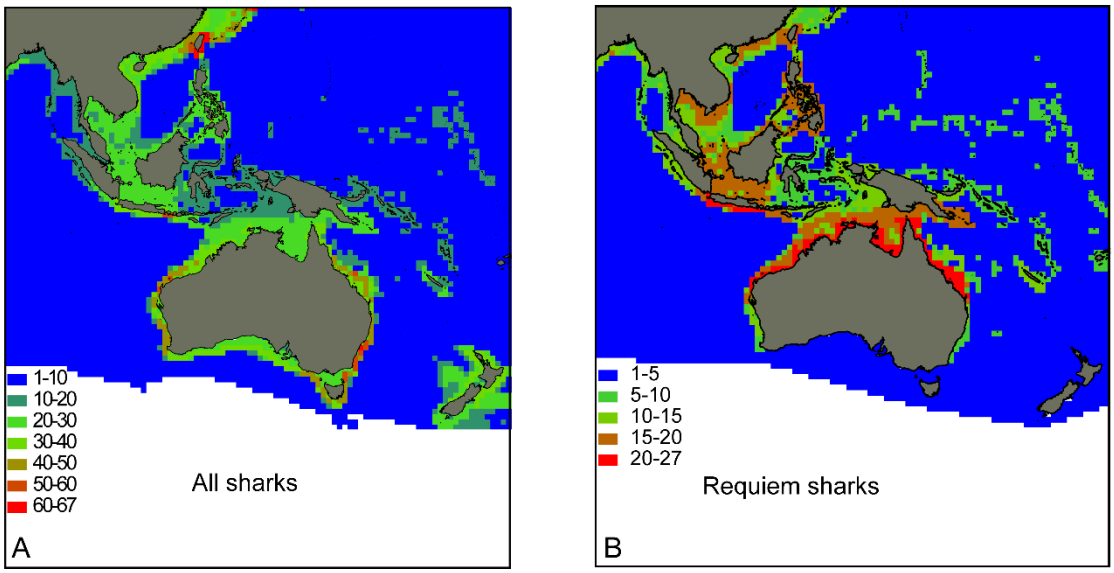

Figure 1*: Heat map representing shark species richness in the Indo-Australasian region. All shark species (Figure 1A) and requiem sharks (Figure 1B). Data on species distribution were obtained from the Global Shark Distribution Database (http://www.globalshark.ca/gs_distribution_db/data.php), and were originally published by Lucifora* et al. *(2011)*

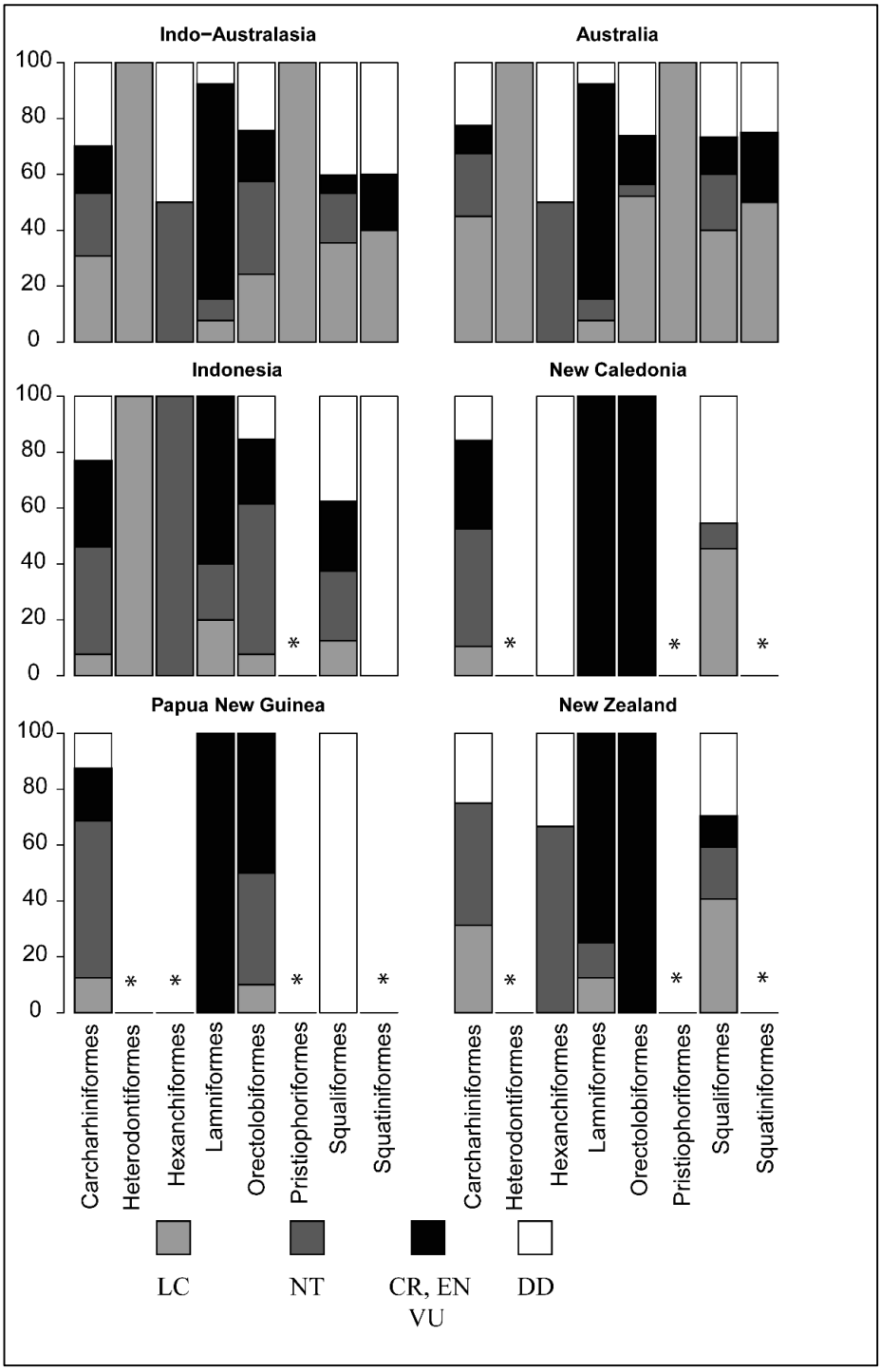

***Figure 2**: Proportion of species Threatened (CR, EN, VU), Near Threatened (NT), Least Concerned (LC) and Data Deficient (DD) in the Indo-Australasian region. Data obtained from the International Union for the Conservation of Nature (IUCN, http://www.iucnredlist.org/).*

Diversity and endemism are very high for a few families of sharks. One of these families are the Orectolobidae (wobbegongs), which are bottom-dwelling sharks that occur mainly in the western Pacific Ocean. This family includes 12 species, 11 of which occur in Indo-Australasia:

seven are endemic to Australia, another four to the Australasian region and only one species (*Orectolobus japonicus*) is known to occur elsewhere (Last & Stevens, 2009). Of approximately 27 species of dogfishes (Squalidae) recorded globally, 19 occur in Indo-Australasia, and 12 are found in Australia (nine of which are endemic). The Indo-Australasian region also supports 36 species of whaler sharks (Carcharhinidae), approximately 70% of global diversity with at least six endemic species. Requiem sharks are a large family that includes many apex predators of commercial importance such as the blacktip shark (*Carcharhinus limbatus*) and the dusky whaler (*Carcharhinus obscurus*) (Last & Stevens, 2009). Sharks belonging to this family tend to favour warm, tropical coastal waters and species richness is highest on the tropical continental shelves of Northern Australia and Indonesia (figure 1B).

Owing to increasing fishing pressure and habitat degradation, shark populations in the region are under threat (figure 2). At the time of writing almost 17% of all shark species in the Indo-Australasian region are listed as Critically Endangered (CR), Endangered (EN) or Vulnerable (VU) by the International Union for the Conservation of Nature (IUCN) and approximately 40% are listed as species of conservation concern (CR, EN, VU and Near Threatened-NT) (data from IUCN: http://www.iucnredlist.org/, see also figure 2). The growing threats to shark biodiversity in the region have potential consequences that go far beyond the extinction of a few shark species. Sharks are an extremely important part of the ecosystem, and fulfil a range of ecological roles which are crucial in maintaining ecosystems. If they disappear, consequences are likely to be far-reaching (Box 1).

Box 1: The ecological consequences of shark declines

Sharks are generally classified as high trophic level predators (figure 3), which are thought to structure marine communities in two main ways: 1) through the removal of prey, and 2) by altering the behaviour or distribution of prey species through predator avoidance, termed 'risk effects' (Heithaus et al., 2009). The role of sharks and the effects of their removal have been modelled based on dietary studies for some economically important species (Kitchell *et al*., 2002). In particular, there has been an increase in studies that have addressed the ecosystem effects of declines in shark populations, which are due to overwhelming increases in shark removal, particularly in coastal areas (Myers *et al*., 2007, Robbins *et al*., 2006). The effects of predator removal have been examined by quantifying the abundance and distribution of sharks and associated prey or benthic communities (Friedlander & DeMartini, 2002). One example found that illegal fishing pressure on north Australian reefs, by artisanal fishers predominantly from Indonesia (see Box 3), led to total absences and decreased numbers of large species of sharks (Field *et al*., 2009b). Studies of pristine vs. fished reef communities in Hawaii have also noted comparably fewer large predators on fished reefs, and an increase in biomass of primary (herbivores) and secondary consumers (planktivores), and primary producers (macroalgae) (Friedlander & DeMartini, 2002, Sandin *et al.*, 2008).

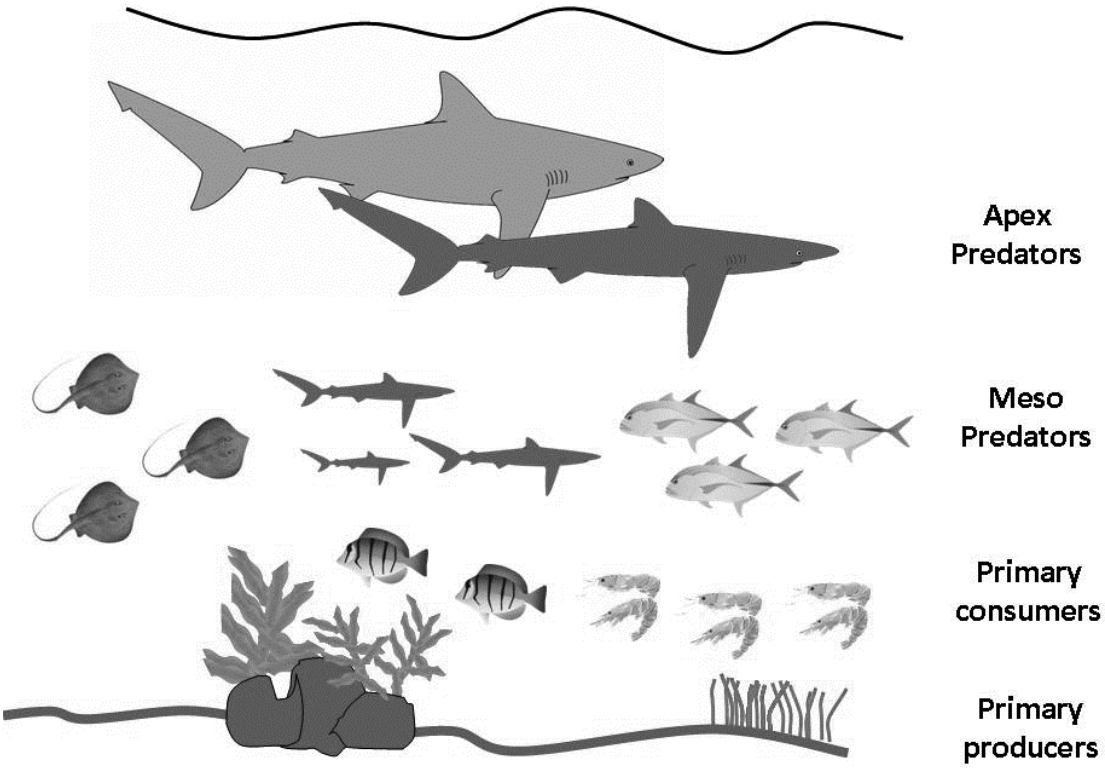

***Figure 3**: Schematic representation of a marine food web, showing sharks as high trophic level predators.*

In some instances, the removal of apex predators has led to an increase in mesopredator populations, known as 'predator release' (Myers *et al*., 2007), or even resulted in trophic cascades (Heithaus *et al*., 2008). A study of coastal elasmobranch (sharks and rays) communities in the northwest Atlantic found that there was a dramatic decrease in abundance of large apex predators (e.g., bull, tiger, scalloped hammerhead sharks) and a concordant increase in mesopredators that are preyed upon by apex predators (e.g., cownose rays, little skates, and chain catsharks) (Myers *et al*., 2007). This increase in mesopredators also coincided with a decrease in abundance of bay scallops, which are a known prey item of the cownose ray, leading to the collapse of the bay scallop fishery, which had been operating for a century.

A more recent study found that the lack of reef sharks on remote fished reefs in north-western Australia led to both predator release and trophic cascades (Ruppert *et al*., 2013), which showed a stark contrast to healthy unfished reefs in the region. Community level responses on fished reefs were shown to be a result of both top down effects due to shark removal and bottom up effects from cyclones and coral bleaching. The authors also provide evidence that shark removal not only created mesopredator release, but also reduced the number of primary consumers (herbivores).

The complex behavioural interactions between sharks and their prey can provide further insights into how sharks indirectly structure prey communities and prey resource

availability. For example, studies on the west Australian coast have found that the presence of tiger sharks affects community dynamics of their preys (dolphins, dugongs, turtles, seabirds), which respond by moving away from prey-rich shallow habitats when sharks are present, into deeper habitats with lower prey abundance (Heithaus *et al*., 2009). This shift in behaviour could potentially affect growth or reproduction potential of prey species due to inadequate use of resources, and also result in a reduction of seagrass grazing in dangerous areas and intensification of grazing in safe habitats (Heithaus *et al*., 2008).

Shifts in prey community structure are possibly a result of both removal of prey as well as risk effects, although there is some controversy surrounding this hypothesis due to non-convergence of dietary links and differences in the distribution of meso- and apex predators (Heithaus *et al*., 2010). The role sharks play in shaping marine ecosystems is therefore likely related to their abundance, distribution and foraging strategies, as well as prey removal. Our current limited understanding of these processes and their importance to marine systems is of concern given rapidly declining shark populations and increasing human populations within the Indo-Pacific region. Determining the functional role of differing trophic groups (secondary consumers, meso-, and apex predators) is essential to assessing how marine systems will cope with reduced numbers of sharks in the future.

SHARKS' LIFE HISTORY STRATEGIES: IMPLICATIONS FOR MANAGEMENT AND CONSERVATION.

Sharks are usually long-lived, slow-growing and take a long time to reach sexual maturity; however, considerable differences in life history traits do exist. The spiny dogfish (*Squalus achantias*) for example can live up to 70 years and reaches sexual maturity at more than 20 years, but age at maturity can be as low as 1 to 2 years for the Australian sharpnose shark (*Rhizoprionodon taylori*) (Last and Stevens, 2009). In general, age at maturity has a bimodal distribution, with most species reaching maturity at 5-6 years or 15-25 years. Fertilisation is internal in all shark species, and embryonic development follows three distinct patterns (Wourms & Demski, 1993). In some species (most whaler sharks and hammerhead sharks) the embryos develop within one of the uteri and are attached to a yolk-sac placenta from which they receive nutrients (placental viviparity). In ovoviviparous species (such as cow sharks-family Hexanchidae), the embryos develop in the uterus but food is supplied by large yolk-sacs, while oviparous species such as horned sharks (Heterodontidae) lay eggs protected in leathery cases where the embryos develop (Last and Stevens, 2009). These strategies all require considerable energetic investments and result in the production of a small number of well-developed pups with high survival rates. Reproductive rates are variable among species. In some taxa females are able to produce a new litter every year, while other species require one or more years of "rest" between pregnancies. The school shark (*Galeorhinus galeus*), a species of low productivity and high commercial importance in Australia, reproduces every third year (Lucifora *et al*., 2004). The vast majority of species produce a small number of pups (5-15) (Wourms & Demski, 1993).

These life history strategies differ substantially when compared to most commercially harvested teleosts (bony fish), and these differences bear important implications for conservation and fisheries management. Teleosts have extremely high fecundity, producing thousands to millions of eggs per year. Mortality is very high and density-dependent, and the result is that recruitment to adult populations is broadly independent of adult population size (Shepherd & Cushing, 1980). As density decreases due to fishing mortality, resources become more abundant and competition decreases, boosting productivity through density-dependent compensations on recruits' survival. Therefore, teleost adult populations can be significantly depleted without negative effects on recruitment. In sharks, however, stock size and recruitment are closely linked and since fecundity and juvenile mortality are low, any density-dependent compensation in terms of survival rates is greatly constrained (Smith *et al*., 1998). Recruitment overfishing, the level of population depletion at which recruitment becomes affected, therefore will start to occur early in the history of a shark fishery (Smith *et al*., 1998).

Due to their life history characteristics, many taxa of commercial importance are particularly vulnerable to overexploitation through fisheries. For example, spurdogs (Squalidae) and gulper sharks (Centrophoridae) have been extensively harvested in south-eastern Australia despite their slow growth and low productivity, resulting in severe declines (refer to Box 2). Therefore, strict management is recommended for species with low rebound potential, but it is also likely that a certain level of protection is necessary to maintain sustainable fisheries of most coastal species. Management actions should take into account the basic life history strategies of sharks, and focus on maintaining reproductive potential, taking into account the strong relationship between adult population size and recruitment.

THE STATUS OF SHARK CONSERVATION IN THE INDO-AUSTRALASIAN REGION

Fishing pressure is the main anthropogenic process threatening shark populations on a global and local scale. According to the United Nations' Food and Agriculture Organization (FAO) landings in the last six decades have nearly tripled, resulting in a worldwide decline of shark populations. The International Union for the Conservation of Nature (IUCN) carried out extensive assessments worldwide on the conservation status of shark species, and recently considerable effort has been placed on local assessments of shark populations in the Indo-Australasian region (Cavanagh *et al*., 2003, White & Kyne, 2010). At the regional level, approximately 17% of the total number of species is under threat, being listed by the IUCN as Critically Endangered (CR), Endangered (EN) or Vulnerable (VU) (figure 2). Approximately

40% are of conservation concern, being listed either as Threatened (CR, EN, VU) or Near Threatened (NT), and will be dependent on the establishment of effective management in the future. Only 29% of the species in the region are listed as Least Concern (LC), and the remaining 31% are data deficient.

The conservation status of a species is the product of the threatening processes to which it is subjected, the extent of its distribution, the level of habitat specialization and the life history characteristics that determine how populations respond to such processes. Species with limited distributions, subjected to high fishing pressure throughout their range and with low rebound potential are under the highest threat of extinction (Field *et al*., 2009b). Sharks exhibit a variety of life history strategies, and fishing pressure varies greatly among shark species and geographic areas within the region. As a result different taxa are inherently more vulnerable than others, and species that may be under imminent threat of extinction in one region may be of no conservation concern in others.

The proportion of species under threat and of conservation concern is highest in Indonesia and Papua New Guinea, where more than 30% of shark species are listed as Threatened and the vast majority of sharks (66 and 78% respectively) are of conservation concern (White & Kyne, 2010). New Zealand and New Caledonia follow with 21 and 35% of threatened species, and 47 and 59% of species of conservation concern respectively (White & Kyne, 2010). Australia is the country with the fewest threatened species, as considerable efforts have been put in place over previous decades in developing effective management strategies. Some species that are listed as threatened in the rest of the region (such as the zebra shark *Stegostoma fasciatum*), are of no conservation concern in Australian waters (Cavanagh *et al*., 2003). A few shark species, however, are under greater threat in Australia than anywhere else. This is the case for Harrison's dogfish (refer to Box 2), and for the critically endangered eastern population of grey nurse sharks (*Carcharias taurus*) which is demographically isolated and continues to decline despite being protected since 1984 (Ahonen *et al*., 2009, Otway *et al*., 2004).

One group, the Lamniformes, have low to moderate rebound potential (Smith *et al*., 1998) and are under fishing pressure throughout the region, where they are either directly targeted for their fins and meat or caught as bycatch in the billfish and tuna long-line fisheries (Francis *et al*., 2001, Dharmadi *et al*., 2007). As a result the vast majority of Lamniformes are currently listed as under threat (figure 2). Within this order are four families for which 100% of the species are listed as under threat throughout the region: Alopiidae (thresher sharks), Lamnidae (mackerel sharks), Odontaspididae (sand tiger sharks) and the monospecific family of the basking shark

(Cetorhinidae). Another family of sharks that is of high conservation concern are the hammerhead sharks (Sphyrnidae, order Carcharhiniformes). Four species of hammerhead shark occur in the region, two of which are Endangered (*Sphyrna lewini* and *Sphyrna mokarran*), one Vulnerable (*Sphyrna zygaena*) and one Near Threatened (*Eusphyra blochii*). Hammerhead sharks are under heavy fishing pressure in the region, particularly in Indonesian waters where they are directly targeted (particularly *S. lewini*) for their fins and meat (White *et al*., 2008); as a result, they were recently listed on Appendix 2 of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Since the stocks of these species are likely shared between Indonesia and neighbouring countries (Ovenden *et al*., 2009) , the effect of overfishing in Indonesian waters may have consequences that go far beyond national boundaries.

Not all sharks are under threat from overfishing. Many species are not under strong fishing pressure, and some have a high rebound potential. Bull-headed sharks for example (Heterodontidae) are relatively fecund (Last & Stevens, 2009) and there seem to be no major threats to these species in the region. Of the 15 species of lantern shark (family: Etmopteridae, order: Squaliformes) present in the region, 11 are not facing any threat of extinction and four are listed as data deficient. While lantern sharks are deep-water species with strongly K-selected life history traits (Kyne & Simpfendorfer, 2007), they are not directly targeted in the region because of their small size and low commercial value. Some species however are caught as bycatch by deepwater trawl fisheries. While they are usually discarded, survival rates from trawl discards are likely to be low (Gordon, 2001) and the current expansion of deepwater trawl fisheries may pose higher risks to these species in the future.

Box 2: Fishing the depths: the collapse of deepwater shark populations in south-east Australia.

Deepwater sharks inhabit the cold depths over and beyond the continental slope. Historically, they were protected from overfishing until the second half of the 20th century, when the decline of shallow water fisheries and the advancement of fishing technologies drove a global shift to deeper fishing grounds (Morato *et al*. 2006). Deepwater fisheries are seldom sustainable; life in the deep has a slow pace and most species have conservative life history strategies that make them extremely susceptible to overfishing (Kyne & Simpfendorfer 2007). Bycatch is high, and discarded catch has little chance of survival (Gordon 2001). Detailed life history data and long-term fishery trends are missing for the vast majority of deepwater sharks, hindering appropriate assessment of the sustainability of current catches. In the few cases for which long-term data are available, the outlook is grim.

A rare case where extensive data are available is the deepwater Commonwealth Trawling Sector (CTS) operating in south-east Australia. The CTS, part of the Southern and Eastern Shark and Scalefish Fishery (SESSF), is one of the principal threats to deepwater shark populations in Australia. Its fishing grounds extend from Sydney southwards to Tasmania, and westwards to Cape Jervis in South Australia. Commercial trawling of the upper slopes started in 1968, primarily targeting gemfish, but dogfishes (Squalidae) and angel sharks (*Squatina* spp.) were a valuable bycatch of the fishery, particularly as demand for squalene (an oil extracted from deepwater shark livers) increased (Graham *et al*. 2001). Species caught included slow-growing gulper sharks (*Centrophorus harrisoni*, *C. zeehani* and *C. moluccensi*) as well as other species of dogfishes and the sharpnose sevengill shark *Heptranchias perlo* (Graham *et al*. 2001). Gulper shark stocks were substantially depleted as the fishery developed, and by the late 1990s stocks of the upper slope of NSW had declined by 98-99% (Graham *et al*. 2001). A decline of >90% was recorded also for the greeneye spurdog (*Squalus chloroculus*), and similar (albeit less dramatic) trends have been reported for other species of dogfishes and the sharpnose sevengill shark.

The broad-scale ecological consequences of such declines are largely unknown. Gulper sharks and sevengill sharks are high trophic level organisms, and a depletion of >90% of apex predator biomass is likely to have far-reaching top-down effects (see Box 1). Both the Harrison's dogfish (*C. harrisoni*) and the southern dogfish (*C. zeehani*) are endemic species, and their entire range falls within the SESSF-managed area. As a result of these declines the Harrison's dogfish has been listed as Critically Endangered by the IUCN, and the southern dogfishe as well as the greeneye spurdog have been nominated to be listed as Threatened by the Environmental Protection and Biodiversity Act 1999 (EPBC Act).

The Australian Fisheries Management Authority (AFMA) implemented a management strategy involving a drastic reduction in fishing effort and the total closure to fisheries of approximately 25% of suitable habitat area, with the aim of rebuilding stocks to 25% of the virgin population size (AFMA 2012). Despite management efforts, a recent review suggests that these measures may not be sufficient to prevent further decline and promote the recovery of these species (Musick 2011). The conservative life history strategies of these organisms, coupled with the strong stock-recruitment relationship that characterises shark populations means that even if appropriate management strategies are in place, recovery will be extremely slow. Many decades (nearly a century for the Harrison's dogfish) may be required for these species to recover to approximately 25% of pre-exploitation levels.

MAJOR THREATENING PROCESSES

Overfishing in Australian waters

Australia has one of the most extensive Exclusive Economic Zones in the world, extending for more than 8 million km^2 and harbouring a very high diversity of shark species. Shark fisheries, however, make up a small fraction of the country's landings. Over the past 20 years, catches ranged between 6,700 and 11,500 t per year. Landings increased in the early 2000s, reaching 11,500 t in 2005 but as a result of the implementation of several fishery restrictions declined to less than 7,000 t in 2010 (data from FAO). This equates to less than 1% of the global catch, and less than 10% of the catch of Indonesia, one of Australia's neighbours. In Australia sharks are caught by commercial, recreational and traditional fisheries. Within commercial fisheries, sharks are both directly targeted and caught as bycatch, which may be retained or discarded. Shark fisheries fall within multiple jurisdictions, with about 50% of shark catches falling within the jurisdiction of Commonwealth Managed Fisheries, while the rest is managed by individual states (Woodhams *et al*., 2012).

Southern and Eastern Shark Fisheries. The Commonwealth managed Southern and Eastern Scalefish and Shark Fishery (SESSF), where sharks are targeted within the Gillnet Hook and Trap and Commonwealth Trawl Sectors (CTS), is responsible for approximately half of the Australian shark catch (Woodhams *et al*., 2012).

The main target of the SESSF is the gummy shark (*Mustelus lenticulatus*), which is harvested by gillnets. Gummy sharks are not currently overfished and there is no major concern about the sustainability of the fishery in the future, however the same fishery has historically targeted school sharks (*Galeorhinus galeus*), a species more prone to overfishing. Since the 1920s, school sharks were directly targeted using longlines, but declining school shark catches and concerns over high mercury content (which culminated in a ban on the sale of large school sharks in Victoria during the mid 1980s), combined with the adoption of monofilament gillnets, resulted in a transition towards higher targeting of gummy sharks in the 1970s and 1980s (Punt *et al*., 2000). School sharks are now considered as bycatch of the gummy shark fisheries, however landings are still substantial (averaged at 325 t per year between 2001 and 2006). Multiple assessments concluded that school shark stocks have been severely depleted, with estimated biomass in the mid-1990s ranging from 15 to 45% of pre-exploitation equilibrium size (Punt *et al*., 2000), and recruitment in 1997 estimated to be only 12 to 18% of pre-exploitation levels (Punt *et al*., 2000). In 2009, the species was listed as conservation dependent under the Environmental Protection and Biodiversity Conservation Act (EPBC Act), and a

rebuilding strategy based on bycatch quotas and closure of some fishing grounds has been set in order to rebuild stock biomass to 20% of virgin biomass by 2020. Adult biomass levels have since stabilised, however the current harvesting level is still too high to allow recovery, and recent biomass estimates range between 8-17% of pristine levels (Woodhams *et al*., 2012).

The deepwater commonwealth trawling sector (CTS) of the SESSF operating in south-east Australia is a major threat to deepwater shark populations (see Box 2). While gemfish are the main target of the fishery, dogfishes and angel sharks are important bycatch. Increased fishing effort in the region in the 1970s, 1980s and 1990s has resulted in a serious depletion of deepwater shark populations, with declines of up to 99% (Graham *et al*., 2001). Despite management effort in the past decade, there are serious concerns that these measures may not be enough to promote the recovery of affected populations (Box 2).

Northern Shark fisheries. On the northern coast of Australia, direct targeting of sharks using gillnets and longlines started in the early 1980s. The main target group are requiem sharks (Carcharhinidae), including blacktip (*Carcharhinus limbatus* and *C. tilstoni*), spot-tail (*C. sorrah*), sandbar (*C. plumbeus*), and dusky sharks (*C. obscurus*), although hammerhead (*Sphyrna* spp.) and tiger sharks (*Galeocerdo cuvier*) are often caught (Woodhams *et al*., 2012). There are concerns over the sustainability of the blacktip shark fishery in the Northern Territory, where catches reached a peak of nearly 900 t in 2003 and were still around 800 t in 2010, but dropped to approximately 400 t in 2011 (Northern Territory Government, 2011). An assessment of the sustainability of the fishery is complicated by the fact that substantial illegal fishing from foreign boats occurs in the area (see Box 3). There is also uncertainty about the sustainability of the blacktip shark fishery in the Gulf of Carpentaria off the Queensland coast (Roelofs, 2012). Furthermore, the fact that Australian blacktip (*C. tilstoni*) and common blacktip (*C. limbatus*) sharks hybridise and can be distinguished only via vertebrae counts or using genetic methods, complicates the assessment of the fisheries for these species (Boomer *et al*., 2010, Ovenden *et al*., 2010).

On the north coast of Western Australia there was a tenfold increase in fishing effort in the period 2000-2005, with catches reaching more than 1000 t in 2004-2005. An assessment by the Western Australian Department of Fisheries (DoF WA) concluded that fishing mortality was unsustainable for the sandbar shark (the principal target), and there were serious concerns about the sustainability of several other species (McAuley & Sarginson, 2011). The DoF WA implemented drastic management measures in order to reduce fishing effort, setting a 20 t per year target. No fishing occurred in 2009/2010, however breeding stock levels of sandbar sharks

remain low, and given the long lifespan and slow growth of this species, future recovery will be slow (McAuley & Sarginson, 2011).

Box 3: Fishing across the border: Indonesian fishing in Australian waters

Illegal, unreported and unregulated (IUU) fishing poses a severe threat to shark populations globally (Bonfil 1994). Within Australia, illegal fishing for sharks is largely attributed to Indonesian and, in smaller numbers, Taiwanese vessels, which venture into the Australian Fishing Zone (AFZ) to harvest shark products (mainly the highly priced fins) (Marshall 2011). Fishing activity by Indonesian fishers in the shallow waters along Australia's northern continental shelf began long before European settlement and it was not until the mid-20th century that it became illegal (Máñez & Ferse 2010). In 1933, Australia gained sovereignty over Ashmore Reef and Cartier Island after the Western Australian Government appealed to the Commonwealth about illegal Indonesian fishing. However, surveillance of the area was infrequent and Indonesian fishing in northern and north-western Australian waters continued relatively unhindered until the late 1960s (Stacey 2007).

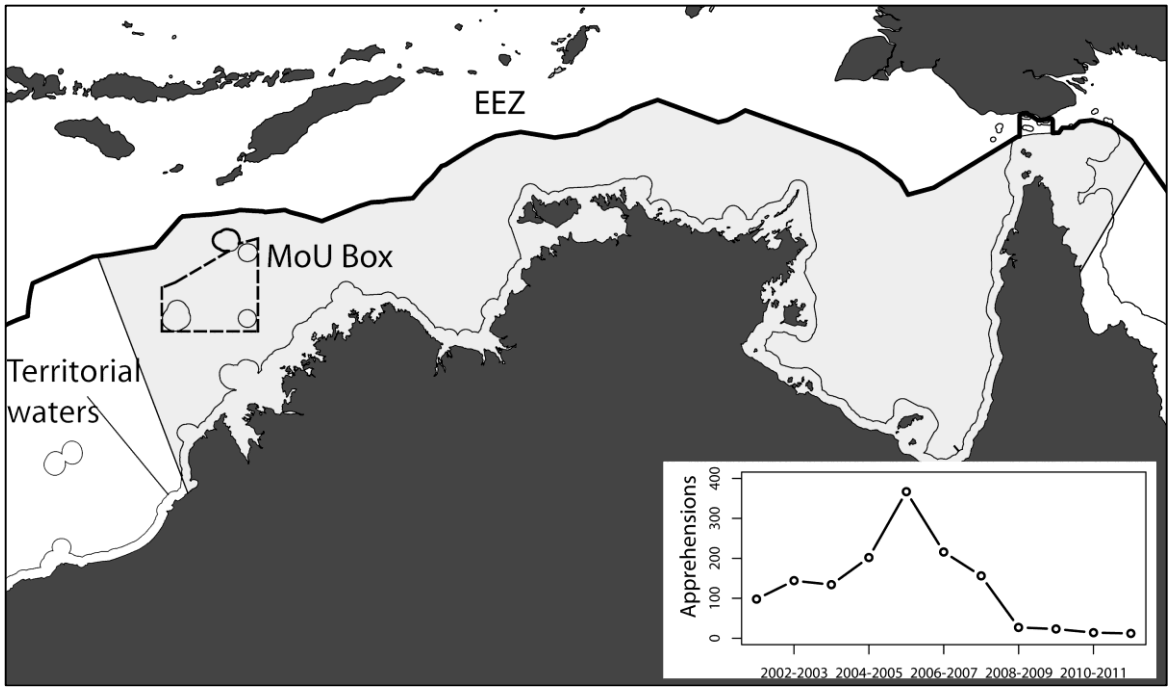

Figure 4: *Map of the Exclusive Economic Zone off the northern Australian coast. The area mainly affected by IUU from foreign vessels is highlighted in grey. The inset shows trends in apprehensions of foreign fishing vessels in the past decade (data from the Australian Fisheries Management Authority).*

The expansion of the AFZ to 12 nm (1968), and later to 200 nm (1979) with the declaration of Australia's Exclusive Economic Zone (EEZ) (figure 4) brought about the loss of vast fishing grounds that had been frequently fished by Indonesian fishers since at least the 1920s (Stacey 2007). A Memorandum of Understanding (MoU) between Australia and Indonesia prohibited Indonesian fishing in the AFZ, but allowed traditional fishing with unmotorised vessels on specified offshore islands and reefs under Australian jurisdiction in the Timor Sea, an area known as the 1974 MoU box (figure 4). Shark fishing in the areas open to traditional fishing became more important from the early 1980s onwards, driven by a sharp increase in the demand for shark fins (Stacey 2007).

The loss of shallow water fishing grounds along the continental shelf and the confinement to the deeper water fishing grounds of the MoU Box led to the development and use of new fishing techniques, particularly longlines in place of traditionally used shark rattles (goro-goro) and hand lines, which proved ineffective in deep water (Stacey 2007). This development of fishing gears demonstrates that the word 'traditional', if used in relation to fishing techniques that are –erroneously – thought to remain consistent over time, is problematic. As conditions have changed, 'traditional' Indonesian shark fishers have adapted their fishing grounds and techniques in response to market demand, catch rates and international regulations (Fox *et al.* 2009).

Three decades of heavy shark fishing in Eastern Indonesia have resulted in depleted local fishing grounds (Field *et al*. 2009a) causing a southward movement of fishing effort into Australian waters, where sharks are reportedly larger in size and more abundant (Field et al. 2009a) (pers. obs. V. Jaiteh 2012). A study of catch composition from 13 Indonesian and two Taiwanese foreign fishing vessels (FFVs) that had entered the AFZ between 2006-2009 identified 1182 individual sharks from 33 species within 8 families (Marshall 2011). Members of the family Charcharhinidae dominated the seized illegal catch, both in terms of numbers and biomass. Silky sharks (*Carcharhinus falciformis*) and blue sharks (*Prionace glauca*) were the most abundant species while blacktip sharks (*C. limbatus*/*tilstoni*) were the third most abundant species in terms of numbers, and the less abundant but larger tiger sharks (*Galeocerdo cuvier*) made the third biggest contribution to the overall biomass of the total catch. Certain species, such as the grey reef shark (*C. amblyrhynchos*), spinner shark (*C. brevipinna*), pigeye shark (*C. amboinensis*), bull shark (*C. leucas*) and scalloped hammerhead (*Sphyrna lewini*) were more common on Indonesian vessels, while tiger sharks and sandbar sharks (*C. plumbeus*) were predominantly recorded from Taiwanese vessels (Marshall 2011). The majority of individuals recorded from the illegal fishing vessels - 59.4% and 44.1% on Indonesian and Taiwanese vessels, respectively - were immature (Marshall 2011). Based on an estimate of 22 FFVs per day in the year 2006 (Salini 2007), Marshall (2011) determined an annual shark catch by Indonesian vessels alone of approximately 290 - 1071 tonnes in 2006, which was comparable to the largest commercial Australian shark fishery operating in northern Australia at the time, the Northern Territory Offshore Net and Line Fishery (Marshall 2011). These numbers do not include the catches of Taiwanese fishing vessels in that year and indicate that shark catches of FFVs in northern Australian waters were equivalent to or larger than those of the largest domestic shark fisheries.

IUU fishing by Indonesian and other south-east Asian vessels poses a serious threat to shark populations in northern Australia, and also to local Aboriginal livelihoods that depend on coastal marine resources (Field et *al*. 2009a). Illegal fishing activities also raise customs and quarantine concerns and issues of national security (Vince 2007). As a result, shark fishing boats entering northern Australian waters are regularly seized and destroyed and their crews arrested (Vince 2007). The number of apprehensions of foreign vessels in northern Australian waters increased steadily in the early 2000s peaking at nearly 367 vessels between 2005- 2006 (figure 4). Since then increased surveillance and tightened enforcement measures by Australian authorities have resulted in a reduction of illegal fishing and the number of apprehensions has steadily and sharply decreased since 2007 (figure 4). Nevertheless, Indonesian shark fishers continue to enter the AFS to fish illegally, often having little choice but to continue fishing for sharks in the absence of alternative livelihoods, due to logistical obstacles that arise from their distance to fresh fish markets, a lack of land rights and the resulting confinement to deriving a livelihood from the sea, or forbidding debt relationships with shark fin traders (Fox *et al*. 2009; Resosudarmo *et al*. 2009).

New Zealand shark fisheries

New Zealand is one of the top 20 shark fishing nations, with an average of approximately 18,000 t landed every year in the first decade of this century (data from FAO). Shark catches were below 5,000 t per year before 1980, but have steadily increased in the two following decades to reach 19,000 t in the late 1990s (Francis, 1998). Commercially important species that are directly targeted by local fisheries include school sharks, gummy sharks and the spiny dogfish (*Squalus acanthias*), as well as various rays and chimaeras. These species are managed under the New Zealand Quota Management System (QMS) through the allocation of Individual Transferable Quotas (ITQs) that are based on estimates of Total Allowable Catch (TAC) to ensure that species are not harvested over the Maximum Sustainable Yield (MSY) levels (Francis, 1998). Species managed under the QMS contribute to more than 80% of the total shark catch.

Direct targeting of other species of sharks is prohibited, however more than 60 other species of sharks, chimaeras and rays are landed as bycatch (Francis, 1998, Francis *et al*., 2001). Of these bycatch species, only three migratory taxa (blue sharks, porbeagle sharks and mako sharks) that contribute to approximately 4% of total shark catch are subject to the QMS regulations (White & Kyne, 2010). Those species are the major bycatch of the pelagic longline tuna fishery, however it is unlikely that the current levels of bycatch are seriously affecting New Zealand's pelagic shark stocks. Of more concern is the status of species that are not managed through the allocation of ITQs, for which there is a paucity of long-term fishery data from which to estimate the sustainability of current catches.

Shark fisheries in Indonesia, past to present

Indonesia has the largest shark fishery in the world, with a reported average annual catch of 110,528 t between 2000-2007, or about 14.09% of global annual shark catches (data from FAO). Elasmobranch catches experienced a boom starting in the mid-1980s in response to a rise in the demand and price for shark products, particularly shark fins (Clarke, 2004). Catches peaked at 118,000 t in 2003 and appear to have experienced a slight but steady decrease since 2006, remaining at or above 100,000 t per year (data from FAO). While shark captures are often reported as bycatch in tuna longline and fish or prawn trawl fisheries (Dharmadi *et al*., 2007, Tull, 2010), a large target fishery that is for the most part under- or unreported also exists (Dharmadi *et al*., 2007, Stacey, 2007). Sharks are caught with a variety of gears, predominantly gill nets (Jaring insang), bottom-set longlines (Rawai dasar) and surface longlines (Rawai hanyut) (Dharmadi *et al*., 2007).

Today, the majority of shark fins are harvested and traded in Eastern Indonesia, where data on catch rates and species composition remain limited (Bonfil, 1994; Resosudarmo *et al*., 2009). Based on a study of shark landings at Indonesian fish markets with a particular focus on Bali, Java and Lombok, Carcharhinidae make up almost 70% of the total number and ca. 60% of the biomass of all sharks landed (White, 2007). Blue sharks (*Prionace glauca*) contribute most to overall carcharhinid biomass (16.3%), while the most abundant species in terms of numbers is the spadenose shark (*Scoliodon laticaudus*) (32.5%). Other important target species include silky sharks (*C. falciformis*) (White, 2007) and scalloped hammerhead sharks (*Sphyrna lewini*), the latter representing up to 12% of landed biomass (White *et al*., 2008). Catches of endangered and protected species are of particular concern, especially where regional information on biological and fishery aspects is insufficient to conduct species assessments and where fishing effort is unreported and unregulated. White *et al*. (2008) found that the vast majority of landed scalloped hammerhead sharks are immature individuals, a clear sign of growth overfishing. These catches are likely to constitute an unsustainable level of fishing mortality and have the potential to cause serious depletions of this species in Indonesian waters (White *et al*., 2008). Another Endangered species listed under CITES Appendix II, the oceanic whitetip shark *Carcharhinus longimanus*, is also caught in the Indonesian shark fishery, although its contribution in numbers and biomass to the total catch is far smaller than that of *Sphyrna lewini* (White *et al*., 2008, White, 2007).

Obtaining data on catch rates and composition is more difficult in the more remote provinces of Nusa Tenggara Timur, Maluku, Papua and West Papua, where ice and storage facilities are not normally available and sharks are usually not sold at markets, but finned at sea and the carcasses returned to the ocean (Marshall, 2011). Shark fishers in Eastern Indonesia generally use small wooden boats of less than 20 GT, often powered by sails and without refrigeration (Tull, 2010). As a result, the fishery is often described as artisanal, which is misleading: unlike other artisanal fisheries, the shark fishery is not limited to near-shore environments, and shark fishers often venture far, undertaking regular voyages across national sea boundaries (see Box 3) (Resosudarmo *et al*., 2009, Stacey, 2007). Rarely, if ever, are sharks targeted for local consumption; the fishery is mostly driven by the international demand for shark fins (Dulvy *et al*., 2008), and only by-products of this fishery may be used locally, such as the salted meat of sharks caught towards the end of a trip. Although there are no available estimates for the number of fishers targeting sharks and rays exclusively and no reliable catch data exist for large parts of the country, the life histories of most commercially valuable species leave little doubt that the Indonesian shark fishery has a serious impact on shark populations and marine ecosystems.

Some shark stocks, including mobile species such as blue sharks and the Endangered hammerheads, are shared between Australia and Indonesia, being affected by the harvest of both nations (Ovenden *et al*., 2009). Overfishing in Indonesian waters, therefore, may have consequences that go beyond the depletion of local stocks, affecting populations in neighbouring countries. Indonesian fishermen from the island of Rote frequently venture into Australian waters, where many have been arrested for fishing illegally in what used to be their traditional fishing grounds (see Box 3). This has created forbidding debt relationships between fishers and shark fin traders who sponsor the fishing trips, as well as conflicts among the two nations over shared marine resources and boundaries.

Habitat loss and climate change

Many shark species rely on a range of different habitats for foraging, mating, and reproduction. Mangrove systems, estuaries and seagrass beds function as important nursery grounds, providing habitats sheltered from predators and inclement environmental conditions (Heupel *et al*., 2007). In addition, some species have strong natal philopatry to specific nursery areas (Hueter *et al*., 2005). Some sharks may be restricted to a single habitat throughout their life-cycle; this is the case of strictly coral reef associated sharks such as the grey reef shark (*C. amblyrhynchos*) and the whitetip reef shark (*Triaenodon obesus*) (Chin *et al*., 2012) and of some deepwater species with restricted distributions. Other species have specific habitat requirements in different stages of their life cycle, showing ontogenetic shifts in habitat use (Grubbs, 2010).

Species showing a high degree of habitat specialisation at any stage of their life cycle can therefore be highly vulnerable to habitat degradation (Chin *et al*., 2010). Even for species without strict habitat associations, the degradation of some habitats may imply the loss of important foraging grounds. The potential effect of habitat loss and habitat degradation on shark populations is not as well understood as the effect of direct fishing mortality.

Box 4: Reef sharks: predators under threat.

Figure 5: The grey reef shark, the quintessential coral reef associated shark. Photo credits: Robert Harcourt

Reef sharks exert important top-down control on coral reef ecosystems, and their removal may have dramatic consequences, accelerating reef degradation through trophic cascades (Bascompte *et al.,* 2005). Declines of reef shark populations have been recorded worldwide, from the Chagos Archipelago in the Indian Ocean to the greater Caribbean (Graham *et al*., 2010, Ward-Paige *et al*., 2010). Even within the Great Barrier Reef Marine Park (GBR), the most well-managed network of marine reserves in the world, reef shark populations have undergone dramatic declines. The two most abundant and commonly caught reef sharks in Australia are the grey reef shark (*Carcharhinus amblyrhynchos*) and the whitetip reef shark (*Triaenodon obesus*) (Heupel et al., 2009). As habitat specialists, they are found exclusively on, or very near to, coral reefs (Chin *et al*., 2012) and exhibit a combination of life history strategies (slow growth, late maturity, low reproductive output) and behavioural characters (site fidelity, habitat specialization), which make them susceptible to even modest levels of fishing pressure. Within the GBR, reef sharks are caught as bycatch in the Coral Reef Finfish Fishery (Heupel *et al*., 2009), and grey reef sharks make up to 6% of the shark catch of the Queensland East Coast Shark Fishery (Gribble *et al*., 2005). Reef sharks are also targeted by Indonesian fishers who hunt for sharks illegally in Australian waters, with grey reef sharks contributing up to 18% of the catch (Box 3).

In the absence of specific legislation regulating the harvest of reef sharks, their conservation relies heavily on networks of marine reserves, although there is evidence that present management is only partially effective. Catch rates in the GBR have been relatively stable in the past 20 years, suggesting declines since 1986 have been subtle (Heupel *et al*., 2009). However, the tendency to under-report discarded sharks, the lack of detailed information prior to the year 2000 (Heupel *et al*., 2009), and temporal changes in target behaviour may seriously compromise the reliability of catch data to infer population trends (Hisano *et al*., 2011). Robbins *et al*. (2006) found strictly enforced no-entry zones afford effective protection to reef shark populations in the Northern GBR, although no-take marine reserves are not as effective. Shark density within no-entry zones was an order of magnitude higher when compared not only to areas open to fisheries, but also to less strictly enforced no-take zones. Another study carried out in the central GBR yielded very similar results, with no-entry zones having 4 times as many sharks as areas open to fisheries (McCook *et al*., 2010). No-take areas in the central GBR seem to afford some protection to reef shark populations,

but densities were still only half of those recorded in no-entry areas. The ineffectiveness of no-take zones in the protection of shark populations is most likely due to direct fishing mortality due to illegal poaching (Hisano *et al*., 2011, Robbins *et al*., 2006). Similar patterns were reported for other coral reef predators targeted by fishermen, such as coral trout and snapper, supporting the hypothesis that no-take zones may be substantially depleted by poaching (McCook *et al*., 2010). While no-take zones make up a substantial part of the GBR marine park (30%), no-entry zones encompass only 1% of the available habitat and are unlikely to play a significant role in the regional conservation of reef sharks. Compliance and effective enforcement of established marine reserve networks will be critical in ensuring the recovery of reef shark populations on the GBR, but this is a challenging task given the scale at which regulations need to be enforced and the remoteness of many areas of the GBR (McCook *et al*., 2010).

Demographic analyses suggest that populations of reef sharks on the GBR are severely depleted and are undergoing rapid declines (Hisano *et al*., 2011, Robbins *et al*., 2006). It is not surprising that shark populations in relatively remote habitats may be threatened by modest levels of poaching. In the Chagos Archipelago, an extremely remote area of the Indian Ocean, reef shark populations collapsed entirely because of illegal shark fishing from foreign fleets (Graham *et al*., 2010). Since reef sharks are facing imminent threats even in the most well-managed network of marine reserves in the world, it is likely that the situation is even more dramatic in areas where similar management strategies are not in place. In Indonesia, according to one study, grey reef sharks make up 2-3% of total landed shark biomass , approximately 3000 t per year (which is equivalent to 30% of total yearly shark landings in Australia) (White, 2007). This is likely a gross underestimate of landings, since shallow water coral reef fisheries in eastern Indonesia are largely unreported and unregulated (Varkey *et al*., 2010). While data to estimate past and future population trends in Indonesia are not available, given the levels of fishing pressure it seems most likely that reef shark populations would be undergoing even sharper declines than on the GBR.

