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Genomic Tools for Conservation and Management of Manta and Devil Rays (Mobula Spp.)

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Genomic Tools for Conservation and Management of Manta and Devil Rays (*Mobula* Spp.)

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Abstract

Effective measures for the conservation of biodiversity require an understanding of the extent and distribution of diversity within and among species. Studies focussed on providing such understanding can benefit from increasingly powerful and accessible genome-wide data. Overexploitation of marine fisheries is a global problem resulting in loss of genetic diversity and declines in many species, and there is increased awareness and uptake of genomic principles in fisheries management and conservation. Manta and devil rays (Mobula spp.) are increasingly threatened by targeted and bycatch fisheries supplying the international demand for their gill plates. Such impacts will likely be met with catastrophic declines exacerbated by their slow life history traits, rendering these fisheries unsustainable. To alleviate threats, all species of mobulid ray are listed on CITES Appendix II to regulate international trade, and on CMS Appendices I and II to coordinate protection and implement conservation efforts. However, the status of mobulid rays is not matched by understanding of stock structure, gene flow, population dynamics, processes driving variation between species, and species boundaries. To date, a lack of representative global tissue samples, ongoing taxonomic ambiguity and ineffectual traceability measures combine to constrain the development and implementation of a coherent and enforceable conservation strategy for these species.

Here, genome-wide Single Nucleotide Polymorphism (SNP) data is generated from an exceptional global collection of mobulid tissue samples, representing all described species across their geographic ranges and is used to target these knowledge gaps. Phylogenomic reconstruction of the Mobulidae combined with species delimitation based on the multispecies coalescent identifies mismatches between currently recognised species, and species units optimal for conservation under international frameworks. Specifically, an undescribed species of manta ray is shown to be present in sympatry with the oceanic manta ray in the Gulf of Mexico, with some evidence of

hybridisation. In addition, these data show two recently synonymised species to be distinct and reproductively isolated and reveals geographically mediated population structure in several species. Substantial incomplete lineage sorting is uncovered and standing variation in extinct ancestral populations is identified as a driver of phylogenetic uncertainty.

These data show that the lineage corresponding to the undescribed species of manta ray is associated with reduced genetic diversity, consistent with a pattern of peripatric speciation in isolation and highlighting conservation concerns for this species. Hybridisation between the oceanic manta ray, Mobula birostris, and the undescribed species of manta ray is confirmed for the first time but shown not to be associated with introgression. Such insights suggest that hybrids may be inviable, with conservation implications where unsuccessful reproductive investment in hybrid offspring is a concern in these species with slow life history traits. Extremely rapid and complete speciation in a marine system is presented, and highlights concerns associated with anthropogenic climate change and accompanying sea level changes on evolution in the oceans.

Population genetic structure is compared between two species of manta ray with contrasting habitat preferences. Whilst the reef manta ray (Mobula alfredi), shows a high degree of population structure among sampling locations, genome-wide SNPs indicate global genetic panmixia in the oceanic manta ray (Mobula birostris). Declining genetic diversity across the Pacific Ocean may be suggestive of successive founder events in the reef manta ray. Global genetic panmixia in oceanic manta rays may relate to past demographic processes, or differential dispersal among life stages. These highly contrasting patterns highlight the importance of evaluating population structure and adaptive divergence individually for related species of conservation concern, rather than relying on an assumption that closely related species display similar patterns. Collectively, our findings provide a substantial contribution to current knowledge pertaining to manta and devil rays.

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Chapter 1

General Introduction

Elements of this Chapter are discussed in a published paper:

Stewart JD, Jaine FRA, Armstrong AJ, Armstrong AO, Bennett MB, Burgess KB, Couturier LIE, Croll DA, Cronin MR, Deakos MH, Dudgeon CL, Fernando D, Froman N, Germanov ES, Hall MA, Hinojosa-Alvarez S, **Hosegood JE**, Kashiwagi T, Laglbauer BJL, Lezama-Ochoa N, Marshall AD, McGregor F, Notarbartolo di Sciara, G, Palacios MD, Peel LR, Richardson AJ, Rubin RD, Townsend KA, Venables SK and Stevens GMW. 2018. Research Priorities to Support Effective Manta and Devil Ray Conservation. Frontiers in Marine Science **5**:314. https://doi.org/10.3389/fmars.2018.00314.

See Appendix I: Published Work for copy.

The PhD candidate is a co-author on this paper and contributed by leading Section 1: Taxonomy and Diversity, which was written in collaboration with Mike Bennett, Tom Kashiwagi, Silvia Hinojosa-Alvarez and Giuseppe Notarbartolo di Sciara, and contributing Section 5.4: Fisheries Impacts on Genetic Diversity, included within Section 5: Fisheries and Bycatch, which was led by Daniel Fernando.

Chapter 1: General Introduction

1.1 Anthropogenic Threats to the Marine Environment

Anthropogenic pressure on the world's oceans is an issue of global concern. The human population now exceeds 7 billion individuals, and pressures on natural resources continue to grow in order to meet international demands for food, water, fuel and other essential services (Ezeh et al. 2012). Covering over two-thirds of the Earths' surface, the oceans are vitally important for global food security (Funk & Brown, 2009; Smith et al. 2010; Rice & Garcia, 2011), with many communities dependent on fish and seafood as their primary source of animal protein (Kent, 1997). Furthermore, healthy ocean ecosystems provide services including absorption of anthropogenic carbon (Sabine et al. 2004; McKinley et al. 2017), coastal protection (Mazda et al. 1997; Kathiresan & Rajendran, 2005), regulation of weather and climate (Macdonald & Wunsch, 1996; Toggweiler & Russell, 2008) and are of cultural importance, in addition to providing further economic value through tourism (Brander et al. 2006; Farr et al. 2014). As such, goods and services provided by marine ecosystems are valued at US$ 2.5 trillion annually (Hoegh-Guldberg et al. 2015).

The relationships between biodiversity and ecosystem functioning, services and stability have been well-studied, with many studies reporting a typically positive relationship (Hector & Bagchi, 2007; Willig, 2011; Mace et al. 2012). In the marine realm, the link between biodiversity, productivity and associated services has been contentious (Duarte, 2000; Covich et al. 2004), although comprehensive evaluation of global data has associated biodiversity loss with resource collapse and declining stability and recovery potential (Worm et al. 2006). As such, targeted management of marine ecosystems for biodiversity is recommended (Palumbi et al. 2009). Healthy oceans are therefore of economic, political, social and environmental importance. However, despite these clear incentives and benefits of the sustainable use and management of marine resources, human activity is widely responsible for marine biodiversity loss (Edgar et al. 2005; McCauley et al. 2015).

Anthropogenic threats to overall ocean health and productivity encompass a broad range of activities and issues. Human-mediated climate change is expected to lead to local extinctions (Cheung et al. 2009) and will continue to disrupt current dynamics (Hoegh-Guldberg & Bruno, 2010), with further implications for highly productive upwelling systems (Bakun et al. 2010; Sydeman et al. 2014) and associated recruitment levels (Brunel & Boucher, 2007). Increased sea surface temperatures have been associated with damage to highly diverse coral reef ecosystems (Sully et al. 2019) and are correlated with decreases in plankton biomass, which represents the base of marine food webs (Hays et al. 2005; Wiafe et al. 2008). Furthermore, unsustainable fishing practices, bycatch and a lack of reliable data threaten commercially and ecologically important fish stocks and constrain the implementation of informed and appropriate management strategies (Pauly et al. 2002; Dulvy et al. 2008). Additional threats include predator loss, which may drive trophic cascades and changes in community structure (e.g. Estes & Palmisano, 1974; Myers et al. 2007a), pollution, particularly involving long-lasting and highly damaging plastics and microplastics (e.g. Andrady, 2017; Worm et al. 2017; Germanov et al. 2018) and habitat destruction (e.g. Althaus et al. 2009; Waycott et al. 2009).

1.1.1 Fisheries and Bycatch

Commercial and small-scale fisheries contribute substantially to the global economy (Dyck & Sumaila, 2010; Teh & Sumaila, 2013). Overfishing, where stocks are removed at a rate that exceeds their ability to recover, is a widespread and ongoing problem (e.g. Jackson et al. 2001; Pauly et al. 2002; Ding et al. 2017), and has led to a number of high profile collapses, such as in North Atlantic cod stocks (Rosenberg et al. 2005; Myers et al. 2007b). Inappropriate management and quotas are predicted to result in further collapses of commercially and ecologically important fish species, such as bluefin tuna (MacKenzie et al. 2009). In addition, fishing techniques such as trawling are highly damaging to ocean ecosystems (Bremner, 2008; Althaus et al. 2009).

To successfully evaluate the effect of fishing on marine ecosystems, realistic and accurate estimations of stock removed are necessary (Pitcher et al. 2002). However, Illegal, Unreported and Unregulated (IUU) fishing compromises the ability to make such estimations and implement appropriate quotas. Illegal and Unreported catches have a global estimated value

of between US$ 10 billion and US$ 23.5 billion annually (Agnew et al. 2009), and it is estimated that IUU fishing makes up between 15-46% of global catch (Pauly et al. 2002; Tinch et al. 2008; Agnew et al. 2009). Compounding the problem, large areas of international waters remain effectively unmanaged (Dulvy et al. 2008). Furthermore, despite being notoriously difficult to assess, bycatch of non-target species is estimated to represent about 40% of marine catches globally (Davies et al. 2009). Fishing vessels may discard catch composed of undersized or non-commercial species in order to maximise returns within their quotas, and logbooks may underreport bycatch in order to feign compliance with fisheries legislation (Lewison et al. 2004). Furthermore, there are obstacles to be overcome when estimating levels of bycatch which could be considered sustainable due to a lack of available data for non-target species (Moore et al. 2013). Bridging this gap in knowledge is constrained by logistical, financial and legislative aspects of accessing highly mobile pelagic wildlife (Lewison et al. 2004; Capietto et al. 2014).

Discard bans, such as that recently implemented throughout the European Union in 2015 and 2016 for pelagic and demersal species respectively, require vessels to land the entirety of their catch, and are intended to address the issue of bycatch and other sources of IUU fishing by reducing uncertainty in available data. However, concerns have been raised regarding the unintended consequences of such policies, especially if unwanted landings are not counted towards total allowable catch, which reduces incentives for selective fishing and increases mortality in non-target species (Sardà et al. 2015). Furthermore, discard bans may result in increased mortality of fish at sensitive life-stages (e.g. Bellido et al. 2017), or have disproportionate impacts on specific functional groups (e.g. Moutopoulos et al. 2018), with further ecological implications. It is therefore widely recognised that such policies should be coupled with other informed management measures (Condie et al. 2014). The issue of quantifying mortality from fisheries is further complicated by the requirement to identify fish and their products down to species and region of origin in order to determine the legality of the catch (Martinsohn & Ogden, 2008; Martinsohn & Ogden, 2009; Nielsen et al. 2012). Furthermore, illegal mislabelling of fish products, often as species which fetch a higher price, is widespread and can result in the unregulated exploitation of additional stocks (Helyar et al. 2014).

Marine megafauna, such as marine mammals, turtles, seabirds and elasmobranchs (sharks

and rays) are considered to be especially vulnerable to the effects of commercial fishing. Larger marine species are particularly threatened by bycatch, due to their propensity to interact with fisheries (Lewison et al. 2004; Senko et al. 2014), and may be further negatively affected by prey depletions caused by intensive fishing (Bearzi et al. 2006). Fisheries bycatch is estimated to affect 67% of all megafauna species, and 86% of elasmobranchs (Žydelis et al. 2009). Overexploitation of elasmobranch megafauna is likely to be met with population declines due to some common life-history traits. Populations are slow to recover from exploitation due to generally slow growth rates, late maturity, long lifespans with associated long generation times, and low reproductive rates (Dulvy et al. 2008; Dulvy et al. 2014; Croll et al. 2016; Pardo et al. 2016a). Indeed, unsustainable fishing practices are considered responsible for various declines in elasmobranch populations (e.g. Robbins et al. 2006; Spaet et al. 2016).

1.1.2 Challenges in Marine Conservation

Conservation of the marine environment is challenging as habitats often span the jurisdiction of multiple governments or fall outside of governed or administrated areas altogether. In addition, a perceived lack of physical barriers in the ocean has historically been thought to result in highly-mobile, homogeneous fish populations of inexhaustible size (Hauser & Carvalho, 2008). Indeed, fish populations commonly migrate (e.g. Kallio-Nyberg et al. 1999; Hunter et al. 2003), highlighting the need for protection and legislation on a regional or international scale. However, there are also now numerous examples of fish stocks that exhibit a high degree of structuring (e.g. Bembo et al. 1996; Henriques et al. 2017; Westgaard et al. 2017; Lehnert et al. 2019), and management units may not align with biological populations (Reiss et al. 2009; Kerr et al. 2017; Mullins et al. 2018).

Conservation of marine megafauna may benefit from mitigating bycatch effects; for example by modifying fishing gear (Senko et al. 2014), or designating important habitats as Marine Protected Areas (MPAs) (Hooker & Gerber, 2004; Game et al. 2009). However, actively protecting marine megafauna by designing spatial management plans is precluded by the high mobility of many species (Agardy et al. 2011; McClellan et al. 2014). Protection also requires the cooperation of multiple governances and legislative bodies, in addition to the expensive

and time-consuming task of enforcing such protective legislation (Rudd et al. 2003). Habitats utilised for foraging and breeding and the migratory corridor between these should be protected (Hooker & Gerber, 2004). However, for many species, the position of these important habitats in space and time are still unknown. Whilst advances in techniques such as satellite telemetry produce data to address these knowledge gaps (e.g. Wilson et al. 2006; Domeier & Nasby-Lucas, 2008; Bonfil et al. 2009; Daly et al. 2018), sample sizes are generally small, and data may only be available for limited life history stages or represent a single sex (e.g. Rohner et al. 2018).

The Convention on the Conservation of Migratory Species of Wild Animals (CMS) requires signatory countries to actively put in place strategies to conserve species listed under the Convention and therefore represents an important example of international cooperation in the conservation and management of highly mobile pelagic species. At the time of writing, the CMS Appendices include 39 elasmobranch species, 77 species of cetacean, all 7 species of marine turtle and over 40 species of seabird. In addition, 52 species of elasmobranch have been listed on the Convention on the International Trade of Endangered Species of Wild Fauna and Flora (CITES) since 2003, which regulates trade in parts or derivatives of listed species (Vincent et al. 2014; Cochrane 2015). Despite the role of CITES in the conservation of marine fish remaining controversial (Vincent et al. 2014; see Section 5.2 for discussion), listings of shark and ray species have attracted support in the last two decades, largely in response to demand for fins (Clarke et al. 2006a; Clarke 2007) and gill plates (Couturier et al. 2012; O'Malley et al. 2017). However, difficulties in identifying parts in trade to species level constrain effective enforcement (Clarke et al. 2006b; Steinke et al. 2017), and indeed, illegal fishing of elasmobranchs remains a significant concern (e.g. Carr et al. 2013).

1.2 Applications of Molecular Ecology in Conservation

Molecular ecology uses discrete, stable and heritable genetic markers to improve understanding of populations and species, through to the structure and functioning of ecosystems (Funk et al. 2012; Shafer et al. 2015; Evans et al. 2016; Flanagan et al. 2018; da Silva & Fabre, 2019). Applications of molecular ecology are diverse, including but not limited

to population and evolutionary genetics, phylogeography, landscape (and seascape) genetics, behavioural ecology, taxonomy and systematics, phylogenetics, biodiversity characterisation and monitoring and wildlife forensics (see Andrew et al. 2013). Recent advances in high-throughput sequencing and associated genomic technologies have facilitated the generation of vast quantities of data with smaller investments in terms of time and cost (Rothberg & Leamon, 2008; Metzker, 2010; Zhang et al. 2011; Reuter et al. 2015; Bleidorn, 2016). Such advances have therefore broadened these disciplines and allowed the examination of large numbers of genome-wide molecular markers, increasing the resolving power of studies (Stapley et al. 2010; Funk et al. 2012; Flanagan et al. 2018), with further applications with respect to informing conservation strategies and policy (Allendorf et al. 2010; Shafer et al. 2015). The progression from DNA barcoding of single genes for applications such as recording biodiversity and detecting cryptic species (e.g. Costa & Carvalho, 2010; da Silva et al. 2011; Mat Jaafar et al. 2012) through to metabarcoding of environmental DNA (eDNA) samples for biodiversity characterisation and monitoring of ecosystems (e.g. Taberlet et al. 2012; Deiner et al. 2017; Ruppert et al. 2019) provides a useful example of a recent advance in molecular ecology fuelled by the availability of high-throughput sequencing technologies.

1.2.1 SNP Markers and RADseq as a Discovery Method

Single Nucleotide Polymorphism (SNP) markers are widespread and numerous across genomes, and meet the evolutionary assumptions of simple mutation models (Morin et al. 2004). SNP discovery methods need not make prior assumptions of the genome, making discovery of large numbers of SNP markers for non-model organisms feasible (Garvin et al. 2010; Helyar et al. 2011; Foote & Morin, 2016; Kang et al. 2017). However, concerns have been raised that SNP markers are liable to ascertainment bias where the SNP discovery step is poorly designed, resulting in datasets that are not representative of the populations they are intended to describe (Garvin et al. 2010). Combining SNP discovery methods with Next-Generation Sequencing platforms through genotyping-by-sequencing approaches and/or utilising samples from across the geographic range of the species of interest has been shown to minimise such bias (Rosenblum & Novembre, 2007; Helyar et al. 2011). As such, SNP markers are widely used in population genetics (e.g. Westgaard et al. 2017; Lehnert et al. 2019), phylogenetics (e.g. Leaché et al. 2015; Leaché & Oaks, 2017) and are useful in the

detection of candidate adaptive loci (e.g. Hemmer-Hansen et al. 2013; Keller et al. 2013; Bekkevold et al. 2016), with further uses in applied fields such as wildlife forensics (Ogden, 2008; Ogden, 2011).

One such approach combining SNP discovery with next-generation sequencing in a single protocol is Restriction-Site Associated DNA Sequencing (RADseq). RADseq methods were originally developed from a micro-array genotyping and polymorphism discovery approach used to map mutations in model organisms with available reference genomes (Lewis et al. 2007; Miller et al. 2007). The technique was then successfully combined with next-generation sequencing, allowing for efficient discovery of large numbers of SNPs (Baird et al. 2008). In brief, the method involves cutting genomic DNA with a restriction enzyme, resulting in multiple fragments which are then ligated to P1 adaptors (modified from the Illumina sequencing adaptor) and a unique molecular identifier, or 'barcode' assigned to each individual sample. Barcoded DNA fragments are then pooled and randomly sheared, before being ligated to a P2 adaptor, PCR amplified, size selected and finally, sequenced on an Illumina platform (Baird et al. 2008; Davey & Blaxter, 2010). The resultant short DNA sequences are then processed bioinformatically (e.g. Catchen et al. 2011) to build loci, discover SNPs and call genotypes.

The RADseq technique carries several advantages over traditional PCR-based methods. First, there is no requirement for any prior knowledge of the genome, since loci can be assembled *de novo,* making SNP markers accessible for studies of non-model organisms (Davey & Blaxter, 2010; Catchen et al. 2011; Hohenlohe et al. 2011). Second, RADseq can be used to effectively sample the genome, generating sequence data for hundreds of thousands of loci and enabling discovery of many thousands of SNPs associated with both neutral and adaptive variation, thereby producing a dataset that is considered a reduced representation of the genome (Davey et al. 2011). Importantly, the data are reproducible, allowing alignment of RADtags across individuals and species for comparison (Baird et al. 2008; Hohenlohe et al. 2011). In addition, the method is very flexible, requiring relatively small amounts of genomic DNA and allowing the number of markers discovered to be closely controlled through choice of restriction enzyme (Davey et al. 2011).

RAD sequencing is not without its limitations, however, and concerns have been raised regarding potential biases introduced by mutations in restriction sites and associated allele

dropout, which may affect estimates of genetic variation (Arnold et al. 2013; Gautier et al. 2013). Further challenges are apparent in studies involving polyploid organisms, where heterozygotes do not necessarily conform to allele frequency assumptions and may therefore be erroneously called as homozygotes (Ogden et al. 2013). In both cases, such challenges may be overcome with sufficient depth of coverage (Gautier et al. 2013; Ogden et al. 2013), highlighting the importance of high-quality genomic DNA, and the need to carefully consider choice of restriction enzyme, the number of individuals to be multiplexed and genome size, all of which have an effect on sequencing depth of RAD markers (Davey et al. 2011).

RAD methods are very amenable to modification for purpose, and a number of variations on the original protocol (Baird et al. 2008) have been published. For example, ezRAD employs a simplified protocol in order to make the methods available in labs with limited facilities (Toonen et al. 2013). Another example of a modified protocol is double digest RAD or ddRAD (Peterson et al. 2012), which involves using a pair of restriction enzymes and excluding the shearing stage of the protocol. The ddRAD method therefore only amplifies tags between restriction sites that sit close together on the genome, so reducing the number of tags sequenced and increasing efficiency and potential to multiplex large numbers of individuals whilst maintaining sequence depth (Peterson et al. 2012).

The flexibility of the approach lends RAD sequence data to a wide variety of applications, and its utility has been demonstrated in studies of population genetics (e.g. Davey & Blaxter, 2010; Hohenlohe et al. 2010; Kang et al. 2017), genetic mapping (e.g. Baird et al. 2008; Baxter et al. 2011), adaptive variation (e.g. Hohenlohe et al. 2010; Gagnaire et al. 2013; Wagner et al. 2013), species delimitation (e.g. Wagner et al. 2013; Pante et al. 2014; Herrera & Shank, 2016) and phylogenetics (e.g. Rubin et al. 2012; Cariou et al. 2013; Cruaud et al. 2014; Viricel et al. 2014; Herrera & Shank, 2016; Tripp et al. 2017).

1.2.2 Species delimitation

Effective measures for the conservation of biodiversity require an understanding and characterisation of diversity within and among species. Biodiversity conservation is enacted through global conventions and regulatory frameworks, including the Convention on the International Trade in Endangered Species of Wild Fauna and Flora (CITES), the Convention

on the Conservation of Migratory Species of Wild Animals (CMS) and the Convention for Biological Diversity (CBD). Such frameworks are implemented through national legislation acting at the species level (Vincent et al. 2014; Donaldson et al. 2016). Effective wildlife management, protection and law enforcement therefore depend on unambiguous classification into biologically relevant species units. Recent examples of proposed taxonomic revisions having far-reaching consequences for biodiversity conservation include giraffe (Fennessy et al. 2016, Fennessy et al. 2017; Bercovitch et al. 2017) and African elephant (Roca et al. 2001), where genetic research has led to possible reclassification and consequent changes to the legal status of these threatened megafauna.

Species delimitation, the process by which populations of individuals are grouped into reproductively isolated and separately evolving units, is therefore a fundamental application of genomic data to biodiversity conservation. However, the field remains constrained by the lack of a single universal species concept (De Queiroz 2007; Frankham et al. 2012), and the criteria for defining species taxa is the subject of fierce debate. For the purposes of effective conservation however, species units that minimise harm and maximise benefit by defining species units on the basis of reproductive isolation are recommended (Frankham et al. 2012). Studies attempting to define such units may benefit from considering named species taxa as explicit hypotheses for reproductively isolated species units in nature (Hey et al. 2003). Reproductive isolation may be demonstrated by very limited gene flow where species occur in sympatry, or by a lack of shared alleles coupled with reduced fitness in hybrids for allopatric populations, thus incorporating both the Biological Species Concept and the Differential Fitness Species Concept (Frankham et al. 2012). The Phylogenetic Species Concept (PSC), which assigns species status to monophyletic groups, is inherently problematic, especially in allopatric populations since monophyletic groups may simply represent population differentiation rather than true speciation. The PSC is therefore sensitive to sampling strategy and risks over-splitting groups that may not be fully reproductively isolated resulting in dangerously small populations and precluding capacity for genetic rescue (Frankham et al. 2012).

Molecular ecology and conservation genetics provide opportunities for quantifying diversity across space and time (Allendorf et al. 2010) and such approaches are increasingly powerful with the growing incorporation of genome-wide data. Accordingly, species delimitation has

received increasing attention in recent years, with numerous methods now available (e.g. Carstens et al. 2013; Zhang et al. 2013; Grummer et al. 2014; Leache et al. 2014; Rannala, 2015; Yang, 2015). Traditional approaches typically relied upon morphological observation, often resulting in artificially broad delineations arising from difficulties in detecting and identifying cryptic species (Frankham et al. 2012). More recently, DNA sequencing has allowed genetic data to be utilised for species delimitation, although early approaches were limited to information from only a few genes or markers. These early approaches left interpretation challenging, particularly in recently diverged groups with substantial incomplete lineage sorting (Maddison, 1997; Maddison & Knowles, 2006). However, coalescent-based approaches and the availability of high-throughput sequencing technologies now allow the detection of lineage separation despite discordant gene trees, demonstrating the utility of genome-wide data for species delimitation irrespective of incomplete lineage sorting (Knowles & Carstens, 2007), and effectively detecting reproductive isolation. As such, genome-wide multi-locus approaches have increased the resolution of species delimitation studies and have been used to clarify contentious relationships and phylogenies (e.g. Leache et al. 2014; Herrera & Shank, 2016) and disclose previously unknown diversity (e.g. Pante et al. 2014). In addition, there are further applications in the characterisation of both Conservation Units and Evolutionarily Significant Units (Funk et al. 2012).

1.2.3 Population Genetic Structure and Adaptive Variation

The declining cost and increasing accessibly of Next Generation Sequencing approaches (e.g. Metzker, 2010; Zhang et al. 2011; Reuter et al. 2015; Bleidorn, 2016) provide insightful opportunities for studying population genetics and applying results to conservation and fisheries management. The preservation of genetic diversity is one of the key goals of conservation research, since a loss of genetic diversity in isolated populations is associated with reduced adaptive potential and increased vulnerability to extinction (Spielman et al. 2004; Frankham, 2005; Sgrò et al. 2011; Hellmair & Kinziger, 2014). As such, understanding of population structure (the extent and distribution of genetic diversity) and the extent and nature of adaptive variation is essential for implementing informed and appropriate management strategies, where genetically discrete and/or locally adapted populations will

likely benefit from independent management (Allendorf et al. 2010; Funk et al. 2012; Shafer et al. 2015; Flanagan et al. 2018). In order to promote the conservation of evolutionarily resilient populations, the importance of coupling an understanding of population boundaries with the extent and patterns of migration, gene flow and connectivity therefore cannot be understated. Such inferences can yield valuable insights into the levels of genetic diversity and adaptive divergence within and among populations of commercially important fish species to enhance detection and study of management units.

DNA-based methodology has been employed previously as a tool to assess fisheries impacts in commercially important fish species. Marine fish were traditionally assumed to have relatively low levels of genetic structuring across essentially panmictic populations, due to a presumed lack of physical barriers to gene flow in the oceans (Hauser & Carvalho, 2008). Notwithstanding, there are now numerous examples of clear population genetic structuring in marine fish (e.g. Henriques et al. 2017; Westgaard et al. 2017; Lehnert et al. 2019). It is therefore important to highlight that stocks cannot simply be split along political or geographic boundaries, and that genetically distinct and demographically independent populations may be better managed at some sub-specific or stock level (Reiss et al. 2009; Kerr et al. 2017; Mullins et al. 2018). Furthermore, within well-defined populations, it is imperative that factors such as genetic diversity are considered, in view of the strong evidence for the positive correlation between genetic diversity and a population's resilience to extinction (Spielman et al. 2004; Frankham, 2005; Sgrò et al. 2011; Hellmair & Kinziger, 2014). Indeed, overexploited fish species have been shown to exhibit lower genetic diversity than closely related species that are not overharvested, and are therefore potentially more vulnerable to environmental change and other pressures (Hauser et al. 2002; Pinsky & Palumbi, 2014). Such considerations are significant, especially given that effective population sizes (N_e), broadly the number of breeding individuals, of a commercially important marine fishery may be much lower than previously anticipated, with associated concerns regarding viability of populations (Hauser et al. 2002).

Importantly, this realisation of population genetic structure in marine fish raises concerns that locally adapted populations which are commercially exploited cannot simply be replenished by migration from refugia whilst maintaining locally adaptive variation; leaving the population more vulnerable to environmental change (Hauser & Carvalho, 2008). Indeed, there are now

several examples of localised genomic regions exhibiting strong population structure in fish (e.g. Hemmer-Hansen et al. 2013; Malinsky et al. 2015; Larson et al. 2017). Furthermore, such genomic regions may also be characterised by reduced genetic diversity, indicating strong positive selection (Hemmer-Hansen et al. 2013). Such studies highlight the need to understand levels of genetic diversity and adaptive divergence within and among populations of commercially important fish species, as well as understand the boundaries between populations for effective management of resources.

Where marine organisms have a larval life stage, population structure and spatial patterning may be influenced by ocean currents or other oceanographic variables with respect to larval dispersal (White et al. 2010; Young et al. 2015). Furthermore, efforts to define population genetic structure in highly mobile marine fish may be complicated by variability associated with spawning, or with other life history stages in space and time (Bozano et al. 2015). In elasmobranch species, many of which give birth to live young and as such do not aggregate to spawn or have a larval life stage, there are increasing examples of population genetic structure (e.g. Castro et al. 2007; Vignaud et al. 2013; Ashe et al. 2015; Veríssimo et al. 2017). However, studies attempting to elucidate population genetic structure of elasmobranch species may still need to account for complex life history dynamics, such as varying levels of migratory behaviour and residency across life history stages, or reproductive philopatry, which may drive age-related population structure (e.g. Feldheim et al. 2009; Chapman et al. 2014; Klein et al. 2019).

1.2.4 Wildlife Forensics

The applied field of wildlife forensics, which combines forensic techniques developed to produce evidence in criminal cases with conservation biology, has grown as a result of the increased availability of sequence data and understanding of evolutionary processes such as speciation and population genetic structure (Ogden et al. 2009). Wildlife forensics aims to answer questions related to the species and population of origin of a biological sample in order to determine whether there is any contradiction of conservation law and regulation, such as that of CITES. Forensics is especially useful in situations where the sample in question has been processed, for example where fish have been filleted, and the ability to identify the

specimen on morphological traits alone is compromised (Ogden, 2008).

More recently, there has been increased interest in using wildlife forensic techniques to assign a sample of interest to a geographic population of origin. This research focus takes advantage of the genetic structure of populations, and may utilise markers which are associated with genetic regions under selection for local geographic conditions (Ogden & Linacre, 2015). FishPopTrace provides a good example of a high-profile initiative aiming to develop traceability tools for fish populations and products. The FishPopTrace Consortium generated SNP data and scanned for those markers with a high level of divergence between populations. Using high-throughput genome scans, the project was able to identify and subsequently design SNP assays whereby populations of hake, herring, sole and cod could be assigned back to the population of origin with a high degree of accuracy (Nielsen et al. 2012). Such tools are of sufficient robustness to provide forensic evidence in a legal context (Martinsohn & Ogden, 2009). Furthermore, the differences in habit and geographical coverage of the target species demonstrates the utility of techniques such as this in tackling IUU fishing and mislabelling of products on a large scale, and the potential to apply such techniques to many other species (Martinsohn & Ogden, 2009; Nielsen et al. 2012).

1.3 Mobulid Rays

The Mobulidae are a family of large, filter feeding pelagic rays, with wingspans of up to seven metres (Figure 1.1) and represent the target group of the current research. Comprising the manta and devil rays, the group are circumglobally distributed in tropical and subtropical waters (Couturier et al. 2012) and consists of a single genus, *Mobula*, within which there are 8 species currently recognised: *Mobula alfredi*, *M. birostris*, *M. mobular*, *M. tarapacana*, *M. thurstoni*, *M. kuhlii*, *M. hypostoma* and *M. munkiana*. Until recently, 3 additional species were also recognised: *Mobula japanica*, *M. eregoodootenkee* and *M. rochebrunei*, now considered to be junior synonyms of *M. mobular*, *M. kuhlii* and *M. hypostoma* respectively (White et al. 2017). Mobulid rays represent an important and charismatic component of marine ecosystems, and provide substantial economic benefits to developing countries through ecotourism (O'Malley et al. 2013). However, they are increasingly threatened by target and

bycatch fisheries supplying the international demand for their gill plates (Couturier et al. 2012; O'Malley et al. 2017). Such impacts will likely be met with catastrophic declines exacerbated by the slow maturation, extended longevity and low reproductive rates of mobulids, rendering these fisheries unsustainable (Dulvy et al. 2014; Croll et al. 2016).

1.3.1 Taxonomy and Evolution

Mobulid rays are elasmobranchs belonging to the Order Myliobatiformes and are most closely related to the cownose rays (Rhinopteridae) (Naylor et al. 2012; Poortvliet et al. 2015). Due to a dearth in biological data, taxonomic relationships within the Mobulidae have been contentious within the community of mobulid researchers in recent years. Until 2017, species were described largely based on morphological and geographic characteristics (Notarbartolo Di Sciara, 1987; Marshall et al. 2009) and as such, the family was split into two genera; *Manta*, including two species of manta ray, and *Mobula*, comprising nine species of devil ray. More recently however, studies have started utilising genetic data to evaluate species relationships within the Mobulidae (e.g. Kashiwagi et al. 2012; Naylor et al. 2012; Poortvliet et al. 2015; White et al. 2017), and recent genetic and morphological evidence suggests that the genus *Manta* may be nested within *Mobula* (Adnet et al. 2012; Poortvliet et al. 2015; White et al. 2017), and therefore invalid.