While direct fishing mortality is at present the highest threat to reef shark populations, habitat degradation is likely to have profound effects on these species in the near future. Coral reefs are declining worldwide at an alarming rate due to the synergistic effects of overfishing, increasing sea surface temperatures, ocean acidification, crown-of-thorns starfish outbreaks, nutrient enrichment and increased tropical storm intensity (De'ath *et al*., 2012, Wilkinsons, 2008). Within the GBR, coral cover has declined by 50% in the past three decades (De'ath *et al*., 2012), and the outlook in other regions is even worse. In Indonesia, nearly 90% of coral reefs are under threat, mostly because of overfishing and destructive fishing (Wilkinsons, 2008). With coral reefs shrinking, so is the habitat on which reef sharks rely for their existence.

The synergistic effects of climate change, coastal development and the use of destructive fishing methods is leading to the degradation of many habitats that are crucial for shark populations. Eutrophication and increased water turbidity due to inadequately managed land use (Brodie *et al*., 2011), the rise in sea surface temperatures (Hoegh-Guldberg & Bruno, 2010) and a reduction in the ocean pH due to increased atmospheric CO2 concentrations (Doney *et al*.,

2009) are likely to have profound effects on ecosystem function, indirectly affecting shark populations that rely on these habitats (Chin *et al*., 2010). Even in relatively well managed areas, such as the coasts of Australia, seagrass beds (Walker & McComb, 1992), mangrove forests and coral reefs (De'ath *et al*., 2012) have been declining at alarming rates. In other areas of the region, such as PNG and Indonesia, the outlook is considerably worse (Wilkinsons, 2008). The effects of habitat degradation on local shark populations are difficult to quantify. The degradation of coral reefs may have dramatic effects on sharks that are strictly coral reef associated (Chin *et al*., 2010) and the loss of coastal nursery areas may have profound effects on future recruitment of shark populations, exacerbating the effects of already unsustainable harvesting regimes (Field *et al*., 2009b) .

CURRENT MANAGEMENT AND CONSERVATION STRATEGIES

Australia and New Zealand have long histories of shark fishery management. Shark fisheries have been traditionally managed using size and bag limits, restrictions in licensing and fishing gear, along with demographic models that are more often than not better suited to highly productive species of fish (Musick *et al*., 2000). The first management actions date back to the late 1950s with the introduction of minimum length requirements in the school shark fishery in New South Wales. Starting from the 1980s and 1990s management strategies based on restrictions of licensing and the introduction of individual transferable quotas (ITQs) of total allowable catch were introduced in Australia and New Zealand. These management strategies, however, were usually restricted to a few commercially important species and were only partially effective. It was not until the last decade that coordinated national strategies for the conservation of shark populations were implemented.

National Plans of Action for the Conservation and Management of Sharks

Given growing concerns regarding the over-exploitation of shark stocks, in 1998 the Food and Agriculture Organization of the United Nations (FAO) launched an International Plan of Action for the Conservation and Management of Sharks (IPoA) (FAO, 1998), with the aim of ensuring the long-term conservation of shark populations and the sustainability of shark fisheries. The IPoA called upon nations to prepare National Plans of Action (NPoA) to ensure the sustainability of shark fisheries with a particular focus on threatened and vulnerable shark stocks by: engaging stakeholders in the development of the plan, creating a shark assessment report (SAR), establishing long-term monitoring of species-specific fishery data, minimising incidental catch, and contributing to the protection of biodiversity and ecosystem function. In the Indo-Australasian region NPoAs have been developed by Australia, New Zealand and

Indonesia. However, only Australia has followed the recommendations outlined in the IPoA closely.

The first Australian SAR was prepared in 2001, and the first NPoA was developed in 2004 following the guidelines and objectives outlined by the IPoA (NPoA Australia, 2004). The NPoA played a major role in the development of coordinated management strategies at the national level, including improvements in observer and monitoring programs, the development of fishery-specific risk assessments for the majority of shark fisheries, and the implementation of specific management responses for threatened and protected species. Most management actions that have been undertaken in the past decade and resulted in improved management of shark fisheries (such as the Commonwealth school shark rebuilding strategy and targeted management of sandbar shark populations in Western Australia) occurred within the framework of the NPoA. In 2009 a new SAR (SAR2) (Bensley *et al*., 2009) was developed and the following year a review of the NPoA was released. These documents identified improvements in the management of commercial harvest as well as the main shortcomings in terms of the development of effective conservation strategies. Among the most important issues was the inadequacy of data reporting, which resulted in the absence of reliable and validated species-specific catch data, particularly for bycatch species, and consequently the need to implement precautionary measures to prevent future shark declines where uncertainties exist. A new NPoA, which was formulated primarily following the recommendations of SAR2 and the 2010 reviews was developed in 2012 (NPoA Australia, 2012).

In 2008, an NPoA was approved in New Zealand (NPoA New Zealand, 2008), however the approval of the NPoA did not follow the development of a nation-wide SAR comparable to the one undertaken in Australia in 2001. New Zealand's NPoA is mostly a description and evaluation of current management strategies for shark fisheries, based on the New Zealand Quota Management System. A few key areas where improvement is necessary are highlighted, including the need for actions to eliminate live shark finning, protect threatened and endangered species that are protected under the Convention of Migratory Species (particularly the basking shark), and a review of management strategies for the spiny dogfish. One of the IPoA recommendations was that NPoAs should be reviewed and revised every 4 years, however New Zealand has yet to produce a SAR or a formal review of the implementation of the actions suggested in the NPoA developed in 2008.

Indonesia has committed to managing its shark fisheries through the formulation of an NPoA; however, rather than proposing management strategies informed by catch data and fishery-

independent assessments, the NPoA released in 2010 outlines the need for increased research effort and data collection to inform management, without providing much guidance on how, where, when and by whom the required data should be collected. Fishery surveys require extended periods of time because in the remote regions of Eastern Indonesia, where there are often no major landing sites, whole sharks are rarely landed and fins are sold directly from fishers to traders, not at fish markets. The lack of reporting requirements and the resulting scarcity of catch data have been an enormous obstacle to species assessments, management strategies and effective conservation initiatives (Blaber *et al*., 2009, Dharmadi *et al*., 2007). Given the many obstacles for an accurate assessment of Indonesia's shark fishery, traditional management based on sufficient fishery-, population- and species-specific data is unlikely to be implemented in time to prevent further species declines and local extinctions (Camhi, 1998). Precautionary measures, implemented strategically and efficiently, are therefore needed if the effects of several decades of overfishing are to be mitigated against and the population declines of some stocks are to be slowed down until better data become available to inform management.

The role of Marine Protected Areas

The establishment of areas permanently closed to fishing has been proposed as an essential tool in the protection of shark populations. Networks of Marine Protected Areas (MPAs) have been established in many countries, including Australia, New Zealand and Indonesia. Possibly the most well-known example of an extensive network of MPAs is the Great Barrier Reef Marine Park, extending for more than 2000 km along the Queensland coast. The effectiveness of MPAs, which are usually not designed specifically for shark conservation, in the protection of shark populations has recently sparked debate (see Box 4) (Heupel *et al*., 2009, Robbins *et al*., 2006). Recent studies have assessed MPAs effectiveness for coastal shark protection, although only a few used empirical evidence that has included long-term movement in and around MPAs (e.g., Knip *et al*., 2012, Speed *et al*., 2016). One study focussed on the effectiveness of an MPA within Ningaloo Marine Park in Western Australia for three species of reef sharks: grey reef sharks (*Carcharhinus amblyrhynchos*), blacktip reef sharks (*C. melanopterus*), and sicklefin lemon sharks (*Negaprion acutidens*) (Speed *et al*., 2016). The authors found that the largest species (grey reef sharks), spent very little (< 1%) of their time within the MPA, whereas juvenile blacktip and sicklefin lemon sharks spent 84-99 % of their time within the protected area. The MPA provided a reasonable level of protection for juveniles, although long-distance movements (10 - 260 km) and larger home ranges by adult grey reef and blacktip reef sharks exposed them to recreational fishing pressure within the Marine Park. These results suggest that

the placement and size of the MPA does not incorporate habitats commonly used by adult grey reef and blacktip sharks, such as the reef slope and channels connecting the reef and lagoon.

Dedicated MPAs, designed especially for shark conservation (shark sanctuaries), have been established in many tropical reef areas over the past few years (e.g., Palau, Honduras, Maldives, The Marshall Islands, Tokelau, Raja Ampat) (PEW, 2012). The effectiveness of shark sanctuaries is currently under debate (Chapman *et al*., 2013, Davidson, 2012), due to scepticism surrounding enforcement. Socio-economic studies of the value of live sharks for tourism compared to fished sharks, have assisted in driving the recent focus on shark sanctuaries as conservation tools (Vianna *et al*., 2012). One of these studies from Palau found that a population of 100 grey reef sharks is estimated to be worth US $10,800 to the fishing industry over their lifetime, which is only 0.006 % of their value to the tourism industry if kept alive (Vianna *et al*., 2012).

In some situations the establishment of a network of MPAs may be the best strategy to protect shark populations. For example, given the obstacles for traditional management of Indonesia's shark fishery, shark management should be approached from different angles, such as the protection of fishing grounds through no-take zones or for eco-tourism purposes, alongside the facilitation of alternative livelihoods. In some regions, tourism has already provided an impetus for shark conservation. For example, the development of Raja Ampat as a dive and nature tourism destination has led to initiatives by resort owners, NGOs and communities to protect the region's shark and ray fauna. These efforts have resulted in the country's first shark and manta ray sanctuary, where shark fishing is prohibited by law. While this is a commendable development, shark fishers that have been displaced from their previous fishing grounds are forced to search for different fishing grounds - which will then experience greater fishing pressure -, or new livelihoods. The identification of alternative livelihoods and the provision of support in the transition phase must therefore form an integral part of shark conservation initiatives. This requires effective communication between scientists, managers, tourist operators, NGOs and government agencies, as well as improved research capacity and increased allocation of management responsibility to provincial and regency levels.

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Chapter 4

Characterisation of 15 novel microsatellite loci for the grey reef sharks (*Carcharhinus amblyrhynchos*).

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ABSTRACT

Grey reef sharks (*Carcharhinus amblyrhynchos*) are important apex predators on coral reefs, and their numbers have declined dramatically as a result of overfishing. Knowledge of environmental factors that shape gene flow is essential for developing appropriate management strategies, but the lack of suitable genetic markers has hindered research on this species. Here, we characterised 15 polymorphic microsatellite loci for grey reef sharks. None of the loci deviated significantly from Hardy-Weinberg equilibrium and there was no evidence of Linkage Disequilibrium. Several loci cross-amplified in other carcharhinid species, and will be useful in future studies of this family.

FINDINGS

Grey reef sharks (*Carcharhinus amblyrhynchos*) exert important top-down control on coral reef ecosystems and their removal may accelerate reef degradation through trophic cascades. Grey reef sharks are categorised globally as a "Near Threatened" species (Smale 2009) however dramatic regional declines, even in areas such as the comparatively well managed Great Barrier Reef, suggest that some populations may be under substantially higher levels of risk (Robbins *et al.* 2006). Understanding what environmental factors shape gene flow is essential for the development of effective management strategies, but the lack of appropriate genetic markers has thus far hindered research effort in that direction. Here we report the isolation of 15 novel, polymorphic microsatellite markers for the grey reef shark.

Genomic DNA was extracted from a fin clip of a male grey reef shark using the ISOLATE II Genomic DNA Kit (Bioline Pty Ltd) following manufacturer's instructions. The extracted DNA (no enrichment step was performed), was sent to the Australian Genome Research Facility where pyrosequencing was performed on a 454 GS-FLX platform (Roche Applied Science) resulting in 186,119 sequences and 68,169,247 bases. A total of 1,882 microsatellite loci (di- tri- tetra- penta- and hexa-nucleotides with more than 5 repeats) with flanking regions suitable for primer design were identified with QDD v 2.0 (Meglécz *et al.* 2010). Of these loci, 28 were chosen following the recommendations from Gardner *et al.* (2011). Primers were designed with similar annealing temperatures (55-60 °C) and for primer compatibility to increase multiplexing potential. All forward primers were labelled with fluorescent dyes (6-FAM, PET®, NED® and VIC®) with the exception of C.amb6, C.amb25, C.amb27 and C.amb28 which were genotyped using the M13-tail approach (Schuelke 2000). Primers were tested on 34 individuals from the northern Great Barrier Reef (Lizard Island, QLD Australia). Microsatellite loci were amplified

using the Type-it Microsatellite PCR Kit (Qiagen) following manufacturer instructions. PCR conditions were as follows: 5 min denaturation 95 °C, followed by 35 cycles of 30 s denaturation (94 °C), 30 s annealing (60 °C) and 30 s elongation (72 °C) and a final elongation of 10 min at 72 °C. For M13 tailed primers, annealing temperature followed a touch-down protocol, starting at 60 °C and decreasing by 0.5 °C per cycle for the first 20 cycles.

Table 1: Novel microsatellite loci isolated in this study, and associated GenBank accession numbers. N=sample size, NA=number of alleles, H_0= observed heterozygosity, H_E= expected heterozygosity, H-W= p value for Hardy Weinberg Equilibrium (none significant after Holm-Bonferroni sequential correction), PID= non exclusion Probability of Identity. The fluorescent dye used is indicated in superscripts: [1]PET®, [2]6-FAM, [3]VIC®, [4]NED®. Loci amplified using the M13 tail protocol (Schuelke 2000) are indicated with *. Combined PID = 7.31×10^{-20}

Locus & Accession n.	Primer Sequence (Forward and Reverse)	Motif	Size (bp)	N	NA	$H_O(H_E)$	H-W	PID
C.amb2 KU877929	F- TCCTACCTGACAAAGGAACTGC[1] R- ATGAACAGAGACAAACAGACCGAC	$(TCTA)_{14}$	328-368	31	11	0.90(0.87)	0.761	0.029
C.amb3 KU877930	F- TGGAGTGCCAATTCTCTTGTCG[2] R- ACTTGGGAGTCTGACTAATCTCC	$(TC)_{18}$	200-272	34	26	0.82(0.90)	0.031	0.013
C.amb4 KU877931	F- GTCGAATGCATTGAGTTTCAGG[3] R- CCAATACAAGCAAAGGGACAAC	$(AC)_{14}$	346-372	32	13	0.78(0.83)	0.335	0.049
C.amb5 KU877932	F- CAGATATGCGGTGTCGTGGC[2] R- TTCCGCGTCTTGTCTCTGC	$(TG)_{13}$	266-284	33	8	0.70(0.71)	0.202	0.117
C.amb6* KU877933	F- TGTGGCTGGGATAAAATGCACG[4] R- TGGCTTGTAAAATCCTGTTCTGCG	$(TGG)_{13}$	251-287	32	11	0.90(0.81)	0.580	0.054
C.amb7 KU877934	F- AGAATGCTGTCTCGTGATGC[4] R- GTTGTCACTGTCGAGATAGAGC	$(AGAC)_{11}$	291-315	34	7	0.79(0.75)	0.039	0.095
C.amb9 KU877935	F- CCCAGGAGCCCTCTCTGTA[1] R- GTCTCTTGCCACGCTCCTAC	$(TG)_{13}$	209-223	34	6	0.56(0.58)	0.885	0.254
C.amb11 KU877936	F- TGAACGCTTTACTGAACCTTGC[4] R- GCAGCCTTTACTCCTCGTCA	$(CA)_{14}$	162-200	33	14	0.90(0.88)	0.373	0.024
C.amb15 KU877937	F- GTATGAGACGAGCATCGTGCC[3] R- AATCGCAGCGTCTGCAATG	$(AC)_{13}$	192-222	33	12	0.88(0.86)	0.380	0.034
C.amb18 KU877938	F- TGCACACGCAGTGATGTTGG[3] R- ATGCCGATTTCTCTGTTAATGAGC	$(AC)_{16}$	143-191	31	21	0.97(0.93)	0.058	0.008
C.amb20 KU877939	F- ATGTGGAGGAGTGATGTTAGCC[2] R- TTAATGTCAGTGTTCACGCTGG	$(GT)_{12}$	314-350	31	14	0.87(0.89)	0.705	0.021
C.amb22 KU877940	F- ATGTCAGTTCTTTAGGAGTAGGG[2] R- CCAATCTACACTTCACTCACTG	$(GA)_{11}$	352-356	32	3	0.22(0.20)	1.000	0.652
C.amb25* KU877941	F- GACTCATCAGGATAGTCTGGATGCT[2] R- GCTCAACTGTCAAAAGAGGAAGCC	$(AGGG)_{8}$	208-248	32	10	0.75(0.79)	0.214	0.071
C.amb27* KU877942	F- AGTCAGTGTCACGATGG[1] R- GCTTTCTATCATTAACATGAGATCC	$(TG)_{11}(AG)_{18}$	169-197	33	10	0.82(0.83)	0.483	0.047
C.amb28* KU877943	F- CACATTGCTATGAGCCTGGAG[3] R- CATCTCTTTCATCACTGCATGATTG	$(AC)_{13}$	286-326	31	10	0.77(0.75)	0.767	0.082

Fragment analysis was performed on an ABI 3730 platform at the Sydney node of the Australian Genome Research Facility. Fifteen loci amplified consistently and showed no excessive stuttering. We used the software MICROCHECKER (Van Oosterhout *et al.* 2004) to test for the presence of null alleles and large allele drop-out and found no evidence of either. Estimates of genetic diversity were obtained and tested for Hardy-Weinberg Equilibrium (H-W) and linkage disequilibrium (LD) using the software package Genepop 4.2 (Rousset 2008)

and Probability of Identity (PID) for each locus was estimated in Cervus 3.0.3. After applying Holm-Bonferroni sequential correction for multiple comparisons all loci were in linkage equilibrium and none departed significantly from H-W (Table1). The average number of alleles per locus was 12.6 (range: 3-26), mean observed (H_0) and expected (H_E) heterozygosities were 0.785 (range: 0.22-0.97) and 0.783 (range: 0.2-0.95) respectively (Table1). The 15 novel loci successfully cross-amplified for a range of other carcharhinid species (Table S1). The loci developed in this study will prove useful for future investigations on the genetic structure of grey reef sharks as well as other Carcharhinidae.

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SUPPLEMENTARY MATERIALS

Table S1: Amplification success of the 15 novel microsatellite markers across eight carcharhinid shark species. Successful amplification is indicated by the sign "+" , unsuccessful amplification by the sign "-".

Species	Camb2	Camb3	Camb4	Camb5	Camb6	Camb7	Camb9	Camb11	Camb15	Camb18	Camb20	Camb22	Camb25	Camb27	Camb28
Triaenodon obesus	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+
Carcharhinus albimarginatus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Carcharhinus leucas	+	+	-	+	-	+	+	+	+	+	-	+	+	+	+
Carcharhinus limbatus	+	+	-	+	-	+	+	+	+	+	-	-	-	+	+
Carcharhinus brachyurus	+	-	-	+	-	-	+	-	+	-	-	-	+	+	+
Carcharhinus plumbeus	+	+	-	+	-	+	+	+	+	+	-	+	+	+	+
Carcharhinus obscurus	+	+	+	+	-	+	+	-	+	+	+	+	+	-	+
Negaprion acutidens	-	+	-	+	+	+	+	-	+	+	+	+	+	-	+

Chapter 5

Connectivity in grey reef sharks (*Carcharhinus amblyrhynchos*) determined using empirical and simulated genetic data.

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ABSTRACT

Grey reef sharks (*Carcharhinus amblyrhynchos*) can be one of the numerically dominant high order predators on pristine coral reefs, yet their numbers have declined even in the highly regulated Australian Great Barrier Reef (GBR) Marine Park. Knowledge of both large scale and fine scale genetic connectivity of grey reef sharks is essential for their effective management, but no genetic data are yet available. We investigated grey reef shark genetic structure in the GBR across a 1200 km latitudinal gradient, comparing empirical data obtained from 121 individuals genotyped at 16 polymorphic microsatellite loci and 108 individual sequences of the mitochondrial ND4 gene with models simulating different levels of migration. The empirical data did not reveal any genetic structuring along the entire latitudinal gradient sampled, suggesting regular widespread dispersal and gene flow of the species throughout most of the GBR. Our simulated datasets indicate that even with substantial migrations (up to 25% of individuals migrating between neighboring reefs) both large scale genetic structure and genotypic spatial autocorrelation at the reef scale were maintained. We suggest that present migration rates therefore exceed this level. These findings have important implications regarding the effectiveness of networks of spatially discontinuous Marine Protected Areas to protect reef sharks.

INTRODUCTION

The conservation of higher order predators is important for the maintenance of marine biodiversity, due to their potentially pivotal role in shaping communities (Heithaus *et al.* 2008). Predator removal may have far reaching consequences on lower trophic levels (Myers *et al.* 2007), including cascading effects on both abundance (Bascompte *et al.* 2005; Myers *et al.* 2007) and behavior (Wirsing *et al.* 2008). This can reduce ecosystem resilience to natural and anthropogenic disturbances (Ruppert *et al.* 2013) and in extreme cases lead to ecosystem phase-shifts (Estes & Duggins 1995). The grey reef shark, *Carcharhinus amblyrhynchos*, the whitetip reef shark, *Triaenodon obesus* and the blacktip reef shark, *C. melanopterus* are abundant mesopredators that occupy the highest trophic level of permanent food webs in coral reefs (Heupel *et al.* 2014; Mourier *et al.* 2013). Recently it has been shown that pristine coral reefs have high biomasses of higher order predators, and reef sharks can make up the majority of that biomass (Friedlander *et al.* 2014; Sandin *et al.* 2008; Stevenson *et al.* 2007). Their potential loss is therefore a major threat to the long term resilience of coral reef ecosystems.

Reef sharks exhibit a combination of life history strategies and habitat specialization that make them particularly susceptible to anthropogenic pressure. They are slow growing, have delayed

sexual maturity, and low reproductive output (Chin *et al.* 2013b; Robbins 2006) and are therefore susceptible to even modest levels of fishing pressure (Hisano *et al.* 2011). As most species are strictly associated with coral reefs (Chin *et al.* 2012; Rizzari *et al.* 2014b), they are further threatened by the ongoing loss of coral reef habitat (Chin *et al.* 2010) due to the synergistic effects of overfishing (Hughes 1994), eutrophication (Fabricius 2005), ocean acidification (Fabricius *et al.* 2011) and increased sea surface temperatures (Hoegh-Guldberg *et al.* 2007). Not surprisingly, their numbers have been shrinking over recent decades (Graham *et al.* 2010; Hisano *et al.* 2011; Rizzari *et al.* 2014a; Robbins *et al.* 2006), particularly in areas open to fisheries, raising concerns about the long-term viability of reef shark populations, even in highly regulated marine parks such as the Great Barrier Reef Marine Park (GBR), Australia (Hisano *et al.* 2011; Robbins *et al.* 2006).

The grey reef shark is the most abundant reef shark in the Indo-Pacific, accounting for up to 50% of higher order predator biomass on healthy reefs (Friedlander *et al.* 2014). Within the GBR the grey reef shark is the most threatened by fishing pressure, having the fastest rate of decline of all reef shark species (Hisano *et al.* 2011; Robbins *et al.* 2006). It has been suggested that grey reef sharks are protected when they inhabit very small (i.e. encompassing a single coral reef) but strictly enforced MPAs, where their density can be an order of magnitude higher than in neighboring areas open to fisheries (Espinoza *et al.* 2014; Rizzari *et al.* 2014a; Robbins *et al.* 2006). However, acoustic telemetry studies show that grey reef sharks may exhibit limited site fidelity in the GBR, with some individuals detected transiting across multiple neighboring reefs, repeatedly crossing the boundaries of different management zones (Espinoza *et al.* 2015a; Heupel *et al.* 2010). Heupel *et al.* (2010) speculated that this apparent contradiction between higher biomass in MPAs and limited site fidelity suggests that the observed differences in biomass may be the result of factors other than fishing pressure such as differences in carrying capacity or behavioral differences between management zones which may have led to bias in surveys- but see also Rizzari *et al.* (2014a).

To be effective in increasing local biomass of target species, MPAs must meet two requirements: 1) they need to protect the target species by encompassing a large proportion of its home range; and 2) they need to be at least partially self-seeding (animals within an MPA need to contribute to the next generation) (Almany *et al.* 2009; Momigliano *et al.* 2015). In this study we use empirical and simulated genetic data to investigate gene-flow for the grey reef shark within the GBR. We hypothesize that if grey reef shark reef fidelity is high and movements limited to neighboring reefs (i.e. a “stepping stone” scenario), patterns of genetic structure will arise over large distances. Similarly, if self-recruitment is high and individual

reefs are largely self-seeding, patterns of genotypic spatial autocorrelation should be detected i.e. genetic distance between individuals should increase with geographic distance. These patterns of genotypic spatial autocorrelation are expected to be most pronounced at small spatial scales and measurable within less than 10 generations (Epperson 2005), and hence could be apparent even in the absence of large scale genetic genetic structure.

MATERIAL AND METHODS

Sample collection

Fin clips from 121 grey reef sharks were obtained along a latitudinal gradient spanning more than 1200 km and 9º of latitude (23º to 14º S, figure 1*)* within the GBR. Specimens from the North GBR (2 reefs, N=32), the Central GBR (9 reefs, N=54), the Coral Sea (1 reef, N=8) and the South GBR (1 reef, N=2) were collected between February 2001 and April 2005 and represent a subsample from Robbins (2006). The remaining individuals from the South GBR (2 reefs, N=25) were collected in April 2013 by hook and line. Sharks were captured in shallow waters (<30 m) on the reef slopes of Wistari Reef and Heron Island reef using heavy handlines and 16/0 tuna circle hooks to reduce the risk of deep hooking. A fin clip of approximately 15 mm^2 was taken and the sex and size (total length and fork length) was recorded before the animals were released. Sampling was conducted in accordance with Macquarie University's approved guidelines and the sampling protocol was approved by Macquarie University's Animal Ethics Committee (AEC reference No.: 2012/044). Samples were collected under permit G13/35796.1 issued by the Great Barrier Reef Marine Park Authority (GBRMPA). Samples were stored in 95% ethanol at -20 ºC. Details of sampling, including geographic coordinates of all sampling locations, are given in the Supplementary Materials (Supplementary Table S1).

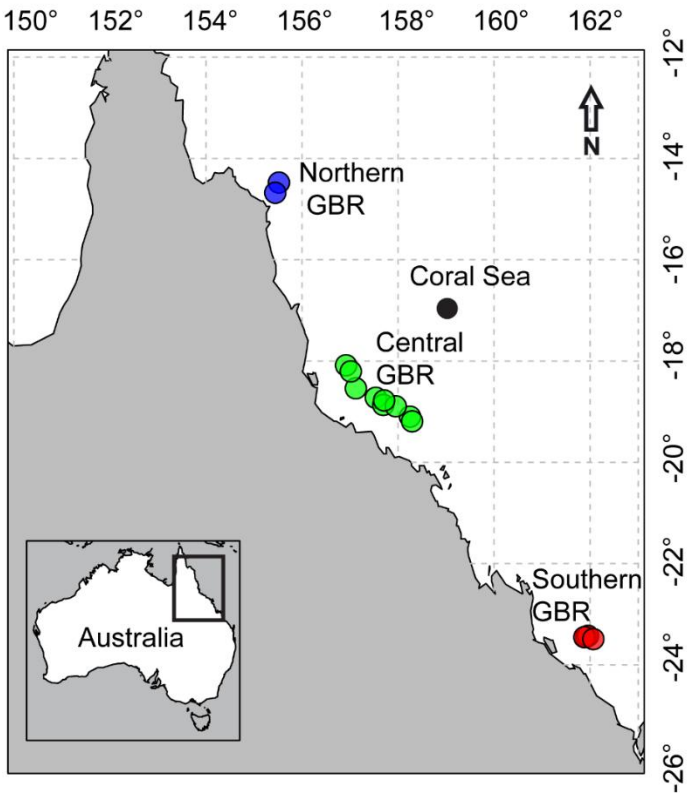

Figure 1*: Sampling design. Genetic samples were collected from 121 grey reef sharks in three distinct regions of the GBR and the Coral Sea (Herald Cays). In the North GBR, 32 individuals were sampled from 2 reefs; in the Central GBR, 54 individuals from 9 reefs and in the southern GBR we sampled 27 individuals from 3 reefs. Furthermore, 8 individuals were sampled from the Herald Cays in the Coral Sea. The map was produced using the package "maps" in the R statistical environment (R Core Team 2014).*

Laboratory procedures

DNA was extracted from fin clips of 121 grey reef sharks using a modified Chelex protocol (Walsh *et al.* 1991). A fragment of the NADH deydrogenase subunit 4 (ND4) gene approximately 850 bp long was amplified by Polymerase Chain Reaction (PCR) using the primers ND4 (Arèvalo *et al.* 1994) and H12293-Leu (Inoue *et al.* 2003). We amplified a suite of 15 microsatellite loci recently isolated for the grey reef sharks by Momigliano *et al.* (2014) and an additional microsatellite locus (Cpl169) which was originally developed for *C. plumbeus* by Portnoy *et al.* (2006). The 16 microsatellite loci used are listed in the supplementary materials (Supplementary Table S2).

Empirical Data Analysis. Mitochondrial DNA sequences were aligned using Bioedit (Hall 1999). Molecular indices, including haplotype diversity (*h*) and nucleotide diversity (π) were estimated in DNAsp (Librado & Rozas 2009). Pairwise F_{st} and ϕ_{st} (the latter based on pairwise

differences) between regions based on ND4 gene sequences were calculated in Arlequin 3.5 (Excoffier & Lischer 2010).

Microsatellites were scored using GeneMapper (Version 4), and alleles were binned using the software TANDEM following the method outlined by Matschiner & Salzburger (2009). The dataset was tested for significant departures from Hardy Weinberg Equilibrium (HWE) in Genepop 4.2 (Raymond & Rousset 1995) and for the presence of null alleles using the software MICROCHECKER (Van Oosterhout *et al.* 2004). Tests for Linkage Disequilibrium (LD) were carried out using the software FSTAT version 2.9.3.2 (Goudet 2001), using a sequential Holm-Bonferroni correction. Because there was no statistically significant departure from HWE and no evidence of LD, all loci were used in the analyses. The microsatellite dataset is publicly accessible through the Dryad repository (doi:10.5061/dryad.362s5).

Allele frequencies and measures of genetic differentiation were estimated in the R statistical environment using the package diveRsity (Keenan *et al.* 2013). Different measures of genetic differentiation -Weir and Cockerham F_{st} (Weir & Cockerham 1984), Hedrick's G'_{st} (Hedrick 2005), and Jost's *D* (Jost 2008)-, and their 95% confidence intervals were estimated using 1000 bootstraps using the same package. Furthermore, Meirman's F'_{st} (Meirmans 2006) were calculated in the software GenALEx 6.5 (Peakall & Smouse 2012).

A Principal Component Analysis (PCA), using region as the grouping factor, was performed using the package Adegenet (Jombart 2008). The principal components obtained from PCA were used as input synthetic variables to perform a Discriminant Analysis of Principal Components (DAPC) -for a full description of the method see Jombart *et al.* (2010) and Horne *et al.* (2011)-. The number of principal components to be retained in the discriminant analysis (N) was determined using alpha-score optimization, a method that finds a trade-off between discriminative power and model over-fitting. The choice for exploring genetic variation with DAPC rather than using Bayesian clustering algorithms was driven by the fact that DAPC is more powerful in resolving weak genetic structure (Horne *et al*. 2013; Jombart *et al*. 2010). To assess the presence of spatial structure, we estimated genotypic spatial autocorrelation across three distance classes; 1) within single reefs 2) within the same geographic region 3) across the entire sampling area. We calculated individual based genetic distances in GenAlEx 6.5, using the codom-genotipic genetic distance option -for a detailed description of the method see Smouse & Peakall (1999)-. Autocorrelation coefficients (*r*) (Peakall *et al.* 2003; Smouse & Peakall 1999) were calculated for each distance class in GenAlEx 6.5. We used 999 random permutations to generate 95% confidence intervals for the null model of no spatial

autocorrelation, and 999 bootstraps to generate 95% confidence intervals of *r* for each distance class. The analysis was performed first for all sampled individuals, and then separately for males and females to detect possible sex-bias in spatial autocorrelation. The overall significance of each correlogram was tested using the non-parametric heterogeneity test outlined in Smouse *et al.* (2008).

Simulations

To determine whether the statistical analyses performed were capable of detecting genetic structuring at different levels of between-reef migration, we carried out a range of simulations using the software EASYPOP (Balloux 2001). We created an 8x8 network of 64 reefs, with each reef encompassing an area of 20 km^2. The census size of each reef was approximated based on the densities provided in the literature from pristine GBR reefs -2 sharks per hectare, see Robbins *et al.* (2006)-. We assumed an effective population size (N_e) to census size (N_c) ratio of 1:10 (Frankham 1995), and created a dataset with the same number of loci and the locus diversity of the empirical dataset. Genetic drift was simulated for 100 generations with a 2-dimensional stepping stone model and levels of between reef migrations were set at 1%, 10% and 25%. Ten simulations were carried out for each migration level. We randomly sampled 10 individuals from three simulated reefs in each region of the simulated seascape, and carried out the same repertoire of analyses that were carried out for the empirical dataset. The value of each of the simulation parameters used was chosen to provide a conservative estimate of genetic drift: that is, the effect of genetic drift and the subsequent genetic structuring within the simulated dataset would be likely less than in our real data set (see Supplementary Materials , pp. 94-95 for details).

RESULTS

Diversity indices

Genetic data were obtained for a total of 121 grey reef sharks sampled along a latitudinal gradient spanning more than 1200 km and 9º of latitude (23º to 14º S, figure 1, Supplementary Table S1) within the GBR. We genotyped all individuals at 16 microsatellite loci. Overall diversity indices for the microsatellite dataset are given in the supplementary materials (Supplementary Table S2), and additional diversity indices for each region are given in Table 1. The number of alleles at each locus ranged from 3 to 34, observed heterozygosity for each locus ranged from 0.356 to 0.958 (Supplementary Table S2). None of the loci significantly

deviated from Hardy-Weinberg Equilibrium (HWE), and there was no evidence of Linkage Disequilibrium (LD) after applying a sequential Holm-Bonferroni correction for multiple comparisons ($p>0.05$). Within each region, allelic richness (A_r) ranged from 5.6 to 6.93, and observed heterozygosity over all loci ranged from 0.79 to 0.8 (Table 1). Fixation indices (F_{is}) were small or negative (Table 1) and non-significant (95% confidence intervals overlapped with 0). We obtained sequence data for 813 bp of the ND4 gene from 109 individuals (Table 1, GenBank accession numbers KT326195-KT326303). A total of 10 polymorphic sites were present, of which 7 were parsimony informative (3 singletons). Ten distinct haplotypes were identified, and haplotype diversity (*h*) ranged from 0.61 to 0.8 (Table 1).

Table 1. Sample sizes and genetic diversity indices. N=sample size, N_h=number of haplotypes, *h*=haplotype diversity, A_r=allelic richness, H_O=observed heterozygosity, H_E= expected heterozygosity. F_{is}= fixation index

Region	**N (mDNA)**	**N_h**	***h***	**N (msat)**	**A_r**	**H_O**	**H_E**	**F_{is}**
North GBR	32	9	0.8	32	6.93	0.8	0.78	-0.023
Coral Sea	8	3	0.61	8	5.6	0.79	0.75	-0.062
Central GBR	42	5	0.66	54	6.89	0.79	0.79	-0.002
South GBR	27	6	0.73	27	6.78	0.79	0.8	0.0124

Spatial structure analyses

Pairwise F_{st} and ϕ_{st} estimates between regions for the mtDNA dataset were small (<0.015) and did not significantly differ from 0 ($p>0.27$). Similarly, all measures of genetic differentiation obtained from the microsatellite dataset were not significantly different from 0 (figure 2). For the whole dataset Meirman's F'_{st} was very small (0.003) and non-significant ($p>0.3$). The results from the PCA analysis (figure 3A) revealed no difference in overall genetic variance among regions; the 95% confidence ellipses of each region were clearly overlapping, and densities along the first two principal components were very similar (data not shown). Even the Discriminant Analysis of Principal Components (DAPC), which is widely used to resolve weak genetic structure (Horne *et al.* 2013; Jombart *et al.* 2010), failed to reveal any clear partitioning between regions (figure 3B).Together these results are strong evidence of extensive gene flow over the entire sampling area.

Spatial autocorrelation analyses did not reveal any spatial structure regardless of whether the dataset was analysed as a whole, or split according to sex (Supplementary figure S1). Autocorrelation coefficients (*r*) were very close to 0 and their 95% confidence interval always overlapped with 0 and fell well within the expectations of the null model. None of the

correlograms were statistically significant according to the non-parametric heterogeneity test developed by Smouse *et al.* (2008) (p>0.1). This complete lack of spatial autocorrelation suggests that reef fidelity persisting for generations is unlikely and is consistent with the results from between region comparisons for both the mtDNA and microsatellite datasets.

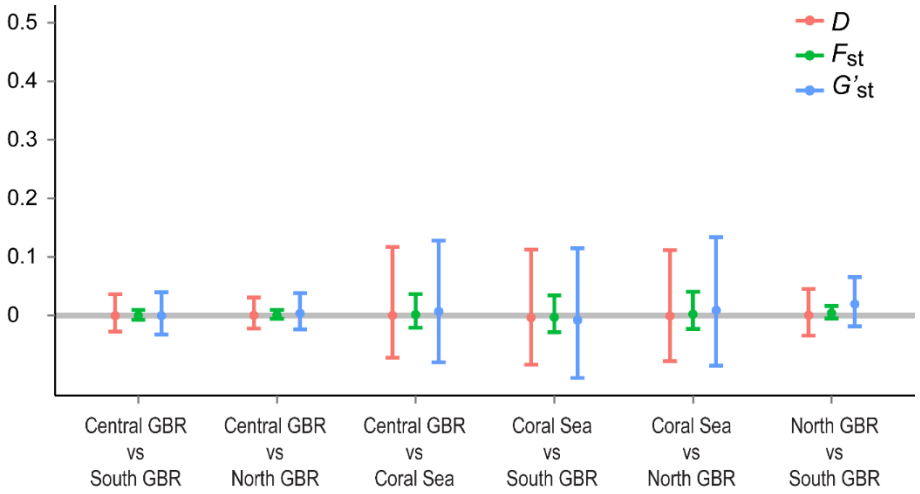

***Figure 2**: Between-region estimates of genetic differentiation with 95% confidence intervals: Weir and Cockerham F_{st} (F_{st}), Hedrick's G'_{st} (G'_{st}) and Jost's D (D). All estimates were very close to 0 and non-significant. Confidence intervals were estimated using 1000 bootstrap pseudo-replicates.*

Simulations

In contrast with the results from the empirical microsatellite and mitochondrial datasets, genetic differentiation between the three main regions in the simulated seascape was clearly evident in the simulations, even when migration between neighbouring reefs was set at 25% (figure 4A). Estimates of overall Meirman's F'_{st} range from 0.49 (1% migration) to 0.08 (25% migration), were all significant (p<0.05) and their confidence interval did not overlap with the F'_{st} value for the observed data. Positive spatial autocorrelation was detected in all simulations, and the extent of spatial autocorrelation was largely dependent on migration rates (figure 4B). Positive spatial-autocorrelation was detected in our simulations despite the fact that simulation parameters were overly conservative, and that we used smaller sample sizes than in our empirical dataset (see Supplementary Materials). These results suggest that the lack of spatial structure observed in the empirical dataset is not the result of limited analytical power, and likely reflects very high levels of connectivity.

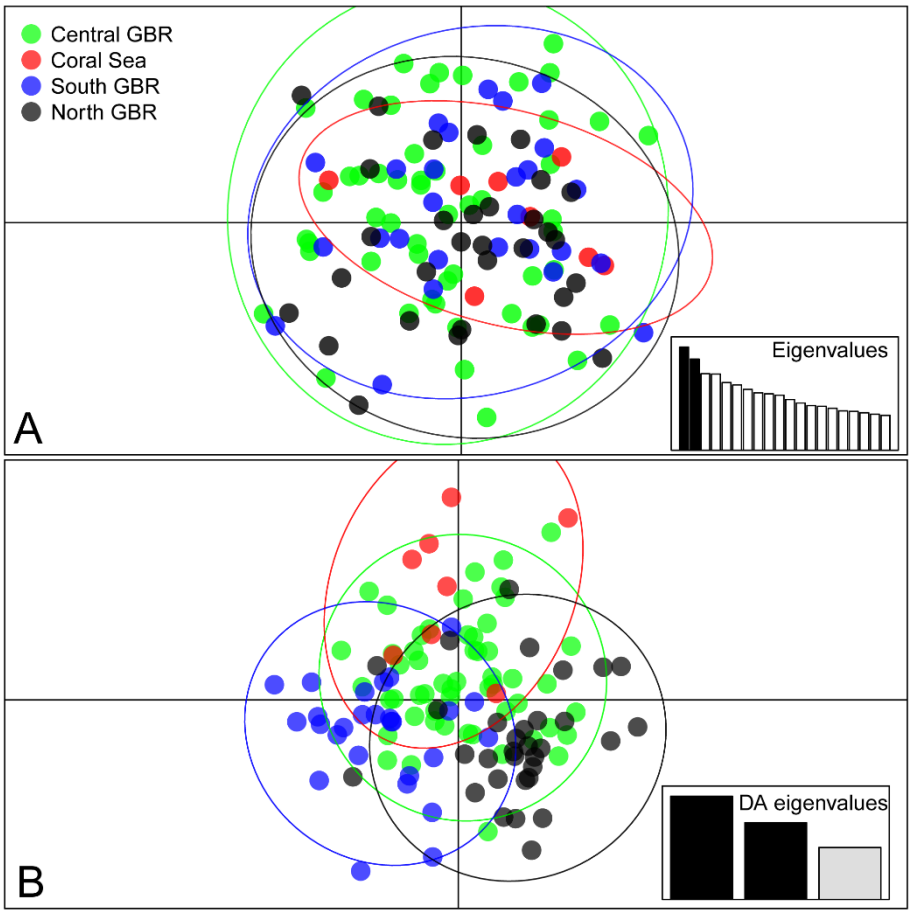

***Figure 3**: Results from PCA (A) and DAPC (B) analyses. Groups were defined as geographic regions within the sampling area (North GBR, Central GBR, South GBR and Coral Sea). Eigenvalues representing the variance explained by principal components (A) and discriminant factors (B) are shown in the scree plots. The x and y axes represent, respectively, the first and second principal components (A) and the first and second discriminant factors (B). Individuals are represented by dots, groups are color-coded and depicted by 95% inertia ellipses.*

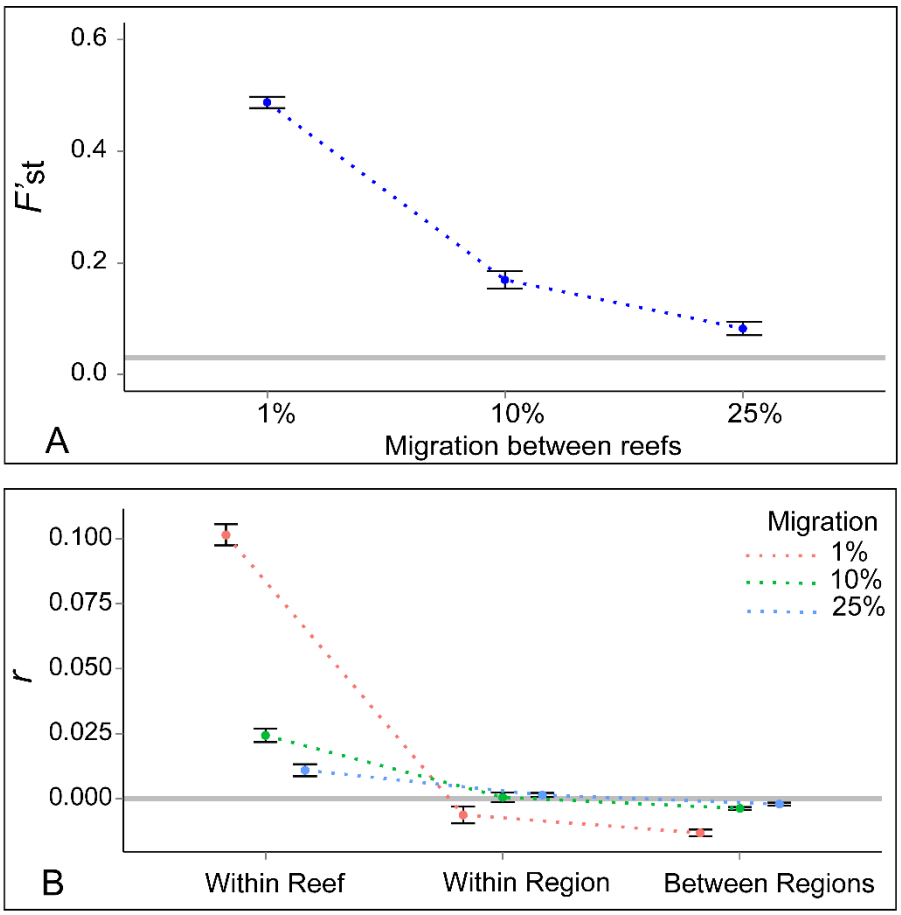

***Figure 4**: Results from the simulated datasets under different migration scenarios. Top graph (A) shows estimated global Meirman's F'*$_{st}$ *estimates between regions. Bottom graph (B) shows spatial autocorrelation estimates (*r *across different spatial scales: within reef, within region and across the entire simulated seascape). All scenarios resulted in positive and significant patterns of spatial autocorrelation. Error bars represent 95% confidence intervals obtained from 10 independent simulations. Grey lines in both A and B represent the estimates obtained from the empirical dataset.*

DISCUSSION

This first assessment of genetic connectivity in grey reef sharks was carried out across a latitudinal gradient spanning more than 1200 km and 9º of latitude i.e. 2/3 of the entire length of the Great Barrier Reef. Despite this spatial scale, we found no evidence of genetic differentiation across the study area and no evidence of fine-scale spatial structure. Our simulated data shows that we had the statistical power to detect structure at the regional geographical scale as well as fine scale spatial autocorrelation, even assuming high migration rates (~25% of individuals) between neighbouring reefs. With respect to temporal scales relevant to ecological processes, we found no genotypic spatial autocorrelation within reefs.

Structuring of allele frequencies via genetic drift may be effectively prevented by a small number of migrants per generation in large populations, and therefore may only become evident across large spatial and temporal scales (Ovenden 2013). It is possible therefore that large scale dispersal by a portion of the migrants resulted in an overestimate of genetic drift in our simulations, particularly given the fact that grey reef sharks in the GBR likely share a recent ancestry. Genotypic spatial autocorrelation on the other hand reflects individual dispersal across a few generations (Stow *et al.* 2001), is most pronounced at small spatial scales, measurable within less than 10 generations and reaches quasi-stationarity within 50-100 generations (Epperson 2005). We can therefore conclude that the lack of spatial autocorrelation reflects sufficient dispersal over a few generations to prevent genotypic structure from accumulating within the GBR for grey reef sharks.

Our results have important implications for the management of this species, and are in sharp contrast with the findings of several previous studies on other reef associated sharks (Mourier & Planes 2013; Vignaud *et al.* 2013; Vignaud *et al.* 2014; Whitney *et al.* 2012). In the closely related *Carcharhinus melanopterus* there are clear patterns of genetic structure between reefs separated by as little as 50 km within French Polynesia (Vignaud *et al.* 2013; Vignaud *et al.* 2014). Similarly, a recent study on gene flow of the other principal reef associated carcharhinid *(Triaenodon obesus*), also revealed very strong genetic structure (Φ_{st}=0.25) between North and Central GBR (Whitney *et al.* 2012). Perhaps more surprising than the lack of spatial structure within the GBR is an absence of genetic partitioning between the GBR and the Coral Sea (Herald Cays). The Herald Cays are isolated coral reefs located approximately 200 km east of the edge of the continental shelf where the outer reefs of the GBR lie. They are separated from the GBR by waters deeper than 1000 m. Therefore, it is evident that grey reef sharks not only disperse between semi isolated reefs within the GBR, but have the capacity for long distance dispersal across oceanic waters. Heupel *et al.* (2010) reported that in June 2008 an individual grey reef shark undertook a large scale movement (124 km) from Osprey Reef in the Coral Sea to the Ribbon Reefs in the North GBR. This same individual returned to Osprey Reef in October of that year (Barnett *et al.* 2012). Our results are in concordance with these observations and suggest that movements on the scale of 100s of km across oceanic waters occur at a rate sufficient to prevent detectable genetic drift between isolated reefs in the Coral Sea and the GBR.

Grey reef sharks are often described as strict habitat specialists, associated exclusively with coral reef habitats (Chin *et al.* 2010; Chin *et al.* 2012; Espinoza *et al.* 2014). Previous telemetry studies suggested that grey reef sharks exhibit a high degree of site fidelity in isolated

seamounts, as most animals show year-round residency within the study area (Barnett *et al.* 2012; Field *et al.* 2011). This has led to the presumption that even within the GBR grey reef sharks will exhibit a high degree of reef fidelity, and therefore will be well protected by strictly enforced but small marine protected areas (Rizzari *et al.* 2014a; Robbins *et al.* 2006). This interpretation has been supported by robust estimates of grey reef shark abundances across different management zones, showing that grey reef shark densities are up to an order of magnitude higher in strictly enforced no access areas compared to areas open to fisheries (Rizzari *et al.* 2014a; Robbins *et al.* 2006). Robbins *et al.* (2006) and Rizzari *et al.* (2014a) concluded that differences in fishing mortality are the main cause of the observed differences, and that therefore even very small MPAs can be very effective in increasing grey reef shark biomass if they are well complied with. In support, Hisano *et al.* (2011) argued that quantitative projections of abundances in fished versus unfished reefs calculated from differences in fishing mortality are also compatible with the observed differences.