In 2017, White et al. published a comprehensive evaluation of genetic and morphological datasets for the 11 previously recognized species of mobulid ray. As a result of this taxonomic review, 8 species are currently recognized: *Mobula alfredi* (previously *Manta alfredi*), *Mobula birostris* (previously *Manta birostris*), *Mobula hypostoma* (with *Mobula rochebrunei* considered a junior synonym), *Mobula kuhlii* (with *Mobula eregoodootenkee* considered a junior synonym), *Mobula mobular* (with *Mobula japanica* considered a junior synonym), *Mobula munkiana*, *Mobula tarapacana* and *Mobula thurstoni*. Prior to this study, the most recent major change came with the resurrection of *Manta alfredi* (now *Mobula alfredi*) and with it, the recognition of two species of manta ray (Marshall et al. 2009). Whilst this speciation event has been confirmed with genetic data (Kashiwagi et al. 2012), there remains evidence of both historic and modern hybridization between the two species. In addition, a third putative species of manta ray is hypothesized to occur in the Caribbean and the Gulf of

Figure 1.1: examples of mobulid ray species. Top left: reef manta ray, *Mobula alfredi*, dorsal view. Top right: reef manta ray, *Mobula alfredi*, ventral view. Centre left: oceanic manta ray, *Mobula birostris*, dorsal view. Centre right: oceanic manta ray, *Mobula birostris*, ventral view. Bottom left: Munk's devil ray, *Mobula munkiana*. Bottom right: Spinetail devil ray, *Mobula mobular cf. japanica*. Photographs © Guy Stevens and are reproduced with permission.

Mexico (Marshall et al. 2009; Hinojosa-Alvarez et al. 2016; Stewart et al. 2018a). It is important to note that manta rays (those species formerly of the genus *Manta*) are far more extensively studied and understood than other devil rays, and the fact that the above changes to taxonomy are so recent is symptomatic of the challenges associated with species delimitation, even with modern techniques. Furthermore, while studies have begun to investigate levels of intraspecific diversity within manta rays, the same cannot be said for the remaining Mobulidae, largely due to difficulties in obtaining samples (Stewart et al. 2018b).

Certainly, the taxonomic study published by White et al. (2017) is severely constrained by a lack of representative samples, with a single sample per putative species (of the previously recognized 11) being compared with respect to approximately 1000 nuclear exons and/or mitogenomes. To address taxonomic uncertainties, and validate or refute recent changes, it is necessary to extend comparable, increasingly robust studies. Indeed, in the case of *Mobula kuhlii* and the now invalid *Mobula eregoodootenkee*, the authors argued that this revision is based on the best available evidence whilst acknowledging that a similar study incorporating population level sampling might resolve them as distinct (White et al. 2017). It is important to note that in all cases of reported synonymy, the close genetic relationship between collapsed species observed by White et al. (2017) is of a comparable magnitude to that detected for *Mobula alfredi* and *M. birostris*. Yet a study encompassing more detailed population level sampling of these species has resolved them as distinct, whilst showing evidence for some historical introgression (Kashiwagi et al. 2012). The challenge therefore is distinguishing between distinct species where hybridization may occur, and single species, and there is a need to consider interactions between species given current evidence for secondary contact in manta rays (Kashiwagi et al. 2012; Walter et al. 2014).

The Mobulidae are characterized by recent divergence times, with divergence from Rhinoptera estimated to have occurred only 30 million years ago (MYA), followed by relatively short bursts of speciation (Poortvliet et al. 2015), of which the most recent event known occurred only 0.5MYA (Kashiwagi et al. 2012). The age of these divergences may allow for widespread secondary contact and introgression between mobulid species, further encumbering efforts to define species boundaries. Furthermore, there is evidence to suggest that periods of rapid speciation within the Mobulidae correspond to episodes of global warming and associated changes in upwelling intensity and productivity, and it is

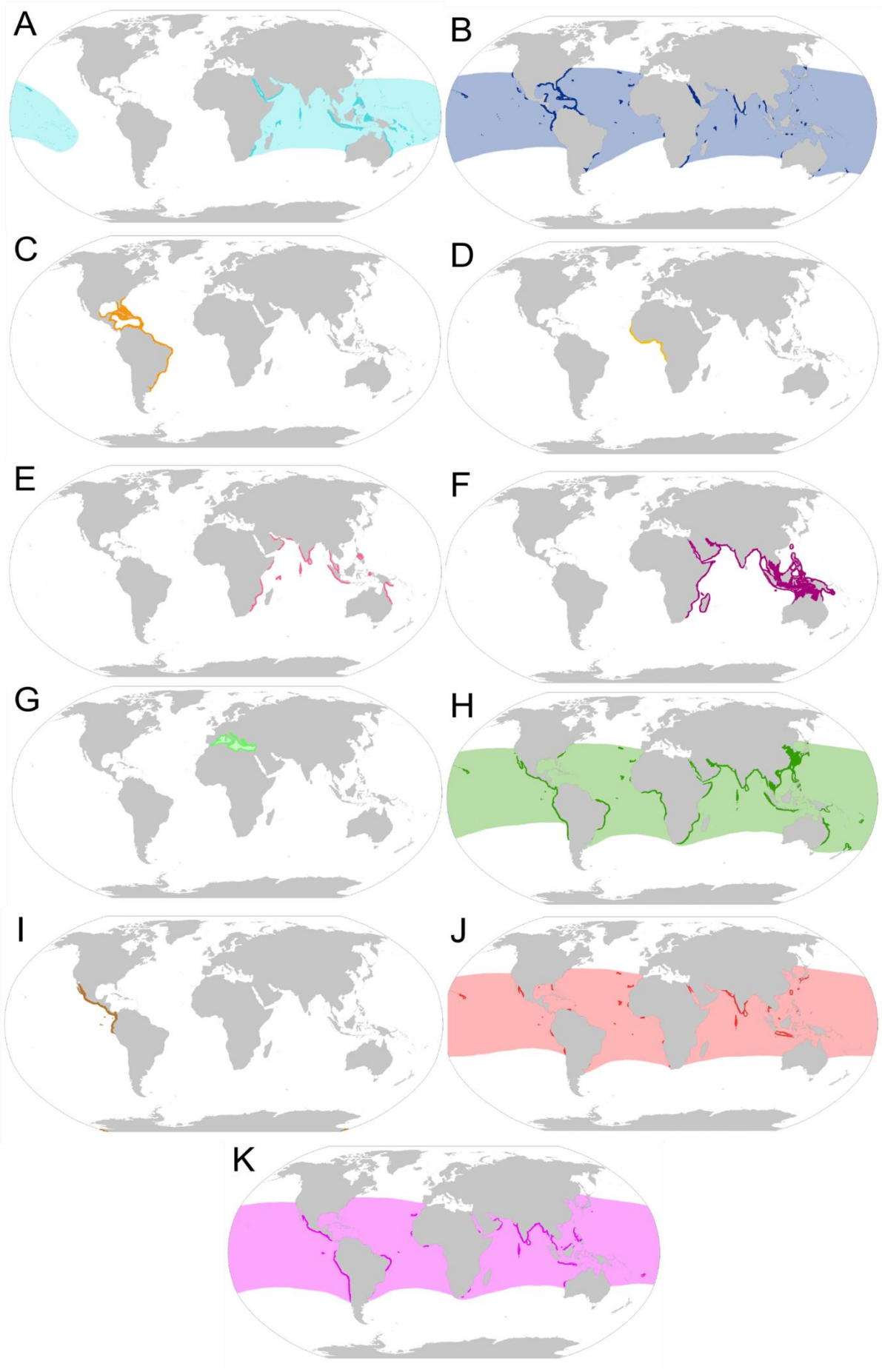
hypothesized that this led to fragmentation and subsequent divergence with respect to feeding strategies (Poortvliet et al. 2015). Indeed, prominent differences in morphology between *Mobula kuhlii* and specimens formerly attributed to *Mobula eregoodootenkee* (Notarbartolo Di Sciara, 1987) and particularly the marked differences in gill plate morphology (Paig-Tran et al. 2013), which are essential to the filter feeding strategy of mobulid rays, may lend support to this hypothesis.

Unless otherwise stated, mobulid species are referred to with nomenclature considered valid following White et al. (2017) throughout this thesis. Where it is necessary to refer to variants that are not considered to be valid species at the time of writing, these are given with *cf*. For example, the previously recognised species *Mobula eregoodootenkee,* recently synonymised with *Mobula kuhlii* (White et al. 2017) will be denoted as *Mobula kuhlii cf. eregoodootenkee*. The term 'manta ray' is used to refer collectively to species formerly of the genus *Manta*: *Mobula alfredi* and *Mobula birostris*, and species identified therein, since this common and globally recognised name is still in regular use.

1.3.2 Distribution and Habitat Use

Mobulid rays are circumglobally distributed in tropical and subtropical waters. Larger species, such as *Mobula alfredi*, *M. birostris*, *M. mobular*, *M. tarapacana* and *M. thurstoni* are found throughout a much broader range than the pygmy species *M. munkiana*, *M. hypostoma* and *M. kuhlii*, which are have more restricted ranges (Figure 1.2).

Manta rays have been shown to exhibit a high degree of residency and site fidelity (e.g. Graham et al. 2012; Jaine et al. 2014; Stewart et al. 2016; Couturier et al. 2018), although relatively large scale movements across regions have been documented (Germanov & Marshall, 2014). Both manta and devil rays are known to be highly aggregative (Couturier et al. 2012) and whilst the reasons for such aggregations remain elusive, suggestions include taking advantage of highly productive feeding grounds, cleaning station visits or coming together for breeding purposes (Notarbartolo di Sciara 1988; Dewar et al. 2008; Armstrong et al. 2016). In addition, size segregation appears to be widespread within the group, with juveniles largely absent from adult aggregation sites (Notarbartolo di Sciara, 1988).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1.3.3 Biology and Ecology

Mobulid rays feed primarily on zooplankton, which they filter from the water using specialised branchial sieving plates, aided by cephalic fins (Notarbartolo di Sciara, 1987; Couturier et al. 2012; Paig-Tran et al. 2013; Rohner et al. 2017). However, small teleost fish may make up a significant portion of the diet for some species (Stewart et al. 2018c). Sympatry of species is suggested to be driven by trophic overlap where different species target similar prey (Stewart et al. 2017).

Detailed data on the life history of mobulid species is distinctly lacking (Stewart et al. 2018b), although life histories are characterised typically by long generation times, late maturity and low reproductive rates (Dulvy et al. 2014; Croll et al. 2016; Pardo et al. 2016a). Indeed, interbirth periods of between 2-7 years have been recorded in the reef manta ray, *Mobula alfredi*, and is likely to be influenced by ocean productivity and resource availability (Deakos, 2012; Marshall & Bennett, 2010). Courtship and mating behaviour has been observed in manta rays, but is rare in other species (Stevens et al. 2018a). Gestation is thought to last approximately 1 year, after which a female will give birth to a single large pup (Marshall & Bennett, 2010). Parturition is thought to be followed immediately by copulation, although direct observations of this behaviour are extremely rare (Uchida et al. 2008; Stevens et al. 2018a).

Despite a sharp increase in the number of articles published in peer-reviewed journals focussing specifically on mobulid rays (see Couturier et al. 2012 for discussion), many aspects of their biology and ecology remains poorly understood, hindering the ability of scientists to advise with respect to management (Stewart et al. 2018b).

Figure 1.2 (opposite): distributions of mobulid ray species and formerly recognised species, a) *Mobula alfredi,* b) *M. birostris*, c) *M. hypostoma*, d) *M. hypostoma cf. rochebrunei* e) *M. kuhlii*, f) *M. kuhlii cf. eregoodootenkee*, g) *M. mobular*, h) *M. mobular cf. japanica* i) *M. munkiana*, j) *M. tarapacana*, k) *M. thurstoni*. Darker areas indicate confirmed range, lighter areas indicate expected range. Note that these maps pre-date the taxonomic revision published by White et al. (2017) and as such, maps c) and d), e) and f), and g) and h) combined can be considered to represent the distributions of currently valid species. Maps reproduced with permission from the Manta Trust.

1.3.4 Threats, Population Trends and Knowledge Gaps

Mobulid rays are threatened by targeted and bycatch fisheries, primarily driven by demand for their gill plates (Figure 1.3), which are used as a health remedy in parts of Asia (e.g. Couturier et al. 2012; O'Malley et al. 2017). Such fishing pressure is considered unsustainable, due to their slow life history traits, hindering recovery from fishing impacts (Dulvy et al. 2014; Croll et al. 2016), and indeed substantial declines have been observed (e.g. Ward-Paige et al. 2013; Lewis et al. 2015). Many species of mobulid ray are listed on the IUCN Red List as Near Threatened or Vulnerable, and many are Data Deficient, highlighting the need for urgent assessment of stock structure (see Table 1.1 for summary of threats and population trends).

To address concerns and regulate the trade in gill plates, the formerly recognised genus *Manta* was listed on Appendix II of the Convention for the International Trade in Endangered Species of Wild Fauna and Flora (CITES) in 2013 and the remaining devil rays (*Mobula* spp.) were added in 2016. Yet morphological similarities and taxonomic uncertainties make identification of mobulid rays and their traded parts challenging (Stevens et al. 2018b); unless customs officials can positively confirm species ID and region of origin, CITES law enforcement by the appropriate country's Management Authority is hindered.

Whilst photographic identification studies which make use of belly-spot patterns unique to each individual have been useful for estimating census population sizes and recording movements (e.g. Deakos et al. 2011; Marshall et al. 2011), a lack of scientific understanding of stock structure, population boundaries and effective population sizes further hinders the ability to implement informed management strategies. In addition, genetic diversity within

Figure 1.3: mobulid gill plates. Left: Spinetail devil ray (*Mobula mobular*) gill plate. Right: Sicklefin devil ray (*Mobula tarapacana*) gill plates. Photographs © Guy Stevens and are reproduced with permission.

Table 1.1: Summary of IUCN assessments of mobulid species. Note that several of these assessments pre-date the taxonomic revision published by White et al. (2017). As such, these species were reassessed during an IUCN workshop for pelagic species in November 2018, although these reports are not yet available.

Species	Common Name(s)	IUCN classification	Threats	Population trend	Reference
Mobula alfredi	Reef manta ray	Vulnerable	Targeted and bycatch fishing, gill plate trade, entanglement, habitat degradation, climate change, pollution, ingestion of plastics and irresponsible tourism practices.	Decreasing	Marshall et al. 2018a
Mobula birostris	Oceanic manta ray or giant manta ray	Vulnerable	Targeted and bycatch fishing, gill plate trade, entanglement, habitat degradation, climate change, pollution, ingestion of plastics and irresponsible tourism practices.	Decreasing	Marshall et al. 2018b
*Mobula hypostoma**	West Atlantic pygmy devil ray or Atlantic devil ray	Data Deficient	Bycatch fisheries	Unknown	Bizzarro et al. 2009a
*Mobula hypostoma cf. rochebrunei**	East Atlantic pygmy devil ray or Lesser Guinean devil ray	Vulnerable	Targeted and bycatch fisheries	Unknown	Valenti & Kyne, 2009
*Mobula kuhlii**	Shorthorned pygmy devil ray or Shortfin devil ray	Data Deficient	Targeted and bycatch fisheries, gill plate trade.	Decreasing	Bizzarro et al. 2009b
*Mobula kuhlii cf. eregoodootenkee**	Longhorned pygmy devil ray	Near Threatened	Bycatch through entanglement in fishing gear.	Unknown	Pierce & Bennett, 2003
*Mobula mobular**	Spinetail devil ray or Giant devil ray	Endangered	Targeted and bycatch fisheries.	Decreasing	Notarbartolo di Sciara et al. 2015
*Mobula mobular cf. japanica**	Spinetail devil ray	Near Threatened	Targeted and bycatch fisheries.	Unknown	White et al. 2006
Mobula munkiana	Munk's pygmy devil ray	Near Threatened	Targeted and bycatch fisheries.	Unknown	Bizzarro et al. 2006
Mobula tarapacana	Sicklefin devil ray	Vulnerable	Targeted and bycatch fisheries, gill plate trade.	Decreasing	Pardo et al. 2016b
Mobula thurstoni	Bentfin devil ray	Near Threatened	Targeted and bycatch fisheries, gill plate trade.	Decreasing	Walls et al. 2016

*Assessment pre-dates taxonomic revision published by White et al. (2017) and so classification may no longer be relevant.

well-defined populations, linked with the ability of populations to adapt to environmental change (Frankham, 2005; Sgrò et al. 2011; Hellmair & Kinziger, 2014) is largely unknown. Indeed, concerns have been raised regarding how this group of filter-feeding megafauna will respond to anthropogenic climate change and associated changes in the oceans (Couturier et al. 2012).

Population genetic structure of mobulid rays is very poorly understood, although there is some evidence to suggest that the oceanic manta ray, *Mobula birostris*, is spatially structured (Stewart et al. 2016). However, at the time of writing, there are no published studies examining population genetic patterns in other species, despite the existence of active fisheries and substantial losses from bycatch (see Stewart et al. 2018b). Such a dearth of information is fuelled by difficulties in obtaining genetic samples and it is for this reason that researchers can benefit from collaboration with other academics and with stakeholders for effective sample sharing (Stewart et al. 2018b). Sampling efforts are often opportunistic, and in many regions only a handful of samples may be available, with vast areas across the known ranges of mobulid species unrepresented in currently available sample sets. Furthermore, samples from across meaningful temporal scales, such as across generations, remain severely limited for mobulid rays, and can be invaluable for establishing demographic trends. The importance of sampling strategy cannot be understated, as it will likely have a huge effect on the outcome of downstream analyses (Meirmans, 2015).

Satellite and acoustic telemetry and similar techniques have however, been embraced by the community of mobulid researchers, although there is currently a heavy bias in current knowledge and research effort towards manta rays over devil rays (see Stewart et al. 2018b). Numerous studies have indicated a high degree of residency in both described species of manta ray, failing to uncover evidence of long-range migrations (e.g. Jaine et al. 2014; Stewart et al. 2016; Couturier et al. 2018). However, extensive connectivity between Marine Protected Areas (MPAs) has been shown in several studies (Deakos et al. 2011; Graham et al. 2012; Germanov & Marshall, 2014), highlighting conservation challenges for these species. To coordinate conservation efforts, in 2014 *Manta alfredi* (now *Mobula alfredi*) and the remaining devil rays joined *Mobula birostris* on Appendices I and II of the Convention on the Conservation of Migratory Species of Wild Animals (CMS), which requires signatories to actively put strategies in place to conserve species listed under the Convention. However,

knowledge gaps are a significant barrier to implementing informative and appropriate management and conservation strategies.

Finally, mobulid rays are known to be affected by ingestion of plastics (Germanov et al. 2018), entanglement in discarded fishing gear, habitat degradation and irresponsible tourism where the ability of rays to visit important feeding habitats and cleaning stations becomes hindered (Couturier et al. 2012).

1.4 Aims and Objectives

The principle aim of this thesis is to evaluate the phylogenetics, population structure, demographic history, speciation and adaptation in manta and devil rays to effectively inform conservation policy. Further, to produce data towards a genetic means of identification of all Mobulids to aid in the enforcement and monitoring of these species under CITES. Specific objectives are to:

1. Perform genome-wide phylogenetic reconstruction of the Mobulidae to address taxonomic ambiguity and clarify recent revisions.
2. Assess differentiation at the genomic level between the recently diverged species of manta ray.
3. Describe population structure and demographic history of manta ray species throughout their range.
4. Apply data from patterns of genomic divergence among populations to generate recommendations for prioritising populations for conservation
5. Produce data to facilitate species identification and traceability of mobulid ray products to address monitoring and enforcement.

These objectives have been achieved using double digest Restriction-Site Associated DNA sequencing (ddRAD) to identify SNP markers of interest.

1.5 Outline of Thesis: Chapter Overview

Chapter 1: General Introduction

This chapter provides a detailed overview of the literature around challenges in marine conservation, with a specific focus on molecular genetic methods and their utility in informing the design of conservation strategies. It also provides an overview of current research and knowledge pertaining to mobulid rays. It highlights the gaps in current knowledge around this charismatic group of marine megafauna and outlines how the current project aims to address some of the key questions, such as the extent and distribution of genetic diversity within the group. Elements of this chapter are discussed in a published paper led by Joshua Stewart: *Front. Mar. Sci* **2018**, 5, 314: doi: https://doi.org/10.3389/fmars.2018.00314. The PhD candidate is a co-author on this paper and contributed by leading Section 1: Taxonomy and Diversity section, which was written in collaboration with four other authors, and contributing Section 5.4: Fisheries Impacts on Genetic Diversity, included within Section 5: Fisheries and Bycatch.

Chapter 2: Phylogenomics and Species Delimitation of Mobulid Rays

In this chapter, genome-wide phylogenomic reconstruction of the Mobulidae is performed using SNP data generated from among the most globally and taxonomically representative set of mobulid tissue samples. This data is used to delimit informative species units for conservation by evaluating the extent of diversity within the group and explicitly testing alternative species delimitation hypotheses. The species tree for the group is estimated and evolutionary processes driving diversity within the group are assessed and discussed. Finally, taxonomic implications of the work are critically evaluated. This chapter has been submitted for publication and is currently in revision with Conservation Biology. A preprint is available on BioRxiv: https://doi.org/10.1101/458141.

Chapter 3: Investigating the Genomic Signature of Speciation in Manta Rays

In Chapter 2, evidence is presented to support a third, currently undescribed species of manta ray in the Gulf of Mexico, with possible hybridisation with *Mobula birostris*. In this Chapter, a SNP dataset representing 217 individual manta rays of all three putative species with broad geographical coverage is generated and used to assess the degree of lineage sorting and divergence to evaluate the extent to which these represent three good species. This putative speciation event has previously been hypothesised to be related to isolation of an ancestral population as a result of historical changes in sea level in the Gulf of Mexico (see Marshall et al. 2009; Hinojosa-Alvarez et al. 2016). To evaluate support for this hypothesis, ancestral effective population sizes are reconstructed along lineages pertaining to these three putative species of manta ray. In addition, the extent of hybridisation and introgression within the manta rays is evaluated, and results discussed in the context of conservation.

Chapter 4: Evaluating Population Genetic Structure of the Reef Manta Ray, *Mobula alfredi* and the Oceanic Manta Ray, *Mobula birostris*.

This chapter evaluates the population genetic structure of the two currently described species of manta ray, which have contrasting habitat preferences. The reef manta ray, *Mobula alfredi*, favours shallower, and therefore more fragmented reef-based habitats, and as such is expected to be more resident. In contrast, the oceanic manta ray, *Mobula birostris*, is more commonly found in offshore pelagic habitats, potentially with higher connectivity and more opportunities for gene flow. However, previous studies have found evidence of residency in both species (e.g. Jaine et al. 2014; Stewart et al. 2016; Couturier et al. 2018). Genetic diversity is evaluated within inferred population clusters and the implications for conservation are discussed.

Chapter 5: General Discussion

This chapter presents an overall discussion of the key findings reported in this thesis in relation to published literature. I discuss the importance of biologically relevant species units, especially with respect to species of conservation concern protected under international legislation acting at the species level, such as CITES. In addition, a paradox of global genetic

panmixia in an apparently resident species, the oceanic manta ray, *Mobula birostris* is discussed in more detail, and several hypotheses to explain this observation are presented, with a focus on conservation. Furthermore, opportunities for developing traceability tools for mobulid rays are discussed in relation to the data produced throughout this project. Finally, suggestions are made for future research questions that will provide further insights into the evolution and diversity of the Mobulidae and assist conservation efforts for the group.

Chapter 2

Phylogenomics and Species Delimitation of Mobulid Rays

This Chapter has been submitted for publication and is in revision with Conservation Biology. A preprint is available on BioRxiv: https://www.biorxiv.org/content/10.1101/458141v2

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JH, EH, GC, MdB, RO, SC and GS designed and conceived of the study and secured funding for consumables relating to laboratory work. EH, GS, DF, AP, MA, JS, SP, SW, RJ, MP, MM, KBH, RB, JS and LP were responsible for sourcing and collecting samples. **JH**, HS and JK carried out laboratory work. **JH**, EH, GC, MdB, RO, SC, HH, AF and HS contributed to analysis of genome-wide SNP data. Figures were designed by EH and **JH** and produced by EH. **JH** wrote the manuscript and all authors contributed to writing and editing the manuscript.

Chapter 2: Phylogenomics and Species Delimitation of Mobulid Rays

Practical biodiversity conservation relies on delineation of biologically meaningful units, particularly with respect to global conventions and regulatory frameworks. Traditional approaches have typically relied on morphological observation, resulting in artificially broad delineations and non-optimal species units for conservation. More recently, species delimitation methods have been revolutionised with High-Throughput Sequencing approaches, allowing study of diversity within species radiations using genome-wide data. The highly mobile elasmobranchs, manta and devil rays (Mobula spp.), are threatened globally by targeted and bycatch fishing pressures resulting in recent protection under several global conventions. However, a lack of global data, morphological similarities, a succession of recent taxonomic changes and ineffectual traceability measures combine to impede development and implementation of a coherent and enforceable conservation strategy. Here, we generate genome-wide Single Nucleotide Polymorphism (SNP) data from among the most globally and taxonomically representative set of mobulid tissues. The resulting phylogeny and delimitation of species units represents the most comprehensive assessment of mobulid molecular diversity to date. We find a mismatch between current species classifications, and optimal species units for effective conservation. Specifically, we find robust evidence for an undescribed species of manta ray in the Gulf of Mexico and show that other species recently synonymised are in fact reproductively isolated. Further resolution is achieved at the population level, where cryptic diversity is detected in geographically distinct populations, and indicates potential for future traceability work determining regional location of catch. We estimate the optimal species tree for the group and uncover substantial incomplete lineage sorting, where standing variation in extinct ancestral populations is identified as a driver of phylogenetic uncertainty, with further conservation implications. Our study provides a framework for molecular genetic species delimitation that is relevant to wide-ranging taxa of conservation concern and highlights the potential for genomic data to support effective management, conservation and law enforcement strategies.

2.1 Introduction

The Anthropocene has been characterised by unprecedented human exploitation of natural resources, resulting in global threats to biodiversity and extinction events across diverse taxa (Dirzo et al. 2014). Effective measures for biodiversity conservation require understanding and characterisation of diversity within and among species. The field of conservation genetics focuses on quantifying diversity across space and time (Allendorf et al. 2010), facilitated by increasingly powerful genome-wide data. Such genomic approaches also have applications in investigating the evolutionary processes generating biodiversity (Seehausen et al. 2014), providing further knowledge towards mitigating declines.

Biodiversity conservation is enacted through global conventions and regulatory frameworks implemented through legislation at the species level. Examples include the Convention on the International Trade in Endangered Species of Wild Fauna and Flora (CITES), and the Convention on the Conservation of Migratory Species of Wild Animals (CMS). In practice however, conservation initiatives and enforcement of regulations typically occur at a more local scale. Species therefore have two important impacts on conservation implementation; as units for inclusion in international conventions designed to coordinate conservation efforts, and representing identifiable targets against which conservation actions are directed and measured (Mace, 2004). Effective wildlife protection, management and law enforcement therefore depend on unambiguous classification of diversity into biologically relevant species units. Recent examples of proposed taxonomic revisions having far-reaching consequences for conservation include giraffe (Fennessy et al. 2016) and African elephant (Roca et al. 2001), where genetic research underpins possible reclassification and changes to the legal status of these megafauna.

Consequently, species delimitation, the process by which individuals are grouped into reproductively isolated and separately evolving units, is a fundamental application of genomic data to biodiversity conservation, with numerous methods now available (e.g. Carstens et al. 2013; Grummer et al. 2014; Leache et al. 2014; Rannala 2015). Traditional approaches typically relied upon morphological observation, often resulting in artificially broad delineations arising from difficulties detecting and identifying cryptic species (Frankham et al.

2012), and impeding conservation efforts. More recently, DNA sequencing has allowed genetic data to be utilised for species delimitation, although interpretation may be challenging in recently diverged groups with substantial incomplete lineage sorting (Maddison & Knowles, 2006). Species delineations should minimise ambiguity by defining species units on the basis of reproductive isolation associated with limited gene flow and a lack of shared alleles (Frankham et al. 2012) and may therefore be optimised with evaluation of genomic data (Shafer et al. 2015). Genome-wide multi-locus approaches have increased the resolution of species delimitation studies, clarified contentious relationships and phylogenies (e.g. Leache et al. 2014; Herrera & Shank, 2016), disclosed previously unknown diversity (e.g. Pante et al. 2014) and elucidated evolutionary processes (e.g. Foote & Morin, 2016; Campbell et al. 2018). In addition, there are further applications in characterisation of Conservation Units and Evolutionary Significant Units to further enhance conservation efforts (Funk et al. 2012).

The importance of judiciously defined species or management units is particularly apparent in fisheries management (Reiss et al. 2009; Kerr et al. 2017; Mullins et al. 2018). Overexploitation of marine fisheries is a global problem (Agnew et al. 2009) resulting in loss of genetic diversity and bottlenecks in many species (Hauser et al. 2002; Pinsky & Palumbi, 2014). One group of heavily targeted fishes are the manta and devil rays (*Mobula* spp.; collectively, mobulids). Despite substantial economic value through tourism (O'Malley et al. 2013), these highly-mobile, circumglobally distributed megafauna are threatened by intense targeted and bycatch fishing pressure driven by demand for gill plates (Couturier et al. 2012; O'Malley et al. 2017). Consumptive exploitation of manta and devil rays is considered unsustainable due to slow life history traits, hindering recovery from fishing impacts (Dulvy et al. 2014; Croll et al. 2016). To alleviate threats, all mobulid species are listed on CITES Appendix II to regulate international trade, and on CMS Appendices I and II to coordinate protection and implement conservation efforts. These fish are poorly studied however, and marked homogeneity in morphology among species, a lack of representative global samples and population-level data, ongoing taxonomic debate, and ineffectual traceability measures constrain classification of optimal species units for conservation (Stewart et al. 2018b). Understanding of evolutionary history and diversification in the Mobulidae derives from few studies, which indicate secondary contact and introgression among lineages may further

impede efforts to delimit species boundaries (Kashiwagi et al. 2012; Poortvliet et al. 2015).

Recent evaluation of eleven previously recognised mobulid species across two genera recognised eight species, and called for the genus *Manta* (manta rays; *Manta alfredi* and *Manta birostris*) to be subsumed into *Mobula* (devil rays) (White et al. 2017). Other recent taxonomic changes include the resurrection of *Manta alfredi* (now *Mobula alfredi*); recognising two species of manta ray (Marshall et al. 2009; Kashiwagi et al. 2012), yet evidence remains of historic (Kashiwagi et al. 2012) and modern (Walter et al. 2014) hybridisation. In addition, a third putative species of manta ray is hypothesised to occur in the Caribbean (Marshall et al. 2009; Hinojosa-Alvarez et al. 2016). To date however, studies have relied on morphological observation (Notarbartolo di Sciara 1987; Marshall et al. 2009; White et al. 2017) and/or been limited to evaluation of a handful of genetic markers, with heavy reliance on uniparentally inherited mitochondrial DNA (Kashiwagi et al. 2012; Hinojosa-Alvarez et al. 2016). Previous studies have also been geographically restricted and reliant on few samples (White et al. 2017), resulting in classifications that fail to encapsulate the extent of diversity within the group and compromise the effectiveness of conservation efforts.

Here, we generate double-digest Restriction-site Associated DNA sequence (ddRAD) data from the largest and most comprehensive set of mobulid tissue samples available. We demonstrate utility in delimiting informative species units for conservation, detecting cryptic diversity, and improving our understanding of associated evolutionary processes in a global radiation of socio-economically important marine megafauna.

2.2 Methods

2.2.1 Sampling and Sanger Sequencing

Tissue samples were obtained from the existing collections and sampling initiatives of researchers and organisations worldwide, yielding samples representing all mobulid species from a broad geographical range (Figure 2.1 and Supplementary Table S2.1), including *Mobula japanica, Mobula eregoodootenkee* and *Mobula rochebrunei,* currently considered to be junior synonyms of *Mobula mobular*, *Mobula kuhlii* and *Mobula hypostoma,* respectively

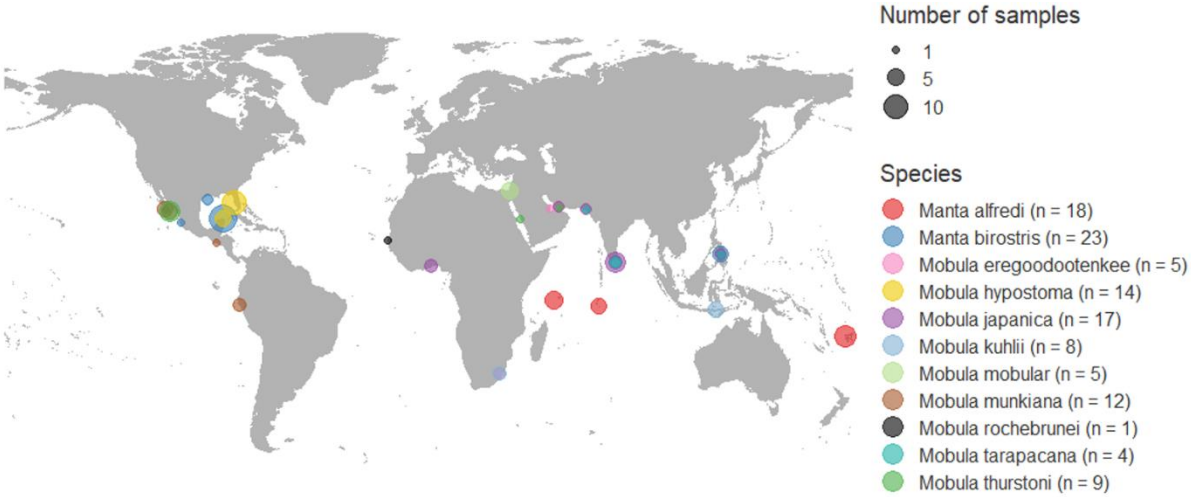


Figure 2.1: Global sampling locations. Species are represented by coloured points, scaled for sample size. Total numbers of samples for each species provided in the key. Further details in Supplementary Table S2.1. Species names are those assigned at time of collection, some now considered invalid (White et al. 2017). Figure designed by JH and EH and produced by EH.

(White et al. 2017), and an outgroup, *Rhinoptera bonasus.* Where this involved taking biopsies from live animals, the procedure was approved by Bangor University's Ethics Committee. Samples were identified to species level based on characteristics described by Stevens et al. (2018b), using original species names assigned and valid at the time of collection.

Genomic DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit and DNA yield measured using a Qubit 3.0 Broad Range Assay. Extracts were quality assessed on 1% agarose gels stained with SafeView. The single sample of *Mobula hypostoma cf. rochebrunei* was from a museum specimen stored in formalin and yielded no detectable DNA.