This interpretation, however, relies on the assumption that grey reef sharks within each reef represent a closed population (Hisano *et al.* 2011). That is, it assumes: 1) that grey reef sharks are protected from fishing throughout most of their home range, and 2) that reef sharks within each MPA contribute to the biomass of future generations within the same MPA. Telemetry studies carried out in the same regions do not support the first assumption. Heupel *et al.* (2010) found that in the North GBR, grey reef sharks show little fidelity to the reef where they were initially tagged, consistently moving across different management zones. Even in the Central GBR, where reefs are isolated by much larger distances, 40% of the grey reef sharks (90% of adult males) were recorded moving between reefs, often crossing different management zones in a period of less than 2 years (Espinoza *et al.* 2015a). Furthermore we found no evidence of genetic spatial autocorrelation, suggesting that that migration rates between neighbouring reefs likely exceed 25%.

Heupel *et al.* (2010) suggested that differences in fishing mortality may not be the only driver of the differences in abundances observed by Robbins *et al.* (2006), and that other factors, such as differences in carrying capacity between areas open to fisheries and protected areas, might also be at play. It is possible that additional factors may affect grey reef shark abundances in strictly enforced protected areas. For example, strictly enforced MPAs have much larger fish biomasses (Edgar *et al.* 2014) compared to areas open to fisheries, and therefore could potentially sustain higher predator abundances. Espinoza *et al.* (2014) also found a positive effect of hard coral cover on grey reef shark abundance. Reefs with high hard coral cover provide higher structural complexity, and this in turn may sustain a higher biomass (Gratwicke

& Speight 2005) of potential prey. It is well established that MPAs that have been enforced for extended periods of times are more effective at preventing losses in hard coral cover (Selig & Bruno 2010). In the GBR, no-take areas have on average higher coral cover than areas open to fisheries (McCook *et al.* 2010), and experience much lower frequencies of crown-of-thorns starfish (*Acanthaster planci*) outbreaks (Sweatman 2008), a leading cause of decline in coral cover in the region (De'ath *et al.* 2012).

The lack of genetic structure in this study may be the result of either high juvenile or adult dispersal, or both, albeit available movement data point to adults representing the dispersal stage. In two acoustic tagging studies conducted on the GBR, adult grey reef sharks were found to exhibit limited reef fidelity and move repeatedly between multiple reefs. Espinoza *et al.* (2015a) reported that approximately 90% of adult males were detected on multiple (up to five), semi-isolated reefs in the Central GBR, while only one out of 12 tracked juveniles was detected at more than one reef. Heupel *et al.* (2010) also found that small juveniles exhibited high reef fidelity, while adults move continuously between reefs. Espinoza *et al.* (2015b) found that larger grey reef sharks and silvertip sharks (*Carcharhinus albimarginatus*), and especially large males, have wider home ranges that encompass multiple reefs. Given that larger sharks can traverse greater distances for comparable energetic expenditure (Parsons 1990), and are less vulnerable to predation, increased dispersal by adults is not surprising. However, a closely related species (*C. melanopterus*) has been shown to exhibit ontogenetic dispersal between inshore nursery and offshore reefs (Chin *et al.* 2013a). While there is no empirical evidence of the existence of nursery areas for grey reef sharks (juveniles co-occur with adults on coral reefs), the hypothesis that ontogenetic dispersal occurs cannot be discarded. Future genetic studies could compare genetic structure in different life-stages (juveniles, sub adults and adults), to test ontogenetic dispersal differences.

Espinoza *et al.* (2015a) suggested that in grey reef sharks high adult male dispersal may provide benefits in terms of genetic and demographic connectivity and/or provide foraging opportunities while reducing competition with large resident females. Our data do not show any evidence of sex-bias in genotypic spatial autocorrelation nor difference in genetic structure estimated from maternally (mtDNA) and bi-parentally inherited (microsatellites) genetic markers. These findings do not necessarily refute the hypothesis of sex-bias dispersal. Firstly, we did not have a large enough sample size of males and females from the same reefs to carry out specific tests for sex/bias dispersal. Secondly, Espinoza *et al.* (2015a) found that > 25% of mature females were detected on two distinct reefs within a period of 2 years. Since they investigated movement patterns between semi-isolated reefs (10s of km apart) within the

Central GBR, it is logical to assume that in other regions of the GBR where reefs are less isolated, movements between reefs would occur at higher frequencies, given that reefs that are separated by small distances may be perceived as a continuous habitat (Heupel *et al.* 2010; Momigliano *et al.* 2015). It therefore seems feasible, particularly given the results of our simulations, that female dispersal is large enough to prevent any detectable genetic drift of the mtDNA genome.

The widespread gene flow documented in this study, along with the findings of recent acoustic telemetry studies on grey reef shark movement suggest that while MPAs play an important role in protecting grey reef sharks, they are no panacea for reef shark conservation. Complementary management strategies appear necessary. The shortcomings of the current model of discontinuous networks of MPAs for the protection of coral reef organisms with adult dispersal have recently been highlighted by Momigliano *et al.* (2015) and Espinoza *et al.* (2015b). Potential solutions that have been proposed include the design of MPAs which include closely spaced (<20 km apart) reefs (Espinoza *et al.* 2015b) and the establishment of protected (i.e. no fishing) connectivity corridors that provide for movements between MPAs, thereby extending protection for animals with larger home ranges (Momigliano *et al.* 2015). Furthermore, complementary management strategies based on fishery restrictions, such the ones implemented in eastern Australia in 2009, appear necessary.

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SUPPLEMENTARY MATERIALS

Simulation parameters

To determine whether the statistical analyses performed would be able detect genetic structuring at different levels of between-reef migration, we carried out a range of simulations using the software EASYPOP (Balloux 2001). EASYPOP is a software that allows simulation of genetic drift using a range of migration models, and is oriented towards the simulation of microsatellite loci. It allows the user to set the number of loci, locus diversity, migration models, as well as mutation rates to mirror empirical datasets.

We created a network of 64 reefs (8x8), each reef with an area of 20 km^2. The census size of each reef was determined based on the densities available from the literature from pristine reefs (2 sharks per hectare) (Robbins *et al.* 2006). We assumed an effective population size (N_e) to census size (N_c) ratio of 1:10 (Frankham 1995), and created a dataset with the same number of loci and the locus diversity of the empirical dataset. Genetic drift was simulated for 100 generations with a 2-dimensional stepping stone model and levels of between reef migrations set at 1%, 10% and 25%. Ten simulations were carried out for each migration level. We randomly sampled from three simulated reefs in each region of the simulated seascape 10 individuals, and carried out the same repertoire of analyses that were carried out for the empirical dataset.

The values for each parameters were chosen so that the simulation would be overly conservative: that is, the effect of genetic drift within the simulated dataset would be likely much smaller than in a real scenario. For example, reef area was set to 20 km^2, while most reefs in the Great Barrier Reefs are much smaller (Almany *et al.* 2009), and the suitable habitat for grey reef shark is likely only a subset of the total area (Espinoza *et al.* 2014). For example, the shark densities used to estimate the census size for each reef were based on abundances in the reef slope of pristine reefs while shark density in the back reefs are known to be lower (Rizzari *et al.* 2014; Robbins *et al.* 2006). This results in inflated N_c and N_e. As the effects of genetic drifts is inversely related to Ne, the rate of genetic drift (and therefore our ability to detect it) will be likely much smaller in the simulated dataset compared to the empirical data. The number of generations (100) assumes reef sharks only colonized the GBR about 1000 years ago. Again, this is a very conservative choice as it implies a very short time scale for genetic drift to act. Furthermore, we only created a network of 64 reefs, while the GBR contains > 3000 reefs, and we sampled only 10 individuals from 3 reefs in each region (a smaller sample size than our

empirical dataset). Therefore, our power to detect genetic differentiation in the real dataset is likely to be much higher than in the simulated dataset.

Table S1 : Sampling locations and sample sizes (N). Note: as sex data were not available for all individuals, the sum of N males and N females is less than the total sample size N.

Location	Region	Latitude	Longitude	N	N males	N females
Day Reef	North GBR	-14,475	145.520	8	0	0
Lizard Island	North GBR	-14,677	145,444	24	12	12
Herald Cays	Coral Sea	-16,967	149,150	8	0	0
Barnet patches	Central GBR	-18,083	146,917	1	0	1
Pith Reef	Central GBR	-18,205	147,016	1	1	0
The Slashers	Central GBR	-18,539	147,121	1	1	0
Centipede Reef	Central GBR	-18,724	147,537	12	6	6
Lynch Reef	Central GBR	-18,777	147,710	14	2	5
Big Broadhurst Reef	Central GBR	-18,867	147,692	7	3	4
Judith Wright Reef	Central GBR	-18,893	147,950	6	3	3
Tobias Reef	Central GBR	-19,097	148,248	4	2	2
Darley Reef	Central GBR	-19,191	148,292	8	4	4
Heron Island reef	South GBR	-23,450	151,917	24	10	14
Wistari Reef	South GBR	-23.453	151.879	1	0	1
One Tree Island	South GBR	-23,489	152,065	2	0	2

Table S2: Microsatellite loci used in this study. N=sample size, N_A=number of alleles, H_0= observed heterozygosity, H_E= expected heterozygosity. The fluorescent dye used is indicated in superscripts: [1]6-FAM, [2]VIC®, [3]NED®, [4]PET®. Loci amplified using the M13 tail protocol (Schuelke 2000) are indicated with *. All loci were isolated by Momigliano *et al.* (2014), with the exception of Cpl169 which was isolated by Portnoy *et al.* (2006). PCR conditions were as described in Momigliano *et al.* (2014)

Locus	**Primer Sequence (Forward and Reverse)**	**Motif**	**Size (bp)**	**N**	**N_A**	**H_O**	**H_E**
C.amb2	F- TCCTACCTGACAAAGGAACTGC[4] R-ATGAACAGAGACAAACAGACCGAC	$(TCTA)_{14}$	161-204	112	15	0.911	0.894
C.amb3	F- TGGAGTGCCAATTCTCTTGTCG[1] R- ACTTGGGAGTCTGACTAATCTCC	$(TC)_{18}$	200-272	120	34	0.892	0.929
C.amb4	F- GTCGAATGCATTGAGTTTCAGG[2] R- CCAATACAAGCAAAGGGACAAC	$(AC)_{14}$	344-380	118	16	0.771	0.814
C.amb5	F- CAGATATGCGGTGTCGTGGC[1] R- TTCCGCGTCTTGTCTCTGC	$(TG)_{13}$	266-284	118	8	0.805	0.784
C.amb6*	F- TGTGGCTGGGATAAAATGCACG[3] R- TGGCTTGTAAAATCCTGTTCTGCG	$(TGG)_{13}$	251-296	120	12	0.808	0.795
C.amb7	F- AGAATGCTGTCTCGTGATGC[3] R- GTTGTCACTGTCGAGATAGAGC	$(AGAC)_{11}$	287-319	121	8	0.758	0.729
C.amb9	F- CCCAGGAGCCCTCTCTGTA[4] R- GTCTCTTGCCACGCTCCTAC	$(TG)_{13}$	209-227	120	8	0.533	0.576
C.amb11	F- TGAACGCTTTACTGAACCTTGC[3] R- GCAGCCTTTACTCCTCGTCA	$(CA)_{14}$	164-204	120	18	0.883	0.883
C.amb15	F- GTATGAGACGAGCATCGTGCC[2] R- AATCGCAGCGTCTGCAATG	$(AC)_{13}$	192-230	119	14	0.882	0.828
C.amb18	F- TGCACACGCAGTGATGTTGG[2] R- ATGCCGATTTCTCTGTTAATGAGC	$(AC)_{16}$	135-195	120	26	0.958	0.944
C.amb20	F- ATGTGGAGGAGTGATGTTAGCC[1] R- TTAATGTCAGTGTTCACGCTGG	$(GT)_{12}$	306-354	119	17	0.891	0.906
C.amb22	F- ATGTCAGTTCTTTAGGAGTAGGG[1] R- CCAATCTACACTTCACTCACTG	$(GA)_{11}$	346-356	118	3	0.356	0.308
C.amb25*	F- GACTCATCAGGATAGTCTGGATGCT[1] R- GCTCAACTGTCAAAAGAGGAAGCC	$(AGGG)_8$	200-252	121	13	0.802	0.802
C.amb27*	F- AGTCAGTGTCACGATGG[4] R- GCTTTCTATCATTAACATGAGATCC	$(TG)_{11}(AG)_{18}$	167-201	121	12	8.818	0.844
C.amb28*	F- CACATTGCTATGAGCCTGGAG[2] R- CATCTCTTTCATCACTGCATGATTG	$(AC)_{13}$	286-348	118	15	0.746	0.799
Cpl169*	F- TGACACAACCATTTATTCCCACG R- GGTTTCCTTGAGTGAAAGAGAGAGC	$(TG)_{42}$	118-198	118	33	0.890	0.944

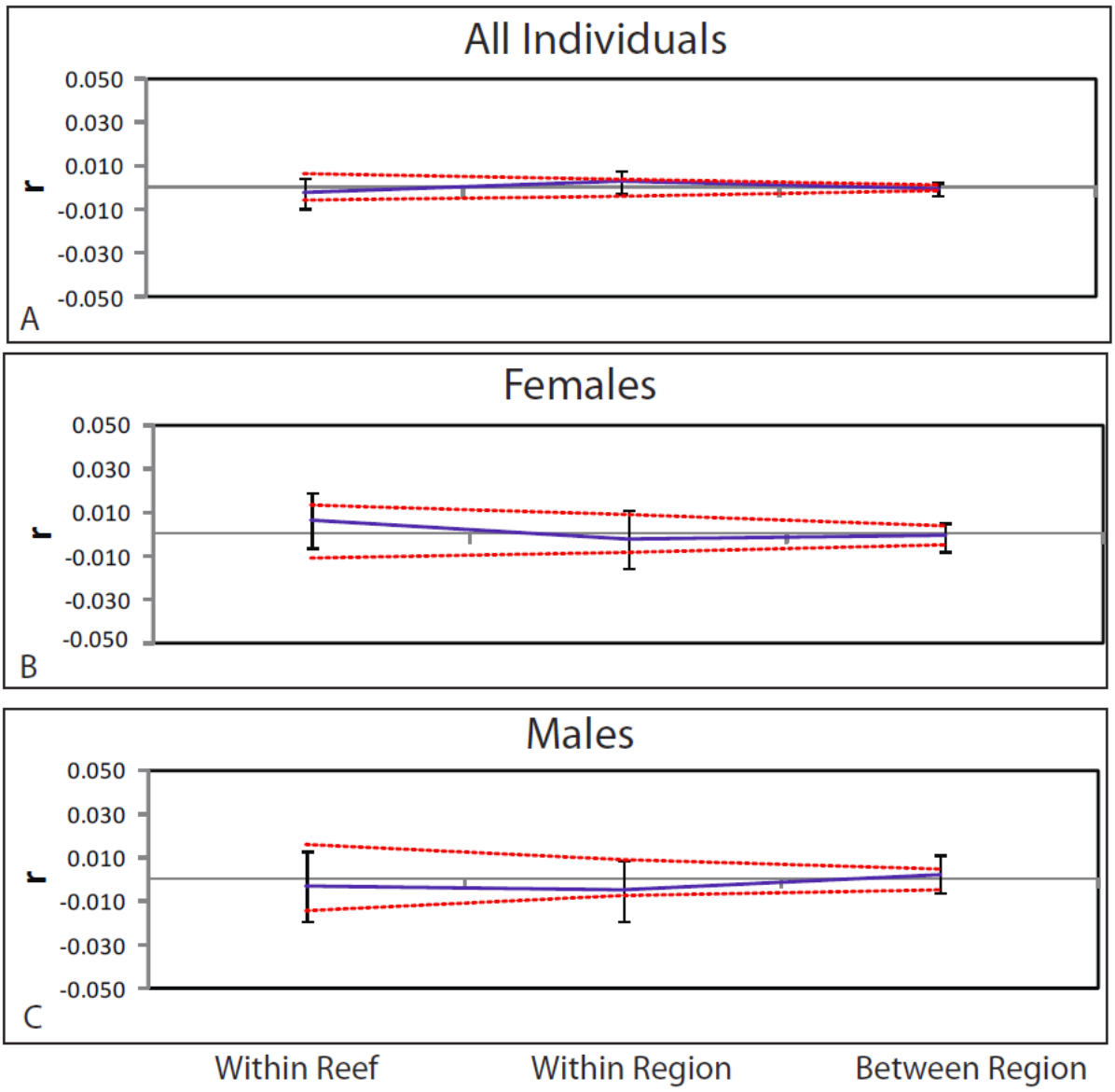

***Figure S1**: Genotypic spatial autocorrelation. Estimates of genotypic spatial autocorrelation (r) across different spatial scales. The x axis represents the different spatial scales (within a single reef, within a geographic region and across the entire sampling area), while the y axis represents the spatial autocorrelation coefficient (r). Analyses were carried out for all individuals (A) and then separately for females (B) and males (C). Error bars represent 95% confidence intervals estimated from 999 bootstraps. Red dotted lines represent the confident intervals of the null model (no spatial autocorrelation) estimated from 999 permutations.*

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Chapter 6

Genome scan reveals signatures of selection in a continuous population of grey reef sharks (*Carcharhinus amblyrhynchos*)

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ABSTRACT

Overfishing has reduced local shark abundance in multiple marine ecosystems, and knowledge of connectivity and local genetic adaptation is required for sustainable management. Despite technological advances allowing for more precise and accurate estimates of neutral and adaptive genetic structure, genetic studies on the oceans' most important predators have focused exclusively on small sets of neutral markers. Here we measured genetic connectivity in a key coral reef predator, the grey reef shark *Carcharhinus amblyrhynchos*, by genotyping 5106 Single Nucleotide Polymorphisms in 142 individuals sampled across Australian and Indonesian waters and 6 individuals from the Cocos (Keeling) Islands in the Indian Ocean. Neutral loci suggest a continuous population along the continental shelves of Indonesia and Australia with isolation by distance and high gene flow, while 3 loci putatively under strong divergent selection show a clear biogeographic pattern, indicating that grey reef sharks in eastern Australia are likely under different selective pressures to those in western Australia and Indonesia. Genetic distance averaged across all loci and at outlier loci were not correlated, suggesting that divergence at putative neutral and outlier loci are likely the results of different processes. Our results have important conservation implications for grey reef sharks, especially given their dramatic decline in eastern Australia. More broadly, our results have important implications for the interpretation of previous genetic studies used to identify fishery stocks and conservation units.

Keywords: Sharks; Population Genetics; Conservation; Selection

INTRODUCTION

Sharks are in decline across the world's oceans due to unsustainable fishing pressure, resulting in large scale changes to ecosystems (Myers *et al.* 2007; Robbins *et al.* 2006). To establish conservation priorities for these apex predators, accurate information on genetic connectivity, stock structure and local adaptation is needed. Over the past quarter of a century, genetic connectivity has been estimated using neutral genetic markers (Dudgeon *et al.* 2012). While these studies can successfully unveil present and past demographic processes, they are often ill-suited to measuring contemporary connectivity in marine vertebrates at temporal scales relevant to conservation (Allendorf *et al.* 2010). Furthermore adaptive genetic variation has never been considered when delineating elasmobranchs' conservation units, thereby preventing the identification of local adaptations that may warrant enhanced conservation measures (Funk *et al.* 2012).

Marine populations usually have large effective population sizes (N_e). While sharks have much lower census sizes (N_c) than teleosts, they have *Ne*:*Nc* ratios which are orders of magnitude higher than most teleosts, whereby up to more than 50% of the adult population can contribute to the next generation (Duncan *et al.* 2006; Portnoy *et al.* 2009). Genetic divergence at neutral loci is a function of the number of generations that have passed since a barrier to dispersal arose (*t*), *Ne*, and the number of migrants per generation (N_em). In the absence of migration, genetic differentiation (F_{ST}) increases over time at a rate roughly equal to $t/2Ne$ (Kalinowski 2002). Accordingly, it may take thousands of generations for neutral genetic structure to arise if *Ne* is high, even where there is complete reproductive isolation (Kalinowski 2002). Similarly when *Ne* is high, a very low migration rate (*m*) will yield a high N_em which will slow down genetic drift. This process is exacerbated in species with long generation times, such as many elasmobranchs. Not surprisingly therefore, sharks rarely exhibit strong neutral genetic structure along continental margins (Dudgeon *et al.* 2012).

By contrast, genes under selection diverge at much faster rates, and recent evidence suggests that selection acting on standing genetic variation may change the genetic and phenotypic makeup of populations in a few generation (Lescak *et al*. 2015). Divergence at loci under selection is a function of the relative values of *m* and the selection coefficient (*s*), a measure of the relative fitness of the individuals having a phenotype under local selection (Allendorf *et al.* 2010). For a given N_em and assuming a constant *s*, the greater N_e, the larger the difference between *m* and N_em becomes, and therefore the larger the difference between divergence at neutral and selected loci. Knowledge of divergence at loci under selection can reveal patterns of local adaptation which can be useful in delineating conservation units (Funk *et al.* 2012). Dudgeon *et al.* (2012) drew attention to the need for a paradigm shift in conservation genetics of elasmobranchs towards studies that address these issues, but as yet this call has gone unheeded.

Here we investigate both neutral and putatively adaptive divergence in the grey reef shark *Carcharhinus amblyrhynchos* in Australia and Indonesia. Grey reef sharks are among the most abundant resident apex predator on coral reefs but have undergone dramatic declines, even in effectively managed networks of Marine Protected Areas (MPAs), such as the Australian Great Barrier Reef Marine Park (GBRMP) (Robbins *et al.* 2006). Recent work has shown that there is no fine scale genetic structure at the regional level (Momigliano *et al.* 2015), however this study was geographically restricted and utlilized a small number of neutral loci, and may therefored have missed cryptic genetic differentiation driven by local selection. Given current estimates of grey reef shark abundance (Robbins *et al.* 2006) and high N_e:N_c ratios in sharks, it

is likely that historical N_e for this species was high (1000s to 10 000s of individuals), which should theoretically have helped maintaining the standing genetic variation on which selection may act upon (Savolainen *et al.* 2013). For these reasons *C. amblyrhynchos* represents an ideal species in which to compare neutral and putatively adaptive genetic variation.

METHODS

We collected fin clips from 142 individuals of *C. amblyrhynchos* at six locations in Australia and one location in Indonesia (figure1), as well as 6 individuals from the Cocos (Keeling) islands which we used as an outgroup population. DNA was extracted using a commercial kit, and DartSeq™ genotyping was performed at Diversity Arrays Technology Pty. Ltd. (Canberra, Australia). DartSeq™ genotyping is a Single Nucleotide Polymorphism (SNPs) discovery and genotyping-by-sequencing approach that combines Diversity Arrays markers (Jaccoud *et al.* 2001) and next-generation sequencing on Illumina platforms (Sansaloni *et al.* 2011) to genotype thousands of SNPs homogenously spaced across the genome (see supplementary materials). It uses a double enzymatic digestion (in this case *PstI* and *SphI*) as a genome complexity reduction approach, similar to double digest RAD sequencing.

Outlier loci were identified using four independent approaches. First, we used the Bayesian approach implemented in the software BAYESCAN (Foll & Gaggiotti 2008), using prior odds of 10 and a False Discovery Rate (FDR) of 0.05. Second, we used coalescent simulations using the hierarchical island model to obtain joint null distributions of *F*-statistics and locus specific p-values (Excoffier *et al.* 2009) in the software Arlequin 3.5, choosing a critical p-value of 0.001. As however both these methods, albeit widely used, may yield many false positives under an isolation by distance (IBD) scenario (Lottheros & Whitlock 2014), we also included two additional analyses which take into account evolutionary non-independence among sampled populations and proved to be much less prone to false positives when population are spatially autocorrelated: FLK and OutFLANK.

FLK (Bohomme *et al.* 2010) is an extension of the original Lewontin and Krakauer test (Lewontin & Krakauer 1973) for the identification of outliers which estimates the population kinship matrix using a phylogenetic approach, and accounts for heterogeneity in genetic drift and evolutionary non-independence in its test-statistics ($T_{F\text{-}LK}$). Cocos (Keeling) was used as the outgroup population in the FLK analyses. OutFLANK (Whitlock & Lottheros 2015) is also based on the observation from Lewontin and Krakauer (1973) that the distribution of F_{ST} at neutral loci is approximated by a χ^2 distrubution with df = n-1 (where n is the number of populations) assuming all populations are evolutionary independent. However, when

populations are spatially auto-correlated the parameter of the χ^2 distribution is often not equal to n-1 . OutFLANK uses the core distribution of F_{ST} (i.e. the part of the distribution excluding the top and bottom 5%, which is unlikely to be under strong balancing or diversifying selection) to estimate via likelihood the df of the χ^2 distribution of neutral F_{ST} and hence predict the entire χ^2 distribution of loci which are unlikely to be under strong diversifying selection. A detailed explanation of each of the outlier test performed is given in the supplementary materials.

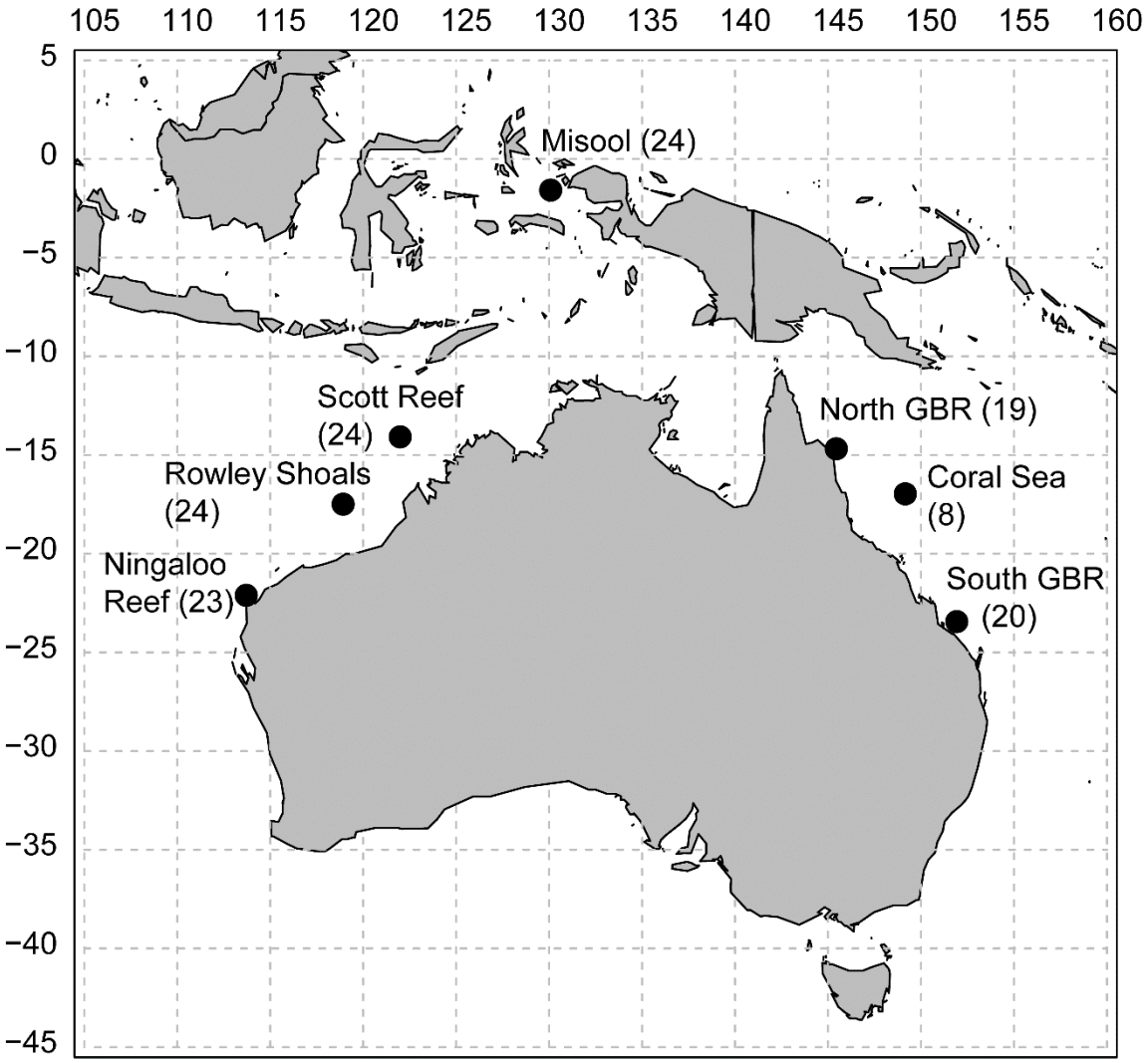

Figure 1: *Sampling locations in Australia and Indonesia. Sample sizes are given in brackets.*

We calculated pairwise Weir and Cockerham F_{st} and their 95% confidence intervals using 100 bootstrap replicates. We tested whether divergence at neutral loci follows an isolation by distance model by performing a major axis regression on pairwise F_{st} and geographic distance and testing for significance of the relationships using a Mantel test (see supplementary materials). Furthermore, we tested whether divergence at neutral loci and loci putatively under selection were correlated, and could therefore be the result of the same processes.

RESULTS

The final dataset consisted of 5106 SNPs. None of the outlier test identified any loci under balancing selection, while the number of identified outliers potentially under spatially diversifying selection varied greatly among tests. As expected, the two tests which do not account for spatial autocorrelation identified the most outliers (Arlequin identified 25 outliers and BAYESCAN 28), yet at least some of these are likely be false positives (figure 2a). FLK identified 17 outlier loci, 8 of which were also identified by both BAYESCAN and Arlequin (figure 2b). OutFLANK was themost conservative of all approaches, reporting only 3 outlier loci, all of which, however, were also identified by the other three method (figure 2c & 2d). Hence all outlier tests yielded a consensus of three loci which exhibit strong signatures of spatially diversifying selection.

Most pairwise F_{ST} averaged across all loci among locations in Australia and Indonesia were not significant (Table 1), but they show a positive, linear relationship with geographic distance (figure 2e) ($p<0.001$), which explained more than 90% of the variation over all locations. On the contrary most pairwise F_{ST} for outlier loci were significant. Divergence was very low between locations within regions in Australia and Indonesia (Table 1, figure 2f), but very high between region. Genetic distance at all loci and at the three outlier loci were not correlated (figure 2 f). Genetic distance at neutral loci was highest, as expected, when comparing the populations from Australia and Indonesia to the outgroup population (Cocos Islands, see Table 1 and figure 2f). Genetic differentiation at outlier loci, however, was highest (F_{ST} up to 0.68) among locations in Eastern and Western Australia which show only very weak genetic differentiation at neutral loci (F_{ST} 0.01)

Eastern Australia location had very high observed heterozygosity (H_O) in the three outlier loci (0.417-0.617), while all locations on the other side of the Torres Straight had very low H_O (0.097-0.2) at the same loci (Table S2), suggesting these loci may be under disruptive selection.

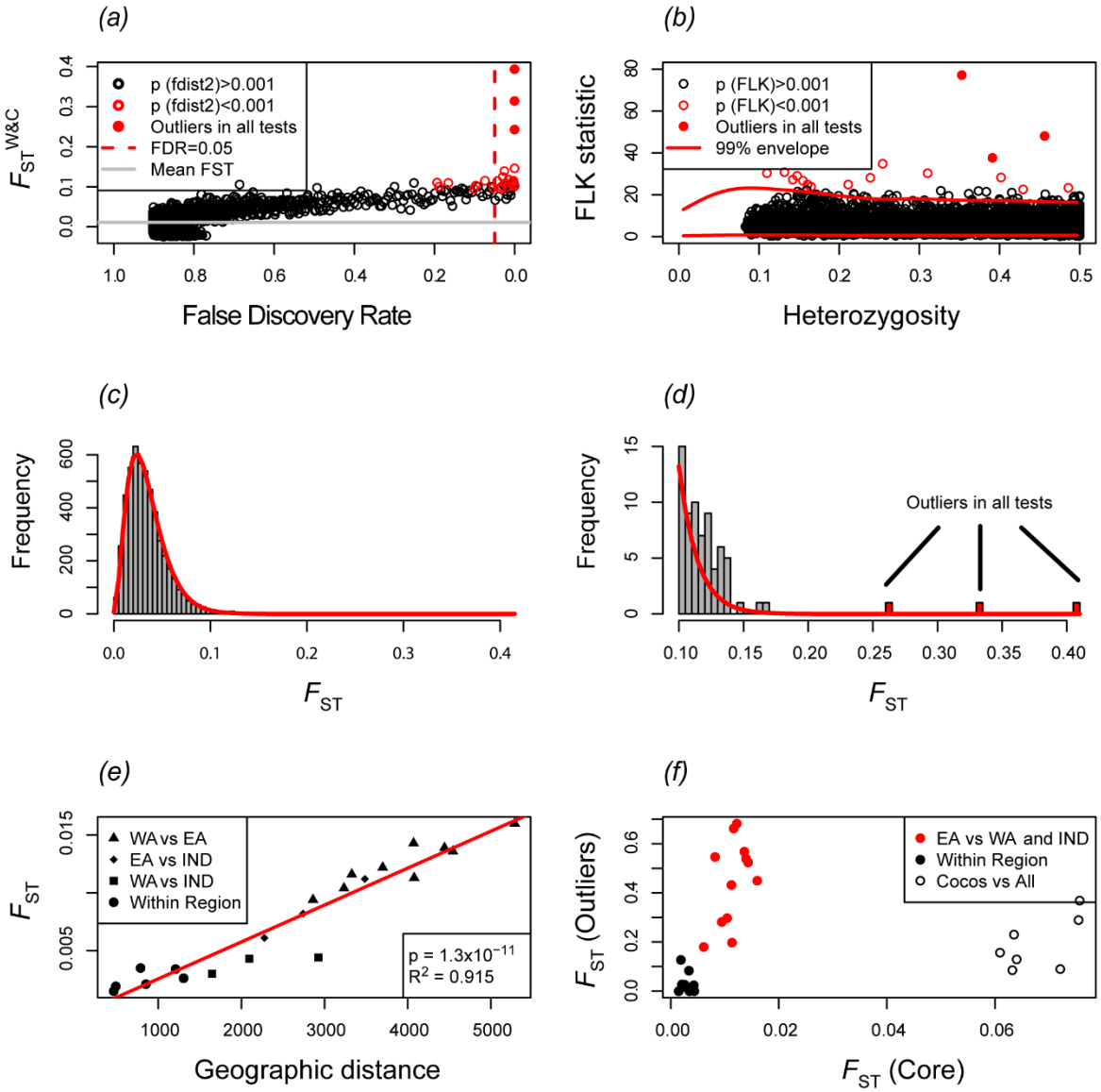

Figure 2: a) *Outlier loci identified as potentially under divergent selection by Arlequin and BAYESCAN. The red dashed line represent the false discovery rate of 0.05, red open circles on the right of the dashed line represent the outlier loci that were identified by both methods, red filled circle represent the outliers identified by all methods* b) *Results from FLK. Red lines define the 99% probability envelope of the neutral distribution of FLK statistics, red open circle show outliers identified by FLK at a critical p value of 0.001, filled red circle represent the 3 outliers which were identified in all analyses* c) F_{ST} *distribution of neutral loci estimated using the OutFLANK method.* d) *Right tail of the neutral FST distribution, showing the 3 outliers identified by OutFLANK (also identified by all other approaches)* e) *relationship between pairwise* F_{ST} *and geographic distance (outgroup population not included)* f) *relationship between "neutral"* F_{ST} *(estimated from the core distribution o FST) and average* F_{ST} *of the three outlier loci for all populations (including outgroup).*

Table 1: Pairwise F_{st} calculated across all loci and using the 3 outlier loci and the associated lower and upper bounds of their 95% confidence intervals. Significant values shown in italics.

Location comparison	F_{st} (all loci)	Lower	Upper	F_{st} (outliers)	Lower	Upper
Cocos vs. Ningaloo	*0.064*	*0.0402*	*0.1126*	0.1295	-0.0104	0.2898
Cocos vs. Rowley	*0.0635*	*0.039*	*0.1048*	*0.23*	*0.0288*	*0.4407*
Cocos vs. Scott	*0.0609*	*0.0371*	*0.1008*	0.1565	-0.0357	0.3891
Cocos vs. Indo	*0.0632*	*0.0376*	*0.1068*	0.0852	-0.0435	0.263
Cocos vs. North GBR	*0.0721*	*0.046*	*0.1121*	0.09	-0.0341	0.2329
Cocos vs. Coral Sea	*0.0757*	*0.0381*	*0.1252*	*0.3689*	*0.1156*	*0.621*
Cocos vs. South GBR	*0.0755*	*0.0505*	*0.1166*	*0.2885*	*0.114*	*0.4642*
Ningaloo vs. Rowley	0.0021	-0.0061	0.0137	0.0268	-0.0248	0.1025
Ningaloo vs. Scott	0.0026	-0.0065	0.0127	0.025	-0.0288	0.1141
Ningaloo vs. Indo	0.0044	-0.0052	0.0149	-0.0152	-0.0346	0.0274
Ningaloo vs. North GBR	*0.0113*	*0.0021*	*0.0247*	*0.1961*	*0.1018*	*0.3098*
Ningaloo vs. Coral Sea	0.0136	-0.0047	0.0448	*0.569*	*0.384*	*0.7278*
Ningaloo vs. South GBR	*0.016*	*0.0059*	*0.0276*	*0.4497*	*0.3354*	*0.563*
Rowley vs. Scott	0.0015	-0.006	0.0119	-0.006	-0.0367	0.0561
Rowley vs. Indo	0.0043	-0.0048	0.0145	0.0229	-0.024	0.0966
Rowley vs. North GBR	*0.0104*	*0.0003*	*0.0247*	*0.2969*	*0.197*	*0.4033*
Rowley vs. Coral Sea	0.0122	-0.0088	0.0447	*0.6827*	*0.545*	*0.8122*
Rowley vs. South GBR	*0.0139*	*0.006*	*0.0267*	*0.5392*	*0.4408*	*0.6357*
Scott vs. Indo	0.003	-0.0042	0.0116	0.0136	-0.0309	0.0872
Scott vs. North GBR	*0.0094*	*0.001*	*0.0202*	*0.2814*	*0.1691*	*0.3942*
Scott vs. Coral Sea	0.0116	-0.0082	0.0401	*0.6631*	*0.5102*	*0.8071*
Scott vs. South GBR	*0.0143*	*0.0059*	*0.0293*	*0.5249*	*0.4205*	*0.6174*
Indo vs. North GBR	0.0061	-0.0027	0.0165	*0.1794*	*0.0718*	*0.2963*
Indo vs. Coral Sea	0.0082	-0.0101	0.0379	*0.5455*	*0.3605*	*0.7159*
Indo vs. South GBR	*0.0112*	*0.0021*	*0.0228*	*0.4321*	*0.3174*	*0.5464*
North GBR vs. Coral Sea	0.0019	-0.019	0.0378	0.1269	-0.0063	0.2875
North GBR vs. South GBR	0.0034	-0.0093	0.0192	*0.0824*	*0.0061*	*0.1787*
Coral Sea vs. South GBR	0.0035	-0.0161	0.0353	-0.0249	-0.0739	0.0795

DISCUSSION

Molecular genetics has played a major role in elasmobranch conservation and management over the last quarter of a century, yet most population genetics studies of sharks have to date focused exclusively on neutral genetic variation using a small number of loci (Dudgeon *et al.* 2012). Here we used thousands of genome-wide SNPs to investigate both neutral and putatively adaptive genetic variation in a key coral reef predator. Divergence measured at neutral SNPs was extremely low and followed an isolation by distance model, while divergence at loci putatively under selection was over an order of magnitude higher and showed a clear biogeographic pattern. Divergence at neutral SNPs and SNPs putatively under strong diversifying selection were not correlated suggesting they are the result of the fundamentally different processes.

The lack of strong genetic differentiation at neutral loci is not surprising. A recent study found that grey reef sharks show no sign of fine scale genetic structure across Eastern Australia (Momigliano *et al.* 2015). Considering the estimates of abundance of grey reef sharks (Robbins *et al.* 2006), and large *Ne*:*Nc* ratios in elasmobranchs (Portnoy *et al.* 2009), historical grey reef shark *Ne* in Eastern Australia was likely in the tens of thousands of individuals. Assuming a historical *Ne* of the same order of magnitude in Indonesia and Western Australia, it could take hundreds to thousands of generations for small levels of genetic drift to arise even if there was complete reproductive isolation (Kalinowski 2002). Taking into account grey reef shark generation time (Robbins *et al.* 2006), this translates into thousands to tens of thousands of years, a time frame similar to that estimated for the major changes in sea level that gave rise to contemporary coastal habitats.

Analysis of loci putatively under selection revealed a sharply contrasting pattern, suggesting that sharks on either side of the Torres Strait may have undergone divergent adaptations. While we have no information on which selective pressures may be responsible, our data are consistent with grey reef sharks in eastern Australia, which have undergone dramatic declines, being under different selective pressures than elsewhere throughout the range sampled. Torres Strait was completely exposed during the last glacial maximum and it wasn't submerged again by the rising seas until 2000 years after the end of the last glaciation (approximately 8000 years ago). Barriers to dispersal are expected to promote local adaptation (Marshall *et al.* 2010) and it is therefore possible that local selective pressures and reproductive isolation have acted in concert to produce the pattern shown here. Future management strategies for this species in the GBRMP and elsewhere need to take into account that grey reef sharks are likely locally adapted and that local declining populations are unlikely to be rescued by migrants, as migrants may exhibit lower fitness due to phenotype-environment mismatches (Marshall *et al.* 2010).

In the past, genetic studies of elasmobranchs have been important in defining fishery stocks and conservation units (Dudgeon *et al.* 2012). Yet they may have largely underestimated the extent of genetic divergence for one of the most important groups of ocean predators. Strong, but cryptic, genetic differentiation driven by local selection may have been overlooked, and it remains questionable whether low levels of genetic differentiation along continental margins are proof of contemporary connectivity, or simply the result of large effective population sizes and sampling of slower-evolving, neutral loci.

ACKNOWLEDGEMENT

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DATA AVAILABILITY

Data will be deposited in the DRYAD Digital Repository upon publication

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SUPPLEMENTARY MATERIALS

Single Nucleotide Polymorphism Genotyping

SNPs discovery and genotyping was performed at Diversity Arrays Technology Pty. Ltd. (Canberra, Australia), using the standard DartSeq™ protocol. DartSeq™ genotyping is a Single Nucleotide Polymorphism (SNPs) genotyping-by-sequencing approach that combines Diversity Arrays (DArT) markers (Jaccoud *et al.* 2001; Luikart *et al.* 2003) and next-generation sequencing on Illumina platforms (Sansaloni *et al.* 2011) to genotype thousands of SNPs homogenously spaced across the genome (Petroli *et al.* 2012). The original DArT method is described in Jaccoud *et al.* (2001) and its combination with next generation sequencing for SNPs genotyping is described by Sansaloni *et al.* (2011). We briefly outline the protocol below.

DNA extraction and library preparation. DNA was extracted using a commercially available spin-column kit (GenCatch TM Blood &Tissue Genomic Mini Prep Kit, Epoch Biolabs), following manufacturer's instructions. Extracted DNA samples were incubated in a 1X solution of Multi-Core™ restriction enzyme buffer (Promega), and subsequently run on a 0.8% agarose gel pre-stained with GelRed™ to determine whether the samples had high molecular weight DNA and were not contaminated with nucleases that may interfere with downstream enzymatic digestions. Approximately 100 ng of each sample was digested using a combination of *PstI* and *SphI* restriction enzymes. *PstI* and *SphI* compatible adapters were ligated to each samples. The *PstI* adapters include Illumina flowcell attachment sequence, sequencing primer and unique barcode sequences for each samples

Following digestion and ligation, samples were cleaned using a spin-column Qiagen PCR clean up kit. Each sample was amplified using a PCR with primers specific to the adaptor and barcode sequences. The PCR conditions were as follows: 1 min initial denaturation at 94 C°, followed by 30 cycles of 20 sec denaturation (94 °C), 30 sec annealing (58 °C) and 45 sec extension (72 °C), and a final extension of 7 min at 72 °C. Equimolar amounts of all samples were pooled, diluted and denatured using NaOH in preparation for hybridization to the flow cell. The library was sequenced on an Illumina HiSeq2500 platform (single read) using 77 cycles, resulting in fragments 77 bp long. A proportion of the samples (60 individuals, ie. >40% of the samples) was carried a second time through the whole library preparation protocol and downstream analyses to create the set of technical replicates which were used to assess the reproducibility of SNPs calls.

Quality Control and SNPs calling. Raw sequence data were converted to .fastq files using the Illumina HiSeq2500 software, and individuals were de-multiplexed based on the ligated barcodes. Each read was assessed using PHRED (Ewing & Green 1998) quality scores (Q-score), and any read containing Q-scores of <25 was removed. All reads were checked against the DArT database (Diversity Arrays Technology Pty. Ltd., Canberra), and GenBank bacterial and viral sequences to identify potential contaminations. Following this primary workflow, SNPs were identified and called by Diversity Arrays Technology Pty. Ltd. following the standard procedure in DArT pipeline DArTSoft14^{TM} (Diversity Arrays Technology). The pipeline workflow is technically similar to the commonly used STACKS pipeline (Catchen *et al.* 2013), yet it differs from it in that sequence clusters are first called for all pooled samples prior to being called for each individual. All monomorphic sequence clusters were removed, and SNPs were called only if they were present in both homozygous and heterozygous forms. The DArT pipeline also removed any locus with very high read depth, retaining only SNPs with a high balance of read counts in allelic pairs (range: 0.35-2.4, average: 0.79), with a reproducibility of >95% and a minimum read depth of 5.

We further filtered the dataset using the following criteria

1- Call rate of >95% i.e. less than 5% missing data over the whole dataset
2- Less than 10% missing data in any sampling location. This means across our locations that in those with the highest sample sizes (n=24) we had no more than 2 individuals with missing data for any locus, in locations with 20 or less individuals we had no more than 1 individual with missing data for any given locus, and no missing data for our smallest sampling area (Coral Sea, n=8; Cocos Keeling, n=6).
3- We only retained the first SNP in each fragment, to avoid creating a dataset containing closely linked loci
4- We required 100% reproducibility across technical replicates
5- A maximum read depth equal to $d + 4 * \sqrt{d}$, whereby *d* is the average read depth. This step has been shown to be very effective in reducing the number of false heterozygotes due to sequencing errors or due to the presence of paralogs (Li 2014). This resulted in a set of SNPs with average read depths between 10 and 40.3.
6- Minor Allele Frequencies (MAF)>0.05

We identified a total of 5106 SNPs after filtering for the above parameters. To further reduce the risk of including false variants in further analyses, all loci were tested for departures from Hardy Weinberg Equilibrium (HWE) using the function hw.test in the R package pegas (Paradis 2010). We tested for departures from HWE in three locations for which we had large sample sizes, one representative of each geographic region: Ningaloo reef (N=23), Misool (N=24) and South GBR (n=19). We maintained a low significance threshold ($p<0.05$), to avoid losing too much statistical power. To control for false positives, we required a locus to deviate from HWE in all three locations to exclude it. Within each location, between 225 (Ningaloo) and 259 loci deviated from HWE, a number which is very close to the number of false positives expected when carrying out 5106 multiple comparisons at an alpha of 0.05 (5106*0.05=255). Only 12 loci deviated from HWE in more than one location (again, roughly the number of false positives that would be expected due to chance alone) and none in all three locations, therefore all loci were retained for further analyses.

DNA sequences and statistics (call rate, heterozygosity, read depth and reproducibility) for all loci as well as genotypes for all individuals will be accessible from the Dryad Digital Repository once the manuscript is published. All data are also deposited at Diversity Array Technology Pty. Ltd. (Canberra, Australia).

Detection of loci under selection

General approach. We utilized outlier tests to identify loci for which genetic differentiation (F_{ST}) is higher than expected by genetic drift alone. There are various methods that can be used to identify loci under selection, and each has different biases, including limited sensitivities (high rate of Type 2 errors) and susceptibility to false discoveries (Type 1 errors) (Narum & Hess 2011). Methods which are commonly used include the Bayesian approach implemented in BAYESCAN (Foll & Gaggiotti 2008),the hierarchical model, coalescent simulation approach implemented in Arlequin (Excoffier *et al.* 2009) as well as Bonhomme *et. al* (2010) extensions of the Lewontin-Krakauer test which accounts for population co-ancestry (FLK). Each of these methods performs well under certain conditions, but both Arlequin and Bayescan can identify a large number of false positives when populations are spatially auto-correlated (Lottheros & Whitlock 2014). The FLK approach is much less susceptible to false positive under scenarios of evolutionary non independence among population (Lottheros & Whitlock 2014). A new method named OutFLANK, which estimates a null distribution of F_{st} for loci unlikely to be under strong positive selection based on the core distribution of a large set of loci, has also proved to be very robust under a number of demographic history scenarios

including IBD, although it is only suited for the identification of loci under strong spatially diversifying selection (Whitlock & Lottheros 2015). In this study we used all the methods outlined above in combination to identify loci putatively under selection, which are defined as the loci which were independently identified as outliers across all tests.