To evaluate traditional markers for mobulid species delimitation, PCR amplification of an approximately 650bp portion of the Cytochrome Oxidase Subunit I (COI) gene was performed using universal Fish primers (Ward et al. 2005). Where these primers failed (for *Mobula munkiana* and *M. hypostoma*), primers MunkF1 (GGGATAGTGGGTACTGGCCT) and MunkR1 (AGGCGACTACGTGGGAGATT) were designed in Primer-BLAST (Ye et al. 2012) using reference sequences published in Poortvliet et al. (2015). PCR reactions were conducted in 15µl using 1x ReddyMix PCR Master Mix (ThermoFisher) with 0.27uM of each primer and 25-50 ng DNA. PCR cycling conditions were: 95°C for 2 min, 35 cycles of 94°C for 30s, 54°C for 30s and 72°C for 1 min and final extension of 72°C for 10 mins. Sanger sequencing was conducted by

Macrogen Europe. Data was aligned using ClustalW and the alignment checked for stop codons in MEGA7 (Kumar et al. 2016). The HKY+G model was identified as optimal for our COI dataset using the Find Best Model option in MEGA7 and a Maximum Likelihood tree was built with 1,000 bootstrap replicates.

2.2.2 ddRAD Library Preparation and Sequencing

ddRAD libraries were designed based on the results of a pilot library sequenced for all available species of mobulid ray (see Supplementary Section S2.1: Method Development) and prepared using a modified version of the original protocol (Peterson et al. 2012; see Palaiokostas et al. 2015 for full protocol) with restriction enzymes *SbfI* and *SphI* (NEB). Unique P1 and P2 barcode combinations were ligated to resulting DNA fragments, which were then size-selected between 400-700bp using gel electrophoresis and PCR amplified. Individual sample replicates within and among libraries were included to assess error rates following the method described by Mastretta-Yanes et al. (2015). The pilot ddRAD library was sequenced on Illumina MiSeq at the Institute of Aquaculture, University of Stirling. Subsequent ddRAD libraries were sequenced by Edinburgh Genomics© on Illumina HiSeq High Output v4, 2 x 125PE read module (see Supplementary Table S2.2 for details).

2.2.3 Data Quality Control and Filtering

Data quality was assessed with FastQC (Andrews, 2010), and processed in Stacks version 1.46 (Catchen et al. 2011). The process_radtags.pl module in Stacks (Catchen et al. 2011) was used to demultiplex the data, filter for adaptor sequences (allowing two mismatches), remove low quality sequence reads (99% probability) and discard reads with any uncalled bases. Since forward and reverse reads of the same amplicon are situated closely together in the genome and are therefore likely to violate assumptions of independence, only forward reads were retained for subsequent analyses to minimise linkage disequilibrium in the SNP data. Short fragments not removed through size-selection were filtered with a custom bash script (8.5% of reads).

The denovomap.pl program in Stacks (Catchen et al. 2011) was used to assemble loci and call

SNPs. The three main parameters for assembly were those generating the largest number of new polymorphic loci shared across 80% of individuals, following the method of Paris et al. (2017). Four identical reads were required to build a stack (-m 4), stacks differing by up to four nucleotides were merged into putative loci (-M 4) and putative loci across individuals differing by up to five nucleotides were written to the catalog (-n 5), giving an average coverage of 105x. The allele error rate, the total number of allele mismatches between replicate pairs as a proportion of the loci being compared (Mastretta-Yanes et al. 2015), for these parameters was 0.055 and 0.036 on average within and among libraries respectively. The SNP error rate, the number of SNP mismatches as a proportion of total SNPs (Mastretta-Yanes et al. 2015), was 0.024 and 0.016 within and among libraries respectively. We then used the populations.pl program in Stacks (Catchen et al. 2011) to generate two VCF files containing SNPs present in at least 10 and 90 individuals, respectively. To remove paralogous loci and mitigate for allele dropout (Arnold et al. 2013; Gautier et al. 2013), loci sequenced at greater than twice or less than one-third the standard deviation of coverage, respectively, were identified and excluded using VCFtools (Danecek et al. 2011). The remaining loci were assessed for excess heterozygosity using VCFtools (Danecek et al. 2011), and those exhibiting a significant probability of heterozygote excess were excluded. Finally, since Stacks (Catchen et al. 2011) ignores indels, SNPs in the last five nucleotide positions were assumed erroneous and excluded. The remaining loci and SNPs were written to a whitelist and filtered for a single random SNP per locus to minimise linkage using the populations.pl program in Stacks (Catchen et al. 2011). This resulted in two final SNP matrices, referred to as ‘‘p10’’ and ‘‘p90’’, with 7926 and 1762 SNPs and 47.1% and 14% missing data, respectively (Supplementary Table S2.3).

2.2.4 Monophyly and Clustering

Relationships among individuals were inferred through Maximum Likelihood phylogenetic analysis using RAxML version 8.2.11 (Stamatakis, 2014). Both ddRAD datasets were analysed since missing data has been shown to influence aspects of phylogenetic inference (Leaché et al. 2015). The GTRGAMMA model of rate heterogeneity was implemented following assessment of best fit models using both the Akaike and Bayesian information criteria in jModelTest2 (Darriba et al. 2015) and support assessed with 1,000 bootstrap replicates.

RAxML (Stamatakis, 2014) identified four highly supported clades separated by long branches. To assess how individuals cluster within these clades, dataset 'p10' was divided by clade (Supplementary Table S2.4) and Principal Components Analysis (PCA) performed for each clade using the R package 'adegenet' (Jombart, 2008). After initial assessment of ten axes, three were retained in all cases. F_{ST} values (Weir & Cockerham, 1984) among inferred clusters were calculated using the R package 'hierfstat' (Goudet & Jombart, 2015) and 95% confidence intervals were obtained by performing 1000 bootstrap replicates over loci of pairwise F_{ST} allowing for significant difference from zero to be established for F_{ST} values.

2.2.5 Bayes Factor Delimitation

Bayes Factor Delimitation (BFD*; Leache et al. 2014) was conducted using the modified version of SNAPP (Bryant et al. 2012), implemented as a plug-in to BEAST version 2.4.8 (Bouckaert et al. 2014). The method allows direct comparison of Marginal Likelihood Estimates (MLEs) for alternative species delimitation hypotheses, hereafter models, under the multispecies coalescent. Path sampling involved 10 steps (1,000,000 MCMC iterations, 20% burnin), implementing the log-likelihood correction. Since MLEs are affected by improper prior distributions, a gamma distribution was implemented on the lambda (tree height) parameter. To assess the effect of priors on the ranking order of models, models were also assessed retaining the default 1/X distribution on lambda, implementing upper and lower bounds (10,000 and 0.00001 respectively), for a proper prior. Bayes Factors ($2\log_e BF$) were calculated from the MLE for each model for comparison (Kass & Raftery 1995; Leache et al, 2014), as follows:

$$2\log_e BF = 2*(MLE_{null} - MLE_{test})$$

Positive $2\log_e BF$ values indicate support for the null model (<10 is decisive; Leache et al. 2014), negative values favour the tested model.

Due to computational constraints, dataset 'p90' underwent Bayes Factor Delimitation and the data were partitioned by clade, as previously described, but including four random individuals from a sister species to evaluate support for interaction from higher phylogenetic levels. Alternative models were informed by the literature and analyses herein (Supplementary

Tables S2.5-S2.8). Models randomly assigning individuals to two or three species were assessed for each clade. Null models matched species defined by White et al. (2017).

2.2.6 Species Tree Inference

Relationships among the Mobulidae were estimated through Maximum Likelihood phylogenetic analysis of both ddRAD datasets as above with RAxML (Stamatakis, 2014). Consensus sequences for each species unit were ascertained using the populations.pl program in Stacks (Catchen et al. 2011), providing a population map assigning individuals to optimal species units based on our previous analyses.

To test tree topology and evaluate uncertainty due to incomplete lineage sorting, species trees were additionally evaluated with SNAPP (Bryant et al. 2012), allowing each SNP to have its own history under the multispecies coalescent, whilst bypassing the need to sample individual gene trees. Due to the computational capacity required to run SNAPP (Bryant et al. 2012), three individuals per species were randomly selected from dataset 'p90' whilst maximising geographical coverage within species. Random sampling of individuals with replacement was repeated a further three times, resulting in four subsampled alignments (Supplementary Table S2.9). MCMC chains consisted of 5,000,000 iterations, sampling every 1,000 and retaining default priors on lambda and theta for each independent analysis. Convergence to stationary distributions were observed after 20% burnin in TRACER (Rambaut et al. 2018), the distribution of trees visualised in DensiTree (version 2.2.6; Bouckaert, 2010) and maximum clade credibility (MCC) trees drawn using TreeAnnotator (version 2.4.7; Bouckaert et al. 2014). Alternative prior combinations produced highly concordant results.

Multispecies coalescent based approaches assume that any discordance of topologies among loci results from incomplete lineage sorting, and do not consider introgression as a source of discordance. TreeMix (Pickrell & Pritchard, 2012) was applied to dataset 'p10' to evaluate evidence for significant introgression events within the Mobulidae by investigating the extent to which variation between user-defined groups is explained by a single bifurcating tree. Given uncertainty identified using SNAPP (Bryant et al. 2012), specifically regarding the placement of *Mobula mobular*, the three-population test (Reich et al. 2009) was additionally used to test for 'treeness' between clades. Similar to TreeMix (Pickrell & Pritchard, 2012), the

three-population test estimates covariance of allele frequencies among groups but is simpler and less parameterised; potentially more powerful for identifying introgression. In addition to *Mobula mobular, M. alfredi* and *M. thurstoni* were randomly selected to represent their respective clades.

2.3 Results

2.3.1 Monophyly and Clustering

Maximum Likelihood phylogenetic trees based on two genome-wide SNP matrices were highly congruent (Figure 2.2 and Supplementary Figure S2.1). Species groups formed well-supported clades separated by long branches. Principal Components Analyses (PCA) within each clade mirrored patterns in phylogenetic trees (Figure 2.3). Putative species, including recently synonymised species *Mobula kuhlii* and *Mobula kuhlii cf. eregoodootenkee* formed both reciprocally monophyletic groups with high bootstrap support (Figure 2.2) and tight clusters separated along axes explaining large portions of variance (63%-74%; Supplementary Figure S2.2). Two reciprocally monophyletic groups were detected within *Mobula birostris*; an Atlantic and a global group, respectively (Figure 2.2), visible as clusters through PCA (Figure 2.3A). One individual was equally well, albeit poorly, placed with each clade in the two phylogenetic analyses (Figure 2.2 and Supplementary Figure S2.1) and in an intermediate position through PCA (Figure 2.3A). *Mobula mobular cf. japanica* and *Mobula mobular* formed a single monophyletic group with 100% bootstrap support (Figure 2.2), with no clear separation through PCA (Figure 2.3C-D). Whilst the first axis provides limited evidence to suggest a clustering of individuals into Indo-Pacific and Atlantic (including Mediterranean) groups, this explained only 8.6% of variance (Supplementary Figure S2.2E), with minimal differentiation between these two clusters (F_{ST} = 0.04 ± 0.008). Geographically separated populations of *Mobula alfredi* and *Mobula kuhlii* formed highly supported monophyletic groups (Figure 2.2) and were demarcated clearly through PCA (Figure 2.3B; Figure 2.3F), showing a high degree of differentiation (F_{ST} = 0.22 ± 0.045 and 0.39 ± 0.046, respectively).

COI sequence data failed to achieve resolution sufficient to discriminate putative species, and

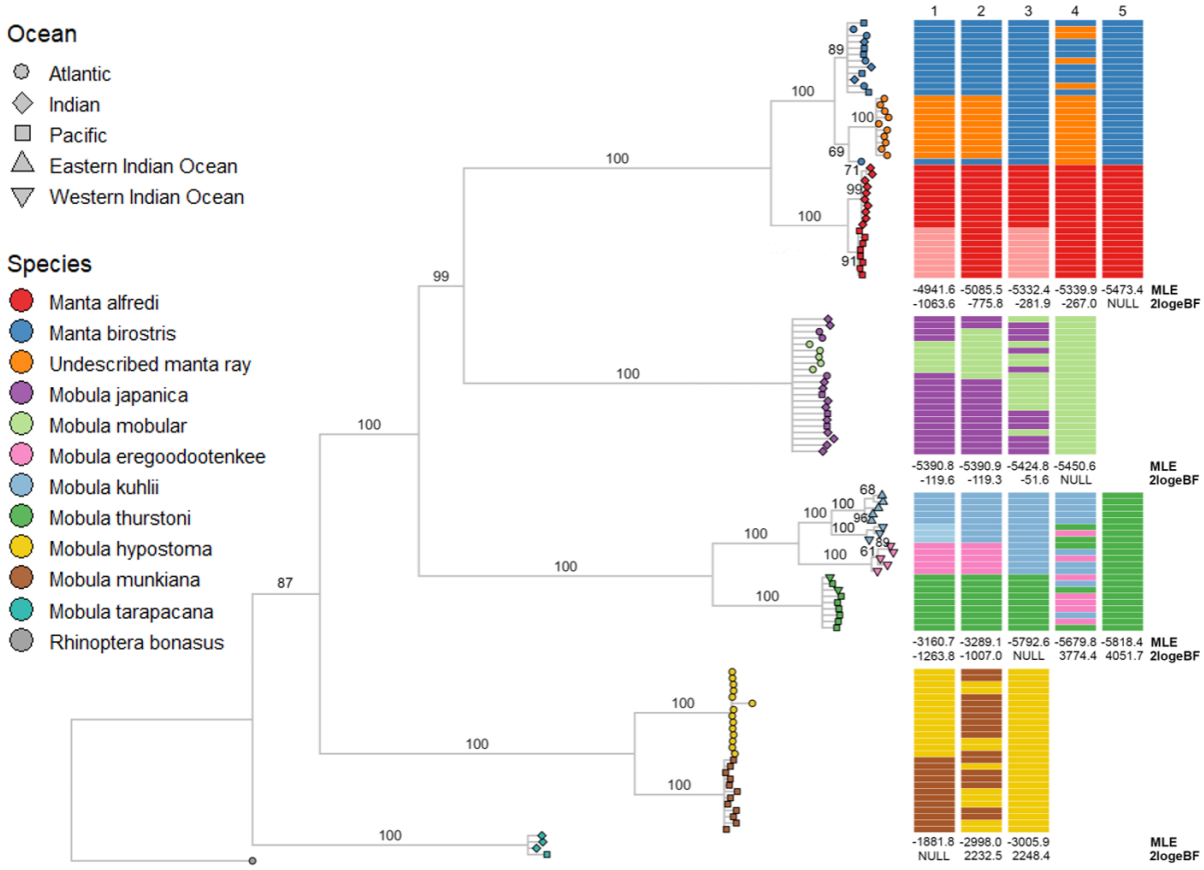


Figure 2.2: (Left) Maximum Likelihood Phylogenetic Tree of mobulid individuals based on 7926 SNPS (dataset 'p10'). Coloured points indicate putative species, and shape indicates geographic origin of samples as specified in the key. Bootstrap values are shown on the branches and nodes with less than 50% support are collapsed. (Right) Bayes Factor Delimitation (BFD*) models with individuals assigned to species groups indicated by coloured bars are also presented, ranked in order of performance from left to right. Marginal Likelihood Estimates (MLEs) and Bayes Factors ($2\log_e BF$) relative to the null model are shown beneath each model for chains with a gamma prior on lambda. Models including individuals from a sister clade are not shown, as these consistently performed poorly (see Supplementary Tables S2.5-S2.8). Species names are those assigned at time of collection, some now considered invalid (White et al. 2017). Figure designed by JH and EH and produced by EH.

phylogenetic analysis showed several multifurcating nodes (Supplementary Figure S2.3).

2.3.2 Species Delimitation

Species models were compared within clades using Bayes Factor Delimitation (Leache et al. 2014; Figure 2.2). Marginal Likelihood estimates were unaffected by lambda priors, with no change in the ranked order of models (Supplementary Tables S2.5-S2.8). We find decisive support for models recognising the Gulf of Mexico and global *Mobula birostris* groups as

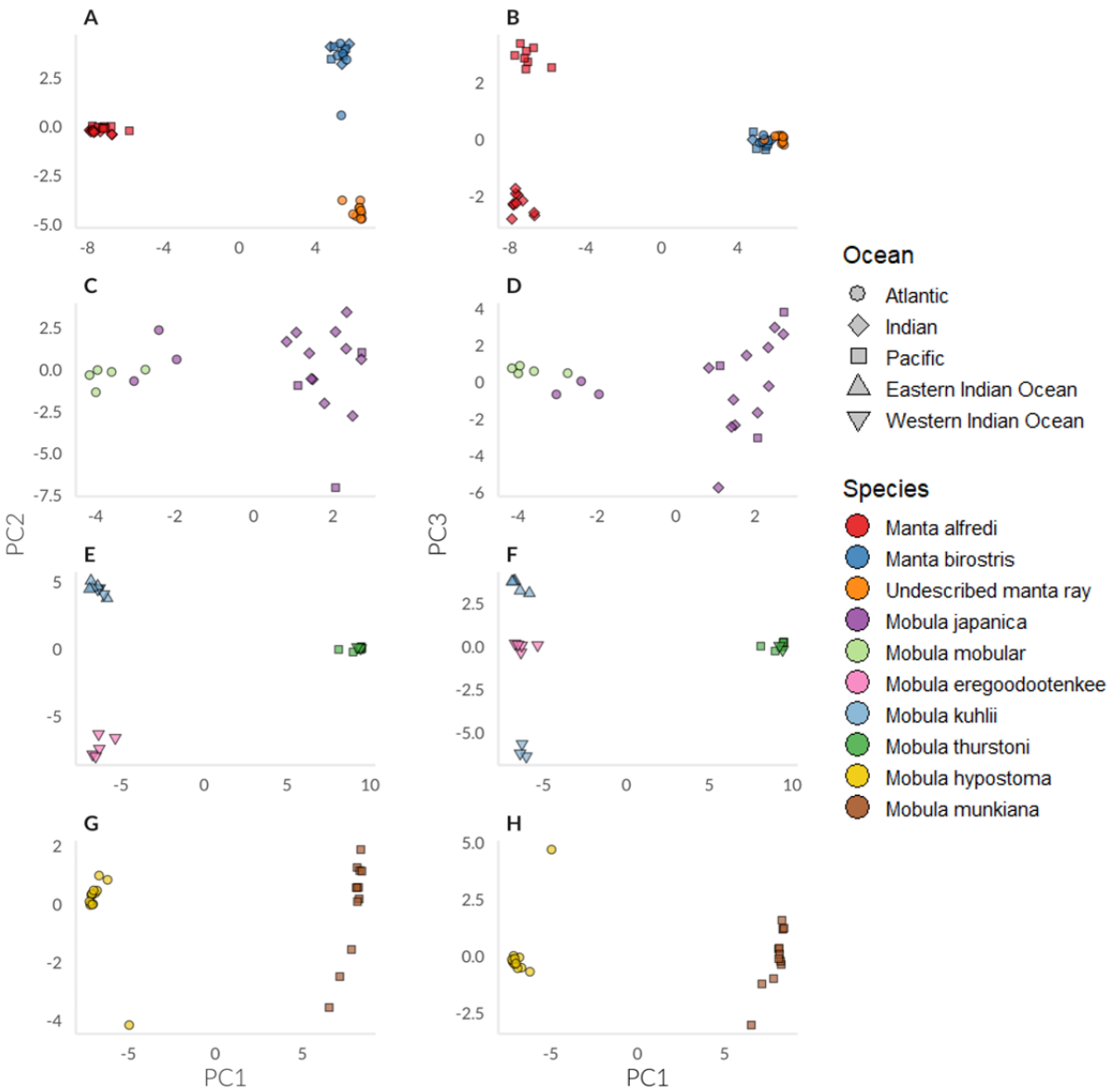


Figure 2.3: Principal Components 1-3 plotted for each mobulid clade. Individuals are represented by a point, colour indicates putative species, and shape indicates geographic origin of samples as specified in the key. Manta rays, *Mobula alfredi*, *M. birostris* and a putative third species, A) PC1 and 2, and B) PC1 and 3; *M. mobular* and *M. japanica*, C) PC1 and 2, and D) PC1 and 3; *M. thurstoni*, *M. kuhlii* and *M. eregoodootenkee,* E) PC1 and 2, and F) PC1 and 3; *M. hypostoma* and *M. munkiana,* G) PC1 and 2, and H) PC1 and 3. Species names are those assigned at time of collection, some now considered invalid (White et al. 2017). Figure designed by JH and EH and produced by EH.

separate species ($2\log_e BF$ = -775.82; hereafter '*Mobula* sp. 1' and '*M. birostris*' respectively) and where individuals identified as *Mobula kuhlii cf. eregoodootenkee* belong to a separate species to *M. kuhlii* as formerly recognised ($2\log_e BF$ = -1007.04). Models splitting *Mobula mobular* and *M. mobular cf. japanica* based on geographic origin marginally outperformed

the null model. Geographically informed models involving *Mobula alfredi* and *M. kuhlii* also performed well, achieving decisive support ($2\log_e BF$ = -1063.58 and -1263.8, respectively). The null model was favoured within the *Mobula hypostoma* and *M. munkiana* clade. Models assessing support for interaction from higher levels and testing random individual assignments consistently performed comparatively poorly (Supplementary Tables S2.5-S2.8).

2.3.3 Relationships Among Species

Maximum Likelihood species trees based on two genome-wide SNP matrices were highly congruent (Figure 2.4 and Supplementary Figure S2.4). Consistent with White et al. (2017), manta rays were nested within the genus *Mobula*, sister to *Mobula mobular* (≥98% bootstrap support) and hereafter all species of manta ray are referred to as *Mobula*. These trees strongly suggest that an undescribed third species of manta ray is sister to *Mobula birostris* (100% bootstrap support). *Mobula tarapacana* was tentatively placed on the group's oldest lineage (84% bootstrap support).

Consensus species trees estimated under the multispecies coalescent exhibited relatively consistent topologies and theta estimates across independent runs, suggesting no major effect of subsampling on species tree topology inferred with SNAPP (Bryant et al. 2012). *Mobula tarapacana* was consistently sister to *M. hypostoma* and *M. munkiana* (highest posterior density (HPD) = 1.0). Topological uncertainty at other nodes is apparent as shown with a cloudogram of gene trees sampled from the posterior distribution (Figure 2.5 and Supplementary Figures S2.5-S2.7). Relationships between sister species within clades remained consistent in alternative topologies within the 95% HPD, but large discrepancies in the placement of *Mobula mobular* (including *M. mobular cf. japanica)* relative to other clades were observed (Supplementary Table S2.10).

TreeMix (Pickrell & Pritchard, 2012) inferred an admixture graph similar to trees produced with RAxML (Stamatakis, 2014; Supplementary Figure S2.8), explaining 99.86% of variance, indicating mobulid species placement is unaffected by admixture. We found no evidence of introgression between clades containing *Mobula alfredi, M. mobular* and *M. thurstoni,* through three-population tests (Reich et al. 2009; Supplementary Table S2.11).

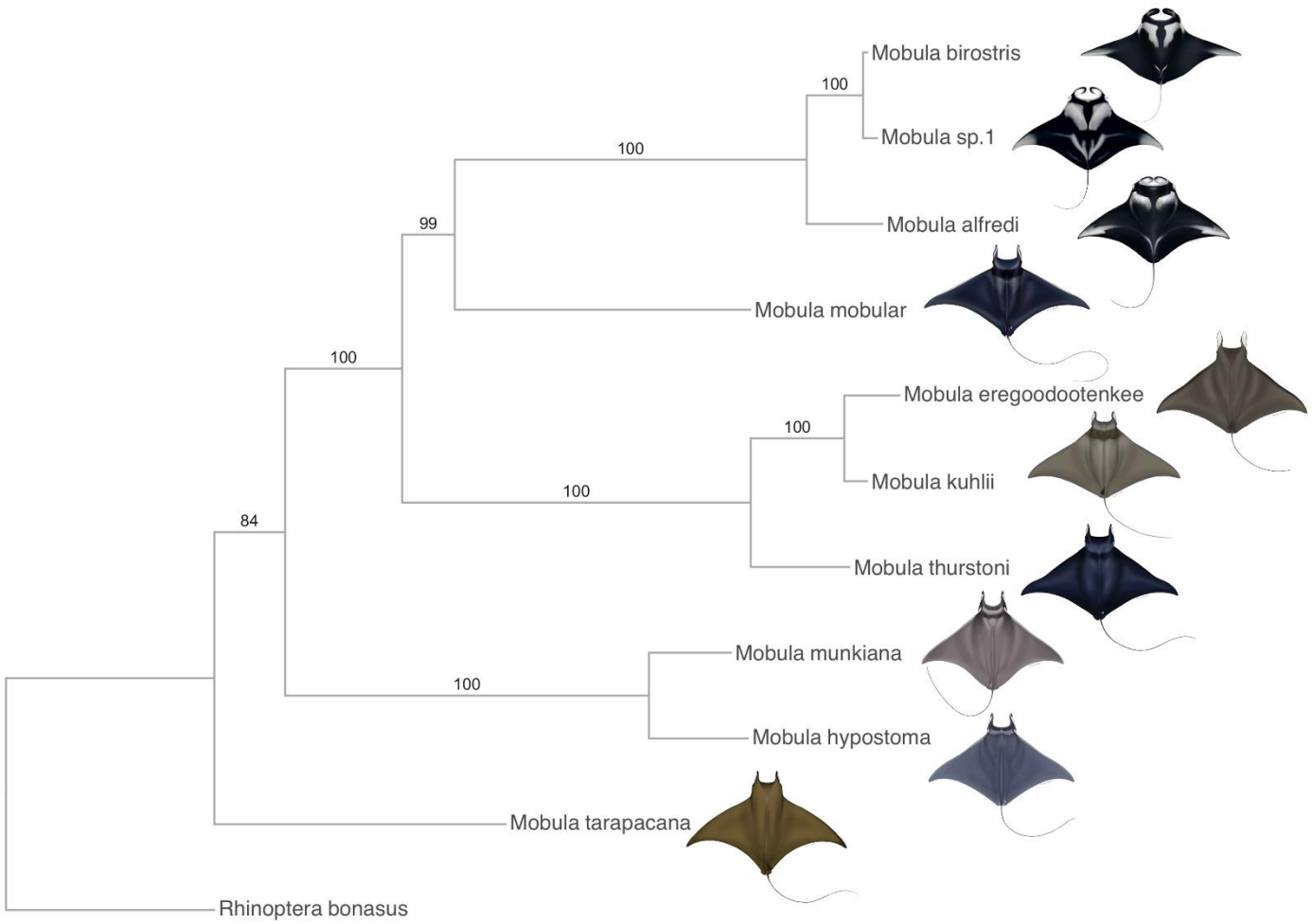


Figure 2.4: Maximum Likelihood tree of inferred mobulid species units based on 7902 SNPs (dataset 'p10'). Bootstrap values are shown on the branches. The drawing of *Mobula* sp. 1 is based on images of dozens of individuals off the Yucatan Peninsula, Gulf of Mexico, where samples analysed here were collected. Illustrations © Marc Dando and are reproduced with permission. Figure designed by JH and EH and produced by EH. Note that *Mobula mobular* here includes specimens identified as *Mobula mobular cf. japanica*.

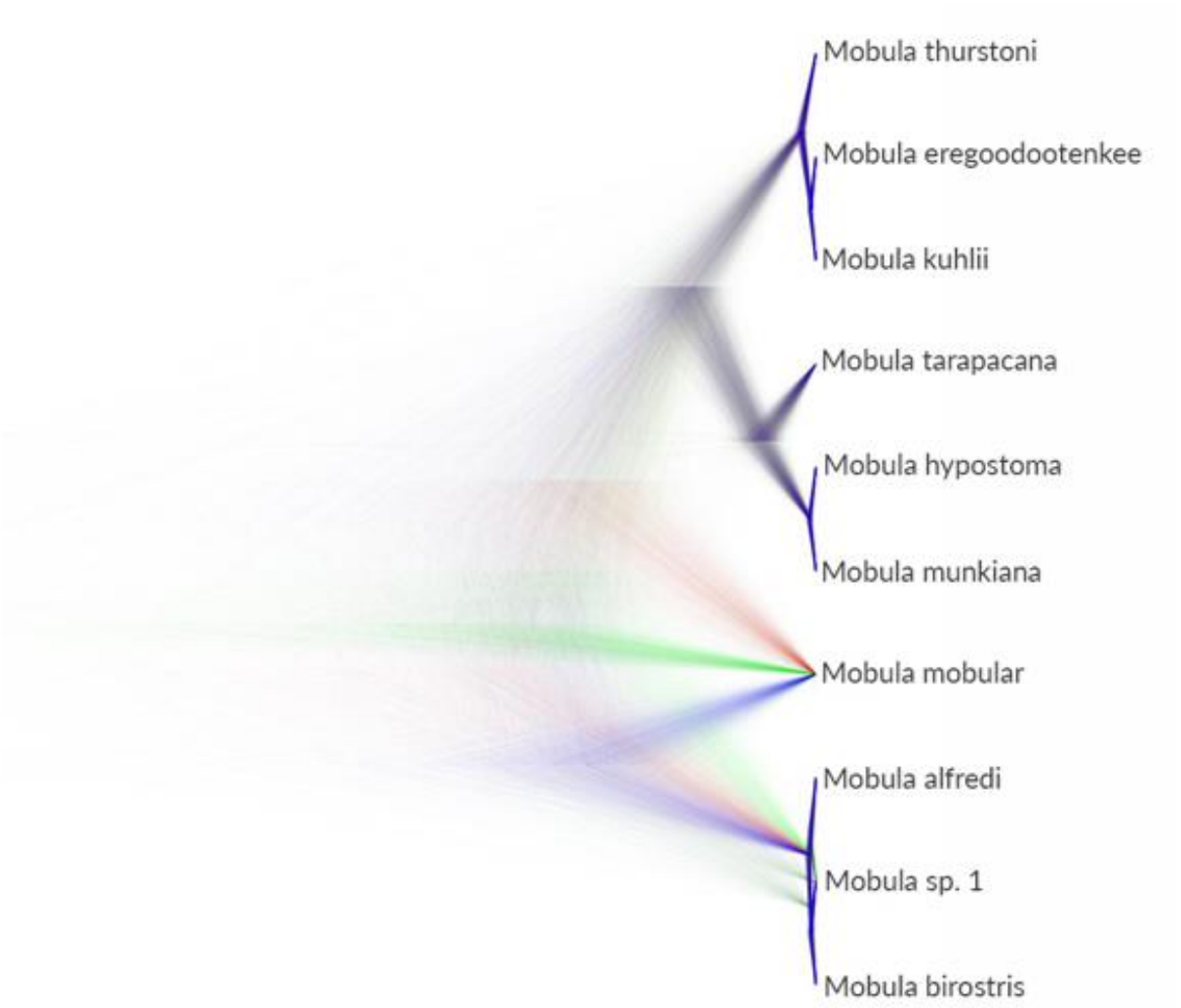


Figure 2.5: SNP phylogeny of 30 randomly chosen individuals assigned to ten species units based on 1242 SNPs (dataset 'p90', individual subsample 1; Supplementary Table S2.9). Tree cloud of sampled trees produced using DENSITREE (representing samples taken every 1000 MCMC steps from 5,000,000 iterations) from SNAPP (Bryant et al. 2012) analysis to visualise the range of alternative topologies. Figure designed by JH and EH and produced by EH. Note that *Mobula mobular* here includes specimens identified as *Mobula mobular cf. japanica*.

2.4 Discussion

Genome-wide SNP data provide unprecedented resolution in a group of conservation concern, and our analyses produced the most extensive phylogeny for the Mobulidae to date. In contrast to previous studies examining mobulid diversity, the global nature of our dataset allowed us to identify reproductive isolation between lineages and distinguish between

population and species units (Sukumaran & Knowles, 2017). We find a mismatch between current classifications and species units optimal for conservation, with implications for management and law enforcement (see Supplementary Section S2.2). We provide robust evidence for a new species of manta ray and demonstrate that individuals identified as recently synonymised species *Mobula kuhlii* and *Mobula eregoodootenkee* are distinct and reproductively isolated. We therefore recommend that such units coincide with enforceable protection (see Supplementary Section S2.2 for critical evaluation). In addition, we detect cryptic diversity between geographically segregated populations of *Mobula alfredi* and *Mobula kuhlii*, which may merit independent management.

These findings have international implications for practical conservation of the Mobulidae since legislation applies to species units and can severely impact anthropogenic pressures on wildlife populations. Our data suggest that the oceanic manta ray (*Mobula birostris*) and an undescribed species of manta ray (*Mobula* sp. 1) occur in sympatry in the Gulf of Mexico, since samples collected within sites fall into both groups, and provides evidence suggestive of hybridisation between these species (Figure 2.2; Figure 2.3A). Management of these similar species as independent units will therefore be challenging, potentially requiring blanket protection of all manta rays in regions where sympatry and/or hybridisation occur, and indeed such protection already exists in Mexico. Notwithstanding, *Mobula* sp. 1 is likely to occur over a broad geographic range, given patterns of distribution of its closest relatives (see Stevens et al. 2018b). To establish effective conservation and traceability measures for this new species, it will therefore be necessary to formally describe *Mobula* sp. 1 and determine the extent of its range, which may extend into international waters or span areas with high fishing pressure or lacking suitable protective measures.

Similarly, *Mobula kuhlii cf. eregoodootenkee*, shown here to be distinct from *M. kuhlii*, shares a geographic range with the latter across a region with intense fishing pressure (Notarbartolo di Sciara et al. 2017). Inference from related species suggests low reproductive output likely resulting in population sizes vulnerable to exploitation (Dulvy et al. 2014; Croll et al. 2016). It is therefore imperative that such units are managed separately. In contrast, species such as *Mobula mobular* may be of lower conservation priority given that *M. mobular cf. japanica* is a junior synonym (White et al. 2017; this study - see Supplementary Section S2.2.4). Significant population structure in *Mobula alfredi* and *M. kuhlii* indicates potential for future

traceability work to determine regional location of catch in these species (Supplementary Section S2.2.7), which is increasingly required to comply with global reporting obligations (Nielsen et al. 2012). Additional population-level studies will allow further assessment of stock structure within fisheries and delineation of mobulid conservation units for effective management.