Description of each method. In BAYESCAN posterior distributions are obtained using a Reversible-Jump Markov Chain Monte Carlo algorithm. We ran 20 pilot runs, followed by 100 000 iterations; that is, 5000 samples with a thinning interval of 10 and a burn-in of 50 000 iterations. For the BAYESCAN analysis, using uninformative prior odds for the neutral model and the model including selection assumes that for each locus the models are equally likely. When sampling a large number of loci this can increase the probability of false discoveries. To minimize the chance of false discoveries we set the prior odds to 10 (corresponding to a prior belief that the selection model is 10 times less likely than the neutral model), which is the value suggested by the authors as appropriate for studies with a few thousands loci. Posterior odds are then calculated from Bayes Factors according to the formula PO=BF*P(M2)/P(M1), where P(M2) and P(M1) represent the prior probabilities for the model under selection and the neutral model respectively. We applied a False Discovery Rates (FDR, qval) of 5 % as a cut off value to identify loci under selection, which resulted in the identification of 28 loci putatively under spatially diversifying selection.

Arlequin uses a coalescent method that is an extension of the method introduced by Beaumont and Nichols (Beaumont & Nichols 1996) to cases where populations are hierarchically structured, in which cases it has been shown to reduce the number of false discoveries (Excoffier *et al.* 2009). In the Arlequin analysis, we grouped locations into 3 groups according to biogeographic regions: Indonesia, western Australia and eastern Australia. We set the number of groups to 10, the number of simulated demes per group to 100 and we performed 50 000 coalescent simulations. We used a critical p value of 0.001 to identify outlier loci, which resulted in 24 loci identified as outliers (19 of which identified by both Arlequin and BAYESCAN).

We implemented the FLK method described by Bonhomme *et al.* (2010) using the R and Python codes which the authors made publically available (https://qgsp.jouy.inra.fr/, accessed on the 12th of January 2016). Shortly, Reynold's distances among populations were computed in the R package Ape and a neighbour joining tree was built using Cocos Islands as the outgroup. A *F* matrix (see Bonhomme *et al.* 2010) was computed from the output of the neighbour joining tree and the FLK statistics and their corresponding χ^2 based p-values were calculated. A null

distribution of the FLK statistic was then estimated using a simulation approach implemented using the Python code provided by the authors. The FLK approach identified 15 outlier loci , 8 of which were also identified by both BAYESCAN and Arlequin.

The OutFLANK outlier test was implemented using the R package OutFLANK (accessed via https://github.com/whitlock/OutFLANK on the 14th of February 2016). The null model was estimated by trimming the lower and upper 5% of the F_{ST} distribution to infer the shape of the entire F_{ST} distribution as outlined in Whitlock & Lottheros (2015). Calculated *p*-values for each locus were transformed in q values to account for multiple comparisons. OutFLANK was the most conservative of all tests, identifying only three loci as outliers (all of which had been also identified by all other tests).

Table S1: List of the 8 outlier loci identified by at least 3 of the four outlier tests. Locus= locus name; Qval= FDR at which the locus is significant in the BAYESCAN analysis ;alpha= alpha values for the BAYESCAN analysis (positive values indicate diversifying selection); F_{ST} (B)= F_{ST} values for each locus as calculated by BAYESCAN; F_{ST} (A)= Weir and Cockerham F_{ST} values for each locus as calculated by Arlequin; *p (Arl)* = *p* values for each locus in the Arlequin analysis; FLK= FLK statistics; p(FLK); p (FLK) =p value of the FLK statistics and Qva (OutFLANK)= q value for the OutFLANK test.

Locus	**Qval (Bayes)**	$F_{ST}^{W\&C}$	***p* (Arl)**	**FLK**	***p* (FLK)**	**Qval (OutFLANK)**
L350	0	0.146183	$8.8x10^{-08}$	23.2757	0.000709	0.053799
L458	0	0.314614	$4.95x10^{62}$	48.1510	$1.1x10^{-8}$	$1.51x10^{-07}$
L479	0	0.393737	$1.53x10^{-98}$	77.1583	$1.38x10^{-14}$	$4.35x10^{-10}$
L1341	0	0.242348	$3.42x10^{-21}$	37.6402	$1.32x10^{-6}$	$3.63x10^{-05}$
L116	0.002526	0.115476	0.00031	22.6717	0.000914	0.223812
L6227	0.015417	0.117735	0.000265	28.3208	$8.18x10^{-5}$	0.223812
L5098	0.026115	0.139202	0.000363	34.8060	$4.70x10^{-06}$	0.073397
L6434	0.02936	0.124416	0.000672	24.9757	0.000345	0.182931

Calculation of diversity indices, pairwise F_{st} and Isolation by Distance

We calculated several diversity indices for all locations: observed heterozygosity (H_O), expected heterozygosity (H_E) and the fixation indices F= (H_E-H_O)/H_E (Table S2). Expected and observed heterozygosities ranged from 0.300 to 0.318 and from 0.307 to 0.314 respectively (Table S2), and there was no sign of heterozygosity deficiency or heterozygosity excess, with the exception of a slight heterozygosity excess in the Coral Sea (which could however be the result of a slight bias in sampling due to the small sample size at this location). We calculated pairwise F_{st} across all loci and across outlier loci, using the function *diffCalc* in the R package

diveRsity (Keenan *et al.* 2013). To estimate 95% confidence intervals for pairwise F_{st} we created a set of 100 pseudo replicates by bootstrapping individuals within each location. We then calculated least cost path oceanic distances between locations using the package *marmap* (Pante & Simon-Bouhet 2013) in R. We used Mantel tests to determine the significance of the relationship between geographic and genetic distance, and major axes regression to fit linear models where there was a significant and linear relationship.

Table S2: Diversity indices obtained from all loci for each sampling location, along with their standard error. N= sample size, H_O= observed heterozygosity, H_E= expected heterozygosity, *F*= fixation index, H_O (Outliers) = observed heterozygosity at outlier loci.

Location	**N**	**H_O**	**H_E**	***F***	**H_O (Outliers)**
Cocos Islands	6	0.314±0.004	0.272±0.003	-0.141±0.005	0.389±0.147
Ningaloo Reef	23	0.314±0.002	0.315±0.002	0.006±0.003	0.203±0.2
Rowley Shoals	24	0.313±0.002	0.314±0.002	0.005±0.003	0.097±0.077
Scott Reef	24	0.314±0.002	0.316±0.002	0.008±0.003	0.111±0.028
Misool	24	0.321±0.002	0.318±0.002	-0.007±0.003	0.222±0.002
North GBR	19	0.312±0.002	0.312±0.002	0.003±0.003	0.596±0.063
Coral Sea	8	0.314±0.003	0.300±0.002	-0.044±0.005	0.417±0.232
South GBR	20	0.307±0.002	0.307±0.002	0.002±0.003	0.617±0.148

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Chapter 7

Genetic connectivity in the grey reef shark (*Carcharhinus amblyrhynchos*) is sex-biased and influenced by the spatial distribution of coral reefs.

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ABSTRACT

Identifying processes that shape connectivity in sharks is frustrated by poor knowledge of dispersal behaviour in concert with often complex patterns of spatial genetic structure. For coral reef associated sharks, high degrees of habitat specialization suggest that the spatial distribution of reef habitats may influence demographic and genetic connectivity. Here we explore this hypothesis, and assess sex-biased dispersal by characterising large scale and fine scale genetic connectivity in the grey reef sharks (*Carcharhinus amblyrhynchos*). We use the largest dataset ever applied to evaluate genetic structure for an elasmobranch, including 5509 nuclear SNPs and a mtDNA gene. We collected samples at nine locations in Australia, Indonesia and from oceanic reefs in the Indian Ocean. Large expanses of oceanic water represent barriers to both male and female dispersal, while on continental shelves genetic differentiation is explained by an isolation by distance model. In Australia and Indonesia differentiation at nuclear SNPs is weak and compatible with a model of dispersal whereby coral reef habitats represent stepping stones through which genetic connectivity can be maintained across large distances. Differentiation at mtDNA sequences was stronger, and more pronounced in females, suggesting dispersal is sex biased. Fine-scale spatial structure in eastern and western Australia suggests that dispersal is influenced by the spatial distribution of coral reefs. We show that habitat structure and sex-biased dispersal are likely important factors in shaping genetic connectivity in the grey reef shark, although further studies employing a full seascape genomics approach will be needed to elucidate the role of habitat in shaping gene flow. Knowledge of connectivity is needed for management, and conserving apex predators in coral reef ecosystems is critical to conserving its biodiversity

INTRODUCTION

Large coral reef predators play a key role in structuring coral reef community, exerting top-down control through direct and indirect effects on the abundance and behaviour of organisms at lower levels (Heithaus *et al.* 2008; Ruppert *et al.* 2013). Recently it has been shown that pristine coral reefs have top heavy food webs, where the biomass of apex predators overwhelms that of the fish assemblages, with reef sharks making up the majority of apex predator biomass (Friedlander *et al.* 2014; Sandin *et al.* 2008; Stevenson *et al.* 2007). The recent declines in reef shark numbers, recorded across multiple ecosystems across the globe (Graham *et al.* 2010; Robbins 2006; Sembiring *et al.* 2015; Ward-Paige *et al.* 2010), is therefore of great concern for the health of coral reef ecosystems.

Knowledge of biological and environmental factors shaping patterns of connectivity in coral reef sharks is fundamental to evaluating the risks of anthropogenic change, and for the development of efficient management strategies (Dudgeon *et al.* 2012). Characterising genetic connectivity in sharks has revealed complex patterns, likely representing high inter-individual variation in dispersal distances, which can be further complicated by sex-bias (Duncan *et al.* 2006; Mourier & Planes 2013; Pardini *et al.* 2001). In addition, patterns of genetic connectivity can be masked by large effective population sizes and, as a consequence, the long temporal scales required for the effects of drift to be detected (Ovenden 2013). Furthermore, in species with high habitat specificity, genetic connectivity is expected to be limited by expanses of unsuitable habitat [for a perspective on the topic, see Momigliano *et al.* (2015b)]. Hence, an holistic assessment of genetic structure in sharks requires investigating patterns of connectivity at multiple spatial and temporal scales. Ideally, the sampling strategy will take into account how complex habitat systems shape dispersal and use genetic markers and analyses with the potential to resolve large scale and fine scale spatial structure so that the biotic and abiotic features influencing dispersal can be identified.

Grey reef sharks (*Carcharhinus amblyrhynchos*) possess features that are likely to yield complex patterns of genetic connectivity. They have a large geographic distribution spanning most of the tropical Indo-Pacific (Last & Stevens 2009), suggesting the potential for wide-range dispersal, yet they are strictly associated with coral reef habitats (Chin *et al.* 2012; Espinoza *et al.* 2014). Grey reef sharks may undertake movements of more than 100 km crossing deep oceanic waters (Barnett *et al.* 2012; Heupel *et al.* 2010; Momigliano *et al.* 2015a), but also show reef fidelity for extended periods of time. Movements of grey reef sharks are influenced by the spatial distribution of coral reefs. They show low levels of reef fidelity in systems where neighbouring reefs are close (Heupel *et al.* 2010), suggesting that coral reefs separated by only a few km may be perceived as continuous habitat (Momigliano *et al.* 2015b). As the distance between neighbouring reefs increases, grey reef sharks show higher residency (Espinoza *et al.* 2015a), and in isolated oceanic reefs they rarely venture far (Barnett *et al.* 2012; Field *et al.* 2011). Espinoza *et al.* (2015a) observed that adult males have larger home ranges than females and juveniles, and speculated that male-mediated dispersal may confer an evolutionary advantage by extending genetic and demographic connectivity beyond individual reefs. Nonetheless, the extent to which these movement patterns observed at reef systems of varying degrees of isolation reflect patterns of dispersal and gene flow remains largely unknown.

A recent population genetics study carried out in the Australian Great Barrier Reef (GBR), where most reefs are located within a distance of <2 km from their closest neighbours (Almany

et al. 2009), revealed no large scale genetic structure at microsatellite loci across a latitudinal gradient of nearly 1200 km (Momigliano *et al.* 2015a). The authors found no genetic differentiation between different regions of the GBR using microsatellite and mtDNA markers, and a complete absence of genotypic spatial autocorrelation at the reef scale for both sexes, suggesting regular migration between neighbouring reefs. Over a larger spatial scale, gene flow and local selection in grey reef sharks was evaluated along the continental shelves of Australia and Indonesia (see chapter 6). Using a panel of more than 5000 nuclear Single Nucleotide Polymorphisms (SNPs) extremely low levels of genetic differentiation which was distributed in accordance with an isolation by distance model was demonstrated, along with signatures of local selection.

In this study we use SNPs and mtDNA sequence data to investigate large scale and fine scale genetic structure of grey reef sharks at multiple spatial scales across a substantial portion of the species range. Based on recent findings from telemetry and population genetics studies, we test four hypotheses:

1- Genetic differentiation of grey reef sharks is explained by a stepping-stone model of dispersal, whereby individual reefs act as stepping stones, maintaining large scale connectivity and resulting in an isolation-by-distance pattern.
2- Large expanses of oceanic waters represent strong barriers to both female and male dispersal.
3- As males have larger home ranges than females, dispersal is male-biased across large distances.
4- Fine scale spatial structure revealed by genotypic spatial autocorrelation analyses is influenced by the degree of habitat fragmentation. Where reefs are separated by short distances (such as the GBR), no clear pattern of spatial autocorrelation at the reef scale should arise. In highly fragmented reef systems, such as the isolated reefs of western Australia, we predict that grey reef sharks will exhibit intergenerational reef fidelity, resulting in significant patterns of spatial autocorrelation.

MATERIAL AND METHODS

Sampling

Most of the samples used in this study have been previously collected by Momigliano *et al.* (2015a) and and for the study reported in Chapter 6. We collected an additional 32 individuals from Scott Reef, three individuals from the Rowley Shoals, 13 individuals from Ningaloo Reef,

five individuals from Misool, and 25 individuals from the Chagos Archipelago (figure 1). Sampling was conducted as described by Momigliano *et al.* (2015a). The additional samples from Ningaloo Reef and the Rowley Shoals were collected under a permit from the Western Australia Department of Environment and Conservation (permit number: CE003632). Samples from Scott Reef were donated by the Australian Institute of Marine Science. Samples from the Cocos Keeling island were donated by Dr. William Robbins, and samples from the Chagos Archipelago where donated by Stanford University and the Bertarelli Foundation. The additional five individuals from Misool were collected by Vanessa Jaiteh under an Indonesian research permit (RISTEK, permit number: #13/EXT/SIP/FRP/SM/I/2013). Genetic analyses of samples from Indonesia were undertaken under the Indonesian RISTEK permit number #03B/TKPIPA/FRP/SM/III/2014.

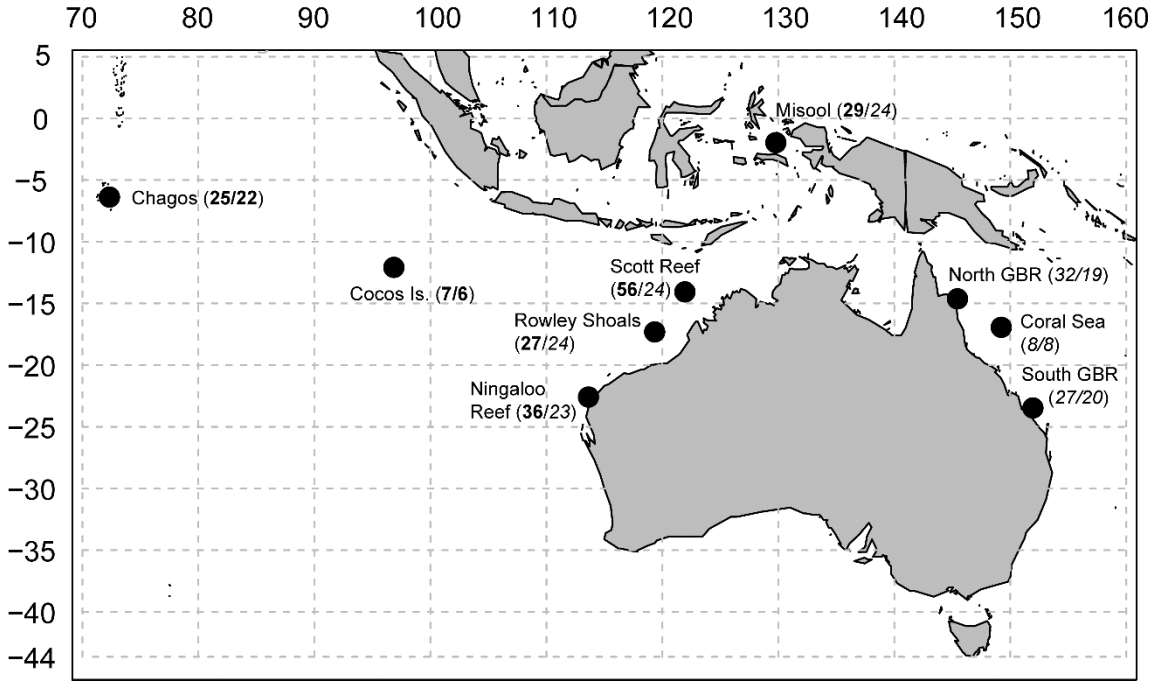


Figure 1*: map showing the sampling locations. In brackets are the number of individuals used for the mtDNA analyses and the SNP analyses, respectively. Numbers in italics represent samples from which data were retrieved from Momigliano et al. (2015a) and Chapter 5. Numbers in bold represent samples for which genetic data were generated in this study.*

mtDNA sequencing and analyses

We extracted DNA from a total of 175 samples (figure 1) and amplified a fragment of 813 bp of the NADH dehydrogenase subunit 4 (ND4) gene as per Momigliano *et al.* (2015a), using the primer set ND4 (Arèvalo *et al.* 1994) and HI12293-Leu (Inoue *et al.* 2003). DNA fragments

were sequenced with the forward primer using a commercial service (Marcrogen Inc., Seoul , Korea) and aligned by eye in BioEdit v. 7.1(Hall 1999). The sequences obtained in this study were analysed together with the ND4 sequences from the north GBR (N=32), south GBR (N=27) and the Coral Sea (N=8) recently published by Momigliano *et al.* (2015a). The final analyses included a total of 242 ND4 sequences. Diversity indices (number of haplotypes N_H and haplotype diversity *h*) for mtDNA sequences were calculated in the software DnaSP (Librado & Rozas 2009). Measures of pairwise genetic differentiation (Φ_{st}, an analogue of F_{ST} for DNA sequence data) were estimated in the software Arlequin v. 3.51(Excoffier & Lischer 2010). A minimum spanning network of ND4 haplotypes was constructed in the software popart v. 1.7 (Leigh & Bryant 2015), using the same parsimony inference method implemented in the software TCS (Clement *et al.* 2000).

To test for sex bias dispersal, we estimated pairwise Φ_{st} for females and males separately. While mtDNA is transmitted only maternally, there is a solid rationale for which mtDNA estimates of genetic differentiation in different sexes can be used to test for sex bias dispersal. Males cannot transmit mtDNA, hence the haplotypes from an immigrant male can only be sampled while it is alive while females have a far greater potential to homogenize mtDNA via migration and successful local reproduction (Lukoschek *et al.* 2008). As O'Corry-Crowe *et al.* (1997) and Lukoschek *et al.* (2008) noted, higher genetic differentiation at mtDNA markers in females is therefore strong evidence of male bias dispersal. We did not have sex information for all our samples, and in most locations we did not have enough samples from each sex to accurately estimate Φ_{st}. We therefore compared three locations for which we had at least ten females and ten males: the Rowley Shoals (10 males and 14 females), the North GBR (11 males and 18 females) and the South GBR (10 males and 15 females).

SNP discovery and filtering

We used the set of SNP genotypes from Chapter 6. Furthermore, we genotyped an additional 22 samples from the Chagos Archipelago (figure 1) following exactly the same procedures outlined in Chapter 6. SNP genotyping and calling was carried out by a commercial service (Diversity Arrays Technology Pty. Ltd., Canberra Australia). SNPs where filtered following the criteria below:

1- No more than 5% missing data in the whole dataset
2- No more than 10% missing data for any locus in any sampling location
3- 100% reproducibility across 60 technical replicates, which were carried through all steps, from DNA extraction to SNP calling, twice.

4- A minimum average read depth of 10, and maximum average read depth defined as $d + 4 * \sqrt{d}$ (where d is the average read depth across all loci), an approach to discard potential false variants and paralogs, see Li (2014)
5- Minor allele frequencies (MAF) >0.05
6- Only one SNP per fragment was retained, to avoid creating a dataset including tightly linked SNPs located within the same fragments.

We removed from the dataset the loci which were identified as under divergent selection in Chapter 6. For a full description of the SNP discovery and filtering methods refer to the supplementary materials in Chapter 6 as we employed exactly the same procedure in this study.

SNP population genetics analyses

We checked that the loci retained did not deviate from Hardy-Weinberg Equilibrium (HWE) in multiple locations. Briefly, we tested for departures from HWE in all locations with a sample size of ≥ 20 using the Monte Carlo procedure implemented in the R package *pegas* (Paradis 2010). We considered a locus to deviate from HWE only if it was found to significantly ($p<0.05$) deviate from HWE in at least three locations. We used this approach to control for the large number of false positives that are inevitable when carrying out tens of thousands of independent comparisons, while avoiding setting an extremely low critical p value for each comparison (necessary to maintain an experiment-wise α of 0.05) which may result in very low power to detect any true deviation from HWE. We calculated the following diversity indices across all loci for each sampling location using the software GenAlEX v. 6.5 (Peakall & Smouse 2012): expected heterozygosity (H_E), observed heterozygosity (H_O) and the fixation index F= 1-(H_O/H_E), along with their standard errors. Pairwise Weir and Cockerham F_{ST} (Weir & Cockerham 1984) and their 95% confidence intervals were estimated in the R package *diveRsity* (Keenan *et al.* 2013) using 100 pseudo-replicate datasets created by bootstrapping individuals within each location. Using confidence intervals determined by bootstrapping individuals was deemed a good strategy to determine the significance of F_{ST} estimates given that two our locations had very low sample sizes (Cocos Is, N=6, and Coral Sea, N=8).

We further investigated patterns of genetic structure by carrying out a Discriminant Analysis of Principal Components (DAPC) using sampling location as the grouping factor (Jombart *et al.* 2010). The analysis was carried out both on the whole dataset, and only including the data from Australia and Indonesia. First, we carried out a PCA, and used the PCs thus produced as synthetic variables for a discriminant analyses, as outlined in Jombart *et al.* (2010). This first step is necessary as two of the main assumptions of discriminant analysis are that variables are

uncorrelated, and that the number of variables is less than the number of observations. Allele frequencies are inevitably correlated, and in large SNPs datasets the number of variables (i.e. number of alleles) can be much greater than the number of sampled individuals. Using principal components obtained from PCA reduces the number of dimensions and creates a set of orthogonal variables which explain exactly the same variation as the original variables. The number of PCs to be retained in the DAPC analyses was determined using cross-validation to avoid overfitting. Simply, 80% of the data were used as a training set and we retained the number of PCs for which the obtained Mean Square Error was lowest (number of PCs=40 for both analyses).

Isolation by distance

We investigated the relationship between genetic distance at both nuclear (F_{ST}) and mtDNA (Φ_{st}) markers and geographic distance using Mantel tests (using 30 000 randomizations) and major axes regression. As genetic differentiation across large oceanic expanses did not show a linear relationship with geographic distance, we only investigated Isolation by Distance across locations in Australia and Indonesia (i.e we excluded samples from the Cocos Islands and Chagos archipelago). First, geographic distance between locations was estimated as the least-cost path across the sea using the package *marmap* (Pante & Simon-Bouhet 2013). We then plotted geographic distance vs genetic distance to ensure the relationship was linear. The significance of the correlation between genetic distances and geographic distance was tested using a Mantel test, therefore taking into account that sampling locations, rather than pairs of sampling locations, are the units of replication. A linear model was the fitted to the data using a major axes regression, a more appropriate method than an ordinary linear regression when both variables (genetic and geographic distance) are sampled with error.

Fine scale spatial genetic structure

Genetic structuring of haplotype and allele frequencies is a function of the migration rate (m) and the effective population (N_e) (Kalinowski 2002). Therefore in large populations a small migration rate can effectively slow down or prevent any detectable level of genetic drift. Hence estimates of genetic differentiation based on allele and haplotype frequencies are often a poor proxy of ecological connectivity at fine spatial and temporal scale. In contrast, estimates of genotypic spatial autocorrelation provide a powerful tool to investigate fine scale spatial structure. Patterns of spatial autocorrelation can appear even in face of migration rates exceeding 10% and are expected to arise within a few generations and reach quasi-stationarity

in less than 50 generations (Epperson 2005; Momigliano *et al.* 2015a; Smouse & Peakall 1999; Stow *et al.* 2001).

We tested for genotypic spatial autocorrelation across 4 distance classes (within reef, within 500km, within 1000km and within 1500km) in two geographic regions for which we sampled multiple reefs: eastern Australia and Western Australia. The spatial structure of these reef systems are broadly different. In eastern Australia coral reefs are relatively close with an average distance between neighbouring reefs of <2 km (Almany *et al.* 2009), while coral reefs in Western Australia are isolated and separated by distances of 100s of km. First, we estimated pairwise genetic distances between individuals using the codom-genotypic measure of genetic distance implemented in GenAlEX v. 6.5 (Peakall & Smouse 2012). The spatial autocorrelation coefficient *r* (Smouse & Peakall 1999) and its confidence intervals were calculated using 999 bootstraps in the software GenAlEX v. 6.5 (Peakall & Smouse 2012) along with the 95% confidence intervals of the null model of no spatial autocorrelation.

RESULTS

mtDNA analyses

The final alignment included 242 individuals for which an 813 bp long fragment of ND4 was obtained, with no missing data. There were a total of 26 polymorphic sites within the alignment, of which 19 were parsimony informative. Twenty six distinct haplotype were present (figure 2), and haplotype diversity across all locations was high (h=0.78). Number of haplotypes and h within each sampling location ranged between 2 and 7 and 0.28 and 0.80 respectively (Table 1). Haplotype diversity was lower in Western Australia and the Cocos Islands compared to the rest of the locations. The haplotype network revealed that no haplotypes were shared between the samples from the Chagos archipelago and any of the other locations. All but one individual from the Cocos Islands shared the same private haplotype (figure 2). The haplotype network revealed two main groups of haplotypes, one including most haplotypes from Western Australia and one including most haplotypes from eastern Australia (figure 2). Samples from Indonesia included haplotypes from both main haplotype groups.

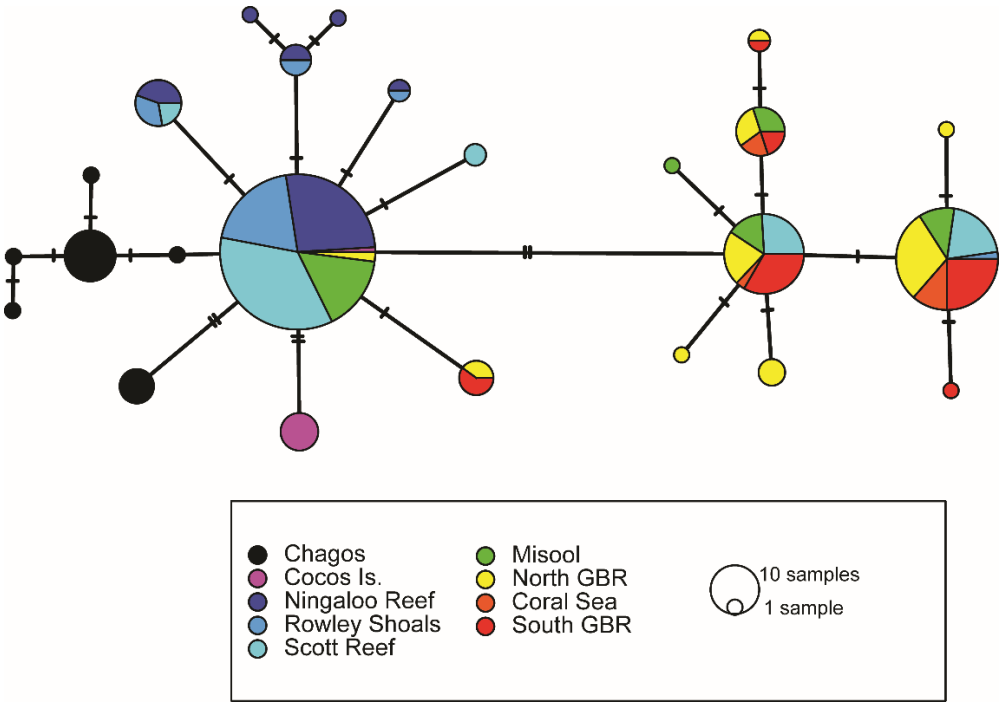
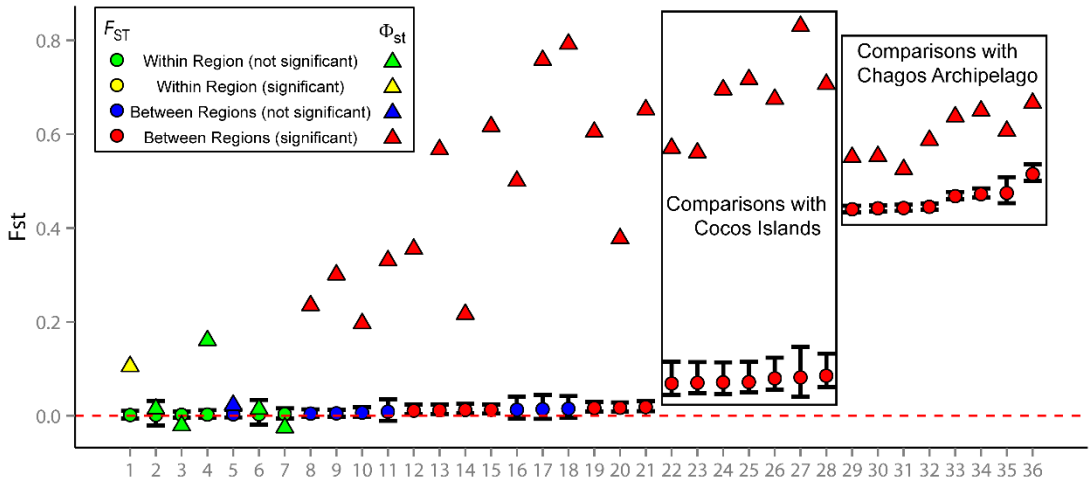


***Figure 2**: Minimum spanning network obtained from 242 individual partial ND4 sequences comprising 26 distinct haplotypes.*

Pairwise Φ_{st} between locations within the same region (eastern and western Australia) were not significant, with the exception of Scott Reef which shows significant pairwise Φ_{st} with other locations in Western Australia (Φ_{st} 0.105-0.160, Table 2, figure 3). All pairwise Φ_{st} between locations in different regions were highly significant and very high (Φ_{st} range: 0.197-0.83), with the exception of the comparison between Scott Reef and Misool. Strong genetic differentiation was observed not only across large oceanic expanses but also between regions along the continental shelves of Australia and Indonesia. Pairwise Φ_{st} estimated separately for males and females show no differentiation between North GBR and South GBR. Pairwise Φ_{st} estimates between Rowley Shoals and North GBR, and Rowley Shoals and South GBR were significant for both sexes, yet the level of differentiation was higher for females (0.65 and 0.67 respectively) than for males (0.49 and 0.50 respectively).



1 Rowleys vs Scott	**13 Rowley Shoals vs North GBR**	**25 Cocos vs Ningaloo Reef**
2 North GB vs Coral Sea	**14 Misool vs South GBR**	**26 Cocos vs North GBR**
3 Ningaloo Reef vs Rowley Shoals	**15 Ningaloo Reef vs north GBR**	**27 Cocos vs Coral Sea**
4 Ningaloo reef vs Scott reef	16 Scott reef vs Coral Sea	**28 Cocos vs South GBR**
5 Scott reef vs Misool	17 Rowley Shoals vs Coral Sea	**29 Chagos vs Scott Reef**
6 Coral Sea vs South GBR	18 Ningaloo Reef vs Coral Sea	**30 Chagos vs Rowley Shoals**
7 North GBR vs South GBR	**19 Rowley Shoals vs South GBR**	**31 Chagos vs Misool**
8 Rowley Shoals vs Misool	**20 Scott Reef vs South GBR**	**32 Chagos vs ningaloo Reef**
9 Ningaloo Reef vs Misool	**21 Ningaloo Reef vs South GBR**	**33 Chagos vs North GBR**
10 Misool vs North GBR	**22 Cocos vs Scott Reef**	**34 Chagos vs South GBR**
11 Misool vs Coral Sea	**23 Cocos vs Misool**	**35 Chagos vs Cocos**
12 Scott reef vs North GB	**24 Cocos vs Rowley Shoals**	**36 Chagos vs Coral Sea**

***Figure 3**: Estimates of pairwise genetic differentiation across all sampled location estimated from SNP data (F_{ST}) and mtDNA data (Φ_{st}). Comparisons are arranged on the x axis in ascending of order of F_{ST} values. The error bars represent 95% confidence intervals estimated by bootstrapping individuals within locations (only for F_{ST}). Symbols are colour coded based on whether the comparisons are within or among distinct regions and whether they are or not statistically significant.*

SNP population genetic analyses and differences across markers

The final SNP dataset included 5509 polymorphic, bi-allelic SNP genotypes for 170 individuals. None of the loci deviated from HWE across three or more locations. Observed and expected heterozygosity ranged between 0.139-0.294 and 0.131-0.291 respectively (Table 1). Fixation indices revealed no significant heterozygosity deficiency, but a significant heterozygosity excess was evident in the Cocos Island, and to a lesser extent in the Chagos archipelago and Coral Sea. However, both Cocos Islands and the Coral Sea had very low sample size, therefore the reported heterozygosity excess could also be due to sampling bias.

Table 1: Diversity indices for all sampling locations. N (ND4): number of samples in the mtDNA dataset. N_H: number of haplotypes. *h*: haplotype diversity. N (SNP): number of samples in the SNP dataset. H_O: average heterozygosity across all loci. H_E: average unbiased expected heterozygosity across all loci. *F*: fixation index 1-(H_O/H_E). Diversity indices for the SNP dataset are given along with their standard error.

Location	**N (ND4)**	**N_H**	***h***	**N (SNP)**	**H_O**	**H_E**	***F***
Chagos	25	7	0.75	22	0.139±0.003	0.131 ±0.002	-0.048±0.003
Cocos Islands	7	2	0.28	6	0.304 ±0.003	0.264±0.003	-0.14±0.004
Ningaloo Reef	36	6	0.43	23	0.287±0.002	0.289±0.002	0.006±0.003
Rowley Shoals	27	5	0.45	24	0.287±0.002	0.287±0.002	0.003±0.003
Scott Reef	56	5	0.55	24	0.288±0.002	0.289±0.002	0.004±0.003
Misool	29	5	0.66	24	0.294±0.002	0.291±0.002	-0.009±0.003
North GBR	32	9	0.80	19	0.285±0.002	0.285±0.002	0.001±0.003
Coral Sea	8	3	0.61	8	0.286±0.003	0.273±0.002	-0.046±0.004
South GBR	27	6	0.73	20	0.280±0.002	0.280±0.002	-0.001±0.003

Pairwise F_{ST} were extremely low (0.0015-0.0187), and mostly not significant when comparing locations along Australia's and Indonesia's continental shelves (with the exception of some comparisons between western and eastern Australian locations, see Table 2 and figure 3). Between these locations pairwise F_{ST} were on average two orders of magnitude lower than pairwise Φ_{st}. Genetic distances across large oceanic expanses however were much higher (0.0688-0.5148), and F_{ST} between samples from the Chagos archipelago and all other locations were similar to estimates of Φ_{st} obtained from the mtDNA data (figure 3, Table2).

The difference between Φ_{st} and $F_{ST,}$ was largely dependent upon the scale considered. The largest difference was for pairwise comparisons between locations far apart but located within Australia and Indonesia's continental shelves, while Φ_{st} and F_{ST} difference were within the same order of magnitude when comparing either locations close by or locations separated by large oceanic expanses (figure 3).

DAPC performed on data from all locations revealed the greatest differentiation in locations separated by large oceanic distances (figure 5A). The first discriminant function clearly shows the great extent of differentiation between samples from the Chagos Archipelagos and the Cocos Islands and all other locations, while locations in different regions of Australia and Indonesia separate along the second discriminant function (figure 5A). The result of the DAPC performed on samples from Australia and Indonesia further revealed more subtle patterns of genetic differentiation (figure 5B).

Table2: Estimates of pairwise differentiation among sampled locations obtained from SNPs (F_{ST}) and mtDNA sequences (Φ_{st}). F_{ST} (LB) and F_{ST} (UB) refer to the lower and upper bounds of the confidence intervals estimated via bootstrapping. Φ_{st} p values as estimated in Arlequin. Significant F_{ST} and Φ_{st} values are show in italics.

Pairwise Comparison	F_{ST}	F_{ST} **(LB)**	F_{ST} **(UB)**	Φ_{st}	**p value(Φ_{st})**
Chagos vs Cocos Islands	*0.4747*	0.4528	0.5085	*0.60646*	$<1x10^{-6}$
Chagos vs Ningaloo Reef	*0.4453*	0.4392	0.4518	*0.58717*	$<1x10^{-6}$
Chagos vs Rowley Shoals	*0.4421*	0.4359	0.4481	*0.55278*	$<1x10^{-6}$
Chagos vs Scott Reef	*0.44*	0.4333	0.4474	*0.55076*	$<1x10^{-6}$
Chagos vs Misool	*0.4427*	0.4364	0.4495	*0.52492*	$<1x10^{-6}$
Chagos vs North GBR	*0.4677*	0.4611	0.477	*0.63719*	$<1x10^{-6}$
Chagos vs Coral Sea	*0.5148*	0.501	0.5352	*0.66606*	$<1x10^{-6}$
Chagos vs South GBR	*0.4722*	0.4653	0.4843	*0.64968*	$<1x10^{-6}$
Cocos Islands vs Ningaloo Reef	*0.0717*	0.0502	0.1147	*0.71649*	$<1x10^{-6}$
Cocos Islands vs Rowley Shoals	*0.0714*	0.0459	0.114	*0.69497*	$<1x10^{-6}$
Cocos Islands vs Scott Reef	*0.0688*	0.0448	0.1154	*0.57037*	$<1x10^{-6}$
Cocos Islands vs Misool	*0.0704*	0.0483	0.1143	*0.56038*	$<1x10^{-6}$
Cocos Islands vs North GBR	*0.0796*	0.0559	0.1237	*0.67473*	$<1x10^{-6}$
Cocos Islands vs Coral Sea	*0.0817*	0.0403	0.1466	*0.82981*	$<1x10^{-6}$
Cocos Islands vs South GBR	*0.0853*	0.0608	0.1326	*0.70658*	$<1x10^{-6}$
Ningaloo Reef vs Rowley Shoals	0.002	-0.0044	0.0092	-0.02104	>0.05
Ningaloo Reef vs Scott Reef	0.0028	-0.0036	0.012	*0.16054*	$<1x10^{-6}$
Ningaloo Reef vs Misool	0.0051	-0.0019	0.0128	*0.30053*	$<1x10^{-6}$
Ningaloo Reef vs North GBR	*0.0127*	0.0046	0.0239	*0.61648*	$<1x10^{-6}$
Ningaloo Reef vs Coral Sea	0.0149	-0.0045	0.0419	*0.79208*	$<1x10^{-6}$
Ningaloo Reef vs South GBR	*0.0187*	0.0097	0.0309	*0.65226*	$<1x10^{-6}$
Rowley Shoals vs Scott Reef	0.0015	-0.006	0.0103	*0.10529*	0.00851
Rowley Shoals vs Misool	0.0049	-0.0015	0.0137	0.23508	0.0001
Rowley Shoals vs North GBR	0.0118	0.0039	0.0236	*0.56711*	$<1x10^{-6}$
Rowley Shoals vs Coral Sea	0.0137	-0.0066	0.0446	*0.75756*	$<1x10^{-6}$
Rowley Shoals vs South GBR	*0.0167*	0.0085	0.0295	*0.60455*	$<1x10^{-6}$
Scott Reef vs Misool	0.0032	-0.0034	0.0127	0.0229	>0.05
Scott Reef vs North GBR	*0.0106*	0.003	0.0234	*0.35523*	$<1x10^{-6}$
Scott Reef vs Coral Sea	0.0132	-0.0062	0.0409	*0.5*	$<1x10^{-6}$
Scott Reef vs South GBR	*0.0169*	0.0089	0.0285	*0.37761*	$<1x10^{-6}$
Misool vs North GBR	0.0066	-0.0015	0.0181	*0.19659*	0.0005
Misool vs Coral Sea	0.0086	-0.0107	0.0348	*0.33061*	0.00158
Misool vs South GBR	*0.0125*	0.005	0.0252	*0.21655*	0.0005
North GBR vs Coral Sea	0.0018	-0.0204	0.0318	0.01491	>0.05
North GBR vs South GBR	0.0038	-0.0056	0.0157	-0.0252	>0.05
Coral Sea vs South GBR	0.0033	-0.0187	0.0339	0.0144	>0.05

Each sampling location appears to be most similar to the two geographically closest locations. Furthermore, the densities along the first discriminant function closely match the longitudinal distribution of the sampling locations (west to east), while densities on the second discriminant function are obviously correlated with latitude. If the order of the second discriminant function

was reversed, the plot would closely match the geographic distribution of the sampling locations, a pattern that suggests a stepping stone model of dispersal (Jombart *et al.* 2010).

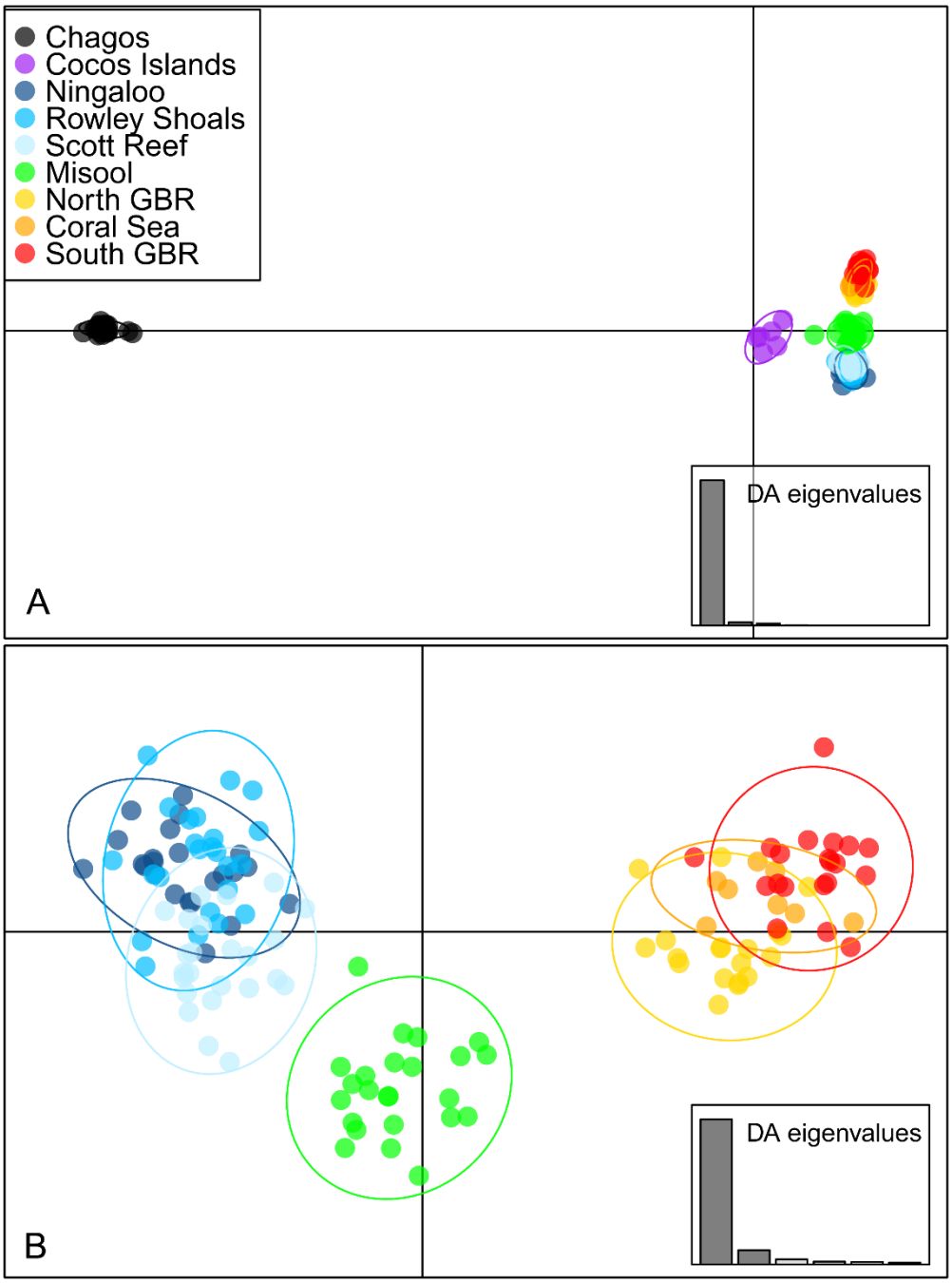


***Figure 4**: Results from DPAC analysis performed on SNP data from all locations (A) and only from locations in Australia and Indonesia (B). The x and y axes represent the first and second discriminant functions, respectively. Ellipse represent 95% confidence inertia ellipses.*

Isolation by distance

There was a clear, linear and significant relationship between geographic distance and genetic distance as measured both by F_{ST} and Φ_{st} (figure 6). Geographic distance explained 92% of the

variation in genetic distance at nuclear loci and 79% of the variation in genetic distance in maternally inherited mtDNA. While genetic differentiation along continental shelves follows an Isolation by Distance model, this linear relationship breaks down when samples from the Chagos Archipelago and the Cocos Islands are included (data not shown). Both nuclear and mtDNA distances follow an Isolation by Distance model, but the slope of the fitted linear model is nearly 2 orders of magnitude higher for mtDNA.

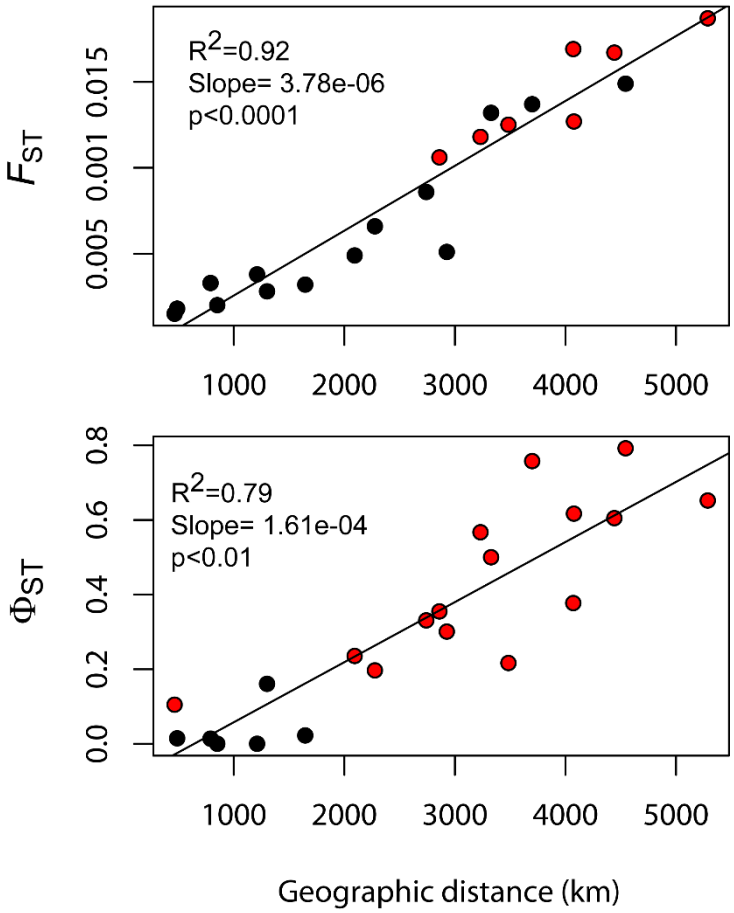


***Figure 5**: Patterns of Isolation by distance using F_{ST} (SNP data) and Φ_{st} (mtDNA data). R^2 and slope of the fitted linear model were estimated by major axes regression, while the significance of the correlation between genetic and geographic distance (p) was estimated via Mantel test using 30 000 randomizations.*

Fine scale spatial genetic structure

Spatial autocorrelation analyses performed separately for samples collected in eastern Australia and western Australia revealed a sharply contrasting pattern. In eastern Australia there was a trend of decreasing spatial autocorrelation with increasing geographic distance (figure 7 A), yet this trend did not significantly deviate from the null model, mostly because of the large variance in spatial autocorrelation within reefs. Conversely there was a clear and significant pattern of

spatial autocorrelation in isolated reefs of western Australia, suggesting that individuals within single reefs are more genetically similar than individuals across any other distance class (figure7 B).

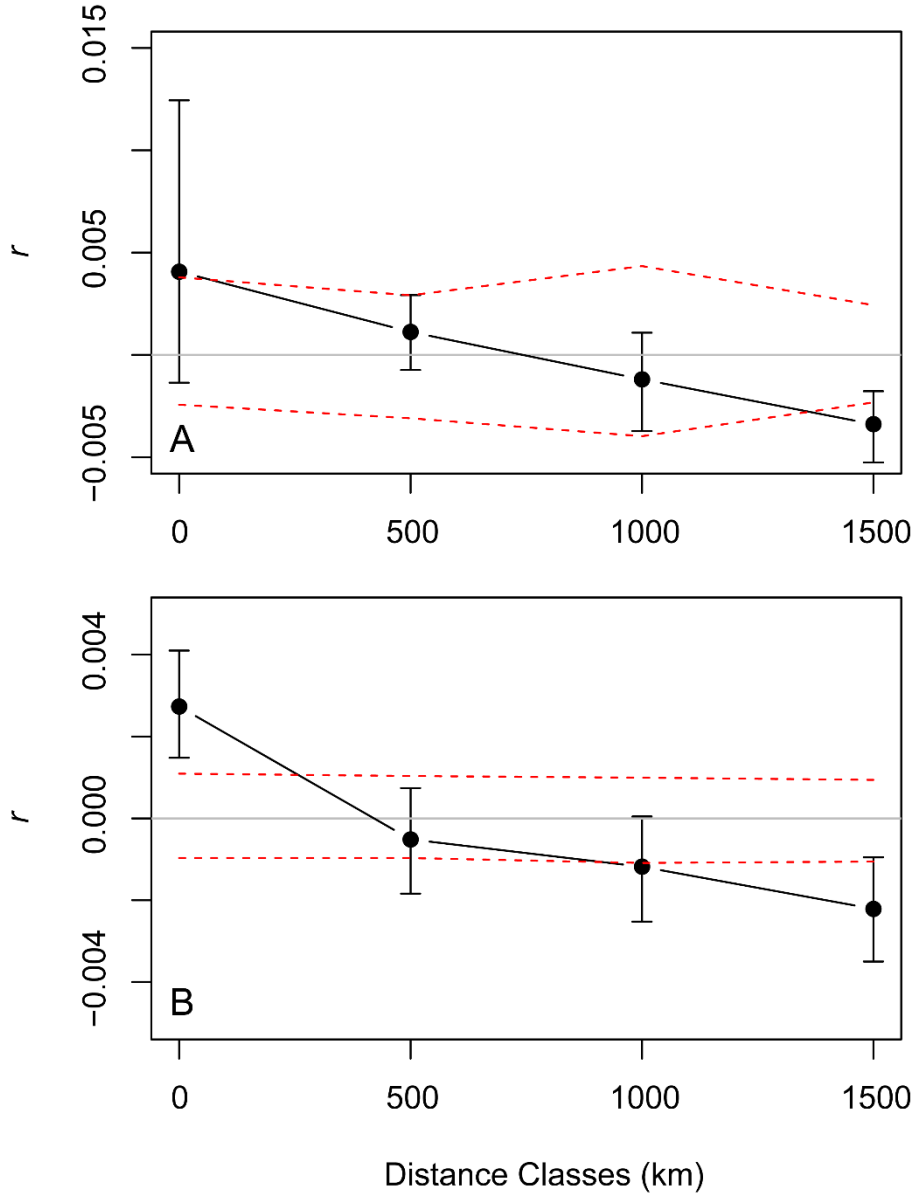


***Figure 6**: Estimates of spatial autocorrelation (*r*) across different distance classes (within the same reef and within 500, 1000 and 1500 km respectively) using samples from eastern Australia (A) and western Australia (B). Error bars represent 95% confidence intervals estimated from 999 bootstraps, and the dotted lines represent the 95% confidence intervals of the null model of no spatial autocorrelation.*

DISCUSSION

We have described both large-scale and fine-scale genetic structure in grey reef sharks across a substantial portion of their distribution. Large expanses of oceanic waters are clearly a strong

barrier to both male and female dispersal, while on the Australian and Indonesia continental shelves genetic differentiation fits an isolation by distance model. Differentiation at mtDNA sequences was much stronger than at nuclear SNPs, and more pronounced in females than males, reflecting male-biased dispersal. Furthermore, fine-scale spatial structure revealed by genotypic spatial autocorrelation in eastern and western Australia suggests dispersal potential may be influenced by the spatial distribution of coral reef habitats.

Large-scale phylogeography

Along the continental shelves of Australia and Indonesia, genetic differentiation at nuclear loci was extremely weak and almost entirely explained by geographic distance, following the same pattern revealed in Chapter 6. Our data strongly suggest the absence of specific biogeographic barriers throughout the sampling area in Australia and Indonesia, and is consistent with very low genetic structure and drift whereby individual dispersal is limited by distance. These results corroborate recent studies which demonstrated that the dispersal potential of grey reef sharks is higher than previously thought (Espinoza *et al.* 2015a; Espinoza *et al.* 2015b; Heupel *et al.* 2010; Momigliano *et al.* 2015a). Grey reef sharks have a continuous distribution in the coral reefs along the northern Australian and Indonesian coasts (Last & Stevens 2009) and there is no contemporary physical barrier to dispersal along the continental shelves of Australia and Indonesia. While the Torres Strait was exposed during the last glacial maximum, it was submerged again 8000 years ago, and given the dispersal potential of grey reef sharks (Heupel *et al.* 2010; Momigliano *et al.* 2015a) and their generation time (Robbins 2006), it seems likely that grey reef sharks in the region may have reached, or are approaching, drift-equilibrium following the disappearance of the Torres Strait barrier.

Our results are nevertheless in contrast with other research concluding that elasmobranchs in eastern and western Australia are genetically isolated, despite the fact they have a continuous distributions along the northern Australian coast. Giles *et al.* (2014) found that *C. sorrah* collected on either side of the Torrest Strait (in Eastern Australia and the Northern territories) exhibited weak genetic structure (Φ_{ST} =0.1, based on mtDNA control region), but they also found evidence of isolation by distance along continental shelves. Keeney & Heist (2006) reported strong (but not statistically significant) differentiation between blacktip sharks (*Carcharhinus tilstoni*) from eastern Australia and western Australia. Yet their study only used a single mtDNA marker, had very low sample sizes (<5 for each of the Australian locations) and their results may have been further influenced by the introgression of *C. tilstoni* haplotypes in *C. limbatus*. Similarly, Phillips *et al.* (2011) reported high genetic differentiation between

individuals of three species of endangered sawfishes (*Pristis* spp.) sampled in nursery areas from different Australian regions using a small number of mtDNA sequences.

We suggest that an isolation-by-distance pattern may characterise some Elasmobranchs previously described with eastern Australian individuals being genetically distinct from those in western Australia. Genetic differentiation between two distinct regions is not necessarily evidence for the existence of distinct genetic populations, particularly if samples collected across large distances are pooled into arbitrary groups. The same exact pattern could arise by sampling along an isolation by distance gradient -see Stowand Magnusson (2012) and Schwartz & McKelvey (2009) for a discussion on the topic. Furthermore, certain behaviours require very careful interpretation of mtDNA data. Many shark species are characterised by natal philopatry whereby females may have large home ranges yet they return to natal grounds to give birth (Hueter *et al.* 2005; Mourier & Planes 2013). Hence, genetic differentiation at mtDNA markers (particularly if individuals are sampled in nursery areas) does not necessarily imply limited female dispersal.

In contrast to the weak genetic differentiation we report for nuclear loci, genetic differentiation at mtDNA markers was strong (up to 77 higher than nuclear F_{ST}), even within locations which are not separated by large oceanic expanses. Divergence followed the same pattern of isolation by distance revealed by nuclear SNPs, but the slope of the relationship between genetic distance and geographic distance was nearly two orders of magnitude higher for mtDNA. Despite the strong genetic differentiation, the three most common haplotypes were represented in eastern Australia, Western Australia and Indonesia, albeit at different frequencies. Samples collected from Misool shared both haplotypes that were common in eastern and western Australia, with intermediate frequencies, as would be expected in the case of isolation by distance with the absence of biogeographic barriers. We therefore conclude that there is no such thing as separate genetic populations of grey reef sharks throughout the sampling area in Australia and Indonesia, but rather that our data provide strong evidence of genetic connectivity following an isolation by distance pattern.

Grey reef sharks, particularly adult males, have the potential to undertake movements of more than 100km across oceanic waters (Heupel *et al.* 2010), yet they are found nearly exclusively in coral reef habitats (Chin *et al.* 2012; Espinoza *et al.* 2014). Therefore, with the absence of very long distances through oceanic waters the most likely mode of dispersal resulting in a pattern of isolation by distance is one where coral reef habitats represent stepping stones allowing for the maintenance of genetic connectivity (at least for the nuclear genome). This

interpretation is strengthened by the fact that genetic differentiation across regions separated by large oceanic expanses is high for both the nuclear and mtDNA datasets. Samples collected from the Chagos Archipelago, and the Cocos (Keeling) islands showed strong patterns of genetic differentiation when compared to all other locations, suggesting that large oceanic expanses represent a strong barrier to both male and female dispersal.

Sex bias dispersal

Genetic differentiation described using mtDNA sequences was substantially larger than for nuclear SNPs. The extent of this difference was largely dependent upon the spatial scale considered. Genetic differentiation at both classes of markers was within an order of magnitude when comparing locations separated by large oceanic expanses. This is not surprising, as when migration approaches zero it is expected that measures of genetic differentiation at nuclear and mitochondrial maker should converge, assuming the sampled populations are at drift equilibrium (Birky *et al.* 1989). Similarly, the extent of genetic differentiation between very close locations was similar for both classes of genetic marker, since there was no evidence of genetic differentiation in neither nuclear nor mitochondrial markers. The difference in estimates of genetic differentiation across markers was however very large when comparing locations separated by large distances along the continental shelf. In these comparisons mitochondrial Φ_{ST} was on average nearly 40 times higher than F_{ST} estimated from SNPs. Drift of mtDNA haplotypes is expected to happen at a faster rate, due to the maternally inherited mitochondrial genome having an effective population size ¼ that of the nuclear genome. Therefore, a higher level of differentiation at mtDNA markers is not necessarily evidence of sex bias dispersal. The expected differences in divergence at mtDNA and nuclear loci is however much smaller than what we observed. O'Corry-Crowe *et al.* (1997) and Lukoschek *et al.* (2008) showed, using simulated and empirical genetic data respectively, that a difference of more than fourfold in levels of genetic differentiation across mitochondrial and nuclear markers can occur in the absence of sex bias dispersal. Our estimated differences however are nearly ten times what would be predicted by differences in effective population sizes alone. Despite using more than 5000 loci, most population comparisons along the continental shelf revealed no or extremely weak genetic differentiation (most comparisons were not statistically significant, and F_{ST} ranged between 0.0018 and 0.0187). In contrast, the highest Φ_{ST} reported for comparisons between Australian locations was 0.79, suggesting a complete lack of mitochondrial gene flow. Furthermore, the level of mtDNA differentiation was higher for females than males, again suggesting male-mediated gene flow (Lukoschek *et al.* 2008; O'Corry-Crowe *et al.* 1997).

These patterns are consistent with a strong sex-bias in dispersal, whereby females are more philopatric and males disperse larger distances. Male-biased dispersal has been observed and documented in many other shark species, including scalloped hammerheads (Daly-Engel *et al.* 2012), lemon sharks (Schultz *et al.* 2008), white sharks (Pardini *et al.* 2001) and black tip reef sharks (Vignaud *et al.* 2013). Sex-biased dispersal has been thus far only demonstrated in shark species which make use of nursery grounds, and it has been linked to female reproductive philopatry. One of the possible reasons for limited female dispersal is that females need to remain within a range from which they can return safely to their natal ground to give birth, in some cases every year. There is however no evidence that grey reef sharks make use of nursery areas, as both juveniles and adults co-occur within the same coral reef habitats and there is no know ontogenetic shift in habitat use, apart from the fact that adults seem to have larger home ranges (Espinoza *et al.* 2015a; Espinoza *et al.* 2015b). Espinoza *et al.* (2015a) observed differences in the dispersal potential of male and female grey reef sharks. In a study carried out within the central GBR the authors found that most tagged females remained within the reef where they were tagged and were detected on a maximum of two reefs, while the vast majority of adult males were detected in multiple reefs (up to five). A possible explanation for this pattern of sex bias dispersal is that male-mediated dispersal may provide an evolutionary advantage by increasing gene flow (Espinoza *et al.* 2015a).

The role of habitat in shaping fine scale genetic spatial structure

Here we estimated patterns of genotypic spatial autocorrelation across multiple spatial scales in two reef systems with different levels of habitat fragmentation, the western and eastern coasts of Australia. In eastern Australia reefs within the GBR, and to a lesser extent reefs in the Coral Sea, are relatively close. Within the GBR the average between-reef distance is less than 2 km, and the sampled oceanic reef in the Coral Sea (the North East Herald Cay) is less than 200 km offshore the outer shelf of the GBR and potentially connected to the GBR through a number of reefs in the Coral Sea that may act as stepping stones (the Flinders Reefs, the Holmes Reefs, Dart Reef and Flora Reef). In contrast, coral reef habitats in western Australia are much more fragmented, and may be separated by hundreds of km of unsuitable habitat.

Our results showed that in eastern Australia there is no strong evidence of genotypic spatial autocorrelation at the reef scale. However, despite the fact that within each spatial class *r* was within the expectations of the null model, there was a clear, albeit very weak, trend of decreasing spatial autocorrelation with increasing distance which is compatible with an isolation by distance model of dispersal. The variance in autocorrelation at the reef scale was

very large, suggesting that within single reefs in eastern Australia there was a mix of local individuals and recent migrants. This pattern is consistent with the high potential for short distance migration in reef sharks, and with results obtained by Momigliano *et al.* (2015a) using microsatellite markers. In western Australia there was a clear pattern of genotypic autocorrelation at the reef scale. Genotypic autocorrelation was highest when comparing individuals from the same reef, and dropped abruptly when moving to the next distance class. This pattern is consistent with a higher degree of intergenerational reef fidelity, suggesting that reefs which are isolated by large distances of unsuitable habitat (even if located within the same continental shelf) may be effectively self-seeding and demographically independent.

These results should however be interpreted with some caution, and further studies will be needed to confirm the effects of habitat quality of connectivity. Employing a full seascape genomic approach, for example by developing Isolation by Resistance models as suggested by Momigliano et al. (2015), will be necessary but this would be very challenging with the current sampling design.

Conclusion

Here we investigated large scale and fine scale genetic structure of grey reef sharks using a combination of more than 5000 nuclear SNPs and a mtDNA marker. We discovered that in Australian and Indonesian waters genetic differentiation at both nuclear and mtDNA is almost completely explained by geographic distance, and that large oceanic expanses constitute strong barriers to both male and female dispersal. Along continental shelves there is evidence of strong sex-bias in dispersal, suggesting that male migration maintains high levels of genetic connectivity. The spatial autocorrelation analyses suggest that intergenerational site fidelity may be influenced by the degree of habitat continuity, although these preliminary results will need to be confirmed employing a full seascape genomic approach. Reefs isolated by large expanses of non-coral reef habitats, even if located within the continental shelf, may be effectively demographically independent while migration is higher in more continuous reef systems. These results have implications for the effectiveness of spatially discontinuous Marine Protected Areas for the protection of grey reef sharks, which may be largely dependent upon the level of habitat fragmentation exhibited by different reef systems (Momigliano *et al.* 2015b).

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DATA ACCESSIBILITY

All data will be made publicly available once the manuscript is published. All mitochondrial DNA sequences will be deposited in GenBank. DNA sequences and statistics (call rate, heterozygosity, read depth and reproducibility) for all SNP loci as well as genotypes for all individuals will be accessible from the Dryad Digital Repository.

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Chapter 8

Conserving coral reef organisms that lack larval dispersal: are networks of Marine Protected Areas good enough?

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INTRODUCTION

Coral reef ecosystems are under increasing threat due to the synergistic effects of habitat destruction, overfishing, eutrophication and climate change (Hughes *et al*., 2003; Hoegh-Guldberg *et al*., 2007; Hughes *et al*., 2007). In response to these threats, management strategies that implement networks of Marine Protected Areas (MPAs) have gained momentum in the past few decades. Networks of MPAs can protect coral reef biodiversity from anthropogenic impacts either by eliminating the impacts of overfishing and habitat destruction, or by increasing ecosystem resilience to other anthropogenic disturbances (Russ and Zeller, 2003; McCook *et al*., 2010).

For networks of MPAs to be effective they must meet three key elements. Individual MPAs must be 1) partially self-seeding (Almany *et al*., 2009; Almany *et al* 2007) , 2) adequately connected to other MPAs via dispersal (Almany *et al*, 2009; Jones *et al*, 2007) and 3) they must protect target organisms during life stages when they are most vulnerable to anthropogenic impact (Zeller & Russ, 1998; Lee *et al*, 2015). Accordingly, MPAs should be large enough to encompass individual home ranges of the target species and to ensure a portion of the larvae produced within a MPA settles within its boundaries (Almany *et al*., 2009). Furthermore networks of MPAs must ensure genetic and demographic connectivity between protected areas. Connectivity is defined as the exchange of individuals between populations. Connectivity bolsters local resilience to stochastic demographic fluctuations and in so doing, minimises genetic erosion, the risk of inbreeding depression and ultimately maximises adaptive potential (Almany *et al*., 2009).

Here we discuss how different life history strategies may affect the feasibility of achieving the three requirements for effective long-term conservation (self-seeding, connectivity, and protection). While sedentary organisms with a pelagic larval phase (most reef fishes and invertebrates), readily achieve this trinity (Planes *et al.* 2009), animals where dispersal only occurs as adults inevitably fail to meet all three requirements simultaneously (figure 1). Here we discuss a potential solution focusing on incorporating information on how habitat shapes adult dispersal to increase connectivity within networks of MPAs.

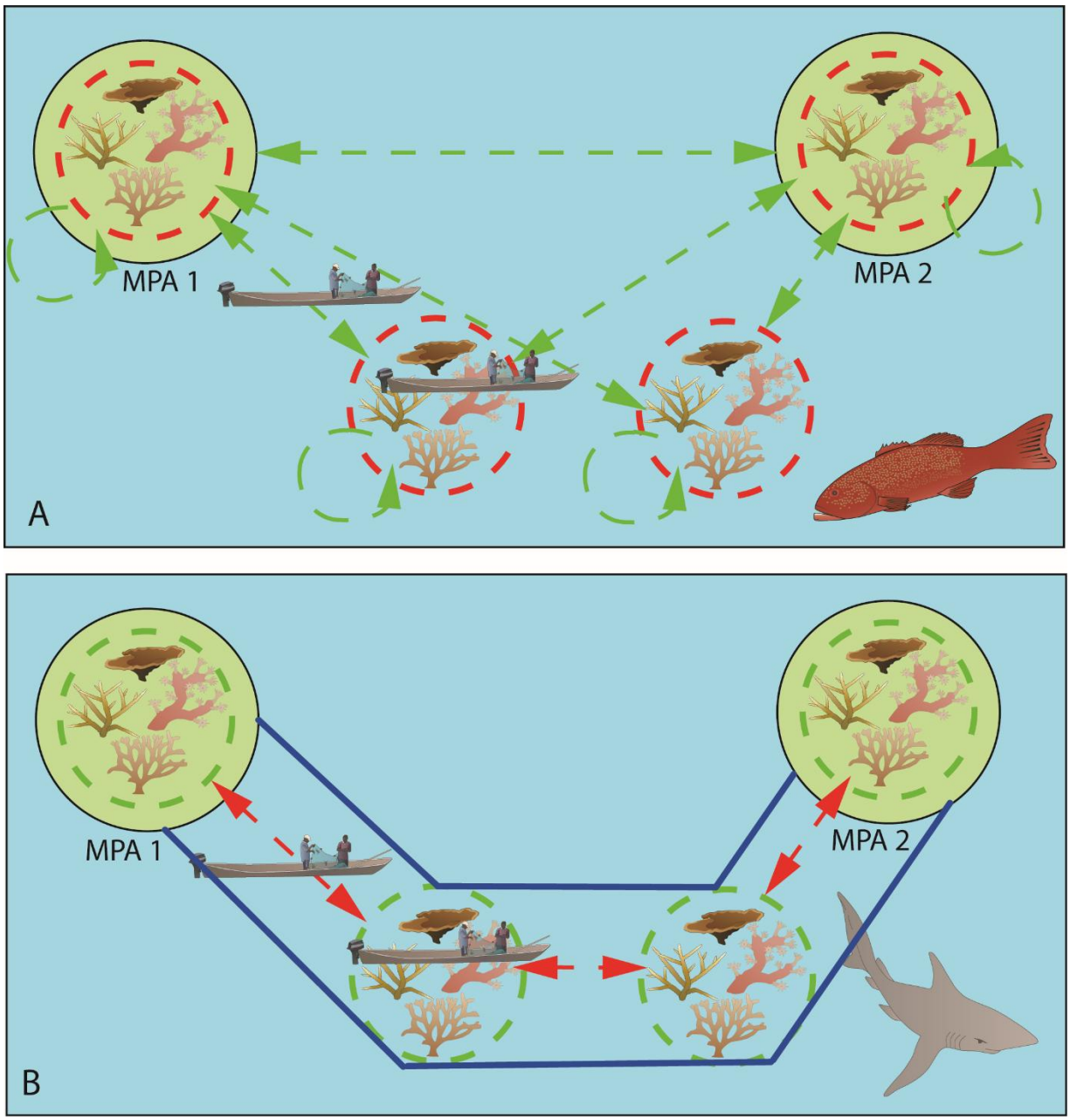

***Figure 1**: Consequences of different life history strategies on the effectiveness of networks of MPAs. A) larval dispersal, sedentary adults. Adult home range is shown in red dashed circles, larval dispersal in dashed green lines. Adults are effectively protected by MPAs (shown in green), and connectivity is ensured via larval dispersal, while a portion of the larvae settles within natal reefs. MPAs also provide recruitment subsidies to fished reefs. B) Adult dispersal, juveniles with site fidelity. Juvenile home range is shown in green dashed circles, adult dispersal by red dashed lines. While juveniles are protected by the MPAs, adults move outside of the reserves' boundaries, crossing to neighboring reefs where they are subject to fishing pressure. A potential solution is the establishment of connectivity corridors (shown in blue), allowing for protected movement of animals between no-take zones.*

PELAGIC LARVAL DISPERSAL

Many reef organisms have a bipartite life cycle: they have pelagic larvae and become sedentary adults. By definition, these adults have often limited home-ranges, and accordingly they are effectively protected even within relatively small MPAs i.e. those encompassing single coral reefs (Zeller & Russ, 1998). Within a single species, larval dispersal often has large variance with some larvae settling close by, within their natal reef, while others disperse to reefs that may be hundreds of km away (Jones *et al*., 2009). Larvae may settle on their natal reefs because they detect good habitat through olfactory cues (Gerlach *et al*., 2007), or simply because they are retained by local oceanographic features such as eddies and density fronts (Sponaugle *et al*., 2002). The results of such high variance in dispersal is that local reefs may be effectively self-seeding (Almany *et al*., 2007), while simultaneously maintaining genetic and demographic connectivity with other MPAs (Jones *et al*., 2009). When their design is most effective, no-take zones may export larvae to fished areas and in so doing help sustain fisheries outside MPAs (Harrison *et al*., 2012).

Although there is large variance in the duration of the pelagic larval stage, many species of reef fishes show similar patterns of self-recruitment and dispersal with the result that larval dispersal for many taxa ranges from a few meters to 10s of km (Jones *et al*., 2009). Accordingly, for many reef fishes, within a network of MPAs, between-reserve distances of < 50 km will be sufficient to ensure connectivity (Almany *et al*., 2009).

ADULT DISPERSAL

While extant MPAs are highly effective for reef fish with pelagic larvae, not all reef organisms share that life cycle. Entire groups of taxa, some of which play pivotal roles in coral reef ecosystems produce highly precocial young with a high degree of natal reef fidelity, for example, most elasmobranchs (Last & Stevens, 2009) and sea snakes (Voris & Jayne, 1979;Ward, 2001). Others, such as many sepiids and octopods, may produce a large number of eggs, but the juveniles that hatch from them often do not disperse widely due to their benthic habits (Boyle, 1990). These life history traits result in important components of biodiversity not being optimally protected by networks of MPAs. Depending on mobility at the adult stage, these taxa will either be largely disconnected from other reef systems, or spend part of their lives unprotected (Heupel *et al*., 2010), with the prospect of self-seeding not being sufficient for any particular MPAs to be demographically independent.

In organisms with pelagic larvae, dispersal occurs at a stage in which mortality rates are extremely high and this mortality is offset by the production of thousands to millions of larvae.

The mortality rates of adult rays, sharks and sea snakes in the absence of anthropogenic threats are on the other hand comparatively low (Fry *et al*., 2001;Ward, 2001;Last & Stevens, 2009). As a result, their potential exposure to fishing pressure when moving between MPAs is of far greater consequence. To muddy the waters for effective conservation even further, the capacity for adult dispersal also varies substantially. For example, the olive sea snake A*ipysurus laevis* rarely ventures far from home reefs and has a home ranges of less than two km^2 (Burns & Heatwole 1998) while adult grey reef sharks (*Carcharhinus amblyrhynchos*) may move > 100 km in a few days (Heupel *et al*., 2010) and turtles may traverse thousands of kilometres between reproductive eisodes (Hays *et al*., 2014). To provide optimum protection for taxa with such diverse dispersal strategies is extremely complex in the absence of detailed information on how and where species disperse.

THE CHALLENGE OF PROTECTING CORAL REEF PREDATORS

Although the number of species with adult dispersal are few compared to the diversity of coral reef organisms with pelagic larvae (Tittensor *et al*., 2010), the proportion of the biomass they account for in healthy reefs is extraordinary. Recent studies suggest that pristine coral reefs have top-heavy biomass pyramids, and apex predators may overwhelm fish assemblages (Sandin *et al*., 2008; Friedlander *et al*., 2014). In remote areas where little fishing has occurred, reef sharks may account for more than 60% of top predator biomass (Friedlander *et al*., 2014). Crucially, their removal may have substantial top-down effects on the whole ecosystem (Ruppert *et al*., 2013; Heithaus *et al*., 2014). This points to the loss of reef sharks being a significant issue for long term sustainability of healthy reef ecosystems, because a very large proportion of the fish biomass may not be optimally protected, even by the most carefully managed networks of MPAs.

MPAs that work best for sharks are very large (>100 km^2) reserves around isolated oceanic islands where no-take policies are effectively enforced (Edgar *et al*., 2014). Under these conditions animals have very little chance of moving out of the reserve because they are surrounded by large stretches of unsuitable habitats, and there is strong evidence that reef sharks will then exhibit a high degree of site fidelity (Field *et al*., 2011; Barnett *et al*., 2012). Unfortunately very few such reserves exist. Even though a number of giant marine reserves and shark sanctuaries have been established in recent years, they vary greatly in terms of which fishing activities are permitted within their boundaries (Cressey 2011; Pala 2013). In the long-term giant reserves are likely to play an important role in conserving the biodiversity of wide ranging animals (Hays *et al*, 2014), but there are enormous challenges both in terms of

enforcement and monitoring which limit their effectiveness (Cressey 2011; Pala 2013) . In multiple use MPA networks, where 'no take' areas are not clearly isolated from areas open to fisheries by large stretches of unsuitable habitat, reef sharks may not be constrained to the MPAs. For example, reefs that are very close are likely perceived as continuous habitats; in these conditions reef sharks may move continuously between neighboring reefs crossing the boundaries of different management zones (Heupel *et al*., 2010). As distance between reefs increases, grey reef sharks exhibit higher site fidelity, but a large proportion of them still hop from reef to reef, moving to (or through) unprotected areas (Espinoza *et al*., 2015). This may be a problem if most reefs in a network of MPAs are small and geographically very close to each other. This is the case in the Great Barrier Reef (GBR) Marine Park in Australia, where the majority of reefs are small (<10 km^2) and separated by distances of < 2km (Almany *et al*., 2007).

UNDERSTANDING CONNECTIVITY IN ADULT DISPERSERS: LESSONS FROM LANDSCAPE GENETICS

For taxa with high adult mobility one potential solution is to include corridors in the design of networks of MPAs, thereby allowing for movement of animals between no-take zones and extending protection for animals with wider home-ranges. Such corridors need not be designated 'all encompassing no-take' policies, but could be targeted specifically to ensure that groups of animals with similar habitat preferences and dispersal abilities are provided effective protection. The concept that connectivity corridors should be included in MPAs networks is fairly new and has so far been advocated nearly exclusively for migratory species en route to protected foraging and breeding grounds, such as sea turtles, pinnipeds and cetaceans (Hooker *et al*. 2011; Pendoley *et al*., 2014). Similar approaches for the co-management of sympatric species of importance have been previously advocated for other iconic animals, such as dugongs and sea turtles (Gredzens *et al*, 2014). Here we argue that such an approach may even be essential for the conservation of meso and apex predators that do not undertake large migrations.

To be effective, designing corridors requires knowledge of how habitat shapes connectivity for the target species and the development of models predicting connectivity through the seascape. On land, these sorts of predictions fall under the umbrella of landscape genetics (Manel *et al*., 2003; Manel & Holderegger, 2013). Seascape genetics, however, has thus far focused nearly exclusively on modelling larval connectivity using biophysical and oceanographic models (Sponaugle *et al*., 2002; Selkoe *et al*., 2008; Galindo *et al*., 2010). Little effort has been placed on modelling connectivity in adult dispersers, and is so far limited to a handful of studies of

apex predators (Schultz *et al*., 2008; Amaral *et al*., 2012; Lowther *et al*., 2012). This dearth of information persists despite the fact that many coral reef organisms without larval dispersal are habitat specialists (for example, grey reef sharks, whitetip reef sharks, olive sea snakes, blue spotted fantail ray) and habitat is likely to have a much stronger influence on their dispersal than oceanographic features.

A key element will be the development of models that test specific hypotheses on how habitat shapes connectivity. Models based on Circuit Theory (such as Isolation-by-Resistance; IBR) and Least Cost Path (LCP) (McRae, 2006; McRae and Beier, 2007; McRae *et al*., 2008) have been successfully used to predict connectivity on land, but their application in marine systems has been largely overlooked. For adult dispersers with specialised habitat requirements, we need to determine whether habitat features are more likely to influence dispersal than oceanographic currents. For example, three environmental variables, distance from the coast, latitude and coral cover, effectively explained much of the differences in abundance of reef associated sharks across the largest network of MPAs in the world, the GBR Marine Park, Australia (Espinoza *et al*., 2014). Similarly, grey reef sharks have been shown to favour offshore areas with high coral cover (Fernandes *et al*., 2005) and, within reefs, they prefer sharp drop-offs over reef slopes (Rizzari *et al*., 2014). All of these habitat features could be easily included in IBR and LCP models.

Connectivity models incorporate pre-existing ecological data, for example patterns of presence/absence, abundance and habitat use, to develop 'resistance surfaces' that describe the relative probability of an animal moving through different seascape features (for example, coral reefs vs. mud flats vs. seagrass beds). These resistance surfaces represent hypothetical relationships between the connectivity of target species and habitat features. The predictions of these models can then be tested using either genetic (McRae, 2006) or empirical data on animal movements (Desrochers *et al*., 2011; St-Louis *et al*., 2014). This approach has the capacity to describe patterns of connectivity through the seascape, and in so doing, identify key areas for protection.

CONCLUSION

Networks of MPAs have transformed our ability to conserve our most precious marine habitats, but are not yet a panacea for biodiversity conservation. Species with sedentary juvenile stages and adult dispersal phases may still be poorly protected due to movement occurring outside of no take areas, or be constrained by limited connectivity. A potential solution may be to adopt conservation management strategies tried and tested for terrestrial organisms. Recent

technological and analytical advances allow us to identify seascape features that facilitate connectivity. Protection for identified corridors may effectively conserve many of those organisms that are key to coral reef ecological function, but as yet not well protected by existing MPA networks.

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Chapter 9

First records of the grey nurse shark *Carcharias taurus* (Lamniformes: Odontaspididae) from oceanic coral reefs in the Timor Sea.

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ABSTRACT

The threatened grey nurse shark (*Carcharias taurus*) is reported for the first time from oceanic coral reefs in the Timor Sea. Generally known from temperate and subtropical coastal reef habitats, this species was encountered by Indonesian traditional fishers on oceanic coral reefs in an area of the Australian Exclusive Economic Zone known as the 1974 MoU Box some 200 km from the Australian mainland. The presence of *C. taurus* on these remote tropical reefs bears important management implications, including the species' protected status in Australian waters and the challenges of regulating catches in areas permitted for traditional Indonesian fishing.

INTRODUCTION

Grey nurse sharks (*Carcharias taurus*) are known to occur on coastal reefs of the continental shelf, from the surf zone to depths of about 230 m (Last & Stevens 2009; Otway & Ellis 2011). They have been reported from inshore regions in temperate to subtropical waters of the Atlantic, Mediterranean and Indo-west Pacific and are generally described as a coastal species (Ahonen *et al.* 2009; Compagno 2001). Within Australia, two main populations of *C. taurus* are recognised: one on the eastern coast and one in Western Australia. The eastern Australian population has a range extending from north of Yepoon in southern Queensland (Latitude: 22° S) to the southern border of New South Wales (Latitude: 37.5° S) (Bansemer & Bennett 2011; Otway & Ellis 2011), while the western population is predominantly found in the coastal waters of south-western Australia, although sightings have been reported from as far north as Exmouth (latitude: 21.5° S) (Chidlow *et al.* 2005). *Carcharias taurus* has been protected in Australia since 1984, after intense fishing caused severe declines particularly in the eastern population. They are currently listed by the International Union for the Conservation of Nature as Vulnerable globally and as Critically Endangered in eastern Australia and in the southwest Atlantic (Pollard & Smith 2009)

Fishers from the island of Rote in Indonesia's East Nusa Tenggara province have long fished Scott Reef as well as the reefs of Ashmore Island, Cartier Island and Browse Island in the Timor Sea (Fox & Sen 2002). Now under Australian jurisdiction, an area encompassing these and other reefs known as the Memorandum of Understanding Box (MoU Box) was declared in 1974, allowing traditional fishing with unmotorised vessels by Indonesian fishers in recognition of their traditional fishing grounds (Stacey 2007). Fishers targeting sharks for local consumption and export of shark fins often sail to Browse Island in the south-eastern corner of the MoU Box, where they catch various species of sharks (Momigliano *et al.* 2015). Recent

fishery surveys (V. Jaiteh, unpublished data) suggested that *C. taurus* forms part of the fishers' catch. Although no surprise to the Indonesian fishers who have fished these waters for centuries, the presence of *C. taurus* on the remote oceanic reefs of the MoU Box was unexpected given the available scientific literature on the distribution of this species.

The presence of *C. taurus* on remote tropical reefs within the MoU Box is described from four specimens collected by Indonesian fishers. These findings highlight the challenges for the management of this species within the MoU Box, where it may be regularly exposed to fishing pressure.

MATERIALS AND METHODS

Tissue samples were collected in the 1974 MoU Box by fishers from the island of Rote, Indonesia (Figure 1) between June and November 2013. All samples were collected from sharks caught on baited demersal longlines during the fishers' normal fishing activities and immediately stored in a NaCl saturated solution containing 20% dimethyl sulphoxide and 0.25 M ethylenediaminetetraacetic acid; no animals were caught specifically for this study. Fishers also recorded sex, total length and fork length for each specimen. Longline sets that included catches of *Carcharias taurus* had soak times of 10 – 12.5 h and were baited with various species of demersal reef fish. Fishers recorded the GPS location and water depth at each location where sharks were caught.

DNA was extracted from fin clips of four specimens that were identified by the fishers as *C. taurus* following a chelex extraction protocol (Walsh *et al.* 1991). A 652 bp fragment of the Cytochrome Oxidase 1 gene was amplified following the protocol outlined by Ward *et al.* (2008). The obtained sequences (GenBank accession numbers: KR003980-KR003983) were matched with sequences deposited in the Barcode of Life Data System (BOLD, http://www.boldsystems.org/) database using the BOLD Identification System (IDS). Furthermore, the sequences from these specimens were aligned with sequences from closely related species of the same family (Odontaspididae) obtained from BOLD to construct a Neighbor-Joining phylogeny using the Kimura's two parameter model (Kimura 1980) and 1000 bootstrap pseudo-replicates using the software MEGA 5 (Tamura *et al.* 2011).

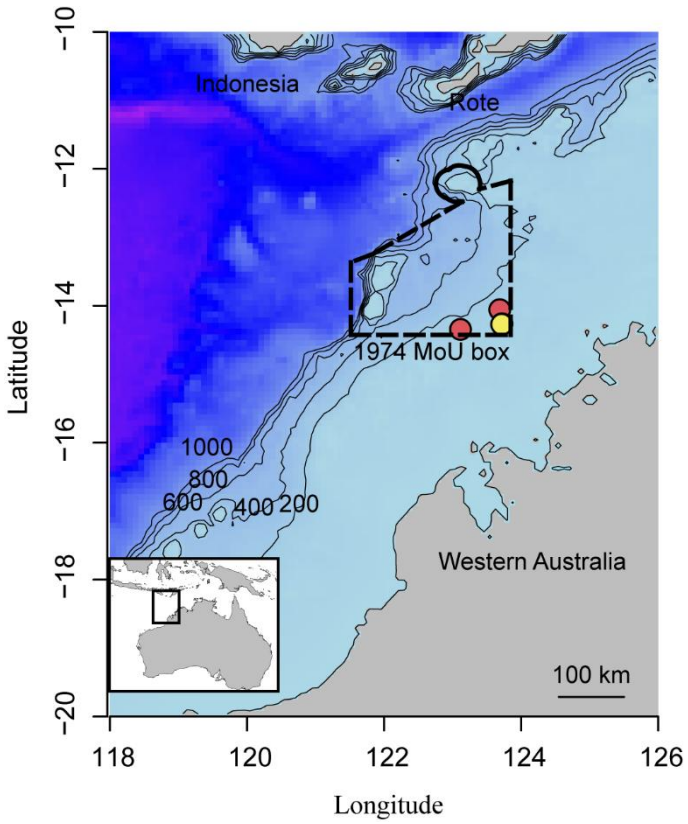

Figure 1: *Map showing locations (circles) where* Carcharias taurus *(n=4) were caught in the 1974 MoU Box, an area opened for Indonesian traditional fishing within the Australian Exclusive Economic Zone. Red circles indicate locations where one shark was caught, the yellow circle represents two individuals. Bathymetric lines at intervals of 200 m are shown.*

RESULTS AND DISCUSSION

Four individuals of *Carcharias taurus* (three females and one male) were caught in the approximate vicinity of Browse Island (14°6'30"S, 123°32'50"E) in the south-eastern corner of the MoU Box (Table 1 and figure 1), on demersal longlines set at depths of 50 - 90 m. Fishers identified the sharks as *hiu lapis gigi* which translates to ‘layered tooth shark’, and matched the species to *C. taurus* when shown an identification guide (Last & Stevens 2009). Total lengths ranged from 209 cm (the only male specimen) to 273 cm (Table 1), suggesting all individuals were adults (Bansemer & Bennett 2011).

Table 1: Sex, lengths and geographical coordinates for each recorded specimen of *C. taurus in* the MoU Box, Timor Sea.

Specimen	Sex	Total Length (cm)	Fork Length (cm)	Latitude	Longitude
GN 1	F	273	223	14° 16' 16" S	123° 42' 56" E
GN 2	F	231	198	14° 3' 27" S	123 41' 16" E
GN 3	M	209	178	14° 20' 44" S	123° 6' 55" E
GN 4	F	270	213	14° 15' 5" S	123° 43' 12" E

The obtained sequences were unambiguously identified as *Carcharias taurus* by the BOLD search engine, yielding matches with sequences of *C. taurus* deposited in BOLD ranging from 99.66% to 100%. The final alignment used for phylogenetic reconstruction included 591 unambiguously aligned positions, including 96 variable sites and 95 parsimony informative sites. Within the phylogenetic reconstruction all individuals were grouped with 100% bootstrap support with other *C. taurus* sequences (figure 2).

Carcharias taurus is listed as Vulnerable globally, as Near Threatened in Western Australia and as Critically Endangered in eastern Australia (Pollard & Smith 2009). The New South Wales government declared *C. taurus* a protected species in 1984, making it the first shark species in the world to be legally protected. *Carcharias taurus* is now protected in all Australian states through fishery legislations and in Commonwealth waters via the Environmental Protection and Biodiversity Conservation Act (1999). The MoU Box forms part of Australia's Commonwealth waters and as such falls under the jurisdiction of the federal government. Since *C. taurus* is a protected species in Australian waters, their protection should extend to the remote reefs of the MoU Box. As with the species recently listed on Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (the porbeagle, oceanic whitetip and hammerhead sharks and manta rays), ensuring the protection of this species in the MoU Box will present challenges not only due to the remoteness of the reefs, but also because of the agreement with Indonesia that allows traditional fishing by Indonesian fishers.

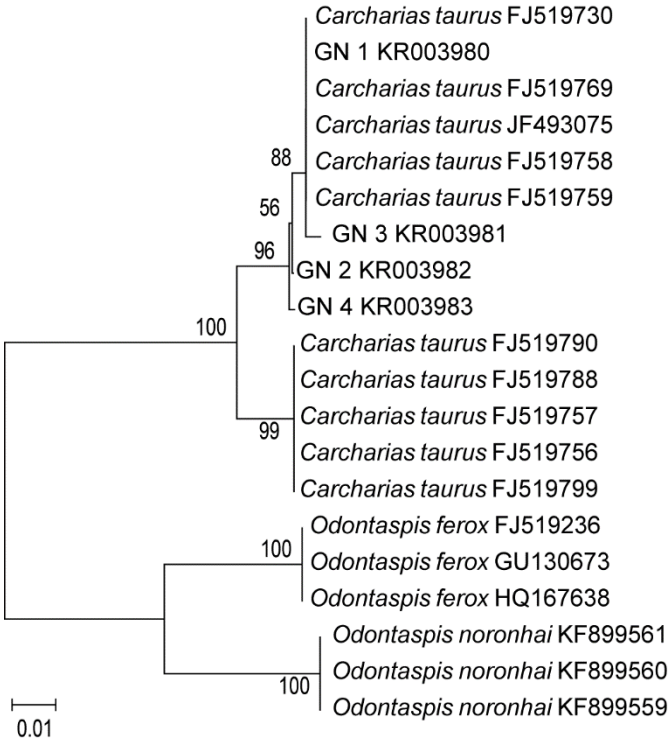

***Figure 2**: Neighbour-Joining tree showing the placement of* Carcharias taurus *samples within a phylogeny of the family Odontaspididae. All sequences obtained in this study are nested within sequences of* Carcharias taurus *and are grouped with sequences of this species with 100% bootstrap support. Internal branch support values represent 1000 bootstrap pseudo-replicate datasets. The tree was mid-point rooted for the purpose of clarity. Scale bar represents number of changes per base pair.*

It is unknown whether *C. taurus* within the MoU Box are part of the Western Australian population or whether they belong to a separate demographic stock. Previous population genetic studies on *C. taurus* suggest that there is negligible migration between the populations along the eastern and western coasts of Australia (Ahonen *et al.* 2009; Stow *et al.* 2006). Within eastern Australia however, individual *C. taurus* have been observed undertaking large-scale unidirectional movements of over 1,100 km (Bansemer & Bennett, 2011). If the individuals in the far north of Western Australia are part of a separate, geographically isolated demographic unit, there would be reason for concern over their conservation status given the species' heavily K-selected life history with only two pups every other year (Bansemer & Bennett 2009) and regular fishing pressure at Browse Island and surrounding reefs (Momigliano *et al.* 2015). An assessment of the genetic population structure of *C. taurus* on the western coast of Australia is recommended to provide insight on the appropriate spatial scale of management for this species in Western Australia.

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Chapter 10

New distribution records of the Vulnerable fossil shark *Hemipristis elongata* from eastern Indonesia call for improved fisheries management.

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ABSTRACT

Genetically verified catch data from fishers in eastern Indonesia provide new distribution records for the fossil shark *Hemipristis elongata* in the Halmahera, Seram and Arafura seas. Previously only recorded from the island of Java, this study reports a range extension for this species of >2000 km across the Indonesian archipelago, suggesting that fossil sharks are subjected to fishing pressure over a much larger geographic area than implied by previous species records. We recommend a review of the current species assessment to reflect the reported range extension and inform management of this fishery-targeted shark.

INTRODUCTION

In the last two decades, shark populations around the world have experienced unprecedented declines driven largely by target fisheries that supply the shark fin trade (Baum *et al.* 2003; Dulvy *et al.* 2008; Ferretti *et al.* 2008). According to the United Nations' Food and Agriculture Organization (FAO), Indonesia has the world's biggest shark fishery with a reported average yearly catch in excess of 100,000 tons over the last decade (FAO 2014).

Global efforts to protect sharks from overexploitation have been informed and recommended in recent years by a substantial rise in the number of scientific studies on various aspects of shark biology and fisheries (Momigliano & Harcourt 2014). However, this global trend of increased scientific, government and non-government attention to the plight of sharks has largely eluded Indonesia for various reasons, such as the size of the Indonesian archipelago, the logistics of accessing remote fishing grounds, the difficulty of obtaining research permits and insufficient allocation of government resources (Momigliano *et al.* 2015). The published scientific studies from the region (Blaber *et al.* 2009; White 2007a; White *et al.* 2008; White & Dharmadi 2010) have focused on relatively easily accessible parts of Indonesia, or were based on fish market surveys where the location of capture was not always known. As a result, essential data on the diversity, biology and distribution of fishery-targeted shark species remain sparse or lacking for large parts of Indonesia, impeding effective management and conservation efforts. Where data are scarce and rigorous fishery-independent assessments not possible, catch data can provide critical information on the status of a species, for example as part of species assessments (Froese *et al.* 2012). Determining the distribution range of a species and the geographic extent of the fisheries targeting it are important components of a species assessment, allowing managers to develop location-specific strategies to protect populations from anthropogenic pressures.

The fossil shark *Hemipristis elongata* (Klunzinger 1871) is a tropical shark known to occur in shallow waters to depths of at least 130 m (Stevens & McLoughlin 1991) in coastal regions of the Indo-western Pacific, including southeastern Africa, the Red Sea, India, Southeast Asia and Australia (Last & Stevens 2009; White 2003). Due to the high quality of its meat and fins, *H. elongata* is targeted by intensive and largely unmanaged coastal fisheries throughout its range with the exception of Australia, where it has little commercial value (White, 2003). Fish market surveys in the Gulf of Thailand and on Java, Indonesia have indicated that the species has declined where it was once common (White, 2003). Despite the strong likelihood that *H. elongata* has experienced significant population declines in the last two decades, biological information for the species is limited. It grows to about 240 cm and females are thought to have two to 11 young every other year (Stevens & McLoughlin, 1991). Size at maturity and growth is not well understood (White, 2007b).

Based on the most recent species assessment (White, 2003), the IUCN classifies *Hemipristis elongata* as globally Vulnerable with a decreasing population trend. Indonesia is one of the countries in which fossil sharks are likely to be heavily exploited, but to date they have only been recorded from fish markets on Java (White 2003; White 2007b). The author of the IUCN assessment of *H. elongata* suggested that market surveys are likely to provide a relatively good representation of the population of this species in Indonesia (White 2003). However, fish market surveys are unlikely to yield reliable data in eastern Indonesia, where sharks are rarely landed whole and sold at markets; instead, fishers often discard the carcasses at sea, bringing back only the fins which they dry and sell directly to traders close to their homeports (Momigliano *et al.* 2015). If not accounted for, this 'invisible' fishing pressure can limit the accuracy and usefulness of regional population assessments for local management purposes. One way to obtain fishery data in the absence of carcasses for species identification is to work directly with fishers to collect catch data at sea. Recent fishery surveys with the objective of describing the species composition of eastern Indonesian shark fisheries (V. Jaiteh, unpublished data) suggested the presence of *Hemipristis elongata* in a region outside of its currently known range. Here we report a range extension of >2000 km for *H. elongata* into far eastern Indonesia from genetically validated catch data collected by shark fishers in the Halmahera, Seram and Arafura Seas. We discuss the implications of this species' occurrence and exposure to fishing pressure in three distinct fishing grounds from where it was not previously described, and recommend a review of the IUCN species assessment for *H. elongata*.

MATERIALS AND METHODS

Fishers from Seram island and the Aru archipelago in Maluku province, Indonesia recorded 19 specimens of *Hemipristis elongata* during four fishing trips between March and December 2012 in their usual fishing grounds: the Halmahera Sea surrounding Halmahera island in North Maluku province; the Seram Sea in the Raja Ampat regency of West Papua province; and the Arafura Sea around Aru regency, Maluku province (figure 1). All three fishing grounds are at a distance of 2000 to 2500 km from the island of Java. Fishers who agreed to take part in this study were trained in data collection and recorded the following information for each longline or gillnet set: the local species names of the first ten sharks caught, their total length and sex. For males, clasper length and a basic assessment of calcification (yes/no) were noted, females were checked for embryos. After each fishing trip, local species names were matched to scientific names using identification guides (Last & Stevens 2009; White *et al.* 2006). Fishers also recorded water depth and, if a GPS was available, the coordinates of each set. Samples of muscle and fin tissue were collected from sharks caught in bottom-set gill nets or baited longlines with a soak time of 10–15 h overnight. Fishers collected tissue samples from 14 specimens: all individuals caught in Halmahera (N=3) and Raja Ampat (N=5), and six individuals from Aru, where a further five specimens were recorded from which no samples are available (figure 1). Fishers from Aru collected tissue samples during their normal fishing activities and stored them in vials that they labeled with the local species name. Fishers in Halmahera and Raja Ampat took tissue samples from the underside of dried fins that they identified to species-level immediately after the respective fishing trips. In all cases, samples were stored in a NaCl saturated solution containing 20% dimethyl sulphoxide and 0.25 M ethylenediaminetetraacetic acid.

DNA was extracted from the 14 specimens following the modified chelex protocol (Walsh *et al.* 1991) described by Ward *et al.* (2008). A 652 bp long fragment of the Cytochrome Oxidase 1 gene was amplified following the protocol outlined by Ward *et al.* (2008), using the primer pair FishF1 and FishR1 (Ward *et al.* 2005). PCR products were sequenced using the forward primer FishF1. All sequencing reactions were carried out by a commercial company (Macrogen Inc.). The obtained sequences were deposited in GenBank (accession numbers: KR003984-KR003997) and matched with sequences deposited in the Barcode of Life Data System (BOLD, http://www.boldsystems.org/) database using the BOLD Identification System (IDS). Sequences from these specimens were aligned with sequences from other Hemigaleidae obtained from BOLD. A neighbour-joining tree was constructed as outlined in Momigliano & Jaiteh (2015).