We find substantial uncertainty in the placement of *Mobula mobular,* and trees within the 95% HPD where the manta rays (formerly genus *Manta*) are nested within *Mobula* are present in approximately equal proportions to trees where the former genera are reciprocally monophyletic (Supplementary Table S2.10). In groups that have undergone rapid speciation with large ancestral effective population size, the effects of incomplete lineage sorting on species tree estimation are particularly prominent (Lischer et al. 2014; Flouri et al. 2018). The Mobulidae have undergone recent rapid bursts of speciation (Poortvliet et al. 2015), and our estimates of mutation-scaled effective population size (theta) were larger on deeper branches of the tree, indicating large effective population size of extinct shared ancestral species (Supplementary Figure S2.9). Thus, standing variation in ancestral populations of mobulid rays is likely to drive uncertainty with respect to the validity of the genus *Manta*. Since we find no evidence of admixture driving these patterns, this uncertainty can be attributed to incomplete lineage sorting. Factors such as similarities in life history and difficulties distinguishing between related species in trade can lead to whole genera being listed on international conventions designed to preserve biodiversity, such as CITES. Our data therefore demonstrates the importance of understanding the extent and nature of incomplete lineage sorting for effective conservation of threatened groups.

Genomic approaches are increasingly informative for inferring phylogenetic relationships among species. Results must, however, be interpreted with caution. Our Maximum Likelihood analysis identified *Mobula tarapacana* as the earliest branching mobulid lineage, coincident with similar analyses of nuclear data (White et al. 2017), yet our Bayesian analyses consistently placed *M. tarapacana* sister to *M. hypostoma* and *M. munkiana*; a previously unreported phylogenetic placement. Analyses employing mitochondrial data support *Mobula tarapacana* as sister to the manta rays and *M. mobular* (Poortvliet et al. 2015; White et al. 2017), an observation we were unable to reproduce. Discordant trees in phylogenomic studies may be attributed to few loci driven by positive selection resulting in convergent

evolution, or evolutionary processes such as incomplete lineage sorting (Shen et al. 2017). Coalescent-based approaches, as applied here, account for the independent history of each gene tree and are therefore less likely to be influenced by single genes, highlighting the suitability of genome-wide data for the inference of species relationships.

Here, genome-wide data considerably enhances delimitation of species units for the conservation of manta and devil rays. These findings have profound implications for the practical conservation of a group threatened by fishing and are relevant to enforcement of CITES regulations by laying the groundwork for species and regional traceability of parts in trade. Furthermore, we demonstrate the ability of genomic data to resolve and identify diversity within organismal radiations and improve understanding of evolutionary processes generating biodiversity. As such, this study provides a framework for molecular genetic species delimitation which is relevant to other wide-ranging taxa of conservation concern and highlights the potential for applied research in supporting conservation, management and law enforcement.

Chapter 2: Supplementary Materials

Chapter 2: Supplementary Materials

S2.1 Method Development

Due to a distinct lack of genomic data representing mobulid species available at the start of this project, a pilot ddRAD library was sequenced using a subset of available samples. The aim of this library was to determine an appropriate number of samples to multiplex within libraries, in order to achieve sufficient sequencing depth to call SNPs with high confidence. This pilot library contained 24 samples, representing 10 species of mobulid ray (of the 11 species recognised at the time, see Section 1.3.1), whilst maximising geographic coverage within species where possible.

In brief, this pilot ddRAD library was prepared using a modified version of the original protocol (Peterson et al. 2012; see Palaiokostas et al. 2015 for full protocol) with restriction enzymes *SbfI* and *SphI* (NEB). The choice of restriction enzymes was informed by the estimated genome size for *Mobula mobular cf. japanica* and *M. tarapacana* (Asahida et al. 1993; Chang et al. 1995), and the required coverage. Unique P1 and P2 barcode combinations were ligated to resulting DNA fragments, which were then size-selected between 400-700bp using gel electrophoresis and PCR amplified. This library was sequenced on an Illumina MiSeq at the Institute of Aquaculture, University of Stirling, producing 18.5 million forward and reverse reads (Supplementary Table S2.2) with an average GC content of 45%.

Data quality was assessed using FastQC (Andrews, 2010) and reads were processed in Stacks version 1.46 (Catchen et al. 2011). The process_radtags module in Stacks (Catchen et al. 2011) was used to demultiplex the data, filter for adaptor sequences (allowing 2 mismatches), remove low quality sequence reads (99% probability) and discard reads with any uncalled bases. The denovomap.pl program in Stacks (Catchen et al. 2011) was then used to assemble loci and call SNPs, using default parameters for the number of identical reads required to build a stack (-m 3), the number of mismatches allowed between stacks to be merged into putative loci (-M 2) and the number of mismatches allowed between putative loci to be written to the

catalog (-n 1).

The resultant catalog contained 81,000 loci, 38,000 of which were polymorphic and a total of 133,000 SNPs were called. The average depth of coverage across individuals was 49x. These results were used to inform the design of subsequent ddRAD libraries, described in Chapters 2, 3 and 4 (see Supplementary Table S2.2), and data from this pilot library were included in the dataset analysed in Chapter 2.

S2.2 Critical Evaluation of Taxonomic Implications

Through the analysis of genome-wide data for a globally and taxonomically comprehensive set of mobulid tissue samples, we produced the most extensive phylogeny for the Mobulidae to date and carried out species delimitation using a multispecies coalescent based approach. As such, our findings have implications for mobulid taxonomy. It is important to recognise speciation as a continuous process, however, where lineage splitting does not necessarily correspond to speciation events. When this is explicitly modelled, the multispecies coalescent has been shown to overestimate species numbers, recovering all structure both at the level of the species and the population (Sukumaran & Knowles, 2017). In contrast to previous studies evaluating mobulid diversity, the global nature of our dataset allows for this conflict to be resolved, where in many cases, individuals from pairs of putative species are sampled within sites (Figure 2.1; Supplementary Table S2.1), thereby allowing for the identification of true reproductive isolation. We summarise and discuss these taxonomic implications below since it will be of general interest to policymakers. Additionally, it is provided as a resource for taxonomists wishing to compliment traditional approaches, such as morphological observation of specimens or ecological and behavioural data, with genomic data to evaluate taxonomy of the Mobulidae.

In brief, our genome-wide SNP data provide evidence supporting ten species within the Mobulidae. Of those species defined by White et al. (2017), our data support *Mobula alfredi, Mobula birostris, Mobula mobular, Mobula thurstoni, Mobula hypostoma, Mobula munkiana* and *Mobula tarapacana*. However, in addition our data strongly suggests that individuals

identified as *Mobula kuhlii* and *Mobula eregoodootenkee* prior to the revision published by White et al. (2017) are distinct and reproductively isolated, thereby belonging to separate species. Furthermore, we find strong evidence for a currently undescribed species of manta ray (referred to as *Mobula* sp. 1) in the Gulf of Mexico. We emphatically urge policymakers, particularly the large conventions (such as CITES and CMS) and the relevant IUCN specialist group to evaluate these as separate units in assessments and when implementing conservation policy.

S2.2.1 Validity of the genus *Manta*

The two species in the genus *Manta* were recently subsumed into *Mobula* (White et al. 2017), meaning that the names *Mobula alfredi* and *Mobula birostris* are considered valid and are in current use. Our Maximum Likelihood phylogenetic analysis indicates that the previously recognised genus *Manta* is nested within *Mobula* and provides further justification for the associated change in nomenclature implemented by White et al. (2017). However, application of a multispecies coalescent-based approach to our data allowed visualisation of the uncertainty in species tree topology and incomplete lineage sorting. Whilst our Bayesian multispecies coalescent analyses do not specifically refute the observation that *Manta* is nested within *Mobula*, we find substantial uncertainty in the placement of *Mobula mobular* (Figure 2.5 and Supplementary Figures S2.5-S2.7). Trees within the 95% Highest Posterior Density (HPD) that place *Mobula mobular* with the manta rays are present in approximately equal proportions to trees placing this species with the remaining devil rays (Supplementary Table S2.10), thereby producing trees where the two formerly recognised genera are reciprocally monophyletic. Our results indicate that this uncertainty can be attributed to incomplete lineage sorting rather than ancient admixture or introgression, and standing variation in extinct ancestral populations is hypothesised to drive taxonomic uncertainty with respect to the validity of the genus *Manta*. Given that recently separated populations or species will pass through stages of polyphyly and paraphyly before becoming reciprocally monophyletic in the absence of additional introgression (e.g. Avise & Ball, 1990; Patton & Smith, 1994), it is reasonable to hypothesise that we are observing this process in the Mobulidae. Based on current information however, our data are in agreement with the

conclusion of White et al. (2017) with respect to the validity of the genus *Manta*, and further support the names *Mobula alfredi* and *Mobula birostris* as valid.

S2.2.2 *Mobula* Sp. 1

We find strong evidence supporting the existence of a third, undescribed species of manta ray in the Gulf of Mexico (hereafter referred to as '*Mobula* sp. 1'). Samples were analysed from two sites within the Gulf of Mexico; offshore of the Yucatan Peninsula and Flower Garden Banks National Marine Sanctuary (FGBNMS) and were initially identified as *Manta birostris* (now *Mobula birostris*) (Figure 2.1). When these Gulf of Mexico samples were analysed alongside *Mobula birostris* samples collected elsewhere (Sri Lanka, the Philippines and the Pacific side of Mexico), individuals were found to fall within two distinct groups; one containing only individuals from the Gulf of Mexico sites, and the other containing additional individuals from these same Gulf of Mexico sites as well as *M. birostris* individuals sampled elsewhere (Figure 2.2 and Figure 2.3A). In addition, we find decisive support for models recognising these groups as distinct species through Bayes Factor Delimitation (Figure 2.2). Given that samples from both groups were collected within Gulf of Mexico sites, *Mobula birostris* can be considered to occur in sympatry with *Mobula* sp. 1, constituting separately evolving lineages (De Queiroz, 2007). Monophyly of groups supports these as separate species under the phylogenetic species concept (Frankham et al. 2012). Furthermore, sympatry of populations suggests reproductive isolation driven either by a factor other than geographical separation, or historical separation followed by modern secondary contact (as hypothesised by Hinojosa-Alvarez et al. (2016)), and these species are therefore further supported under the Biological Species concept (Frankham et al. 2012). In addition, we report on a single individual which could be considered genetically intermediate between the two groups (Figure 2.2 and Figure 2.3A), indicating that hybridisation may occur between the two species, as between *Mobula alfredi* and *M. birostris* (Walter et al. 2014), although this does require confirmation through further testing.

In addition to previous observations of possible morphological differences (Marshall et al. 2009), novel mitochondrial DNA haplotypes have also been reported from manta rays off the Yucatan Peninsula, and a speciation event hypothesised (Hinojosa-Alvarez et al. 2016). Our

study is the first analysis of genome-wide data to suggest that there are two species of manta ray in the Gulf of Mexico; a finding that is consistent with previous studies (Hinojosa-Alvarez et al. 2016; Stewart et al. 2018a). Monophyly of groups indicate that some *Mobula birostris* individuals using sites in the Gulf of Mexico are more closely related to *M. birostris* in Sri Lanka and the Philippines than to individuals of *Mobula* sp. 1 using those same Gulf of Mexico sites. It is therefore likely that these species occur in a state of mosaic sympatry, as with *Mobula alfredi* and *M. birostris* elsewhere (Kashiwagi et al. 2011). For effective conservation and management, it will be necessary to formally describe this new species and determine the extent of its range.

S2.2.3 *Mobula kuhlii* and *Mobula kuhlii cf. eregoodootenkee*

A recent taxonomic review concluded that *Mobula eregoodootenkee* is a junior synonym of *Mobula kuhlii* based on mitogenome and nuclear data for a single sample per putative species (White et al. 2017). In direct contrast, our phylogenetic analysis of genome-wide SNPs for multiple individuals per species from a broad geographic range placed individuals of *Mobula kuhlii* and *M. kuhlii cf. eregoodootenkee* into discrete monophyletic clades with very high bootstrap support (Figure 2.2). This pattern was also mirrored in the results of our Principal Components Analysis (PCA; Figure 2.3E). Bayes Factor Delimitation models that recognised individuals identified as *Mobula eregoodootenkee* as a distinct species from *M. kuhlii* were consistently favoured over the null model where these individuals are considered a single species (Figure 2.2; Supplementary Table S2.7). Given that both species groups included samples that were collected within the same ~120km stretch of South African coastline, the divergence reported here between *Mobula kuhlii* and *M. kuhlii cf. eregoodootenkee* cannot be attributed to geographic population structure (Sukumaran & Knowles, 2017). There is evidence to suggest that periods of speciation within the Mobulidae correspond to episodes of global warming and associated changes in upwelling intensity and productivity, and it is hypothesized that this led to fragmentation and subsequent divergence with respect to feeding strategies (Poortvliet et al. 2015). Differences in morphology between specimens identified as *Mobula kuhlii* and *M. kuhlii cf. eregoodootenkee* (Notarbartolo di Sciara, 1987; Notarbartolo di Sciara et al. 2017), and particularly differences in the length of the cephalic fins and gill plate morphology (Paig-Tran et al. 2013) that relate directly to the filter feeding

strategy of mobulid rays may lend support to this hypothesis. Notwithstanding, the present study provides robust evidence from genomic data that individuals identified as *Mobula kuhlii cf. eregoodootenkee* belong to a distinct species to individuals identified as *Mobula kuhlii* as recognised prior to White et al. (2017).

S2.2.4 *Mobula mobular* and *Mobula mobular cf. japanica*

A recent taxonomic review concluded that *Mobula japanica* is a junior synonym of *Mobula mobular* (White et al. 2017). In agreement with this conclusion, we find no evidence from genome-wide SNPs to support *Mobula mobular cf. japanica* as a distinct species to *M. mobular* as formerly recognised. Individuals identified as *Mobula mobular* (as formerly recognised; with a distribution restricted to the Mediterranean Sea) do not form a reciprocally monophyletic group to the exclusion of individuals identified as *M. mobular cf. japanica* (circumglobally distributed with the exception of the Mediterranean Sea), and instead these individuals form a single clade with high bootstrap support (Figure 2.2). Clustering analyses indicate a degree of population structure (Figure 2.3C-D), with some modest differentiation between Indo-Pacific and Atlantic (including Mediterranean) groups (F_{ST} = 0.04 ± 0.008). Results from Bayes Factor Delimitation are far less conclusive than those for other clades (Figure 2.2), and support for split models being driven by geographic segregation of populations cannot be ruled out (Sukumaran & Knowles, 2017). Our data therefore supports *Mobula mobular* as a single species unit, with *Mobula mobular cf. japanica* a junior synonym.

S2.2.5 *Mobula hypostoma* and *Mobula munkiana*

In their recent taxonomic review, White et al. (2017) reported a close relationship between *Mobula hypostoma* and *Mobula munkiana* observed through analysis of nuclear exon data for a single sample per putative species and commented that further research is required to ascertain whether these are truly separate species. Our genome-wide SNP data provide evidence to support *Mobula hypostoma* and *M. munkiana* as distinct species units (Figure 2.2 and Figure 2.3G-H). Whilst these species are geographically segregated in the Atlantic and Eastern Pacific Oceans respectively, our data indicates divergence of a similar magnitude to

that of other distinct species groups within the Mobulidae (Figure 2.2 and Figure 2.3, Supplementary Figure S2.1).

S2.2.6 *Mobula hypostoma cf. rochebrunei*

A recent taxonomic review concluded that *Mobula rochebrunei* (a pygmy devil ray species described off the coast of West Africa) is a junior synonym of *Mobula hypostoma,* based on mitogenome data for a single sample per putative species (White et al. 2017). However, mitogenome data is considered unsuitable for species delimitation or phylogenetics when used in isolation (Petit & Excoffier, 2009), and previous studies have concluded *Mobula hypostoma cf. rochebrunei* is a distinct species based on morphological differences (Cadenat, 1960; Notarbartolo di Sciara, 1987). In this study, we were unable to generate molecular data representing *Mobula hypostoma cf. rochebrunei*, due to the only available sample being from a museum specimen stored in formalin, yielding no DNA. Notwithstanding, the revision published by White et al. (2017) is consistent with equivalent mitochondrial sequence divergence estimates for mobulid groups where further study has resolved separate species: *Mobula alfredi* and *M. birostris* (Marshall et al. 2009; Kashiwagi et al. 2012; this study), and *M. kuhlii* and *M. kuhlii cf. eregoodootenkee* (this study). Given the extent of Illegal, Unreported and Unregulated (IUU) fishing pressure in the West African region (Agnew et al. 2009) and the high vulnerability to extinction which exists for mobulid species with restricted ranges (Atta-Mills et al. 2004; Doumbouya, 2009) efforts to evaluate mobulid diversity in West Africa should be given a high priority (see Stewart et al. 2018b).