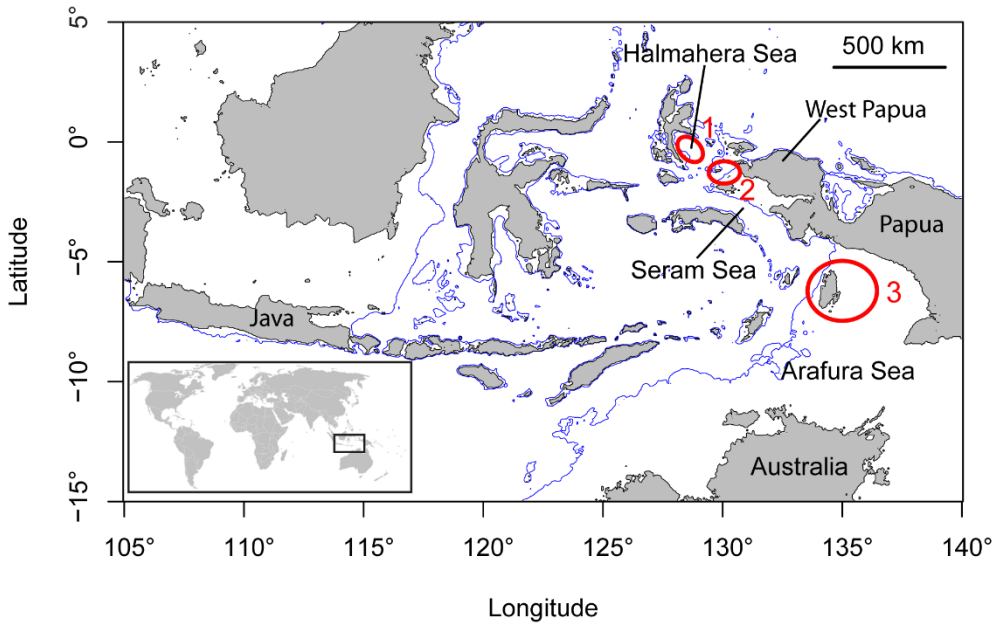

***Figure 1**: Map showing the fishing grounds (red circles 1-3) where 19 individuals of* Hemipristis elongata *were recorded, 14 of which were sampled and genetically verified. Fishing grounds are numbered: 1 = Halmahera Sea in North Maluku province; 2 = Seram Sea, Raja Ampat in West Papua province; 3 = Arafura Sea, Aru in Maluku province. The 200 m bathymetric depth contour is shown in blue.*

RESULTS

All of the 14 samples for which genetic analysis was carried out were unambiguously identified as *Hemipristis elongata* by the BOLD IDS system. In the phylogenetic tree all individuals grouped with 100% bootstrap support with known sequences of *Hemipristis elongata* (figure 2), therefore verifying the suspected occurrence of this species in far eastern Indonesia. Fishers identified the species as *hiu putih*, or 'white shark' when referring to identification images of *H. elongata* in White *et al.* (2006) and Lastand Stevens (2009). All fossil sharks recorded by the fishers were caught at depths between 17 and 125 m. Of the ten genetically verified individuals that were measured and sexed by the fishers, all females (N=6) were immature with no embryos while all males (N=4) were mature with calcified claspers (Table 1).

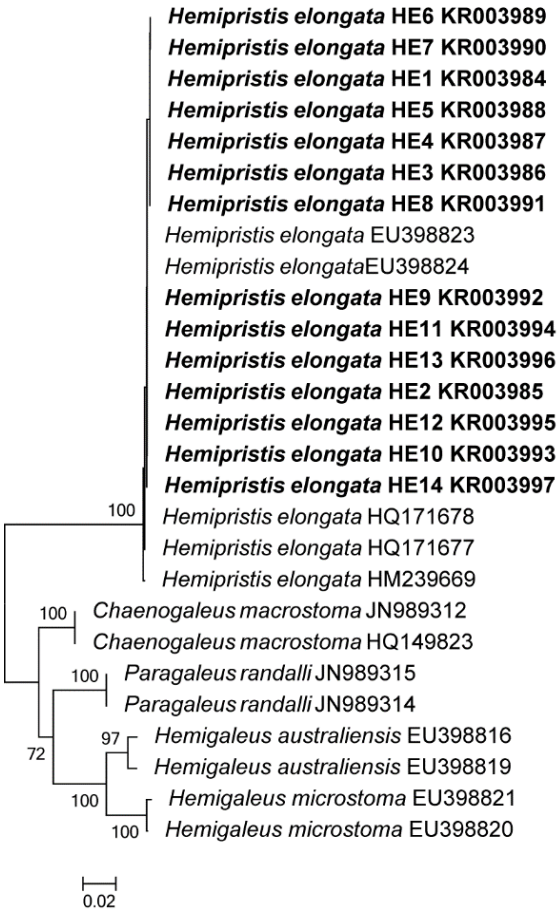

Figure 2: *Neighbour-Joining tree of the family Hemigaleidae based on a 652 bp long fragment of the Cytochrome Oxidase 1 gene. Individuals analysed in this study are shown in bold. Internal branch labels represent bootstrap support based on 1000 pseudo replicate datasets. Scale bar represents number of changes per base pair.*

Table 1: Sex, lengths and geographical coordinates recorded by fishers in eastern Indonesia for ten genetically verified specimens of *Hemipristis elongata.*

Specimen	**Sex**	**Total length (cm)**	**F: Number of embryos M: Claspers calcified (y/n)**	**Latitude**	**Longitude**
HE 1	F	102	0	1° 44.223' S	129° 57.586' E
HE 2	F	109	0	1° 42.630' S	129° 46.944' E
HE 3	F	89	0	1° 50.971' S	129° 38.125' E
HE 4	F	85	0	0° 37.991' S	130° 10.625' E
HE 5	F	102	0	1° 38.638' S	129° 54.744' E
HE 6	F	112	0	6° 57.239' S	134° 46.505' E
HE 7	M	145	y	6° 43.29' S	133° 51.129' E
HE 8	M	210	y	6° 43.29' S	133° 51.129' E
HE 9	M	220	y	6° 43.29' S	133° 51.129' E
HE 10	M	210	y	6° 27.197' S	133° 57.021' E

DISCUSSION

In Indonesia, fossil sharks have previously only been recorded from Java (White 2003), which is bounded by the Indian Ocean to the South and the Java Sea to the North (figure 1). This study provides new information on the distribution of *Hemipristis elongata* across the archipelago, extending its known range by >2000 km into the Halmahera, Seram and Arafura Seas. The IUCN assessment for *H. elongata* states that "the intensive and largely unmanaged net and trawl fisheries that occur throughout its range (…) fish heavily in its known habitat and are likely to catch this species if present" (White 2003). Our results confirm that this is the case in eastern Indonesia, where *H. elongata* has not been documented before and shark fisheries have barely been described. This has important implications for the management and conservation of *H. elongata* and other species caught in the eastern Indonesian shark fishery, where catches are generally not reported.

Shark management has been slow to develop in Indonesia, partly due to a lack of information about the species occurring in Indonesia's waters, their distributions and the level of threat they are exposed to through target fisheries and bycatch (Blaber *et al.* 2009; White & Kyne 2010). Their more charismatic relatives, manta rays (Mobulidae), have the same IUCN threat status as fossil sharks but owing mostly to their value for tourism, are now protected throughout Indonesian waters with violation of the law incurring a fine of 250 million rupiah (approx. US $20,000). *Hemipristis elongata* however, along with the majority of species caught in Indonesia's shark fisheries, is not protected by national law, and there are currently no fisheries management plans in place for this species. The Raja Ampat shark sanctuary declared in 2013 is the exception to the current lack of effective shark protection in Indonesia, and the fishers that provided data for this study from their Raja Ampat fishing grounds no longer fish there.

Species assessments are important sources of information for management plans, making the availability of accurate biological and fisheries data essential for effective conservation of threatened species. The IUCN assessment for *H. elongata* lists as recommended Conservation Actions, "Recent species composition and catch data for fisheries within its range are required to assess the population trends, especially in areas where there is a very high level of fishing pressure." This study contributes recent catch data from eastern Indonesia, a region with extensive shark fisheries from which *H. elongata* was not previously known to occur. The length and sex data collected by the fishers imply that all caught females were immature, with lengths <120 cm and no embryos while all males were mature at lengths >136 cm (Last & Stevens 2009) and calcified claspers (Table 1). Our results highlight the value of catch data recorded by fishers, in combination with genetic verification of their species identifications, to

provide fundamental information on the distribution and status of a Vulnerable species. This information calls for an update of the IUCN species assessment, as well as improved fisheries management strategies in one of the world's most significant shark fisheries.

Indonesia lies within the global center of marine biodiversity known as the Coral Triangle. Despite this, the available data suggest that this region harbors a surprisingly lower biodiversity of elasmobranchs than its neighbour Australia (White & Kyne 2010), which is regarded to have the highest abundance of elasmobranch species in the world (Last & Stevens 2009). However, Indonesia has far less resources to study the shark species in its waters and the threats they face. Nevertheless, in recent years previously unobserved species have been reported from various parts of the island archipelago (Allen *et al.* 2013; White & Last 2006; White *et al.* 2005), suggesting that elasmobranch biodiversity in the region has likely been underestimated. A more extensive study of the catch composition of eastern Indonesian shark fisheries would help to identify further knowledge gaps and support the development of management plans for these fisheries.

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Chapter 11

General Discussion

Authors:

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This thesis focused on two main themes: first, I summarized the challenges and successes of shark conservation and management, both globally (chapter 2) and specifically for Australia and Indonesia (chapter 3), and second, I applied genetic methods to obtain conservation relevant information for coral reef associated sharks (chapters 4 to 10).

In the first part of my thesis I critically reviewed scientific literature on shark conservation, and analysed trends in scientific output, with a particular focus on Australia and Indonesia. I identified major geographic and taxonomic biases in the allocation of scientific effort, which had an effect on the implementation of effective management strategies. In the second part of my thesis, I unravelled how a complex seascape and local selective pressures likely influence patterns of genetic variation in the grey reef shark, a coral reef predator which has undergone dramatic declines, and discussed the implications of these findings for the management of reef shark populations.

Below, I provide a synthesis and discussion of the main findings of my thesis, and point to future research directions to deal with some of the issues that my research has highlighted.

THE STATUS OF SHARK CONSERVATION SCIENCE

In the second and third chapters of my thesis, I undertook a critical review of the status of shark conservation and analysed trends in shark conservation research by evaluating 20 years of scientific output. I discovered that while scientific effort has increased substantially in the past two decades, it has done so with a strong geographic bias. Australia and the United States made an overwhelming contribution to research outputs, while countries like Indonesia, which is home to the largest shark fishery in the world, lagged well behind. This geographic bias in scientific efforts has important consequences in terms of shark conservation. Australia is a world leader in shark conservation science and has implemented an adaptive management framework for the conservation of elasmobranchs. This is reflected in a continually reviewed and updated Nation Plan of Action (NPoA) for the Management of sharks and this follows the guidelines of the United Nations International Plan of Action for the Conservation and Management of Sharks (IPoA). While there are still major concerns for many shark species in Australia (Chapter 3), and a lack of data for many bycatch species (Chapter 2), overall the advancement in shark conservation science and management in Australia has been remarkable. In contrast, the implementation of an effective NPoA in Indonesia has been thus far a failure (see Chapter 2 and 3). In part this is due to a lower research capacity, but it may also be driven by other intrinsic factors (see Chapter 2).

In Chapter 2 and Appendix A I noted how supporting Indonesian research capacity through establishing fruitful international collaborations has the potential to play an important role in improving shark management, and in the course of this thesis I have actively contributed to this. In recent years, the number of collaborative projects between Indonesian scientists and foreign researchers (principally from Australia, France and the USA) has increased. Australian institutions (Macquarie University, the University of Queensland, Murdoch University and CSIRO), French institutions (Institute de Research pour le Development (IRD)) and American institutions (the University of California Los Angeles (UCLA)) have formally established collaborations in elasmobranch research with their Indonesian counterparts Lembaga Ilmu Pengetahuan Indonesia (LIPI, Indonesian Institute of Science), the Indonesian Biodiversity Research Center (IBRC) and the Indonesian branch of Conservation International. The IBRC, which was initially funded by USAID, aims specifically to build research capacity in Indonesia. I initiated the collaboration between Macquarie University and IBRC for my research in Indonesia. These collaborations have already yielded important findings on shark conservation issues including fishery surveys (Sembiring *et al.* 2015), studies on species distributions (Fahmi & White 2015; Jaiteh & Momigliano 2015; Momigliano & Jaiteh 2015), phylogenetic and taxonomic work (Arlyza *et al.* 2013a; Borsa *et al.* 2013), research on Marine Protected Areas (Stacey *et al.* 2012) and population genetic studies of sharks and rays (Arlyza *et al.* 2013b; Giles *et al.* 2014; and this thesis, Chapter 6). In some cases a local researcher led the collaborative projects. Furthermore, in recent years there has been an increase in research conducted solely by Indonesian researchers and published in international scientific journals (Dharmadi *et al.* 2015; Dharmadi *et al.* 2014; Fahmi & Dharmadi 2014, 2015), suggesting that there has been an increase in Indonesian research capacity. It is hoped that this increase in research capacity, along with the stronger commitment to shark conservation and management now being expressed by the Indonesian government will result in future improvements in shark conservation and management.

CONSERVATION GENETICS

In chapters four to seven I investigated genetic connectivity and local selection in the grey reef shark, using the most extensive genetic dataset ever used for any elasmobranch species, including 16 microsatellite loci, more than 5000 SNPs and a mtDNA gene. This work revealed complex patterns of genetic structure, highlighting the major roles of sex-bias dispersal, the spatial distribution of coral reef habitats and local selective pressures in shaping gene flow. These findings, together with the results from recent acoustic telemetry studies carried out in the Great Barrier Reef (GBR) and in Western Australia (Espinoza *et al.* 2015a; Espinoza *et al.*

2015b; Field *et al.* 2011; Heupel *et al.* 2010), provide crucial information for the conservation management of grey reef sharks in Australia and Indonesia.

Grey reef shark connectivity

My first important finding is the absence of any evidence of large and fine scale genetic structure and local selection within the majority of the GBR, irrespective of the type of genetic marker used (Chapter 5, 6 and 7). These results are compatible with recent findings from acoustic telemetry studies in the GBR, which suggest that grey reef sharks can move great distances, despite evidence of prolonged site fidelity by some individuals (Espinoza *et al.* 2015a; Heupel *et al.* 2010). Both genetic and acoustic telemetry studies therefore suggest that individual reefs within the GBR are most likely not demographically independent units. This may have implications for the effectiveness of discontinuous MPA networks for the protection of grey reef sharks in the GBR. For example, grey reef sharks, particularly adult males which have a higher dispersal potential, may be subjected to fishing pressure outside of their natal reefs. As such, migration outside of MPAs may limit the benefits of protection in sustaining biomass across generations (Momigliano *et al.* 2015). It appears that the limits of MPAs in the protection of grey reef sharks are not solely related to potential problems with compliance to regulations, as suggested by Robbins (2006) and Rizzari *et al.* (2014b), and MPAs should only be seen as a component of more structured management strategy, one which includes ecosystem-wide regulation of catches.

In contrast, both genetic (Chapter 7) and acoustic telemetry studies (Field *et al.* 2011) conducted among the more isolated coral reefs of Western Australia revealed a very different pattern. I found evidence of both large scale genetic differentiation and genotypic spatial autocorrelation at the reef scale in Western Australian reefs, suggesting that coral reef systems isolated by large expanses of unsuitable habitats, even if located within the same continental shelf, are likely demographically independent. While genetic differentiation at nuclear markers was low, there was evidence of mitochondrial genetic differentiation between Scott Reef and other locations in Western Australia, suggesting female migration is low. Furthermore, individual genotypes were spatially auto-correlated, suggesting that sharks sampled within the same reef were genetically more similar. This pattern is consistent with high self-recruitment, and with the results of acoustic telemetry studies which recorded very high site fidelity in Western Australian reefs (Field *et al.* 2011).

The results from chapter five and seven suggest that genetic connectivity is influenced by the spatial distribution of coral reef habitats which act as stepping stones, maintaining genetic

connectivity via male dispersal across large distances. These results are corroborated by the fact that locations separated by large oceanic expanses are highly genetically differentiated, suggesting that in the absence of coral reef "stepping stones" dispersal is unlikely. More detailed information on how habitat shapes connectivity will likely benefit future conservation decisions. In chapter eight I suggest possible strategies to use acoustic telemetry and genetic data to address this question. New theoretical developments based on graph theory show great promise to create spatially explicit, predictive models of dispersal based on habitat data. For example, Isolation by Resistance models (McRae 2006; McRae & Beier 2007; McRae *et al.* 2008) may provide a framework to assess how the spatial distribution of coral reef habitats shape connectivity. Fine scale genetic data as well as acoustic telemetry data could be used to tests different hypothesis on the effects of habitat on connectivity, therefore identifying potential corridors which warrant high conservation priority.

Integrating adaptive genetic variation in conservation genetic studies

In this thesis I investigated, for the first time, putatively adaptive genetic variation in a shark. By screening a very large number of nuclear SNP in sharks from different locations, I identified 3 loci putatively under divergent selection, and therefore potentially implicated in local adaptation. The patterns of genetic variation at these loci followed a significantly different pattern from those loci that behave according to neutral expectations and suggest the presence of local adaptation. The identification of local adaptation has important implications for management. In particular, the fact that sharks in the GBR are likely under different selective pressures than in the rest of the sampled range has important implications for their conservation in eastern Australia. Grey reef sharks have undergone dramatic declines in the GBR, most likely due do fishing pressure (Hisano *et al.* 2011; Rizzari *et al.* 2014a; Robbins *et al.* 2006). If sharks in eastern Australia are locally adapted, it is possible that migration from other regions may not be advantageous for local populations. If grey reef sharks were to become locally extinct in the GBR, recolonization may prove difficult, as migrants may show lower fitness due to phenotype-environment mismatches (Marshall *et al.* 2010). Hence, I suggest that grey reef sharks in eastern Australia warrant very high conservation priority.

Investigating adaptive genetic variation in sharks may provide important information for the management of other elasmobranchs species. For example, identifying similar patterns of local adaptation for multiple species may assist with prioritising conservation activities at regional scales (Dudgeon *et al.* 2012). It is also important that integrating adaptive genetic variation into population genetic studies does not suffer from some of the shortcomings encountered thus far

when using population genetic approaches to identify management units and fishery stocks (Ovenden 2013). Shark populations have often very large effective populations sizes, particularly in wide ranging species (Duncan *et al.* 2006), and therefore often exhibit very little genetic drift even when migration is low. This makes it difficult to identify management units and fishery stocks using population genetics tools, because a lack of genetic structure does not necessarily suggest demographic connectivity (Ovenden 2013).

Large effective population sizes however favour the evolution of local adaptations (Allendorf *et al.* 2010; Savolainen 2011; Savolainen *et al.* 2013). More individuals result in a higher chance of mutations arising and low genetic drift in large populations maintains high levels of standing genetic variation, the raw material on which natural selection acts (Allendorf *et al.* 2010; Savolainen *et al.* 2013). In addition, low genetic drift means that adaptive genetic variation is less likely to be stochastically lost (Savolainen *et al.* 2013). Divergence at loci under selection is a product of the relative values of the migration rate (*m*) and the selection coefficient (*s*) which represents the strength of local selective pressures (Allendorf *et al.* 2010). Therefore, in large populations even weak selection may result in genetic differentiation if *m* is low, since *s* is likely to be greater than *m*. Furthermore, changes in allele frequencies at loci under selection are expected to happen at a much faster rate than changes due to genetic drift. The potential of investigating adaptive genetic variation in order to identify cryptic genetic structure has been clearly demonstrated in studies on other marine fish. Corander *et al.* (2013) found evidence of strong genetic differentiation at outlier loci in herring (*Clupea harengus*) of the Baltic Sea (a region with stark environmental gradients in salinity, pollution and water quality). Similarly, Bradbury *et al.* (2013) were able to detect cryptic genetic divergence in the Atlantic cod (*Gadus morhua*) by including potentially adaptive genes in their analysis.

In addition to detecting cryptic genetic structure driven by local selection, markers under selection are also an extremely powerful tool for assigning individuals to their population of origin. Bradbury *et al.* (2013) were able to assign 100% of their individuals to the location of origin using a combination of neutral and adaptive markers. Nielsen *et al.* (2012) tested whether the high discriminative power of adaptive genetic markers could be used to monitor the provenance of four commercially important marine species: the Atlantic cod (*Gadus morhua*), the Atlantic herring (*Clupea harengus*), the sole (*Solea solea*) and the European hake (*Merluccius merluccius*). In all cases they were able to correctly assign with high certainty more than 90% (in some cases 100%) of the individuals to their sampling location. This unprecedented assignment precision provides a powerful tool for the independent control of the

provenance of international traded species, and may prove very useful for monitoring the international, and partially illegal and unreported, shark fin trade (Shivji *et al.* 2005).

CONCLUSION

In the first part of this thesis I reviewed the status of conservation research and management of sharks, with a particular focus on Australia and Indonesia. I identified biases in scientific effort, identifying both geographical regions and groups of taxa which will likely require more scientific attention in the future. Furthermore, I identified some of the major threatening processes to shark populations in the region. This work will help shaping the future directions of shark conservation science in Australia and Indonesia.

In the second part of this thesis, I focused on the conservation genetics of reef sharks. I integrated for the first time the analysis of loci putatively under local selection within elasmobranch conservation science. By using multiple types of genetic markers (nuclear and mitochondrial, neutral and adaptive), and employing analytical frameworks suited to investigate genetic connectivity at multiple spatial and temporal scales, I was able to identify the roles that sex-bias, habitat and local selection play in shaping genetic variation. The integration of adaptive genetic variation into conservation genetic studies opens up new horizons for shark conservation, enabling to detect cryptic genetic differentiation driven by local selection helping to identify local populations which warrant high conservation priorities. Furthermore, the use of markers under natural selection has the potential to provide a powerful tool for monitoring the international fin trade.

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Appendix A

Scientists focusing on the wrong sharks in the wrong places.

Authors:

Momigliano, Paolo and Harcourt, Robert

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https://theconversation.com/scientists-focusing-on-the-wrong-sharks-in-the-wrong-places-26245

THE CONVERSATION

Scientists focusing on the wrong sharks in the wrong places

May 15, 2014 5.18am AEST

Paolo Momigliano
PhD Student, Marine Biologist at Macquarie University
Rob Harcourt
Professor of Marine Ecology at Macquarie University

Blacktip whaler, a heavily targeted species for shark fisheries. Rob Harcourt

Sharks are in danger in most parts of the world, with a quarter of all sharks and rays now threatened with extinction. This ongoing collapse of shark populations has already had far-reaching effects on marine ecosystems. To arrest this trend we desperately need scientific data that effectively inform conservation management. But are we focusing our research effort in the right areas?

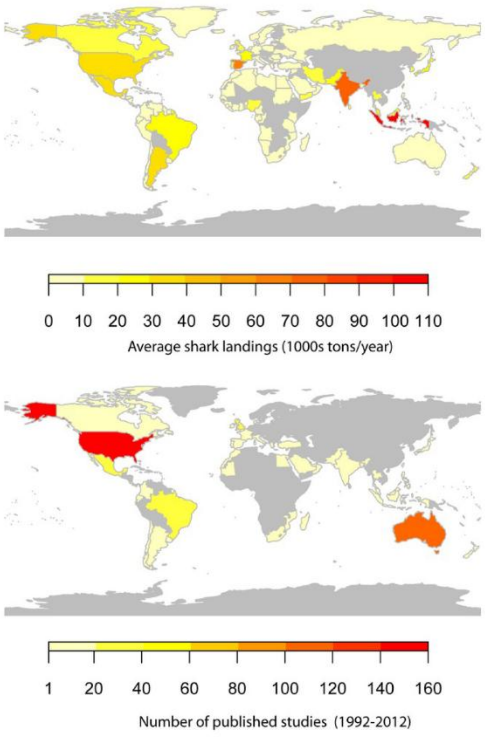
We looked at 20 years' worth of scientific papers on shark conservation to see if shark science is meeting the needs of conservation. Our results, included in a forthcoming book, suggest that the species most in need, and the places where most damage is done, aren't getting the attention they deserve.

Are we looking in the right places?

For science to have the best chance of helping to protect sharks, it should be focused on places where the need for conservation is greatest. As fishing is the most serious threat to

sharks, research efforts should therefore correspond to the places with the most fishing.

Pleasingly, our study found that although shark fishing has increased, scientific effort has also increased substantially in the past few decades. In 1992 only 2 peer-reviewed studies specifically on shark conservation and management were published, by 2011, 80 were published. However, the increase in scientific output is largely driven by two countries: Australia and the United States. In the countries where most shark landings occur (Indonesia, India, Taiwan and Spain) there is almost no research on shark conservation or management.



National contributions to shark landings (above) and scientific output (below) (source: shark landing data were obtained from the FAO). Modified from Figure 5.2 in : Momigliano, P. and Harcourt, Robert (in press). The Science-Law Disconnect. In: Klein, N. and Techera, E. (eds.) Sharks: Conservation, Governance and Management. Earthscan Series, Routledge

This lack of research effort is mirrored by the failure of these countries to implement national shark management plans along United Nations guidelines. There is therefore very little information on the conservation status of some of the world's most vulnerable sharks, and few plans in place to improve the prospects of these populations.

Can we change the situation?

This geographic bias can be at least partly explained by wealth. Research is expensive, and countries that catch the most sharks are relatively poor. But as sharks are key players in marine ecosystems all over the world, we all have a vested interest in their conservation.

Moreover, many shark species are highly mobile. Sharks caught in one country's waters may well have spent much of their lives elsewhere. That means that effective conservation in one region can be spoiled by poor management in a neighbouring country.

We suggest that countries that already invest heavily in conservation and management research have a powerful incentive to cooperate with those that cannot afford it.

For example, Indonesia catches more sharks than any other country in the world. If Australia were to focus some of its shark research on its nearest neighbour, the collaboration could deliver big gains in sustainable fishing management.

Carcasses of requiem sharks at a landing site in Indonesia. Reporting official landings of approximately 100 000 tons of sharks per year, Indonesia is the leading shark fishing nation in the world. Copyright: Vanessa Jaiteh

Are we doing the most effective science?

Australia publishes more studies on shark conservation research, per capita, than any other country. But we cannot afford to be complacent about the type of science that we do. Our study found major biases in which species of shark attracted the attention of researchers.

If conservation research is to be useful, it should prioritise species that are most at risk. Yet of 479 scientific articles on shark conservation and/or management published worldwide over the past 20 years, only 22 (less than 5%) focused on globally endangered species. And 16 of those were focused on a single species that is also of economic importance: the scalloped hammerhead. That leaves just six papers (on four species) in two decades which looked at all of the other globally endangered sharks (24 species).

More than 200 species (over 40%) of sharks are classified by the International Union for the Conservation of Nature as "data deficient": we simply do not have enough data to make a call as to whether they are endangered or not. Yet, in the past two decades only 30 studies focusing on 14 data deficient species have been published. Many of these species are likely to be threatened or endangered but without hard data we just don't know, so effective management is impossible.

Specifically referring to shark conservation, of the 20 most studied species, only the scalloped hammerhead is endangered and just two are listed as data deficient. We found that the vast majority of research is highly skewed towards species that are either economically important (such as the blacktip shark), or charismatic, such as the whale shark and the great white shark.

This skew may be explained by the advantages charismatic species confer in leveraging funding, combined with evidence that publishing in high-impact journals is easier with popular species, with obvious implications for researchers' careers.

Importantly, bycatch species are largely ignored, despite the fact that most sharks are caught as bycatch. This has serious consequences. In southeastern Australia, for example, no data on bycatch of deepwater sharks were available for many decades. By the time this data became available, some species – such as the now critically endangered Harrison's dogfish – had declined by nearly 99%.

We have a long way to go to improve shark conservation science. While the research effort has increased over the last 20 years, it has done so in only a few countries and for a handful of species – and not always those most in need in either case.

If we want to ensure that sharks are properly managed right around the world, we need to start paying closer attention to the "where" and "what" of shark science.

Appendix B

Multiple paternity in captive grey nurse sharks (*Carcharias taurus*): implications for the captive breeding of this critically endangered species.

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Multiple paternity in captive grey nurse sharks (*Carcharias taurus*): implications for the captive breeding of this critically endangered species

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Abstract. The grey nurse shark (*Carcharius taurus*) is listed as threatened throughout much of its global distribution, and as critically endangered in eastern Australia. Captive breeding programs have thus far been largely unsuccessful and little is known of its mating system in this context. Here we carry out a paternity analysis to determine if the mating system in captivity is characterised by multiple mating, and whether poor offspring survival is associated with a particular male. Tissue samples from grey nurse sharks were collected from three potential sires, the two dams and nine pups housed at Manly SEA LIFE Sanctuary in eastern Australia. Each individual was genotyped at seven microsatellite markers and three cases of multiple paternity were inferred. No paternal link to stillborn (5), or scoliotic (2) pups was indicated. For the first time, we show the natural wild phenomenon of multiple paternity occurring in a captive environment.

Keywords: captive breeding, elasmobranch, microsatellite, multiple paternity

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Introduction

Grey nurse sharks (*Carcharias taurus*) make ideal aquarium specimens, being large and long-lived, and adults cope well within the confines of an aquarium (Gordon 1993; Smith *et al.* 2004). Wild animals spend much of their time in 'gutters' and near-shore environments (Otway *et al.* 2003), and these conditions are easily replicated in captivity. It is for these reasons, coupled with their conservation status, that grey nurse sharks are widely held in public aquaria around the world (Gordon 1993; Smith *et al.* 2004).

The eastern Australian population of grey nurse sharks was fished to near-extinction in the 1960s and 1970s and is now listed as Critically Endangered in New South Wales (NSW) waters and protected under the *Environmental Protection and Biodiversity Conservation (EPBC) Act (1999)*. Since 2002, there has been a no-take policy from both eastern and western populations for aquarium display purposes (enforced by the Australian Department of the Environment through the EPBC Act), which in combination with very poor captive breeding success has resulted in an aging captive population. The average estimated age of east coast animals in captivity is 26.4 years ($n = 9$). Given that the maximum known age for grey nurse sharks is 35 years (Goldman *et al.* 2006), and a lifting of the moratorium on collection for aquaria is not likely in the near future, an increased effort to maximise breeding success is needed if this species is to be held in aquaria into the future. Only a handful of aquaria worldwide have had breeding events (J. Choromanski, pers. com.) and there is no record of a captive-born animal producing viable offspring. A first step in the process of establishing captive-bred populations is to gather information on current breeding behaviour in captivity, such as whether multiple mating occurs and whether poor offspring survival can be linked to particular parents.

Grey nurse sharks have an unusual reproductive mode involving intrauterine cannibalism and oophagy (Grant *et al.* 1983). Females have two uteri in which multiple eggs are fertilised, often by multiple males (Chapman *et al.* 2013). The largest embryo in each uterus consumes all other embryos (Grant *et al.* 1983), and the mother continues to ovulate in order to nourish the surviving embryo (Grant *et al.* 1983). Each mature female produces two pups every second year, very rarely up to four pups, resulting in one of the slowest reproductive rates of all shark species (Grant *et al.* 1983). This low fecundity coupled with relatively slow maturation rates of 6–7 years for males and 9–10 years for females (Goldman *et al.* 2006) has hampered the recovery of the eastern Australian population of grey nurse sharks (NSW Fisheries 2002) and emphasises the requirement for successful breeding in captivity for display purposes. To facilitate this we aim to provide the first genetic characterisation of the mating system in a captive situation. Here we carry out paternity analysis to ascertain whether multiple paternity occurs in captivity and assess whether any relationship exists between the paternity of pups and stillborn young.

Materials and methods

Sample collection

All adults (3 males and 2 females) were collected between 1985 and 1996 from wild stocks in Eastern Australia, and housed in the same aquarium at Manly SEA LIFE Sanctuary (MSLS). Tissue samples were obtained from the three living candidate sires, one living dam, one deceased dam, one living male that had been born in captivity and eight deceased captive-born pups that were all held at MSLS between 2000 and 2013. The nine samples from captive-born animals were from six separate pregnancies to the two known dams. Samples from live individuals were taken from the dorsal or pectoral fins using a sterile biopsy punch during routine interactions at MSLS. All tissue samples were stored in 70% ethanol at −20°C.

DNA extraction and PCR

DNA was extracted from each individual tissue sample using a Bioline DNA extraction kit following manufacturer's instructions. DNA quality was checked via gel electrophoresis, and DNA concentration was quantified using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc.). Seven microsatellite loci were amplified by PCR: Cta45-69, Cta56-72 (Feldheim *et al.* 2007), Iox12 (Schrey and Heist 2002), Ct243, Ct417, Ct432 and Ct655 (Chapman *et al.* 2013). The primers we used for PCR amplification were either M13-labelled primers (Schuelke 2000) or the forward primer was directly labelled using the fluorescent dye 6-FAM™ (Macrogen Inc.). PCR conditions varied in accordance with the primers used (Table 1). For directly fluorescently labelled primers we used 50 ng of template DNA, 200 μM dNTPs, 0.5 μM of each primer, 2.5–3 mM $MgCl_2$, 1 unit of Promega Go Taq DNA Polymerase and PCR buffer (following manufacturer's instructions) in a total volume of 20 μL. For M13 primers we used 50 ng of template DNA, 200 μM dNTPs, 0.1 μM forward primer with attached M13 tail, 0.5 μM reverse primer, 0.5 μM M13 primer labelled with 6-FAM™, 2.5–3 mM $MgCl_2$, 1 unit Promega Go Taq DNA Polymerase and PCR buffer (following manufacturer's instructions), in a total volume of 20 μL.

Thermocycling conditions were as follows: initial denaturation for 3 min at 94°C, then 35 cycles of 30 s denaturation at 94°C, 30 s annealing (at the specific primer annealing temperature) and 30 s at 72°C followed by a final 10-min extension at 72°C. PCR products were sent to Australian Genome Research Facility for fragment analysis using an ABI 3730XL analyser. Allele sizes were determined using an internal size standard LIZ (GeneScan™ 500 LIZ) and the Peak Scanner™ 1.0 software (both from Applied Biosystems).

Analysis

To evaluate the suitability of the loci selected to estimate paternity via genetic exclusion, we calculated the non-exclusion probability for the second parent given the observed allele frequencies and Probability of Identity (PID) using the software Cervus 3.0 (Kalinowski *et al.* 2007). PID is the probability of two unrelated individuals sharing the same genotype (Waits *et al.* 2001). For each litter we confirmed mother–offspring relationships by visually inspecting genotypes to ensure that for each locus a pup shared at least one identical allele with its mother. Paternity was then assigned both by exclusion and using the likelihood approach adopted by the software Cervus 3.0. Given the size of the dataset, genetic exclusion could be determined by visually identifying mismatches. CERVUS assigns paternity while taking into account user-defined scoring errors and the proportion of candidate parents sampled (100%). The program calculates a logarithm-of-the-odds (LOD) score for all candidate parents and carries out simulations to estimate the critical difference in LOD score between the most likely and second most likely candidate parent, at a confidence level that can be set by the user (in this study 99% and 99.9%).

Table 1. Summary statistics for the loci used for paternity analysis
N, sample size; K, number of alleles; PID, probability of identity. Forward primers were either fluorescently labelled with 6 FAM™ (Labelled) or had a M13 tail at their 3′ end (M13)

Locus	Repeat	*N*	K	Size range	PID	Labelled/M13
Cta45-69	$(TG)_{20}$	14	5	149–173	0.16	M13
Cta56-72	$(AG)_{12}$	14	2	184–190	0.60	M13
Iox12	$(GT)_8$ GAGT$(GA)_4$	14	4	325–331	0.18	Labelled
Ct243	$(TAGA)_{16}$	11	5	195–237	0.14	Labelled
Ct415	$(TAGA)_{27}..(AGAC)_8$	9	8	341–381	0.04	Labelled
Ct432	$(TAGA)_{26}$	14	7	260–324	0.09	Labelled
Ct655	$(TATC)_{15}$	14	7	147–207	0.07	Labelled

Results

Genotypes for five of the seven selected loci were obtained for all individuals, while for the remaining two loci there were some missing data (Tables 1, 2). The number of alleles per locus ranged between 2 and 8, and PID ranged from 0.004 to 0.6 (Table 1). The combined non-exclusion probability for the second parent was 8×10^{-3}, suggesting that even with the limited number of loci, we can be confident of genetic exclusion. Genotypes for each individual are given in Table 2.

The paternity of all nine pups was determined. The exclusion method and the maximum-likelihood approach yielded identical results. The maximum-likelihood approach determined paternity of each pup with confidence levels higher than 99% (Table 3). We identified three separate cases of multiple paternity, whereby siblings from the same pregnancies were sired by different fathers (Maia–Apollo, GNS4–GNS5 and Atlas–Murdoch) (Table 3).

Discussion

This is the first time that multiple paternity has been identified in captive grey nurse sharks. Multiple paternity has been described in several elasmobranch species (Saville *et al.* 2002; Daly-Engel *et al.* 2007; Fitzpatrick *et al.* 2012) and increases genetic diversity among offspring (Fitzpatrick *et al.* 2012). Multiple paternity in grey nurse sharks, while long suspected (Grant *et al.* 1983), has only recently been demonstrated in wild populations (Chapman *et al.* 2013), where 60% of gravid females were fertilised by at least two males. Captive observations of matings at MSLS have shown that an individual female may mate multiple times with the same or different males over the course of the mating season (Gordon 1993; authors' obs.).

Table 2. Microsatellite genotypes for each individual

Individual	Sex/pup	Iox12		Cta45-69		Cta56-72		Ct243		Ct415		Ct655		Ct432	
Artemis	F	327	331	163	171	184	184	0	0	0	0	167	179	264	302
Pallas	F	329	331	167	167	184	190	195	203	341	381	147	179	268	314
Patches	M	331	331	167	167	184	184	203	203	365	373	167	187	284	324
Trio	M	329	331	167	167	184	184	203	207	349	357	203	207	268	284
Huey	M	325	329	173	149	184	190	203	237	0	0	147	167	260	268
Maia	P	327	331	163	167	184	184	0	0	0	0	167	187	264	284
Apollo	P	329	331	163	167	184	184	203	203	357	369	167	207	284	302
Murdoch	P	325	329	149	167	184	190	195	237	0	0	147	167	268	268
Atlas	P	329	331	167	167	184	190	203	207	349	381	179	207	268	268
GNS4	P	331	331	163	167	184	184	203	233	369	373	179	187	264	284
GNS5	P	325	331	163	173	184	184	0	0	0	0	147	179	268	302
Phoebos sib	P	327	331	167	171	184	184	203	203	353	373	167	167	284	302
GNS77	P	331	331	167	171	184	184	207	233	349	353	167	207	268	302
GNS86	P	327	331	167	171	184	184	207	233	349	369	167	207	264	268

Table 3. Survival and paternity of grey nurse shark pups at Manly SEA LIFE Sanctuary

Pups from the same litter are identified by the same superscript (1, 2 or 3). The combined non-exclusion probability for the second parent (exclusion method) was 8×10^{-3}. Confidence refers to confidence in paternity assignment when the mother is known using the likelihood-simulation approach implemented in Cervus 3.0

Pup name	Sex	Date of birth	Date of death	Survival	Mother	Candidate Father	Confidence
Maia[1]	Female	23 Dec 2001	25 Jan 2012	10 years[A]	Artemis	Patches	>99.9%
Apollo[1]	Male	23 Dec 2001	29 Nov 2004	3 years[A]	Artemis	Trio	>99.9%
Atlas[2]	Male	29 Jun 2006 (premature)	06 Jul 2006	7 days	Pallas	Trio	>99.9%
Murdoch[2]	Male	06 Feb 2007	Alive	7 years	Pallas	Huey	>99.9%
GNS86	Unknown	04 Oct 2011	04 Oct 2011	Stillborn	Artemis	Trio	>99%
GNS77	Unknown	19 Sep 2009	19 Sep 2009	Stillborn	Artemis	Trio	>99%
GNS4[3]	Unknown	22 Feb 2000	22 Feb 2000	Stillborn	Artemis	Patches	>99.9%
GNS5[3]	Unknown	22 Feb 2000	22 Feb 2000	Stillborn	Artemis	Huey	>99.9%
Phebos sib	Unknown	03 Nov 2003	03 Nov 2003	Stillborn	Artemis	Patches	>99.9%

[A]Died from complications associated with scoliosis.

All available males sired at least one offspring that survived longer than one year; however, each of the males also sired stillborn young. These results suggest that reduced survival times of captive-born grey nurse sharks may not be due to genetic factors and that the captive environment may be responsible. Nonetheless, it has also been demonstrated that grey nurse sharks from eastern Australia have especially low genetic variation (Stow *et al.* 2006; Ahonen *et al.* 2009), and low genetic variation might reflect inbreeding (Frankham *et al.* 2002). Inbreeding depression may lead to poor fertility and low survival rates, therefore a worrying explanation might be that the poor survival being observed in captivity reflects survival rates in the wild eastern Australian population.

Captive-born eastern Australian grey nurse sharks frequently succumb to scoliosis, with two out of the three pups at MSLS that survived longer than a year eventually dying from complications associated with the condition. Recent work on scoliotic animals in captivity suggests that external influences such as manual handling of animals, swim patterns and vitamin intake, rather than genetic factors, are responsible for scoliosis in captive grey nurse sharks. Wild-caught individuals brought into captivity at a smaller size while the cartilage skeleton is still developing show a greater rate of scoliosis (Tate *et al.* 2013). The same factors may be responsible for the scoliosis rate in captive-born animals because young sharks are often moved several times in the first few years of life, a process that often involves an element of manual handling that may initiate scoliosis (Anderson *et al.* 2012). The factors responsible for the high frequency of stillborn young observed in captivity are difficult to guess at. Analysis of the genetic characteristics of the stillborn young provide no indication of any association between genetic variation and stillborn young. For example, mean multilocus heterozygosity of the stillborn young (0.73) was only marginally lower than that of the rest of the sampled individuals (0.78). Nonetheless, measures of genetic variation at neutral markers can be a poor proxy for genetic variation at functionally important parts of the genome (Hoffman *et al.* 2006) and, as such, we cannot conclude that genetic factors are not responsible.

It is conceivable that the environmental needs for successful reproduction of grey nurse sharks are not being replicated in captivity. Grey nurse sharks on the east coast of Australia

partake in long seasonal migrations and are subject to various changes in water temperature as a result (Bansemer and Bennett 2009). There is evidence that female grey nurse sharks segregate from males while gravid and move towards the northern (warmer) end of their range (Bansemer and Bennett 2009). Aquaria holding grey nurse sharks do not have the capacity to mimic the temperature changes that would be experienced by female sharks during these migrations nor do many have the ability to segregate gravid females from males. These unnatural conditions may place undue stress on gravid animals that could result in the high rate of stillborn pups in captivity. Other potential stressors for gravid sharks in captivity could be stray voltage, unnatural photoperiods, noise and the close proximity of possible predators (Smith *et al.* 2004).

The display of sharks by aquaria may play a role in conserving this taxonomic group through public education. Awareness of the critically endangered status of grey nurse sharks has been heightened by their display in aquaria, but to perpetuate this message the survival of pups born into captivity is critical. Determining the environmental and, potentially, genetic factors associated with pup survival is fundamental to achieving this goal.

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Appendix C

Molecular phylogenetics and morphology of *Gambierdiscus yasumotoi* from tropical eastern Australia.

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Molecular phylogenetics and morphology of *Gambierdiscus yasumotoi* from tropical eastern Australia

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ABSTRACT

Here the occurrence of the species *Gambierdiscus yasumotoi* is reported for the first time along a latitudinal gradient spanning more than 1550 km of the Australian Great Barrier Reef (GBR), a region with endemic ciguatera fish poisoning. *G. yasumotoi* was found at three tropical and sub tropical coral reef sites, Raine Island (northern GBR), Nelly Bay (central GBR) and Heron Island (southern GBR), indicating a wide-ranging distribution in tropical and subtropical eastern Australia. Specimens from Australia broadly fitted the original description of *G. yasumotoi*, but differed in some aspects, showing some similarities to *Gambierdiscus ruetzleri*. Molecular phylogenetic analyses based on nuclear rRNA gene sequences and morphological analyses showed specimens to be intermediate between the two species *G. yasumotoi* and *G. ruetzleri*. The full intraspecific diversity of these two species appears to be incompletely known, and these two species may represent a species complex. Strains of this species from other sites around the world have been found to produce an as yet unknown toxin, possibly an analogue of maitotoxin.

1. Introduction

The genus *Gambierdiscus* Adachi and Fukuyo (Gonyaulacales, Dinoflagellata, Alveolata) is one of more than 550 known genera of dinoflagellates. Species of this genus are the main causative agents of Ciguatera Fish Poisoning (CFP), caused by the consumption of fish contaminated with toxins produced by these dinoflagellates, which are biomagnified along the food web. CFP is the most common non-bacterial seafood poisoning disease in the world (Fleming et al., 1998) and mainly occurs in tropical countries. Cases have been reported in coral reef areas in Australia (Gillespie et al., 1985; Lewis, 2006), the Pacific and Indian oceans as well as the Caribbean (Tosteson, 2004). In Australia, there were more than 1400 documented cases between 1965 and 2010, including two fatalities (Gillespie et al., 1985; Hamilton et al., 2010).

Gambierdiscus are epibenthic on seagrass, macroalgae, sand and coral rubble, however, they can also occur in the plankton (Nakahara et al., 1996; Parsons et al., 2011). Species of this genus have the plate formula: Po, 3′, 7″, 6c,?s, 5‴, 1p, 2⁗ (Chinain et al., 1999; Faust, 1995; Fraga et al., 2011; Holmes, 1998; Litaker et al., 2009) and are distinguished from one another based on characters such as surface patterns, cell flattening (apical or dorso-ventral), size and shapes of the plates and of the cells.

The type species, *Gambierdiscus toxicus*, was first identified as recently as 1979 (Adachi and Fukuyo, 1979), and until 15 years ago, only three species were known in the genus. It was recognised that the type description of *G. toxicus* probably incorporated several species, and *G. toxicus* was redescribed more narrowly, based on a lectotype (Litaker et al., 2009). Currently, 12 species of *Gambierdiscus* are recognised: *G. australes*, *G. belizeanus*, *G. caribaeus*, *G. carolinianus*, *G. carpenteri*, *G. pacificus*, *G. polynesiensis*, *G. ruetzleri*, *G. toxicus*, *G scabrosus*, *G. yasumotoi*, and *G. excentricus* (Chinain et al., 1999; Faust, 1995; Fraga et al., 2011; Holmes, 1998; Litaker et al., 2009; Nishimura et al., 2014). At least five additional unnamed clades that are clearly genetically distinguishable have been found, indicating that further species may be distinguished in future (Litaker et al., 2009; Richlen et al., 2008; Xu et al., 2014).

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Published reports initially indicated the presence of only a single species, *Gambierdiscus toxicus* in Australia, from tropical regions of Queensland (Holmes et al., 1990, 1991, 1994), and more recently, from New South Wales (Murray, 2010). Cultures of this taxon from sites in Queensland were made in the 1990s (Babinchak et al., 1994; Holmes et al., 1990, 1991, 1994) and subsequently lost. Since these initial reports, our understanding of the morphological and genetic diversity present in the genus *Gambierdiscus* has increased significantly (Chinain et al., 1999; Fraga et al., 2011; Litaker et al., 2009; Nishimura et al., 2014; Richlen et al., 2008; Xu et al., 2014), and it is therefore necessary to revisit initial sites in order to assess the presence of these species. Recently, a second species of *Gambierdiscus*, *G. carpenteri*, has been reported from several sites in Australia for the first time (Kohli et al., 2014a,b).

The species *Gambierdiscus yasumotoi* was originally described from Singapore waters (Holmes, 1998), and was found to produce an uncharacterised toxin that was lethal to mice, producing symptoms similar to those of maitotoxin (Holmes, 1998). This species has since been reported from additional sites around the world, including from the Pacific and Caribbean waters. Strains isolated from New Zealand were found not to produce known analogues of ciguatoxins (CTXs) or maitotoxin (MTX) 1, but were likely to produce an as yet uncharacterised analogue of maitotoxin (Rhodes et al., 2014a).

In this study the presence of *Gambierdiscus yasumotoi* is shown for the first time along a latitudinal gradient of more than 1550 km in waters of the northern, central and southern Great Barrier Reef (GBR), Queensland, Australia. *G. yasumotoi* is described from Australia based on scanning electron microscopy of field material, as well as scanning electron microscopy and phylogenetic analysis of a culture of *G. yasumotoi* isolated from the central GBR region. The occurrence of two other species of *Gamberdiscus* is also documented: *Gamberdiscus carpenteri* and *Gambierdiscus* cf *belizeanus*.

2. Materials and methods

2.1. Sample collection

Between 3rd and 8th December 2003, eight samples were taken at low tide at sites on Raine Island (11° 45′.501 S, 144° 02′.309 E), a remote uninhabited tropical coral cay in the northern GBR, in the vicinity of the tower end of the beach (Fig. 1), as part of annual sampling coordinated by the Raine Island Corporation. On 15th June 2003, four samples were collected from Heron Island (23° 25′ S, 151° 55′ E), a tropical coral cay in the southern GBR (Fig. 1). Samples were taken from encrusting macroalgae in shallow (less than 30 cm deep) tidal pools at low tide.

Macroalgal samples were obtained from central GBR waters on low tide in the area between Townsville (Pallarenda at the mouth of Three Mile Creek, 19° 12′ 34″ S, 146° 46′ 36″ E) and Magnetic Island (Nelly Bay, 19° 10′ S, 146° 50′ E; Great Barrier Reef Marine Park Authority Permit G06/20234.1) in August 2004 and March 2008 (Fig. 1). Macroalgal material was washed in filtered (0.45 μm) seawater to flush epiphytic dinoflagellates off their surface and the wash water was sequentially concentrated by filtration through 53 and 20 μm nylon mesh filters.

2.2. Culture establishment

Dinoflagellates were isolated from Pallarenda and Nelly Bay macroalgal wash waters at the North Queensland Algal Identification/Culturing Facility (NQAIF) at James Cook University, Townsville, Australia using the microcapillary-capturing-technique (Andersen and Kawachi, 2005) at 10× magnification on an inverted light microscope (Olympus CKK 41, Olympus Australia Ltd., Mt Waverley, VIC 3149). Cells were dispensed in autoclaved and filtered (0.45 μm) seawater. Cells were allowed to swim for 10 min and were then recaptured. This procedure was repeated 10 times to ensure that nano- and pico-plankton were no longer in the vicinity of the cell to be isolated. Cultures of NQAIF116 (Pallarenda) and NQAIF210 (Nelly Bay) were established in L1 medium (Guillard and Hargraves, 1993) prepared in natural seawater and maintained at 24 °C, a 12:12 h photoperiod and light intensity of 45 μmol photons m^{-2} s^{-1} in a Contherm cross-flow phytoplankton growth chamber. Cultures were subcultured in L1 medium every 4 weeks.

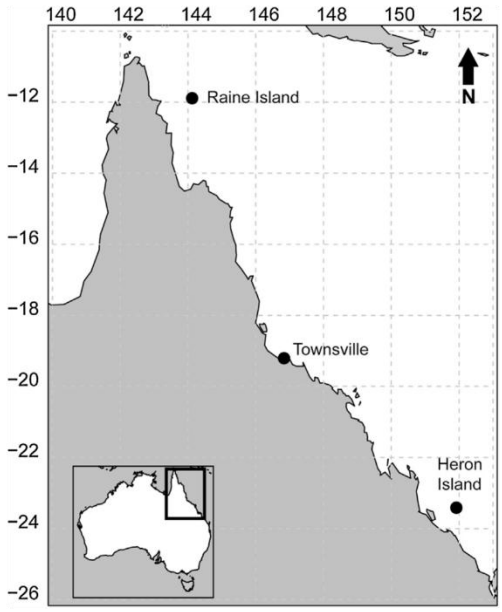

Fig. 1. Map of the sampling sites in Great Barrier Reef (GBR) region, east coast, Queensland, Australia. Inset box, showing the location of the sampling area within Australia. The three regions in which sampling took place: Raine Island in the far northern GBR; Pallarenda and Nelly Bay, Townsville, in the central GBR; Heron Island in the southern GBR.

2.3. Microscopy

Samples were immediately examined using a dissecting light microscope. Individual cells were isolated onto 5 μm millipore filtersor 1 cm round glass coverslips that had been treated with poly-L-lysine (Marchant and Thomas, 1983), to make preparations to observe in the scanning electron microscope. Cells were dehydrated in a series of increasing ethanol concentrations (15%, 30%, 50%, 70%, 90%, and 100%), followed by 50% and 100% hexamethyldisilizane (HMDS). The filters were air dried before being sputter coated with gold or gold/palladium. They were observed using a Philips 505 scanning electron microscope (Philips, Eindhoven, the Netherlands) at 10–15 kV and a JEOL JSM-5410LV scanning electron microscope (JEOL, Sydney, Australia).

2.4. DNA extraction and sequencing

DNA was extracted from cultures NQAIF210 and NQAIF116 using a modified Chelex® protocol (Walsh et al., 1991) as outlined in Momigliano et al. (2013). The near-complete nuclear 18S rRNA gene, and the D1–D3 and D8–D10 regions of the 28S rRNA gene were amplified and sequenced using the primers listed in Table S1.

PCR reactions were set up as follows: 1 μl of template DNA, 800 μM each DNTPs, 10 pmol of each primer, 1X Green GoTaq® Flexi Buffer and 1 unit GoTaq® Flexi Polymerase (Promega, Inc.). PCR reactions underwent an initial denaturation of 3 min at 94 °C, followed by 35 cycles of 30 s denaturation (94 °C), 30 s annealing (see Table S1 for details), and 1 min extension (72 °C), and a final extension of 5 min at 72 °C. PCR products were cleaned by isopropanol precipitation and sequenced using a commercial service (Macrogen, Inc.). All sequences were deposited in GenBank (accession numbers: KM272970–272974).

2.5. Sequence alignment and phylogenetic analysis

Overlapping fragments of the D1–D3 and D8–D10 regions of the 28S and the near-complete 18S genes were assembled using the software Chromas Pro (Technelysium Pty Ltd.). The sequences obtained from culture NQAIF210 and NQAIF116 were aligned with available sequences of *Gambierdiscus* spp. (for a list of the strains used see Table S2) using Clustal W (Thompson et al., 1994) and the alignments were visually refined using BioEdit (Hall, 1999). Phylogenetic analysis was performed on the datasets including the D8–D10 region of the 28S rRNA and the near complete 18S rRNA gene. Substitution models were selected for each dataset in jModelTest2 (Darriba et al., 2012), using Bayesian Information Criterion as a measure of the relative quality of the models. Phylogenetic reconstructions were carried out using both a Maximum Likelihood (ML) and Bayesian Inference (BI) approach.

ML trees were produced in PhyML 3.1 (Guindon et al., 2010), using a TrN+G substitution model with 4 gamma categories (alpha = 0.46) and a TIM2+G model with 4 gamma categories (alpha = 0.46) for the D8–D10 region of the 28S gene and the 18S dataset respectively, tree improvement by using the best of nearest neighbour interchanges (NNI) and subtree pruning and regrafting (NPR), starting with five random trees. Bayesian analysis was performed in MrBayes 3.2.2 (Ronquist and Huelsenbeck, 2003). The substitution model used for the D8–D10 region of the 28S gene was the same as in the ML analysis. However as MrBayes does not implement the TIM2 substitution model, we used the next-best available model, the HKY+G model. The number of Markov Chain Monte Carlo generations was set to 2,000,000, but set to automatically stop when the standard deviation of split frequencies fell below 0.01. Trees were sampled every 100 generations, and convergence of the two independent runs was checked by analysing the stability of each parameter estimate in the software Tracer v1.6.

In addition, for each locus p-distances for all available strains belonging to the *Gambierdiscus yasumotoi*/*ruetzleri* clade were estimated in the R statistical environment using the ape package (Paradis et al., 2004). The distance matrices were further analysed using Principal Coordinates Analysis (PCoA) implemented in the package ade4 (Dray and Dufour, 2007).

3. Results

3.1. Phylogenetic analyses

The final alignments of the D8–D10 region of the 28S rRNA gene based on 45 sequences included 929 unambiguously aligned nucleotides and 348 variable sites, of which 286 were parsimony informative. The 18S alignment included 36 sequences, 1746 unambiguously aligned nucleotides and 696 variable sites of which 603 were parsimony informative. Both phylogenies show strong support for all terminal branches (Figs. 2 and 3), and largely support previously published phylogenies of the genus *Gambierdiscus*. Small differences in the trees presented here relative to previously published phylogenies are due to the fact that

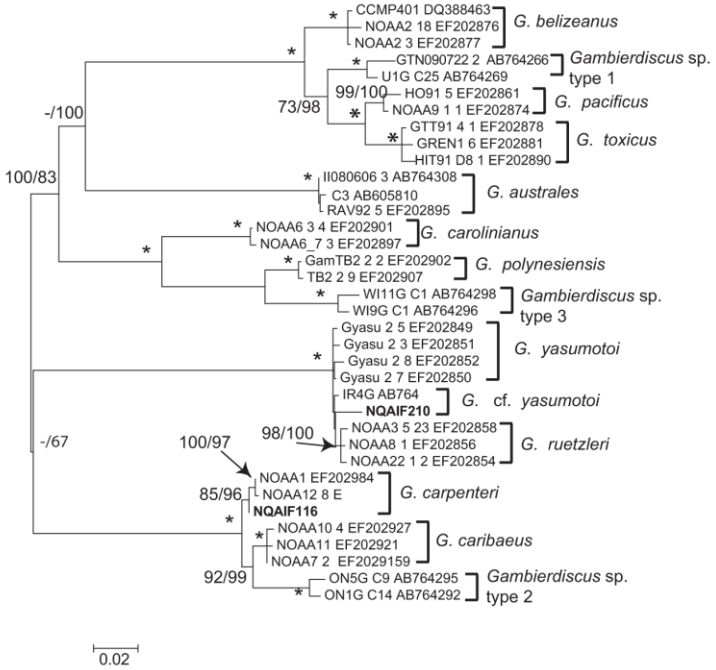

Fig. 2. Maximum likelihood phylogeny of the genus *Gambierdiscus* based on near-complete 18S gene sequences. Branch support values (ML/BI) represent bootstrap support based on 1000 pseudo-replicate datasets and Bayesian clade credibility values respectively. Branches with 100/100 support are indicated by an asterisk. Scale represents number of changes per nucleotide. Strains isolated in this study are shown in bold.

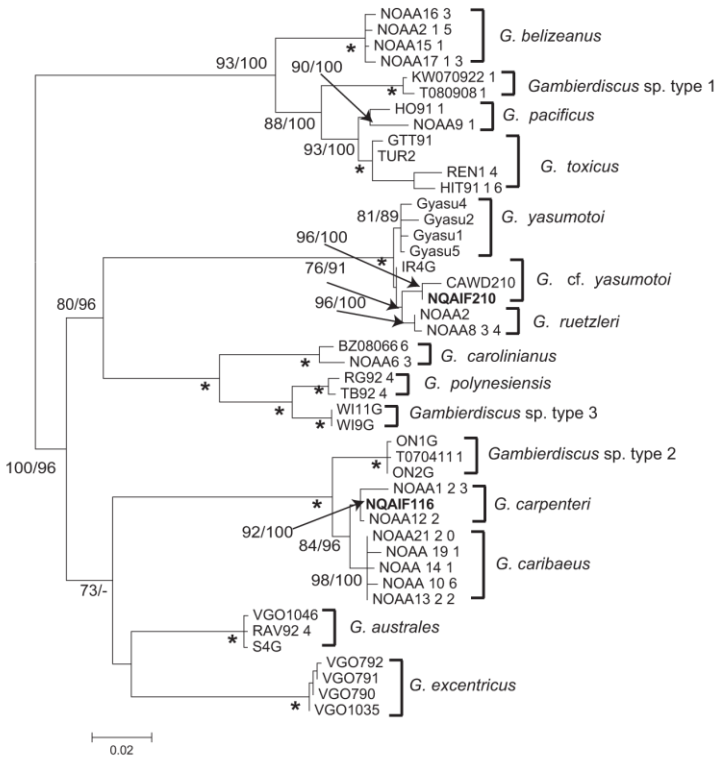

Fig. 3. Maximum likelihood phylogeny of the genus *Gambierdiscus* based on the D8–D10 region of the 28S rRNA gene. Branch support values (ML/BI) represent bootstrap support based on 1000 pseudo-replicate datasets and Bayesian clade credibility values respectively. Branches with 100/100 support are indicated by an asterisk. Scale represents number of changes per nucleotide. Strains isolated in this study are shown in bold.

out-group taxa were not used in phylogenetic reconstruction and disappear if the trees are rooted with the most basal *Gambierdiscus* clade (*G. yasumotoi* clade).

NQAIF210 grouped with high support within the *Gambierdiscus ruetzleri*/*Gambierdiscus yasumotoi* clade – Clade I in Nishimura et al. (2013) in both phylogenies (Figs. 2 and 3). NQAIF210 could not be unambiguously identified as either *G. yasumotoi* nor *G. ruetzleri* based on 18S sequences. In the phylogeny reconstructed using the D8–D10 region of the 28S gene, NQAIF210 grouped with high support with the New Zealand strain CAWD210, and both NQAIF210 and CAWD210 grouped with *G. ruetzleri* with moderate bootstrap support (Fig. 3). The culture NQAIF116 consistently grouped with high support within the *Gambierdiscus carpenteri* clade (Figs. 2 and 3). Representations of p-distance matrices in reduced space (Fig. 4) showed that NQAIF210 and CAWD210 have identical sequences for the D1–D3 region, and have small p-distances for the D8–D10 region but the analysis also failed to show any clear grouping of NQAIF210 with other previously identified strains in this clade, from which NQAIF210 is roughly equidistant (Fig. 4). However, it must be remembered that all sequences of *G. yasumotoi* utilised in the phylogenies and p-distance analyses are clones of the same strain (Gyasu), and therefore they cannot be used as a proxy of intra-specific variation. Furthermore, p-distances within this clade, and between putative species within it, are very small when compared to between-species distances elsewhere in the genus. p-Distances between *G. yasumotoi* and *G. ruetzleri* are as little as 0.01 (D1–D3 and D8–D10 regions of the 28S rRNA gene) and 0.004 (near complete 18S gene).

Interestingly, p-distances among clones of the same strain of *Gambierdiscus yasumotoi* are comparable to those observed between clones of *G. yasumotoi* and strains of *Gambierdiscus ruetzleri* (Fig. 4, particularly 4A, 4C and 4E).

3.2. Morphological analyses

The morphology of *Gambierdiscus yasumotoi* from field samples and from the cultured strain is shown in Figs. 5–7. Cells from field samples were laterally compressed in ventral view, and oval to round in lateral view, with a depth of 57 μm (range 54–59 μm), width 48 μm (range 46–50 μm) and length 56 μm (range 53–59 μm) (Figs. 6 and 7). The depth to width ratio was 1.18, while the length to width ratio was 1.16. Cultured cells were slightly smaller, more globular and less obviously laterally compressed, with a depth of 49 μm (range 44–54 μm), width 45 μm (range 40–49 μm) and length 51 μm (range 49–54 μm) (Fig. 5). The depth to width ratio was 1.08, while the length to width ratio was 1.13 (Table 1).

The thecal plates were smooth with many small round pores, approximately 0.3 μm diameter (Figs. 5b and 7a,b). The epicone had the plate formula of *Gambierdiscus*, with three apical plates and seven precingular plates (Figs. 5a and 6b,g,h,i). The apical pore plate was tear drop shaped, while the pore was long and fish hook shaped at the cell apex, approximately 8–9 μm long, with 33–45 pores surrounding (Figs. 5b, 6g, and 7a). The hypocone consisted of five postcingular plates, two antapical plates and one posterior intercalary plate (Figs. 5f, g, i and 6f).

The cingulum was deeply excavated and descending, displaced about 1–2 cingular widths (Figs. 5c, 6a–c and 7b–d).

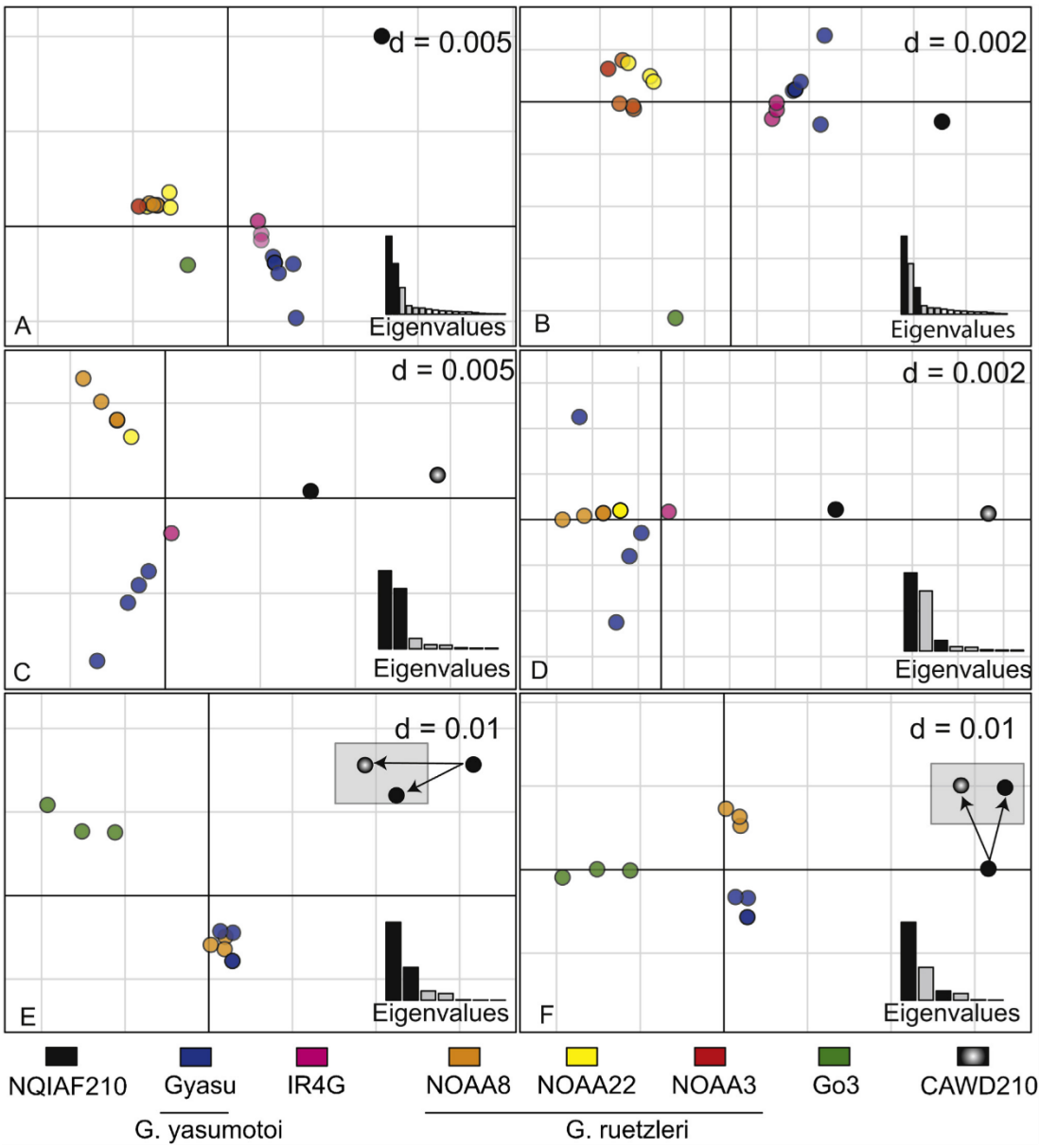

Fig. 4. Principal coordinate analyses (PCoA) based on p-distance matrices among sequences within the *Gambierdiscus yasumotoi*/*Gambierdiscus ruetzleri* clade. PCoA based on near complete 18S gene sequences (A, B) and on the D8–D10 (C, D) and D1–D3 (E, F) regions of the 28S rRNA gene. Clones from the same strain are represented by partially transparent symbols with the same colour, allowing visualisation of density of points via colour saturation. Eigenvalues of the plotted axes are shown in black.

Prominent cingular lists were present in all cells (Figs. 5i, 6a,c and 7b–d). In cultured cells, the cingulum was positioned slightly higher than the mid point of the cell in left lateral view, approximately in the mid point of the cell in dorsal view, and just below the mid point of the cell in right lateral view (Fig 5c–e). In cells from field samples, the cingulum was positioned higher, approximately 1/3 of the way down the cell from the apex in left lateral view, and slightly above the mid point of the cell in right lateral view (Fig 6a, c, f).

The sulcus has been reported to consist of seven plates, the Sdp, Sda, Ssp, Ssa, t, Sma and Smp (Litaker et al., 2009). Of these, the Ssp, Ssa, Sdp, t and Sda plates were clearly visible in this study (Fig. 7b–d). The tiny, oval shaped Sma plate, which is adjacent to the Sda plate, was also visible in several images (Fig. 7c and d). The Smp plate could not be observed in our images. Prominent sulcal lists were present in all cells from the field (Figs. 5i, 6a,c and 7b–d), and some cultured cells (Fig. 5c), but absent in other cultured cells (Fig. 5h). The sulcal lists consisted of a long list on the left side of

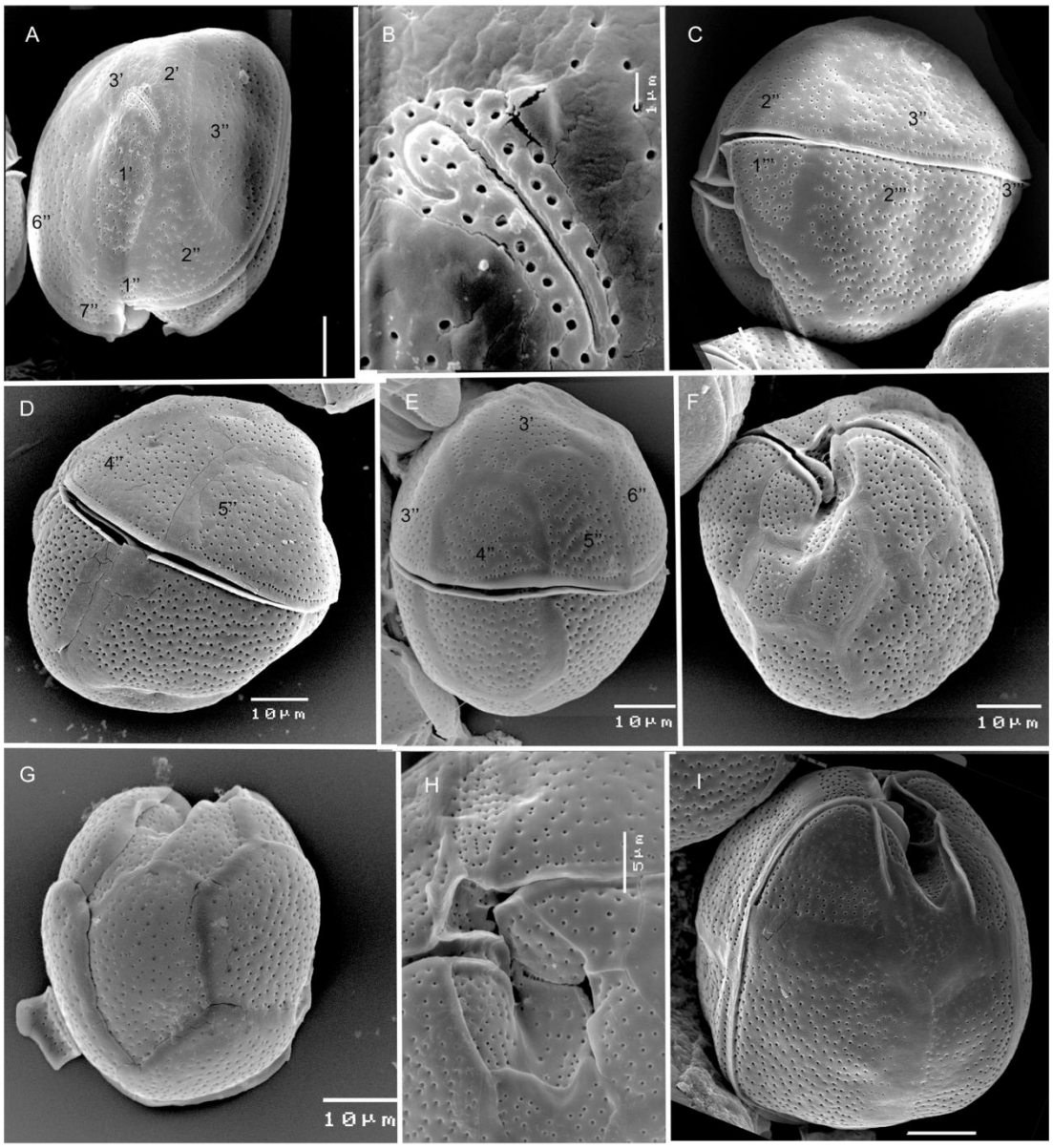

Fig. 5. Scanning electron microscope images of *Gambierdiscus yasumotoi* from the cultured strain, NQAIF210. Scale bars are shown. (A) Apical view, showing the epithecal plate pattern and shape of the epicone. (B) The apical pore plate, showing the comma and pores. (C) Cell in left lateral view. (D) Cell in dorsal view. (E) Cell in dorsal view. (F) Antapical-ventral view, showing antapical plates and part of the sulcus. (G) Antapical view, showing substantial plate overlaps. (H) Ventral view, showing the sulcus and a lack of sulcal lists. (I) Antapical/lateral view, showing the sulcal lists.

the Ssa and Ssp plates, and a shorter list on the right side of the Sdp plate (Fig. 7b–d). The 2′′′′ plate was fork shaped and invaded the sulcus (Figs. 5g–i and 6a).

Substantial plate overlaps, particularly in the hypothecal cells, were found in some cells in the cultured material (Fig. 5g).

Two other species of *Gambierdiscus* were found in field samples: *Gambierdiscus* cf *belizeanus*, and *Gambierdiscus carpenteri*. These species were only partially documented from the sites, and the morphological information about them is shown in the Supplementary figures.

4. Discussion

Gambierdiscus yasumotoi has previously been reported from subtropical Japan (Nishimura et al., 2013), the Persian Gulf and the Red Sea (Saburova et al., 2013), Singapore (Holmes, 1998), the Mexican Caribbean (Hernández-Becerril and Almazán Becerril, 2004), and even the northern coast of New Zealand (Rhodes et al., 2014a), but not from Australian waters. Here the occurrence of *G. yasumotoi* at three sites along a latitudinal gradient spanning more than 1550 km of the Australian GBR is reported, a region with endemic CFP.

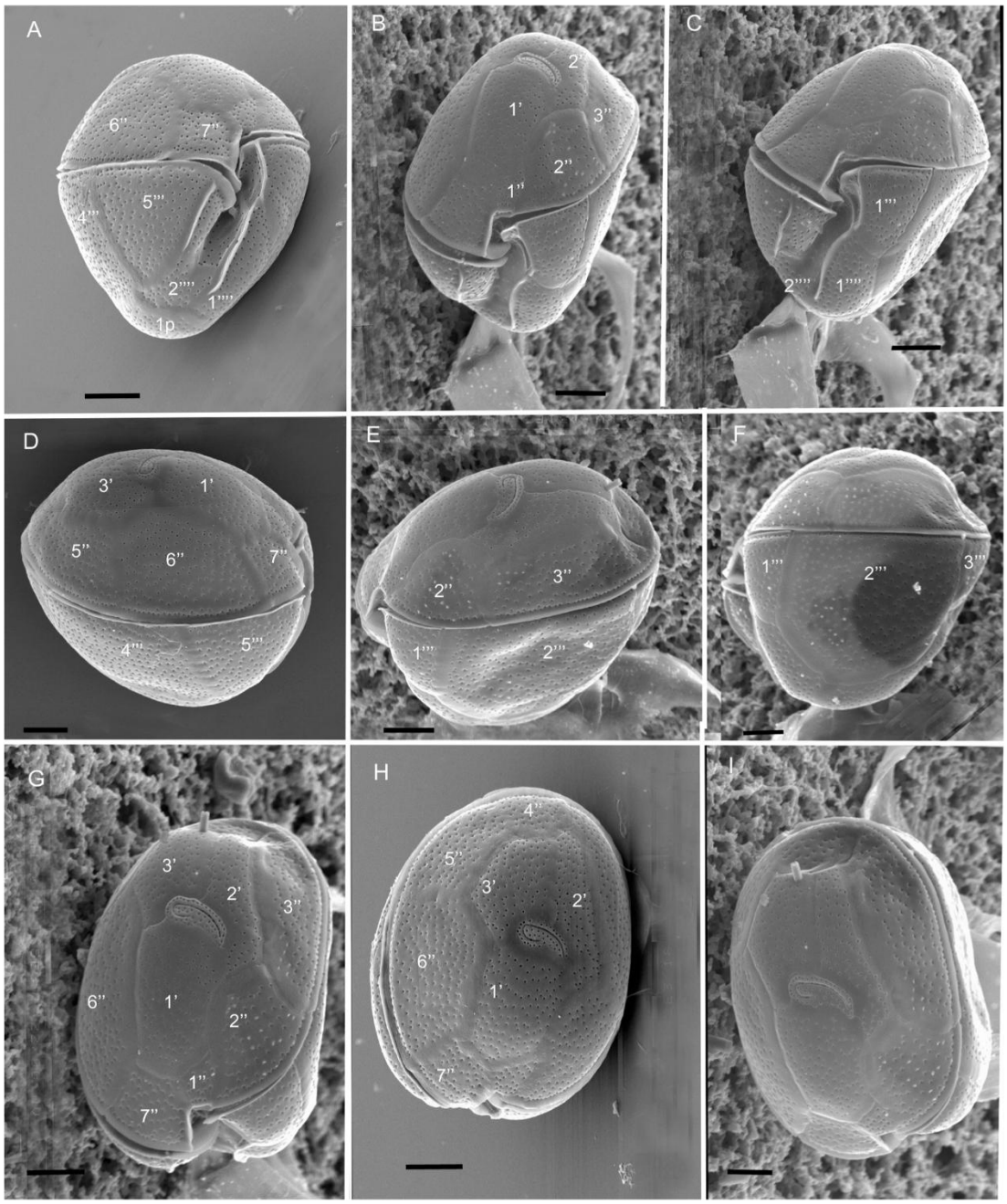

Fig. 6. Scanning electron microscope images of *Gambierdiscus yasumotoi* from the field material from Raine Island and Heron Island. Scale bars are 10 μm. (A) Cell in ventral/lateral view, showing sulcal lists. (B) Cell in apical/ventral view, showing part of the epicone. (C) Cell in ventral view. (D) Cell in right lateral view. (E) Cell in left lateral view. (F) Cell in left lateral view. (G) Cell in apical view, showing the epicone, including the apical pore plate and comma. (H) Cell in apical view, showing the epicone. (I) Cell in apical view, showing the epicone.

Records of *Gambierdiscus yasumotoi* in tropical and sub-tropical regions in the Indian, and Pacific Oceans, both in the northern and southern hemispheres, suggest that this species has a circum-global distribution in tropical and sub-tropical waters. Interesting is the occurrence of *G. yasumotoi* in northern New Zealand, reported by Rhodes et al. (2014a), as sea surface temperatures (SST) fall well below 15 °C in this region, the lowest thermal limit for any species of *Gambierdiscus* reported to date (Kibler et al., 2012). However, as (Rhodes et al., 2014a) noted, the specimens were collected in summer, when SST was 23 °C. It is possible that

S. Murray et al. / Harmful Algae 39 (2014) 242–252

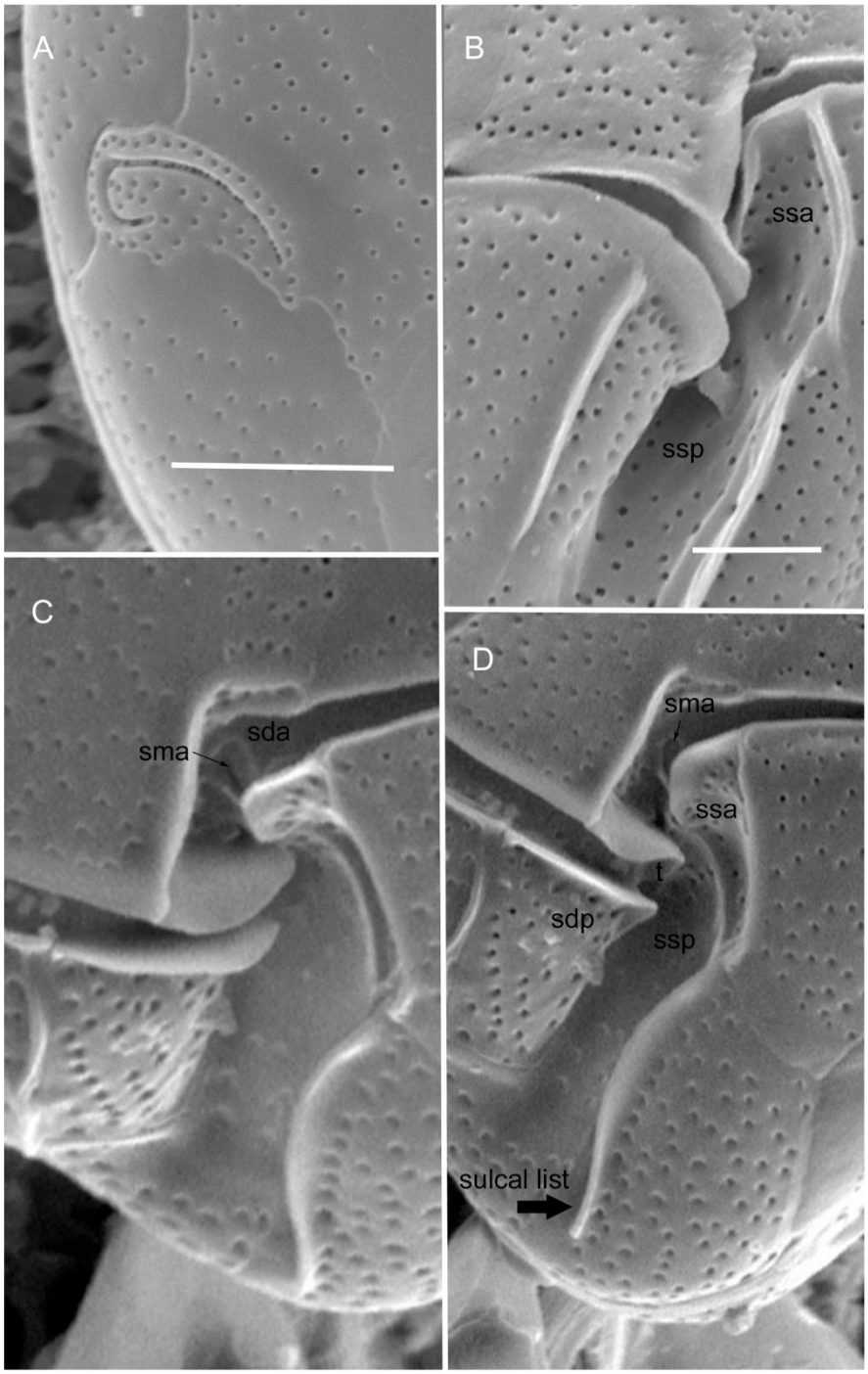

Fig. 7. Scanning electron microscope images of *Gambierdiscus yasumotoi* from the field material from Raine Island and Heron Island, showing the apical pore region, and sulcus. Scale bars represent 10 μm. (A) The apical pore plate. (B) The sulcal plates, showing the list, and ssa and ssp. (C) Showing the Sma and Sda plates. (D) Showing the Sma plate and sulcal list.

Table 1
Cell sizes and comparison of other morphological features of *Gambierdiscus yasumotoi*, from our data and previous reports.

	G. yasumotoi (this study field cells)	*G. yasumotoi* (this study culture)	*G. cf yasumotoi* (Rhodes et al., 2014a)	*G. yasumotoi* (Saburova et al., 2013)	*G. yasumotoi* (Saburova et al. 2013)	*G. yasumotoi* (Holmes, 1998), type description	*G. yasumotoi* (Litaker et al., 2009)	*G. ruetzleri* (Litaker et al., 2009)
Site	Heron Island, Raine Island	Townsville	Northern New Zealand	Kuwait	Jordan	Singapore		
Depth	Mean = 57.2 Range 54–59	Mean = 49 Range 44–54	Mean = 54.8 ± 5.7	Mean = 62.9 ± 4.4 (n = 30)	Mean= 62.6 ± 6.1, N = 7	Mean = 50 (n = 17) Range 43–61	Mean = 56.8 (n = 14)	Mean = 45.5 Range 41–55
Length	Mean = 56 Range 53–59	Mean = 51 range 49–54	Mean = 59.8 ± 7.5	Mean = 61.7 ± 6.2, N = 20	Mean = 62.9 ± 6.8, N = 7	Mean = 53 (n = 17) 45–63	Mean = 62.4 (n = 14) 45–59.5	Mean = 51.6
Width	Mean = 48 Range 46–50	Mean = 45 Range 40–49	Mean = 42.5 ± 4.1	Mean = 54.1 ± 5.1, N = 9	Mean = 54.7 ± 1.5, N = 3	Mean = 44 38–50	Mean = 51.7 (n = 14)	Mean = 37.5 30.9–42.2
Epitheca to hypotheca ratio	~0.5	~1.0		~1.0	0.5–0.6	~1.0		
D:W ratio	1.18	1.08	1.29	1.28	1.14	1.13	1.1	1.35
L:W ratio	1.16	1.13	1.41	1.14	1.15	1.20	1.2	1.45

the occurrence of *G. yasumotoi* in New Zealand is seasonal, with cells being transported from tropical and sub-tropical Australia during the warmer months via the East Australian Current (EAC) and the Tasman Front. The EAC is known to affect phytoplankton communities in the eastern Australian coast, transporting tropical species into sub-tropical and temperate areas (Armbrecht et al., 2014).

4.1. *Comparison between* Gambierdiscus yasumotoi *and* G. ruetzleri

Interestingly, most of the strains of *Gambierdiscus yasumotoi* which have been recently isolated have intermediate morphologies between *G. yasumotoi* and the more recently described *Gambierdiscus ruetzleri* (Table 1). According to the original description of *G. ruetzleri*, the features that distinguish this species from *G. yasumotoi* are: size (*G. ruetzleri* is significantly smaller and has a cell width less than 42 μm); a smaller, narrower and differently shaped 2′′′′ plate; a greater depth to width ratio, meaning they are proportionally narrower; a smaller length to width ratio, and a smaller epitheca to hyptheca ratio, as the cingulum is closer to the centre of the cell, rather than closer to the cell apex (Litaker et al., 2009).

There was some variability in these features in both individual cells from the culture, and between cells found in field samples as compared to cultured cells (Table 1). Both the field and cultured material had L:W and D:W ratios that were more similar to those in the original description of *Gambierdiscus yasumotoi* than that of *Gambierdiscus ruetzleri* (Table 1). However, the epitheca to hypotheca ratios appeared to differ between the field and cultured material. For the cultured strain, this ratio seems to be smaller than that reported for *G. yasumotoi*, but the difference is not nearly as pronounced as in the original description of *G. ruetzleri* (Litaker et al., 2009). In previous studies, the D:W and L:W ratios of material from New Zealand were most similar to those of *G. ruetzleri*. However in overall cell size, these specimens more closely resembled *G. yasumotoi* (Rhodes et al., 2014a, Table 1). In the material from Kuwait (Saburova et al., 2013), the D:W ratio and epitheca:hypotheca ratio appeared to be most similar to those of *G. ruetzleri*, while the cell width and overall cell size were most similar to those of *G. yasumotoi*.

In terms of the shape of the 2′′′′ plate, variability was found in the Australian samples. In some cells this appeared to have an almost horizontal posterior side (Figs. 5f and 6a), similar to the shape as described for *Gambierdiscus yasumotoi*. Other cells had a 2′′′′ plate with a 45° angle on the posterior side of this plate (Fig. 6c), a shape more similar to that of *Gambierdiscus ruetzleri* (Litaker et al., 2009).

Several specimens of *Gambierdiscus yasumotoi* with aberrant plate pattern have been observed among cultured cells (Saburova et al., 2013) and cells with substantial plate overlaps were found in this study (Fig. 5g), which would tend to distort the plate size or shape. For this reason, interpreting species-level differences based on minor differences in plate sizes or shapes could be misleading.

In our molecular analysis, NQAIF210 grouped with high support within the *Gambierdiscus yasumtoi/ruetzleri* clade, but it was not clearly grouped with either based on 18S gene sequences (Figs. 3 and 4). The phylogeny constructed using the D8–D10 region of the 28S rRNA gene suggests that NQAIF210 and the *G.* cf. *yasumotoi* isolated from New Zealand constitute a highly supported sub-clade, and the two strains had identical sequences for the D1–D3 region of the same gene (Fig. 3). The genetic data support the morphological observations, suggesting that these strains are indeed very similar. Interestingly, while both strains were morphologically closer to the original description of

G. yasumotoi, in the D8–D10 phylogeny they grouped with high support with *Gambierdiscus ruetzleri* (Fig. 3). There are now reports of a number of strains, including NQAIF210 (this study), IR4G and Go3 (Nishimura et al., 2013) and CAWD210 (Rhodes et al., 2014a), which always group within the *G. yasumotoi*/*G. ruetzleri* clade, but cannot be clearly identified based on molecular and morphological data as either species.

Our analyses of p-distances among strains (Fig. 4) belonging to the *Gambierdiscus yasumotoi* clade suggest that caution is needed in trying to delineate species boundaries within this group. Firstly, the erection of *Gambierdiscus ruetzleri* as a new species was based on a comparison with a single strain of *G. yasumotoi*, the strain used in the original description (Litaker et al., 2009). The estimates of within-species p-distances for *G. yasumotoi* are based on multiple sequences from this same strain, and therefore it could be considered a form of pseudo-replication to use such differences to recognise *G. yasumotoi* and *G. ruetzleri* as distinct, well supported entities in the phylogenetic analysis. Furthermore, our PCoA analyses of p-distance matrices for 18S and 28S rRNA gene sequences seem to suggest that p-distances among clones of *G. yasumotoi* are in some cases comparable to p-distances between strains of *G. yasumotoi* and *G. ruetzleri* (Fig. 4). In addition, the genetic differences between these two species are less than those determined for within-species divergence for other *Gambierdiscus* species (Nishimura et al., 2013; Rhodes et al., 2014a). Morphological and genetic distances similar to, or even larger than the ones reported between putative species within the *G. yasumoti/ruetzleri* clade have been described within species of other dinoflagellate genera, such as *Coolia* (Fraga et al., 2008; Momigliano et al., 2013; Rhodes et al., 2014b) and *Amphidinium carterae* (Murray et al., 2012). Within the *G. yasumotoi*/*ruetzleri* clade, the discovery of a number of strains which show intermediate morphologies and cannot be unambiguously identified as either *G. yasumotoi* or *G. ruetzleri* (Nishimura et al., 2013; Rhodes et al., 2014a) throws into doubt the status of these as distinct species. It is possible that *G. yasumotoi* and *G. ruetzleri* are conspecific, and the minor genetic and morphological differences reported are at the population level, or that they form an as yet unresolved species complex.

4.2. Toxicity

In the original description of *Gambierdiscus yasumotoi*, methanol extracts were found to be toxic to mice with symptoms similar to those of maitotoxins (MTX) (Holmes, 1998). The strain of *G. yasumotoi* from New Zealand was found by LC–MS analysis not to produce known analogues of MTX or of the monitored analogues of CTXs at detectable levels (Rhodes et al., 2014a). However, a putative MTX analogue which had the same mass as MTX-3 (Holmes and Lewis, 1994; Lewis et al., 1994) was found (Rhodes et al., 2014a). A study using the human erythrocyte lysis assay (HELA) showed significant toxicity in *Gambierdiscus ruetzleri* (Holland et al., 2013). Experiments which used specific inhibitors of the MTX pathway and purified MTX, *Gambierdiscus* whole cell extracts, and hydrophilic cell extracts containing MTX, were consistent with MTX as the primary hemolytic compound produced in those studies (Holland et al., 2013). MTX was generally considered not to be related to CFP symptoms, which are thought to be solely caused by ciguatoxins (CTX) (Lewis and Holmes, 1993). However, the propensity for MTX to accumulate in fish flesh following exposure to *Gambierdiscus* spp has been re-examined experimentally, and its role in fish toxicity remains incompletely known (Kohli et al., 2014c). A strain of *G. yasumotoi* was recently found to increase mortality and the expression of stress related genes in copepods (Lee et al., 2014).

Author contributions

Conceived and designed the experiments: PM, SM, KH, DB. Performed the experiments: PM, SM, KH. Analysed the data: PM, SM. Contributed reagents/materials/analytical tools: KH, SM, DB. Wrote the paper: PM, SM. Provided feedback on the manuscript: KH, DB.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.hal.2014.08.003.

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Appendix D

Symbiosis in a giant protist (*Marginopora vertebralis*, Soritinae): flexibility in symbiotic partnerships along a natural temperature gradient.

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Symbiosis in a giant protist (*Marginopora vertebralis*, Soritinae): flexibility in symbiotic partnerships along a natural temperature gradient

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ABSTRACT: Benthic foraminifera of the family Soritinae are important members of coral reef communities, contributing to carbonate deposition on coral reefs. These giant protists form photosymbiotic associations with microalgae of the genus *Symbiodinium*. The extent of flexibility in foraminefera–*Symbiodinium* partnerships is not well understood. While some studies suggest foraminifera exhibit strong specificity with regard to symbiont choice, recent work illustrated that at least a few taxa are able to host >1 symbiont type. We explored the symbiont diversity of a widely distributed soritid foraminifera (*Marginopora vertebralis*), sampling 369 individuals from 16 populations distributed across a wide latitudinal gradient (31 to 9° S) in the western Pacific Ocean using the internal transcribed spacer region 2 (ITS2) of rDNA. We discovered that *M. vertebralis* forms symbiotic associations with a high diversity of *Symbiodinium* types, which encompassed 27 unique ITS2 rDNA haplotypes from 4 major *Symbiodinium* clades. Distance-based redundancy analysis revealed that the observed geographic variation in symbiont community composition was correlated with several sea surface temperature parameters. Symbiont diversity was highest at the inshore Great Barrier Reef, in marginal habitats characterized by high seasonal fluctuations in environmental parameters. In those areas we found evidence of mixed infections, with individual hosts harboring multiple symbiont lineages. These findings suggest a high degree of flexibility in foraminifera–*Symbiodinium* partnerships and highlight the importance of environmental variables in shaping symbiotic associations. We discuss the results in light of the hypothesis that within-population symbiont polymorphism and mixed infections may be a mechanism to cope with temporal environmental fluctuations.

KEY WORDS: *Symbiodinium* · Temperature · Foraminifera · Symbiosis · Diversity

INTRODUCTION

Large benthic foraminifera are important members of tropical coral reef communities, contributing to ca. 5% of a coral reefs' carbonate deposition (Hallock 1981, Langer et al. 1997). Approximately 80% of this contribution can be attributed to large symbiont-bearing taxa (Langer et al. 1997). A small group of benthic discoidal soritid foraminifera (Soritinae), including the genera *Amphisorus*, *Sorites* and *Marginopora*, forms symbiotic partnerships with a diversity of dinoflagellate types belonging to the genus *Symbiodinium* (Pawlowski et al. 2001, Pochon & Pawlowski 2006, Pochon et al. 2007). Members of this genus form symbiotic associations with many diverse taxa, including corals, sponges, foraminifera and mollusks (Rowan 1998), and, through their association with hermatypic corals, play a fundamental role in coral-reef formation processes (Muscatine & Porter 1977).

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The genus *Symbiodinium* was long thought to be monospecific, but molecular phylogenetic studies based on nuclear (18S, 28S, ITS1 and ITS2 regions) and chloroplast (23S) ribosomal DNA markers revealed that this genus is extraordinarily diverse (LaJeunesse 2001, Baker 2003, Pochon et al. 2006, Pochon & Gates 2010). *Symbiodinium* spp. are hierarchically classified in clades (A to I), subclades (e.g. F1 to F5), and types, the latter being usually identified by a single ITS1 or ITS2 haplotype (LaJeunesse 2001, Coffroth & Santos 2005, Pochon & Gates 2010). While there is evidence of physiological and ecological differentiation among subclade types of *Symbiodinium* (Ulstrup & van Oppen 2003, Sampayo et al. 2007), determining what constitutes distinct ecological groups (ecotypes) based solely on DNA sequence is a challenging task since mutations in fast-evolving, non-coding regions are not always indicative of distinct physiologies. Correa & Baker (2009) described a promising approach to classify *Symbiodinium* diversity in a meaningful way at the ecological and physiological levels based on genetic data. The authors showed that ecotypes of symbionts usually can be defined as clusters of closely related haplotypes within parsimony networks based on ITS2 sequences (Correa & Baker 2009). This genetic cluster approach, based on population theory, provides a useful framework to investigate specificity and flexibility in symbiosis.

Soritid formanifera are known to host a high diversity of *Symbiodinium*, including members of 6 of the 9 known clades (C, D, F, G, H, I), 4 of which (F, G, H, I, with the exception of Subclade F2) occur exclusively in Soritinae (Garcia-Cuetos et al. 2005, Pochon et al. 2007, Pochon & Gates 2010). The first extensive investigation of *Symbiodinium* diversity in foraminifera suggested a high degree of specificity in terms of symbiotic partnerships, whereby a large number of host phylotypes exhibit strict specificity with respect to the symbiont types harbored (Garcia-Cuetos et al. 2005). More recent studies provide additional evidence of host-specificity, but also hint that some host phylotypes are flexible with regards to symbiont choice (Pochon et al. 2007) and that individual foraminifera may host mixed and dynamic symbiont communities (Fay et al. 2009). The sampling strategy of these studies (Garcia-Cuetos et al. 2005, Pochon et al. 2007), while covering a large number of host phylogenetic lineages, was geographically restricted to a few very close locations, and often all members of a host phylotype were sampled from the same site (Garcia-Cuetos et al. 2005, Pochon et al. 2007). As a result these studies may have largely underestimated symbiont diversity and flexibility in symbiont choice, as plasticity in symbiotic associations due to local selective pressure and biogeography may have been overlooked.

Studies on anthozoan symbioses show that, while there is a certain degree of specificity at the clade level (Goulet 2006, 2007), flexibility in symbiotic partnerships both at the clade and subclade level is common and might be overlooked if the sampling design and detection techniques are not appropriate (Baird et al. 2007, Baker & Romanski 2007, Silverstein et al. 2012). When sampling is carried out in different geographic regions (Ulstrup & van Oppen 2003, Macdonald et al. 2008), along environmental gradients (Rodriguez-Lanetty et al. 2001, Ulstrup & van Oppen 2003, LaJeunesse et al. 2004a, Cooper et al. 2011) or before, during and after an environmental impact occurs (Little et al. 2004, Jones et al. 2008), flexibility in host–*Symbiodinium* associations seems to be common. Some coral species can harbor many unrelated symbiont types simultaneously (mixed infections) (Fay & Weber 2012, Silverstein et al. 2012), and shuffling low-level background symbionts may be an acclimatization strategy to deal with environmental stress (Rowan et al. 1997, Berkelmans & van Oppen 2006).

Based on recent observations of a few foraminiferal hosts (Pochon et al. 2007, Fay et al. 2009) and of anthozoan–*Symbiodinium* associations (for a review, see Fay & Weber 2012), we hypothesized that flexibility is an important factor in explaining symbiotic partnerships in foraminifera and that foraminifera–*Symbiodinium* associations are shaped by environmental factors. To test this hypothesis, we investigated symbiont community composition in a widely distributed soritid (*Marginopora vertebralis*) across a gradient spanning >20° of latitude in the western Pacific Ocean. We demonstrated that *Marginopra vertebralis* harbors a high diversity of *Symbiodinium* lineages. Most of the symbiont types are not specific to this host lineage; spatial differences in community composition are likely being shaped by variation in temperature, and diversity is highest in marginal habitats subject to high environmental fluctuations.

MATERIALS AND METHODS

Sample collection and microscopy

Individuals of *Marginopora vertebralis* were collected on a latitudinal gradient from Lord Howe Island (31° 33′ S), Australia to Milne Bay, Papua

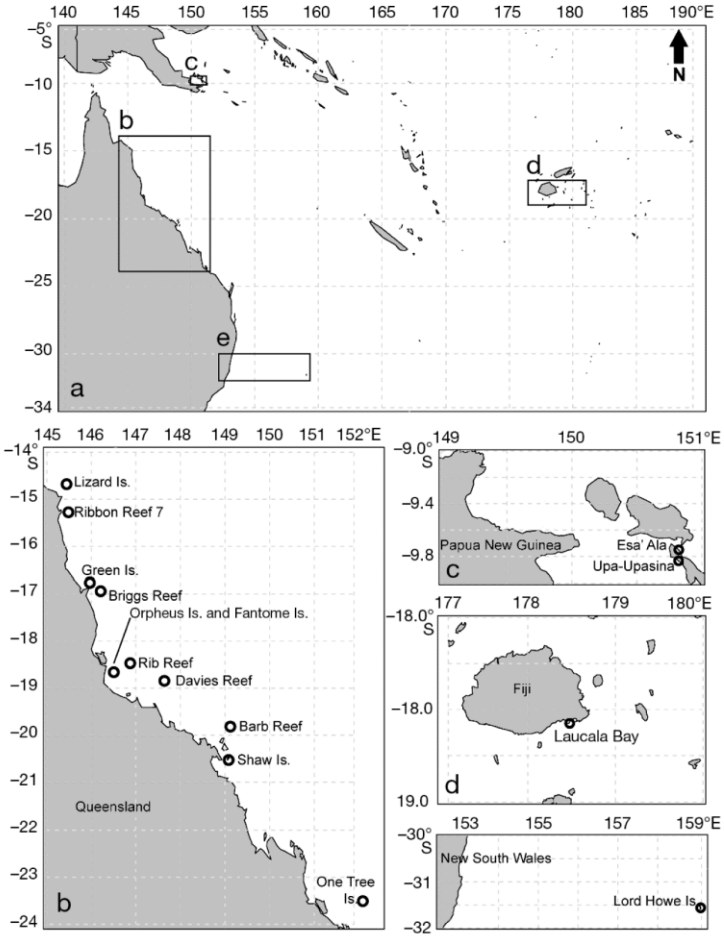

Fig. 1. Sampling areas within (a) the western Pacific Ocean, and detailed maps of collection sites (b) in the Great Barrier Reef, Australia, (c) Papua New Guinea, (d) Fiji, and (e) New South Wales, Australia

Table 1. Sampling locations. Date is given as dd/mm/yy. PNG: Papua New Guinea; GBR: Great Barrier Reef; NSW: New South Wales; N: no. of samples

Location	Region	Latitude (S)	Longitude (E)	Date	N
Upa-Upasina	PNG	9°49.69′	150°49.13′	23/04/11	24
Esa'Ala	PNG	9°44.29′	150°49.27′	25/0411	23
Lizard Is.	GBR	14°40.94	145°26.79′	06/10/04	22
Ribbon Reef No. 7	GBR	15°16.50	145°45.85′	16/07/11	24
Green Is.	GBR	16°45.71	145°58.33′	0/01/12	24
Briggs Reef	GBR	16°56.33	146°12.68′	04/01/12	24
Laucala Bay	Fiji	18°10.56	178°28.40′	27/07/11	18
Davies Reef	GBR	18°50.47	147°38.39′	17/09/08	24
Barb Reef	GBR	19°48.806	149°07.20′	20/02/07	18
Rib Reef	GBR	18°28.32	146°52.59′	26/01/11	24
North Bay, Fantome Is.	GBR	18°39.617	146°30.51′	25/01/11	24
Hazard Bay, Orpheus Is.	GBR	18°38.93	146°29.25′	24/01/11	22
Yank's Jetty, Orpheus Is.	GBR	18°39.08	146°29.20′	24/01/11	24
Shaw Is.	GBR	20°31.05	149°4.81′	06/08/09	47
One Tree Is.	GBR	23°30.33	152°5.61′	17/01/11	19
Lord Howe Is.	NSW	31°31.55	159°3.60′	24/02/05	8

New Guinea (PNG) (9° 44′ S, Fig. 1, Table 1). This species is epiphytic, and all samples were collected on either seagrass or macroalgal substrata adjacent to coral reefs, at a depth of 1 to 3 m. *M. vertebralis* were identified by light microscopy and phylogenetic analysis (see next subsections), and several representative samples were visualized by scanning electron microscopy (SEM; Fig. 2). Samples to be visualized by SEM were bleached for 1 h at 60C°, dehydrated in 100% ethanol, mounted on stubs and sputter-coated with gold. Samples were visualized on a JEOL JSM-5410LV scanning electron microscope at the Advanced Analytical Centre at James Cook University (Australia).

DNA extraction, amplification, sequencing and DGGE profiling

Total DNA was extracted from individual foraminiferal samples using a modified Chelex protocol (Walsh et al. 1991). As different symbiont types have been found to occupy different areas along the radius of foraminiferal tests (Fay et al. 2009), DNA extractions were performed in such a way to avoid bias. For smaller samples, DNA was extracted from half of the test. For larger samples a fragment extending from the center to outer edge of the test was used. Each fragment of foraminiferal test was placed in 100 µl Chelex solution (5% Chelex100®, 10 mM Tris-HCl) to which 5 µl of 20 mg ml^{-1} Proteinase K was added. Samples were digested at 55°C for 2 h, and following digestion proteinase K was denatured by heat-shocking at 95°C for 15 min. Samples were spun at 9200 × *g* for 5 min, and 0.5 µl of the supernatant was used as

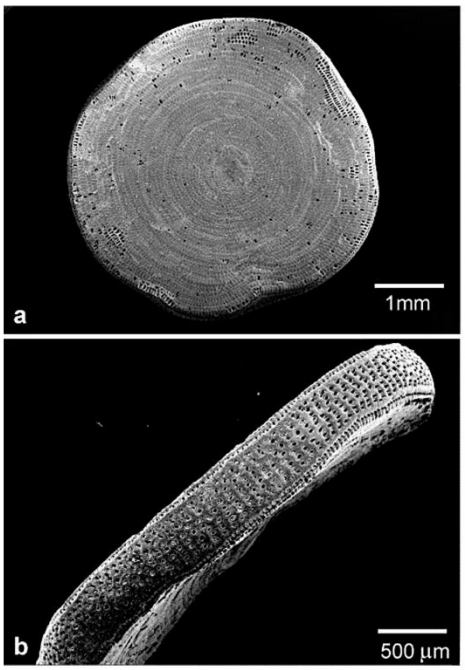

Fig. 2. *Marginopora vertebralis*. Scanning electron micrographs: (a) top view, (b) details of the lateral pores

template for PCR. All PCR reactions were prepared using the Qiagen Multiplex Kit, following manufacturer instructions.

For foraminiferal species confirmation, approximately 1700 bp of the 18S rRNA gene was amplified from representative samples of *Marginopora vertebralis* from each location using the primer pairs Sa10/LyS1 (Pawlowski 2000, Holzmann et al. 2001) and s6r/s17 (Pawlowski 2000) (Table 2). PCR cycling conditions were as follows: 15 min initial denaturation at 94°C, followed by 40 cycles of 40 s denaturation at 94°C, 40 s annealing at 56°C (Sa10/LyS1) or 50°C (s6r/s17), and 40 s elongation at 72°C and a final elongation step of 5 min at 72°C. PCR products were direct-sequenced with the amplification primers (Macrogen).

PCR and denaturing gradient gel electrophoresis (DGGE) of the ITS2 region of the *Symbiodinium* ribosomal RNA operon was conducted as per LaJeunesse & Trench (2000). Multiple representative bands of each DGGE profile were excised and reamplified using the primer pair ITSintFor2/ITSReverse (see Table 2) (Coleman et al. 1994, LaJeunesse & Trench 2000). PCR products were direct-sequenced using the primer ITSintFor2. Additionally, the D1 to D3 region of the 28S rRNA gene was amplified and sequenced for representatives of each ITS2 haplotype cluster. The approximately 900 bp long fragment of the 28S rRNA gene was amplified using the primer pair ITS2intFor/D3B (Scholin et al. 1994) (Table 2). PCR conditions were as for ITS2 amplification, with the difference that elongation time was 75 s, and the final elongation step was 10 min instead of 30 min.

Sequencing was carried out using a commercial service (Macrogen).

Sequence alignment, parsimony networks and phylogenetic analysis

Nucleotide sequence chromatograms were visually checked and assembled using the software ChromasPro (Technelysium). Sequence alignments were performed in ClustalW (Thompson et al. 1994) and visually refined using BioEdit (Hall 1999). All host sequences and representative sequences from each

Table 2. Primers used for amplification and sequencing. gc clamp underlined

Primer	Sequence	Organism	Region	Direction	Source
Sa10	CTCAAAGATTAAGCCATGCAAGTT	Foraminifera	18S	Forward	Holzmann et al. (2001)
LyS1	CTCCAACTATCTCCATCGA	Foraminifera	18S	Reverse	Pawlowski (2000)
S6r	GGGCAAGTCTGGTGC	Foraminifera	18S	Forward	Pawlowski (2000)
S17	CGGTCACGTTCGTTGC	Foraminifera	18S	Reverse	Pawlowski (2000)
ITSintFor2	GAATTGCAGAACTCCGTG	*Symbiodinium*	ITS/28S	Forward	LaJeunesse & Trench (2000)
ITSReverse	GGGATCCATATGCTTAAGTT CAGCGGGT	*Symbiodinium*	ITS	Reverse	LaJeunesse & Trench (2000)
ITS2Clamp	CGCC....GCCCGGGATCCATATGC TTAAGTTCAGCGGGT	*Symbiodinium*	ITS	Reverse	LaJeunesse & Trench (2000)
D3B	TCGGAGGGAACCAGCTACTA	*Symbiodinium*	28S	Reverse	Scholin et al. (1994)
D1R	ACCCGCTGAATTTAAGCATA	*Symbiodinium*	28S	Forward	Nunn et al. (1996)

Symbiodinium type were deposited in GenBank (Accession Numbers: KC802023 to KC802083).

Marginopora vertebralis

Near-complete 18S host sequences were aligned with *Marginopora* sp. sequences retrieved from GenBank (AJ842190, AJ842188 to AJ842190) and 2 *Sorites* sp. sequences (AJ842193, AJ404311) to be used as outgroups in the phylogenetic analysis. Substitution models were tested in Modeltest (Posada & Crandall 1998). A phylogeny based on 18S gene sequences was estimated by Bayesian inference (BI) and by analyzing 100 bootstrap datasets by maximum likelihood (ML). The ML phylogeny was estimated in PHYML 3.0 (Guindon et al. 2005, 2010) by ML using the GTR+I+G substitution model, 4 gamma categories, tree improvement by using the best of the nearest neighbor interchange (NNI) and subtree pruning and regrafting (SPR) using 5 random starting trees. The Bayesian analysis was performed using the same substitution model in MrBayes 3.1 (Ronquist & Huelsenbeck 2003). The number of Monte Carlo Markov chain generations was set to 4 000 000, and trees were sampled every 100 generations. Convergence of 2 independent runs was tested by checking that standard deviation of split frequencies (as estimated for the last 75% of sampled trees) fell below 0.01, and by visually analyzing the stability of each parameter estimate in the software Tracer (Rambaut & Drummond 2003). The first 25% of sampled trees were discarded as burn-in.

Symbiodinium

ITS2 haplotypes obtained were aligned with sequences retrieved from GenBank, and separate ITS2 alignments were produced for each *Symbiodinium* clade represented in our samples (C, D, F, H). Sequences were trimmed to the length of the shortest sequence. Parsimony networks were produced using the software TCS (Clement et al. 2000), and genetic clusters were identified following the method outlined by Correa & Baker (2009). Clusters are here defined as groups of sequences of minimal sequence divergence within a parsimony network, where average within-cluster pairwise sequence divergence is <50% of the average between-cluster divergence (Palys et al. 1997, Correa & Baker 2009). Between- and within-cluster divergence were calculated using the 'DNA divergence between populations' function in DNAsp v.5.10 (Librado & Rozas 2009). The 28S partial sequences obtained from representatives of each genetic cluster were aligned with *Symbiodinium* sp. sequences representative of each known clade and subclade retrieved from GenBank. Model selection and 28S phylogenetic reconstruction followed the same procedure outlined above for *marginopora vertebralis* phylogenetic analysis, with the difference that the substitution model selected was GTR+G (instead of GTR+I+G).

Statistical analysis of *Symbiodinium* communities

We investigated the influence of various temperature metrics on the composition of symbiont communities hosted by *Marginopora vertebralis*. This analysis was restricted to the *M. vertebralis* Phylotype Mar II to avoid possible confounding effects of *Symbiodinium*–host specificity among distinct host phylotypes. Temperature series data from the last 11 yr for each of the sampled locations were obtained from the NOAA Comprehensive Large Array-Data Stewardship System (CLASS, www.class.noaa.gov). We used the SST50 surface temperature dataset, which consists of sea-surface temperature (SST) data at a 50 km resolution, obtained from a composite gridded-image derived from 8 km resolution global SST observations, and is generated twice weekly (every Tuesday and Saturday). From temperature series data, a number of metrics were derived: mean 11 yr temperature, maximum recorded temperature, minimum recorded temperature, mean winter temperature, mean summer temperature and mean yearly range (see Table A1 in Appendix 1). Because samples over a large geographic scale could not be sampled during the same season, we also statistically tested (see below) whether mean temperature in the month preceding sampling influenced the *Symbiodinium* type(s) present within samples.

The relationship between *Symbiodinium* type and temperature metrics was investigated with constrained analysis of principal coordinates (also known as distance-based redundancy analysis, db-RDA), which is an ordination method that tests the effects of individual or combined environmental variables on a community dataset using non-Euclidean distance matrices (Legendre & Anderson 1999). *Symbiodinium* community abundance data for each population were obtained by scoring symbionts (classified at the cluster level) for each individual host. Abundance data were row-standardized to account for different sample sizes. To test the effect of the

obtained temperature metrics, db-RDA was applied on Bray-Curtis distance matrices obtained from *Symbiodinium* community abundance data of all *Marginopora vertebralis* populations belonging to Phylotype Mar II from all survey sites (i.e. all samples with the exception of the Fiji population, see 'Results'). As different temperature metrics are likely to be strongly correlated, variables were assessed for collinearity, and only metrics with correlation coefficients of <0.9 were used. The significance of individual metrics was tested by permutation tests (10000 permutations). Only variables significant in the individual tests were included in the final model.

The effect of the experimental design on estimates of symbiont diversity was explored by creating a rarefaction curve showing the effect of the number of locations sampled on the number of genetic clustered detected (Fig. A1 in Appendix 1). These analyses were conducted using the Vegan package (Oksanen et al. 2011) of the R statistical environment (R Development Core Team, http://R-project.org).

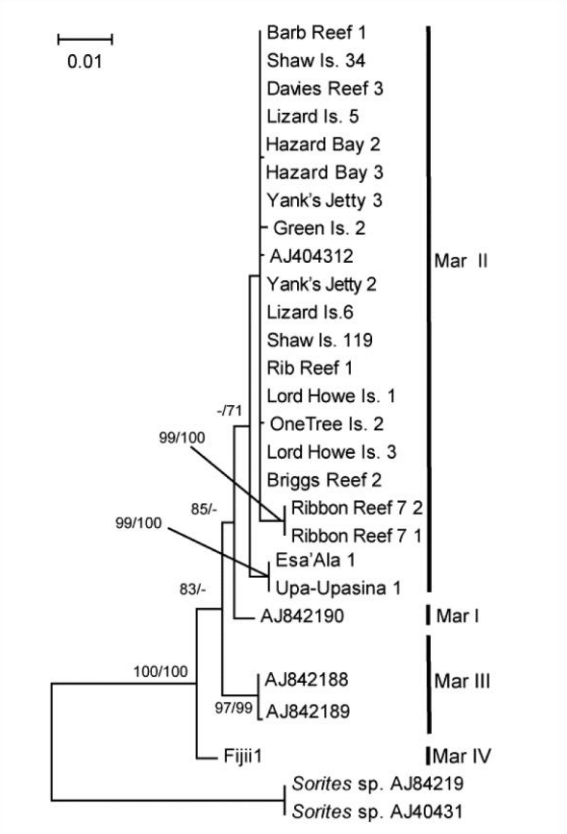

Fig. 3. Phylogeny of *Marginopora vertebralis* phylotypes based on near-complete 18S rRNA gene sequences. Branch support values (ML/BI) represent 100 bootstrap datasets by maximum likelihood (ML) and Bayesian clade credibility values (BI), respectively. Clades are named following Garcia Cuetos et al (2005)

RESULTS

Marginopora vertebralis genetic analysis and morphology

The final alignment of the near-complete 18S rRNA gene sequences included 27 individuals, 6 of which were obtained from GenBank. The alignment consisted of 1716 unambiguously aligned positions, 122 variable sites and 115 parsimony informative sites. The phylogenetic analysis (Fig. 3) supports the classification of *M. vertebralis* in distinct phylotypes (Mar I, Mar II and Mar III) (Garcia-Cuetos et al. 2005). However, while the distinction between Mar III and the other phylotypes is very clear and well supported, in our analysis the distinction between the Phylotypes Mar I and Mar II was not as obvious as that described by Garcia-Cuetos et al. (2005), despite the fact that we used a larger fragment of the 18S gene. Furthermore, we report a fourth phylotype (Mar IV), represented by an individual from Fiji (Fig. 2). The Phylotype Mar II shows the morphological features of *M. vertebralis* as described in Holzmann et al. (2001) by SEM (Fig. 2). With the exclusion of the individuals collected from Fiji, all our samples belonged to Phylotype Mar II and all representatives of Phylotypes Mar I and III represented sequences from GenBank.

Symbiodinium genetic analysis

Twenty-seven unique *Symbiodinium* ITS2 haplotypes were identified from 369 *Marginopora vertebralis* samples from the southwestern Pacific Ocean. Of these 27 haplotypes, some differed by a single base pair mutation or by a single insertion or deletion (Fig. 4). If 2 or more haplotypes were found to always co-occur in the same individuals, they were assumed to be intragenomic variants (circled by dotted lines in Fig. 4). Haplotype networks identified 23 haplotypes, 19 of which were novel (i.e. not represented in GenBank; Fig. 4). The reason for the observed incongruence is that sequences were trimmed to the length of the shortest sequence and gaps were treated as missing data; some variable sites were excluded as a result. The 23 unique haplotypes identified by the TCS analysis spanned 4 clades (C, D, F, H) and 10 genetic clusters. Intragenomic variants were always assigned to the same genetic cluster. Of the 10 gen-

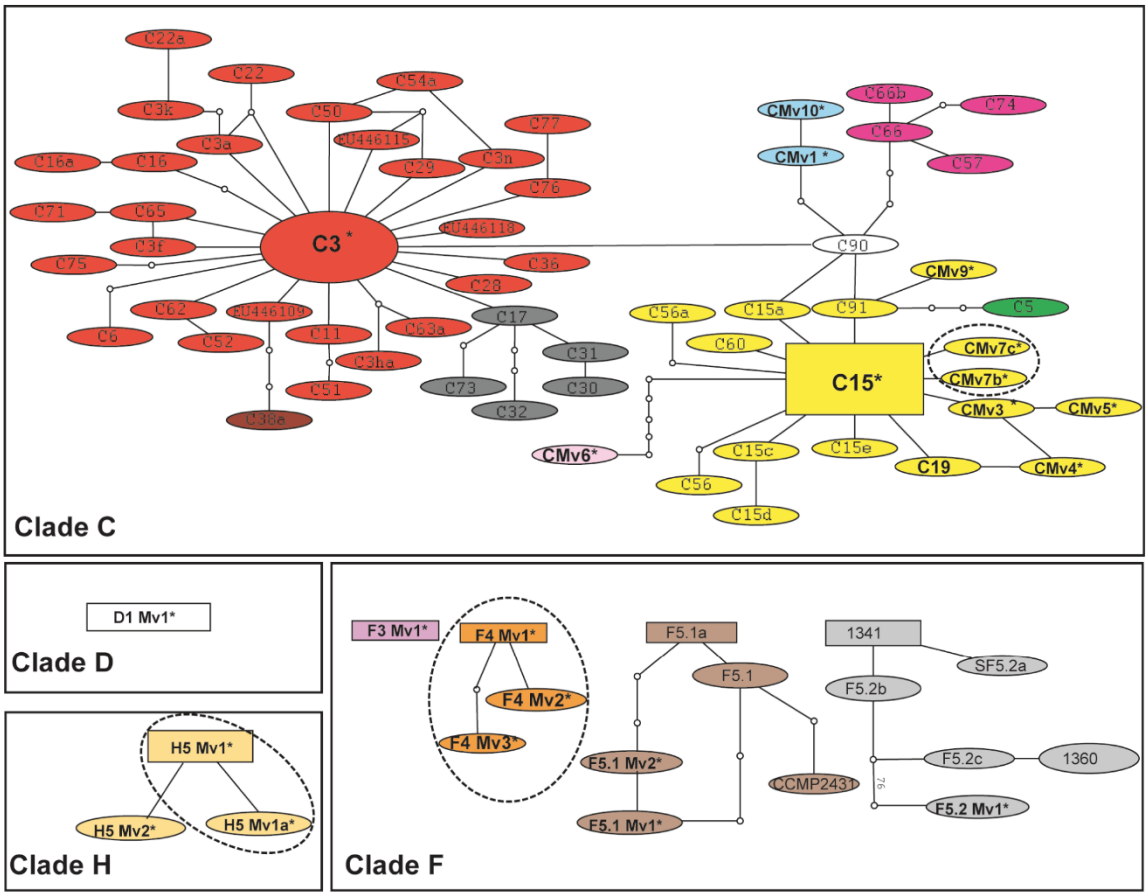

Fig. 4. Parsimony networks of *Symbiodinium* ITS2 haplotypes. Genetic clusters within each clade are shown in different colors. Haplotypes isolated in this study are shown in bold and marked with an asterisk. Intragenomic variants are circled in dotted lines

etic clusters, 6 represented novel clusters and 4 belonged to novel ITS2 networks (Fig. 4). The 4 known genetic clusters included symbiont groups previously identified in both coral (C3, C15, F5.1) and foraminiferal lineages (C3, C15, F5.1, F5.2).

A subset of *Symbiodinium* samples (76 sequences) was also run using 28S rRNA to confirm taxonomic conclusions derived by ITS2. The 28S rRNA gene alignment included 782 unambiguously aligned sites, 373 of which were variable and 336 parsimony informative. The phylogeny based on 28S sequences (Fig. 5) provided adequate identification of representative genetic clusters to the clade and subclade level, including the genetic clusters that belong to novel ITS2 networks. The phylogenetic tree based on 28S rRNA gene sequences was consistent with phylogenies produced in previous studies (Pochon et al. 2006, Pochon & Gates 2010) using both 28S rRNA and 23S cpRNA gene sequences, identifying 9 well-supported major clades (A to I), 2 subclades within Clade D and 4 within Clade F (Fig. 5). While each subclade in Clade F was well supported in both the ML and BI analyses, Clade F as a whole was not; thus, it is likely that this clade is polyphyletic and in need of taxonomic revision. Symbionts isolated from *Marginopra vertebralis* (Phylotype Mar II) spanned 4 major clades (C, D, F and H) (Fig. 5). Within Clade F symbionts isolated from our samples were well grouped within Subclades F4 and F5. Within Clade D symbionts from *M. vertebralis* grouped most closely with the symbionts isolated from the sponge *Haliclona koremella* (Carlos et al. 1999). The length of the ITS2 of this subclade was longer (>400 bp) than that of other *Symbiodinium* types.

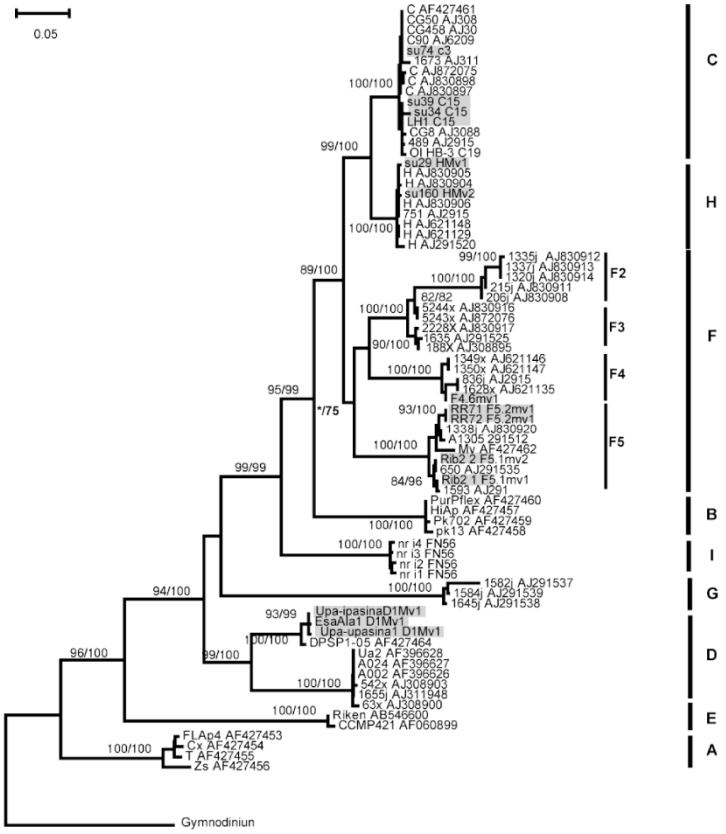

Fig. 5. *Symbiodinium* phylogeny based on partial 28S rRNA gene sequences (D1 to D3 region). Sequences obtained in this study are highlighted. Branch support values (ML/BI) represent 100 bootstrap datasets by maximum likelihood (ML) and Bayesian clade credibility values (BI), respectively

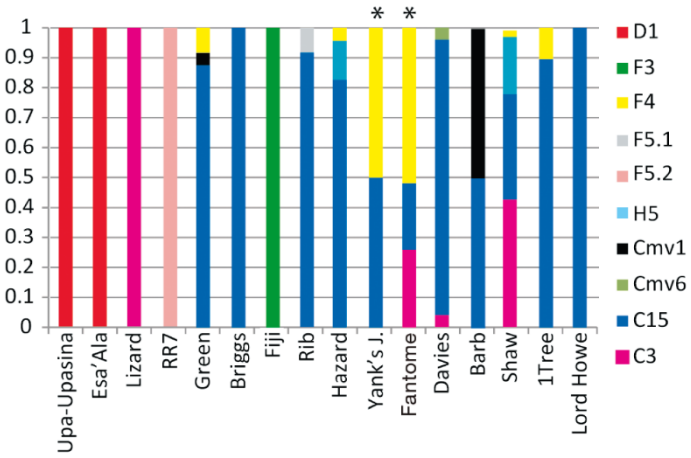

Fig. 6. *Marginopora vertebralis*. Frequency distribution of symbiont genetic clusters across symbiont communities in *M. vertebralis* populations. Populations are in order of decreasing latitude (north to south). Populations in which individual hosts were found to harbor multiple symbiont clusters are marked with an asterisk, and details on the occurrence of multiple infections are given in the 'Results'

Statistical analysis of *Symbiodinium* communities

Symbiont community composition varied among *Marginopora vertebralis* populations (Fig. 6). In the central and southern Great Barrier Reef (GBR) region and off Lord Howe Island, symbiont communities were largely dominated by the genetic Cluster C15, with some contributions from Clusters C3 and F4 in inshore reefs of the central GBR (Yank's Jetty, Fantome Island and Shaw Island) (Fig. 6). All populations in the central and southern GBR (with the exception of Briggs Reef) harbored >1 group of symbionts, with the highest diversity reported at Shaw Island (where 4 groups representing 3 major clades were present). In contrast, populations in the northern GBR (Lizard Island and Ribbon Reef No. 7) and outside of the GBR (PNG and Lord Howe populations) were dominated by a single genetic cluster of symbionts. The symbiont Clusters C15 and F4, common in central GBR, were entirely absent on reefs further north than Green Island (16°45S). Symbiont communities in the PNG populations were dominated by a single *Symbiodinium* cluster belonging to Clade D (Fig. 6). Only a few host individuals harbored multiple symbiont clusters. Multiple symbiont clades (C and F) were harbored by a small number of individuals in the inshore reefs of Orpheus Island (2 out of 22 at Yank's Jetty) and Fantome Island (3 out 24 individuals collected in North Bay).

Db-RDA was used to assess the relationship between *Symbiodinium* clusters and locations and several temperature-related metrics as environmental variables. As expected, most temperature-related parameters were highly correlated, and we chose 4 parameters which were less correlated ($R < 0.9$) for further analysis: mean winter temperature, mean summer temperature, mean temperature range and average temperature in the

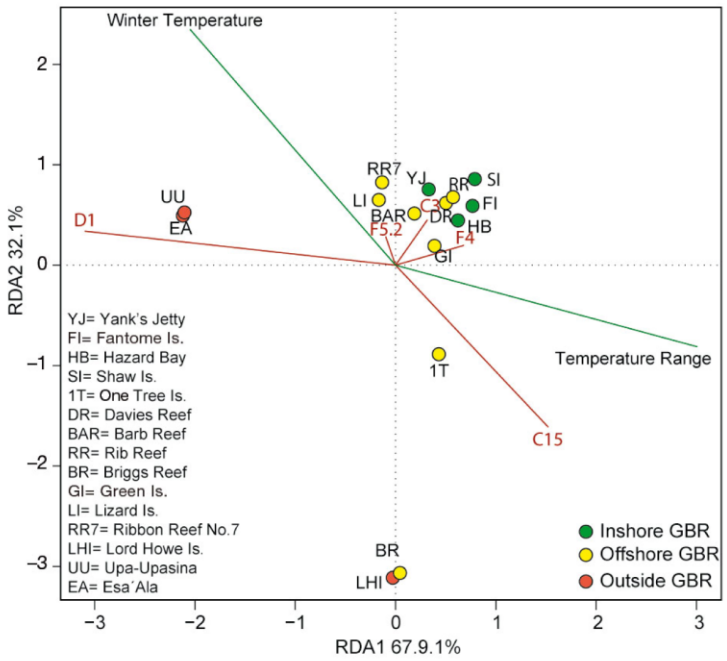

Fig. 7. *Marginopora vertebralis*. Distance-based redundancy (RDA) analysis of symbiont community composition among populations of *M. vertebralis*. To be noted: the strong negative correlation of *Symbiodinium* Cluster C15 with winter temperature, the positive correlation between C15 and temperature range and the positive correlation of Cluster D1 with low temperature range and high winter temperature. GBR: Great Barrier Reef

month before sampling). Permutation tests indicated that the winter temperature (pseudo-F = 3.49, p < 0.0026) and temperature range (pseudo-F = 6.73, p = 0.0001) explained a significant amount of the variation; results for the other 2 variables were not significant. In a db-RDA the 2 variables combined explained 39.7% of the distances observed between sample populations. The individual variables mean winter temperature and mean temperature range explained 22.8 and 35.2% of the distance, respectively.

The presence of *Symbiodinium* Cluster C15 was strongly negatively correlated with high winter temperature minima and positively correlated to a higher temperature range (Fig. 7). In contrast, Cluster D1 was highly correlated with a low temperature range and high winter temperatures. Thus, this analysis confirmed findings from the frequency analysis.

The rarefaction curve (Fig. A1) suggests that more intensive sampling would likely yield additional symbiont genetic clusters and that previous studies, which included only 1 or a few locations, may have undersampled clusters.

DISCUSSION

Symbiodinium-bearing foraminifera are subject to similar stresses as corals: they are susceptible to bleaching under high temperatures (Uthicke et al. 2012) and other stressors including reduced pH through ocean acidification (Uthicke & Fabricius 2012, Reymond et al. 2013, Uthicke et al. 2013). Nevertheless *Marginopora vertebralis* has a wide latitudinal distribution, being present in habitats with very different temperature regimes (such at Lord Howe Island and PNG). It is therefore possible that the type of symbionts they associate with plays a significant role in local acclimatization. We sampled *M. vertebralis* across a wide (>20°) latitudinal gradient and investigated symbiont community composition in each population. Our data suggest that *M. vertebralis* exhibits high diversity and flexibility in symbiotic associations, which are at least partially explained by environmental factors. The diversity of symbionts harbored by *M. vertebralis*—Phylotype Mar II sensu Garcia-Cuetos et al. (2005)—is remarkable, with 27 unique haplotypes belonging to 9 genetic clusters representing 4 clades, and more intensive sampling is likely to yield even higher symbiont diversity estimates (Fig. A1). While some of the detected genetic clusters are relatively rare, 6 (C3, C15, Cmv1, F4.6mv1, F5.2, DMv1) contribute to at least 50% of symbiont communities in local populations of *M. vertebralis* (Mar II).

This level of diversity is very high when compared with other *Symbiodinium*-bearing taxa. While symbiont polymorphism is a common phenomenon, most symbiont-bearing taxa associate only with a limited number of *Symbiodinium* types, and just a few are able to harbor many distinct lineages (Fay & Weber 2012). Many symbionts associated with *Marginopora vertebralis* (C3, C15, C19, F5.1, F5.2) in our study have been previously isolated from various species of corals and foraminifera, suggesting they are to some extent generalists (van Oppen et al. 2001, LaJeunesse et al. 2004b, 2009, Pochon et al. 2007, Bongaerts et al. 2010, Venera-Ponton et al. 2010). The Cluster D1Mv1 was novel, but closely related to symbionts previously isolated from another phylotype of *M. vertebralis*

(Phylotype Mar I) (Pochon et al. 2007) and from a sponge (Carlos et al. 1999).

Flexibility in symbiotic partnerships

The findings presented here suggest that foraminifera–*Symbiodinium* partnerships can be flexible. *Marginopora vertebralis* (Phylotype Mar II) can associate with a variety of symbiont types, and different symbionts may dominate depending on the location sampled. Because the marker used to explore hosts genetic diversity (18S) may not carry enough genetic variation to distinguish between subspecies, it cannot be excluded that some degree of specificity may exist at a lower host taxonomic level. However, of the 16 populations sampled, half of them harbored >1 distinct symbiont cluster, suggesting that even at the population level flexibility seems to be common.

The fact that low sampling effort may affect the perceived specificity of symbiotic partnerships has sparked debates in the past (Baker 2003, Goulet 2006, Baker & Romanski 2007). Previous studies of foraminifera–*Symbiodinium* associations considered a large number of foraminiferal phylotypes, but were limited to either a few individuals or a few locations per phylotype (Garcia-Cuetos et al. 2005, Pochon et al. 2007), and, therefore, most likely underestimated the level of diversity and flexibility in foraminifera–*Symbiodinium* partnerships. At single points in space and time, symbiont communities appear to be dominated in several populations by a single type (Garcia-Cuetos et al. 2005, Pochon & Gates 2010). Symbiont lineages may differ in physiological traits (van Oppen et al. 2001, Little et al. 2004), and, therefore, some symbiont–host associations may be positively selected in specific environmental conditions. Under stable environmental conditions a host species may show local preference for a particular symbiont lineage, at least until environmental conditions are perturbed. Since preferences may be host specific, local selective pressure could create localized patterns of host–symbiont associations that may be misinterpreted as evidence of host specificity. Garcia-Cuetos et al. (2005) interpreted such patterns as evidence of strict specificity in foraminifera–*Symbiodinium* partnerships. However, we argue that this is not evidence of fidelity, as it does not provide insight into whether a host has the flexibility to form symbiotic associations with multiple symbiont lineages under different environmental conditions (Baker 2003). This process may account for both the high degree of specificity previously reported in soritid–*Symbiodinium* symbioses at a local scale (Garcia-Cuetos et al. 2005), and the high flexibility in symbiotic partnerships exhibited by *Marginopora vertebralis* across a wide latitudinal range.

Spatial differences in symbiont community composition

The changes observed in symbiont community composition were not random, and symbiont types varied predictably with temperature regimes. No effect of temperature in the month prior to sampling was detected, suggesting that differences in sampling times in different regions were unlikely to be a confounding factor in our analysis. These results should however be interpreted with caution; since the effect of temperature was not investigated in controlled conditions, the interpretation of the results is necessarily made on the basis of the correlation that exists between temperature parameters and latitude.

Type C15 was negatively correlated with high winter temperature and positively correlated with a high average temperature range. This type is the dominant type in most populations in the central and southern GBR (mean winter temperatures: 19 to 22°C; mean yearly temperature range: 4.6 to 5°C), as well as off Lord Howe Island (mean winter temperature: 19.2°C; mean yearly range: 5°C), but is completely absent from the warmer populations. This pattern cannot be explained in terms of symbiont biogeographic distribution, as C15 is widespread and found in many other hosts across the Indo-Pacific (LaJeunesse et al. 2003). This symbiont type may confer a higher tolerance to lower temperatures, or offer a physiological advantage over the wider temperature range experienced by populations at higher latitudes. Pochon et al. (2007) reanalyzed the dataset from Pawlowski et al. (2001) and reported C15 in *Marginopora vertebralis* from Lizard Island, several hundred kilometers north of where we detected C15 on the GBR. However, it is unclear whether the population sampled by Pawlowski et al. (2001) belonged to the Phylotype Mar II. Both Phylotypes Mar II and Mar III have been reported from Lizard Island (Garcia-Cuetos et al. 2005), the latter being found in deeper waters and being associated with Clade C *Symbiodinium*. In light of the results of the present study, it is likely that the C15 *Symbiodinium* reported by Pochon et al. (2007) might have been associated with the deeper-water Mar III phylotype.

The low-latitude D1Mv1, on the other hand, is found exclusively in PNG populations, where winter

temperature is highest (27.4°C) and mean yearly temperature fluctuation lowest (2.3°C). A warm-water population of another phylotype of *Marginopora vertebralis* (Mar I from Guam; latitude: 13.5N; mean winter temperature: 27.7°C; mean yearly range: 1.75°C) was found to associate with a symbiont type closely related to D1Mv1 (Pochon et al. 2007), suggesting that temperature tolerance may be a shared character of this D subclade. Clade D symbionts have been associated with thermal tolerance in a range of coral species (Rowan et al. 1997, Rowan 2004, Berkelmans & van Oppen 2006, Ulstrup et al. 2006, LaJeunesse et al. 2009).

Symbiont diversity in *Marginopora vertebralis* decreases towards the latitudinal edges of the study area (latitudes: 9 to 10°S at PNG and 31 to 32°S at Lord Howe Island); communities at those locations consisted of a single symbiont type. In the central region of the GBR, symbiont communities were more heterogeneous and diversity was highest in inshore reefs. In those inshore reefs we also found evidence of multiple symbiont lineages in single *M. vertebralis* individuals. These inshore populations experienced yearly temperature fluctuations similar to higher latitude locations (see Table A1). For example, Shaw Island has a mean yearly temperature range of 5.3°C, a range comparable to the higher latitude populations of One Tree Island (5.3°C) and Lord Howe Island (5°C). In addition, inshore reefs are subject to much higher seasonal fluctuations in salinity, nutrient levels and water clarity when compared to offshore reefs and can experience elevated sea surface temperature, low salinity and increased nutrients levels in the summer months (Schaffelke et al. 2012). These reefs are marginal habitats for *M. vertebralis*, which is not found any closer inshore in the GBR lagoon (S. Uthicke pers. obs.). On inshore reefs *M. vertebralis* grows more slowly (Reymond et al. 2011), and experimental studies have confirmed that elevated nutrients and temperatures inhibit growth and photosynthesis in this species (Uthicke et al. 2012). It is possible that both the higher within-population and within-individual diversity observed in inshore reefs is related to the instability of environmental conditions which characterizes these marginal habitats. These results are consistent with the hypothesis proposed by Cooper et al. (2011) that, while SST parameters are important predictors of *Symbiodinium* types at a broad geographical scale, water quality parameters play an important role in shaping symbiotic partnerships at a local scale.

The hypothesis that temporal variability in environmental conditions can favor heterogeneous symbiont communities, both in host populations and in host individuals, is not new (see review by Fay & Weber 2012). *Acropora millipora* populations were found to harbor mixed symbiont (C2, C1 and D1) communities in the inshore reefs of the Whitsunday Islands (central GBR), characterized by higher fluctuations in water quality parameters, while corals in the outer zone of the Whitsundays harbored exclusively C2 symbiont types (Cooper et al. 2011). Rowan et al. (1997) showed that corals that harbor multiple symbiont types may be more resistant to bleaching, and concluded that temporal variability in environmental conditions may favor the coexistence of multiple symbiont types in a single host. Rowan et al (1997) investigated temperature stress, but other stressors (changes in salinity, light availability and nutrient levels due to run-off) may act in the same way.

Flexibility in symbiotic partnerships may provide a mechanism to cope with environmental stress, but this hypothesis has been challenged by Putnam et al. (2012). These authors found that coral species exhibiting higher flexibility in symbiont choice are often susceptible to environmental stress, while corals that are often classified as short-term 'ecological winners' show greater specificity in symbiotic partnerships (van Woesik 2001). Putnam et al. (2012) propose that flexible symbioses are disadvantageous for coping with environmental stress. However, the data presented are correlative, and the authors do not show any causality between flexibility specificity and susceptibility to environmental stress. The taxa compared have different life-history traits, and these differences are likely to play a key role in determining susceptibility to environmental stress and bleaching (van Woesik 2001). We argue that there is an alternative, equally parsimonious, hypothesis to explain the data by Putnam et al. (2012): hosts that are physiologically more susceptible to stress evolve greater flexibility in symbiotic partnerships to cope with environmental fluctuations.

It is important to keep in mind that DGGE is not able to reliably detect the presence of symbionts at background levels (<10% of symbiont community). More powerful molecular techniques, such as real-time PCR, are needed to assess the occurrence of background communities (Mieog et al. 2007). Therefore, the results presented in this study likely underestimate symbiont diversity within individual formanifera. Furthermore, while DGGE and the clustering method we adopted perform well in delineating symbiont ecotypes (Correa & Baker 2009), symbiont adaptation at the population level may occur before mutations in the ITS region arise and sym-

bionts of the same ITS2 type may be adapted to different thermal regimes (Howells et al. 2012). Finer scale genetic methods, such as the use of microsatellite markers, may reveal higher symbiont diversity at lower taxonomic levels that could be physiologically relevant.

In conclusion, *Marginopora vertebralis* harbors a high diversity of symbiont lineages, representing 4 distinct *Symbiodinium* clades. Symbiont community composition varies in a non-random fashion along a wide latitudinal gradient, and the occurrence of several symbiont types is correlated with temperature. These results highlight the importance of environmental factors in shaping symbiotic associations, and suggest that at a broad geographical scale foraminifera–*Symbiodinium* partnerships are more flexible than previously thought.

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Appendix 1.

Table A1. Sea-surface temperature (*T*) parameters for each sampling location

Location	Mean *T* (°C)	Mean summer *T* (°C)	Mean winter *T* (°C)	Min. *T* (°C)	Max. *T* (°C)	Mean *T* in month before sampling (°C)	Mean range (°C)	Latitude
Upa-Upasina	28.7	29.7	27.4	24.6	31.8	30.0	2.3	9.83
Esa'Ala	28.7	29.7	27.4	24.6	31.8	30.0	2.3	9.74
Laucala Bay	27.5	28.8	26.9	24.5	30.5	26.9	2.0	18.18
Ribbon Reef 7	27.0	29.0	24.9	22.7	30.9	23.9	4.1	15.28
Lizard Is.	26.9	29.0	24.9	22.8	31.3	24.7	4.1	14.68
Briggs Reef	26.8	28.9	24.7	22.2	31.1	29.3	4.3	16.94
Green Is.	26.8	28.9	24.7	22.2	31.1	29.3	4.3	16.76
Rib Reef	26.4	28.7	24.1	19.3	32.0	29.0	4.7	18.47
Fantome Is.	26.4	28.7	24.1	19.3	32.0	29.0	4.7	18.66
Hazard Bay	26.4	28.7	24.1	19.3	32.0	29.0	4.7	18.65
Yank's Jetty	26.4	28.7	24.1	19.3	32.0	29.0	4.7	18.65
Davies Reef	26.2	28.6	23.7	19.4	31.7	23.1	4.9	18.84
Barb Reef	26.0	28.3	23.6	21.9	30.1	28.7	4.8	19.81
Shaw Is.	25.7	28.3	23.1	21.4	30.3	23.1	5.3	20.52
One Tree Is.	24.7	27.2	22.0	20.3	29.7	27.6	5.3	23.51
Lord Howe Is.	21.6	24.2	19.2	17.8	26.5	24.3	5.0	31.53

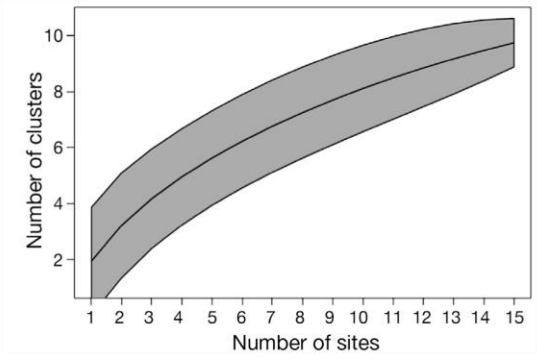

Fig. A1. Rarefaction curve showing the effect of the number of sites on the number of symbiont genetic clusters detected. Grey areas represent 95 % confidence intervals. The slope of the line suggests that while most of the variation has been sampled, additional sites would likely result in the discovery of unsampled genetic clusters. This indicates that previous studies, which only included 1 or a few locations, may have been severely undersampled

Editorial responsibility: Christine Paetzold, Oldendorf/Luhe, Germany

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Appendix E

Ethics Approval

MACQUARIE UNIVERSITY

ANIMAL RESEARCH AUTHORITY (ARA)

AEC Reference No.: 2012/044 - 2 **Date of Expiry:** 18 August 2013

Full Approval Duration: 19 August 2012 to 18 August 2015 (36 Months)

This ARA remains in force until the Date of Expiry (unless suspended, cancelled or surrendered) **and will only be renewed upon receipt of a satisfactory Progress Report before expiry.**

Principal Investigator:
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In case of emergency, please contact:
the Principal Investigator / Associate Investigator named above
or Animal Welfare Officer 9850 7758 / 0439 497 383

The above-named are authorised by MACQUARIE UNIVERSITY ANIMAL ETHICS COMMITTEE to conduct the following research:

Title of the project: **Grey Reef Sharks: patterns of habitat use and connectivity in Australia and Indonesia**

Type of animal research: **7 – Research: Environmental Study**

Aims: To investigate movement patterns, habitat utilisation and spatial dynamics of sharks within Ningaloo Marine Park using arrays of acoustic listening station.

Surgical Procedures category: **4 – Minor surgery with recovery**

All procedures must be performed as per the AEC-approved protocol, unless stated otherwise by the AEC and/or AWO.

Maximum numbers approved (for the Full Approval Duration):

Species	Strain	Sex	Weight	Age	Total	Supplier/ Source
23 - Fish	*Grey Reef Shark (Carcharhinus amblyrhynchos)*	Male and female	N/A, up to 2 meters in length	N/A	163	wild
				TOTAL	163	

Location of research:

Location	*Full street address*
Ningaloo Reef Marine Park	Western Australia, Australia
Heron, One Tree Island and surrounding reef, located in the Capricorn Bunker, Southern Great Barrier Reef	Queensland, Australia
Clerke and Imperieuse Reefs	North Western Australia, Australia

Amendments approved by the AEC since initial approval:

1. Addition of two new locations and addition of approximately 103 more animals (March 2013)

Conditions of Approval: **To satisfy compliance with The Code, for monitoring of research in a remote location, please supply the AEC with unedited video footage of the experimental procedure, from capture to final release of the animal, on the first three animals.**

Being animal research carried out in accordance with the Code of Practice for a recognised research purpose and in connection with animals (other than exempt animals) that have been obtained from the holder of an animal suppliers licence.

Professor Mark Connor (Chair, Animal Ethics Committee) **Approval Date:** 14 March 2013

Adapted from Form C (issued under part IV of the Animal Research Act, 1985)