S2.2.7 Other diversity within the Mobulidae

We identify substantial geographically mediated population structure within *Mobula kuhlii* and *Mobula alfredi* with genome-wide SNPs. In *Mobula kuhlii*, individuals sampled across the Indian Ocean fall into reciprocally monophyletic groups with high bootstrap support (Figure 2.2). Consistent with this, individuals from the East and West Indian Ocean are separated into distinct clusters through PCA (Figure 2.3F), with substantial differentiation (F_{ST} = 0.39 ± 0.046). Models recognising these populations as distinct species were favoured through Bayes Factor

Delimitation (Figure 2.2; Supplementary Tables S2.5 and S2.7). Indeed, there are anecdotal suggestions of morphological differences occurring in *Mobula kuhlii* across the Indian Ocean (Stevens et al. 2018b). We identified similar patterns in *Mobula alfredi*. Individuals sampled in the Indian and Pacific Oceans formed distinct monophyletic groups (Figure 2.2), visible as clusters through PCA (Figure 2.3B) and exhibited substantial differentiation (F_{ST} = 0.22 ± 0.045). Furthermore, models recognising these populations as distinct species were consistently favoured through Bayes Factor Delimitation (Figure 2.2, Supplementary Tables S2.5 and S2.7).

However, approaches based on the multispecies coalescent, such as Bayes Factor Delimitation, recover all structure both at the level of the species and the population (Sukumaran & Knowles, 2017). In *Mobula kuhlii* and *M. alfredi*, we cannot rule out a geographic driver of the patterns observed, which should therefore be tentatively attributed to population structure over speciation. Our study aimed to delimit species units for conservation and understand phylogenetic relationships. It is therefore important to recognise that the set of samples analysed here is limited to a few individuals per population, and a limited number of populations are sampled across the broad geographic ranges of these species, within which large areas are unrepresented. Detailed inferences regarding population genetic structure are therefore not possible since capturing population level diversity requires a much more comprehensive sampling regime, and results must be interpreted with caution. Further research is therefore required to determine whether the large differences within *Mobula kuhlii* and *M. alfredi* observed here are due to barriers to gene flow, isolation by distance across ocean clines or other factors. Nonetheless, we find sufficient intra-specific diversity to indicate potential for determining regional location of catch in these species, and indeed this is increasingly required to comply with global requirements to include capture location and species in trade (Nielsen et al. 2012). Such further study to assess population genetic structure of both species, and other species of mobulid ray, would be prudent to support effective management.

S2.3 Supplementary Figures and Tables

Supplementary Table S2.1: Sample information. We use species names assigned to samples at the time of collection, some of which are now considered invalid following White et al. (2017). CITES information is provided in footnote.[a]

Sample codes	Species	Location	Contacts	Previous Publications	Total	No. in COI Dataset	No. in ddRAD Dataset
0130, 0131, 0132, 0135, 0136, 0138	*Manta alfredi*	D'Arros, Amirante Islands, Seychelles	L. Peel, G. Stevens		6	6	6
0140, 0141, 0144, 0145, 0146, 0147, 0148, 0149	*Manta alfredi*	Barefoot Channel, Yasawas Islands, Fiji	S. Pollett, D. Bowling, H. Pacey		8	8	8
0685, 0686	*Manta alfredi*	Egmont, British Indian Ocean Territory (BIOT), Chagos Archipelago	D. Fernando		2	2	2
0687, 0688	*Manta alfredi*	Diego Garcia, British Indian Ocean Territory (BIOT), Chagos Archipelago	D. Fernando		2	2	2
0731	*Manta birostris*	Mirissa, Sri Lanka	D. Fernando	Poortvliet et al. (2015); Stewart et al. (2016); Stewart et al. (2017)	1	1	1
0732, 0736	*Manta birostris*	Negombo, Sri Lanka	D. Fernando	Poortvliet et al. (2015); Stewart et al. (2016); Stewart et al. (2017)	2	1	2

1102, 1110, 1114, 1122	*Manta birostris*	Jagna, Bohol landing site, Philippines	A. Ponzo	Stewart et al. (2017)	4	4	4
0980[b], 0981[b], 0982[b], 0983[b], 0984[b], 0985, 0986, 0987, 0988, 1239[b], 1240[b], 1241[b], 1242[b]	*Manta birostris*	Yucatan Northern tip, Mexico Caribbean	R. Bonfil	Hinojosa-Alvarez et al. (2016)	13	9	13
1168	*Manta birostris*	Bahia de Banderas, Jalisco, Mexico Pacific	J. Stewart	Stewart et al. (2016)	1	1	1
1327[b], 1328[c]	*Manta birostris*	Flower Garden Banks National Marine Sanctuary, Texas, USA	J. Stewart		2	2	2
0684	*Mobula eregoodootenkee*	Al Khor, Qatar	A. Moore	Moore (2012)	1	1	1
0696	*Mobula eregoodootenkee*	Arabian Gulf, United Arab Emirates	R. Jabado		1	1	1
0697	*Mobula eregoodootenkee*	Fujeirah, United Arab Emirates	R. Jabado		1	1	1
0810, 0813	*Mobula eregoodootenkee*	Zinkwazi, South Africa	S. Wintner	Poortvliet et al. (2015)	2	2	2
0873, 0874, 0886, 0888, 0891, 0900, 0924, 0929, 0933, 0938	*Mobula hypostoma*	Sarasota, Florida	K. Bassos-Hull		10	10	10
0990, 0991, 0992, 0993	*Mobula hypostoma*	Yucatan Northern tip, Mexico Caribbean	R. Bonfil		4	4	4
0003, 0024	*Mobula japanica*	Jagna, Bohol landing site, Philippines	A. Ponzo	Stewart et al. (2017)	2	2	2

0219, 0229, 0234, 0283	*Mobula japanica*	Negombo, Sri Lanka	D. Fernando	Poortvliet et al. (2015); Stewart et al. (2017)	4	4	4
0300, 0329, 0343	*Mobula japanica*	Mirissa, Sri Lanka	D. Fernando	Poortvliet et al. (2015); Stewart et al. (2017)	3	3	3
0707, 0711	*Mobula japanica*	Ras Al Khaimah, United Arab Emirates	R. Jabado		2	2	2
0757, 0771, 0773	*Mobula japanica*	Lome, Togo	M. Poortvliet	Poortvliet et al. (2015)	3	3	3
0793	*Mobula japanica*	Puerto Adolfo Lopez Mateos, Mexico Pacific	M. Poortvliet	Poortvliet et al. (2015)	1	1	1
0853, 0862	*Mobula japanica*	Karachi, Pakistan	M. Moazzam		2	2	2
0524	*Mobula kuhlii*	Negombo, Sri Lanka	D. Fernando	Stewart et al. (2017)	1	1	1
0582, 0677	*Mobula kuhlii*	Durban, South Africa	S. Wintner	Poortvliet et al. (2015)	2	2	2
0605	*Mobula kuhlii*	Warner Beach, South Africa	S. Wintner	Poortvliet et al. (2015)	1	1	1
0774, 0776, 0777, 0778	*Mobula kuhlii*	Maumere, Indonesia	M. Poortvliet	Poortvliet et al. (2015)	4	4	4
0101, 0104	*Mobula mobular*	Gaza City Fishing Landing Site, Gaza, Palestine	M. Abudaya, J. Salah		2	2	2
0111, 0112	*Mobula mobular*	Khanyounis Fishing Landing Site, Gaza, Palestine	M. Abudaya, J. Salah		2	2	2
0129	*Mobula mobular*	Middle area fishing landing site, Gaza, Palestine	M. Abudaya, J. Salah		1	1	1
0816, 0821, 0824, 0827	*Mobula munkiana*	El Pardito, Mexico Pacific	M. Poortvliet	Poortvliet et al. (2015)	4	4	4

0830, 0833, 0840, 0843	*Mobula munkiana*	Puerto Adolfo Lopez Mateos, Mexico Pacific	M. Poortvliet	Poortvliet et al. (2015)	4	4	4
0844	*Mobula munkiana*	Buena Vista Port of San Jose, Guatemala	R. Brittain		1	1	1
1006, 1009, 1019	*Mobula munkiana*	Peru	K. Forsberg, J. Stewart	Stewart et al. (2017)	3	3	3
0943	*Mobula rochebrunei*	Musee de la Mer, Goree, Senegal	F. Doumbouya		1	0	0
0086	*Mobula tarapacana*	Jagna, Bohol landing site, Philippines	A. Ponzo	Stewart et al. (2017)	1	1	1
0408, 0411	*Mobula tarapacana*	Mirissa, Sri Lanka	D. Fernando	Poortvliet et al. (2015)	2	2	2
0847	*Mobula tarapacana*	Karachi, Pakistan	M. Moazzam		1	1	1
0159	*Mobula thurstoni*	Jeddah Fish Market, Saudi Arabia	J. Spaet	Spaet & Berumen, (2015)	1	1	1
0712	*Mobula thurstoni*	Fujeirah, United Arab Emirates	R. Jabado		1	1	1
0780, 0782, 0783, 0784, 0785, 0786, 0787	*Mobula thurstoni*	El Pardito, Mexico Pacific	M. Poortvliet	Poortvliet et al. (2015)	7	7	7
1366, 1367, 1368, 1369, 1370	*Rhinoptera bonasus*	Sarasota, Florida, USA	K. Bassos-Hull		5	0	5
Totals					121	110	120

[a] Where relevant, samples are associated with CITES permit numbers 4980, 551058/01, 10/0004/2014/01, 531703/01, MX80544, 542913/01 and 548594/01 or were exchanged between institutions under CITES scientific exemption codes GB015 and US150(A).

[b] Samples identified as belonging to *Mobula* sp. 1 in our analyses.

[c] Likely hybrid (*Manta birostris* x *Mobula sp. 1*).

Supplementary Table S2.2: Sequencing information for ddRAD libraries used in the phylogenomics study presented in Chapter 2. Each library was sequenced in a single lane of the relevant platform.

Library	Sequencing Platform	Number of Reads (forward and reverse)	Number of Samples Multiplexed
1	Illumina MiSeq	18,586,221	24
2	Illumina HiSeq	230,459,465	96

Supplementary Table S2.3: Information on two SNP matrices generated using ddRAD, referred to as 'p10' and 'p90'. The number of SNPs in individual level matrices are given, with numbers of SNPs in species level matrices in brackets.

Dataset	Minimum individuals possessing a locus	Loci excluded with >2x SD coverage	Loci excluded with <1/3x SD coverage	Loci excluded with >95% probability of heterozygote excess	SNPs retained	Missing Data
'p10'	10	1761	7661	24	7926 (7902)	47%
'p90'	90	789	0	0	1762 (1755)	14%

Supplementary Table S2.4: Details of SNP matrices used to run PCAs. Dataset 'p10' (7926 SNPs) was split into clades for ease of visualisation, resulting in sites not sampled within clades to drop out.

Clade	SNPs retained
Mobula alfredi and *Mobula birostris* (including *Mobula* sp. 1)	5730
Mobula mobular (including samples identified as *Mobula mobula cf. japanica*)	5428
Mobula thurstoni and *Mobula kuhlii* (including samples identified as *Mobula kuhlii cf. eregoodootenkee*)	5384
Mobula hypostoma and *Mobula munkiana*	5063

Supplementary Table S2.5: Details of species delimitation models tested with Bayes Factor Delimitation (BFD*) within the first clade identified, the manta rays; *Mobula alfredi* and *Mobula birostris*, including *Mobula mobular cf. japanica* sister clade individuals 0707, 0283, 0771 and 0024.

Rank	SNPs retained	MLE ($2\log_e BF$) - gamma prior	MLE ($2\log_e BF$) - default 1/X prior	Description	Rationale	Reference
1	1746	-4941.58 (-1063.58)	-4946.44 (1054.72)	Split *Mobula alfredi* into two species units in Indian and Pacific Oceans. In addition, another species of manta ray is present in the Atlantic Ocean, sharing most recent common ancestor with *Mobula birostris.*	Monophyly of *Mobula alfredi* individuals in these ocean basins, and distinguishability through PCA. *Mobula birostris* split hypothesised in several studies.	This study
2	1749	-5085.46 (-775.82)	-5086.94 (-773.72)	In addition to *Mobula alfredi* and *Mobula birostris*, a third species of manta ray is present in the Atlantic Ocean, sharing most recent common ancestor with *Mobula birostris.*	Hypothesised in several studies. Some showing divergence on phylogenetic trees following this pattern.	Marshall et al. (2009); Hinojosa-Alvarez et al. (2016); this study.
3	1748	-5332.41 (-281.92)	-5335.11 (-277.38)	Split *Mobula alfredi* into two species units in Indian and Pacific Oceans but recognise all individuals identified as Mo*bula birostris* as a single species.	Monophyly of *Mobula alfredi* individuals based on these ocean basins, and distinguishability through PCA. Potential hybridisation between *Mobula birostris* groups identified herein.	This study

4	1750	-5339.87 (-267)	-5341.8 (-264)	Split *Mobula birostris* based entirely on geography (Atlantic/Gulf of Mexico individuals a separate species unit). Recognise all individuals identified as *Mobula alfredi* as a single species.	To assess whether all *Mobula birostris* individuals sampled in the Atlantic could be considered a distinct species from *Mobula birostris* sampled elsewhere.	Marshall et al. (2009)
5	1751	-5473.37 (Null)	-5473.8 (Null)	Null model and current arrangement – two species of manta ray: *Mobula alfredi* and *Mobula birostris.*	Current taxonomic arrangement.	Marshall et al. (2009); Kashiwagi et al. (2012)
6	1754	-7348.39 (3750.04)	-7350.44 (3753.28)	Random assignment of individuals into two species units.	To assess relative support for other models.	
7	1754	-7360.08 (3773.42)	-7359.33 (3771.06)	Recognise a single species of manta ray (lump *Mobula alfredi* and *Mobula birostris*).	Similar levels of sequence divergence as species that have previously been lumped based on said low sequence divergence.	White et al. (2017)
8	1760	-11864.6 (12782.46)	-11864.51 (12781.42)	Lump *Mobula mobular cf. japanica* with *Mobula birostris. Mobula alfredi* distinct.	To assess evidence for interaction from higher up the tree.	

Supplementary Table S2.6: Details of species delimitation models tested with Bayes Factor Delimitation (BFD*) within the second clade identified; *Mobula mobular* and *Mobula mobular cf. japanica*, including *Mobula alfredi* sister clade individuals 0135, 0131, 0685 and 0146.

Rank	SNPs retained	MLE ($2\log_e BF$) - gamma prior	MLE ($2\log_e BF$) - default 1/X prior	Description	Rationale	Reference
1	1752	-5390.81 (-119.58)	-5391.65 (-118.44)	*Mobula mobular cf. japanica* and *Mobula mobular* are distinct species units, where the latter is restricted to the Mediterranean Sea.	Taxonomy recognised prior to revision by White et al. (2017).	(Notarbartolo di Sciara (1987); Adnet et al. (2012); Bustamante et al. (2016)
2	1755	-5390.93 (-119.34)	-5393.24 (-115.26)	Split individuals into Atlantic (including Mediterranean) and Indo-Pacific species units.	Distinguishability of these two groups through PCA.	This study
3	1755	-5424.82 (-51.56)	-5426.71 (-48.32)	Random assignment of individuals into two species units.	To assess relative support for other models.	
4	1757	-5450.6 (Null)	-5450.87 (Null)	Null model and current arrangement - *Mobula mobular cf. japanica* is a junior synonym of *Mobula mobular,* recognising a single species.	Current taxonomic arrangement.	Poortvliet et al. (2015); White et al. (2017)
5	1759	-9150.56 (7399.92)	-9153.48 (7405.22)	Lump *Mobula alfredi* with *Mobula mobular (*as formerly recognised; not including specimens formerly attributed to *Mobula mobular cf. japanica*).	To assess evidence for interaction from higher up the tree.	

Supplementary Table S2.7: Details of species delimitation models tested with Bayes Factor Delimitation (BFD*) within the third clade identified; *Mobula thurstoni, Mobula kuhlii* and *Mobula kuhlii cf. eregoodootenkee*, including *Mobula hypostoma* sister clade individuals 0874, 0933, 0924 and 0992.

Rank	SNPs retained	MLE ($2\log_e BF$) - gamma prior	MLE ($2\log_e BF$) - default 1/X prior	Description	Rationale	Reference
1	1710	-3160.68 (-1263.8)	-3159.35 (-1270)	Split *Mobula kuhlii* into two species units in East and West Indian Ocean, and recognise *Mobula thurstoni* and *Mobula kuhlii cf. eregoodootenkee* as distinct species.	Monophyly of *Mobula kuhlii* individuals based on geography, and distinguishability with PCA. Monophyly and distinguishability of *Mobula thurstoni* and *Mobula kuhlii cf. eregoodootenkee.*	This study
2	1717	-3289.06 (-1007.04)	-3291.85 (-1005)	*Mobula kuhlii cf. eregoodootenkee, Mobula kuhlii* and *Mobula thurstoni* are three distinct species.	Taxonomy recognised prior to revision published by White et al. (2017).	Notarbartolo di Sciara (1987)
3	1731	-3792.58 (Null)	-3794.35 (Null)	Null model and current arrangement – *Mobula kuhlii cf. eregoodootenkee* is a junior synonym of *Mobula kuhlii. Mobula thurstoni* is distinct.	Current taxonomic arrangement.	White et al. (2017)
4	1756	-5679.77 (3774.38)	-5683.06 (3777.42)	Random assignment of individuals to 3 species units.	To assess relative support for other models.	
5	1759	-5818.44 (4051.72)	-5816.29 (4043.88)	Single species within this clade (lump *Mobula thurstoni*, *Mobula kuhlii* and *Mobula kuhlii cf. eregoodootenkee*).	For completeness.	

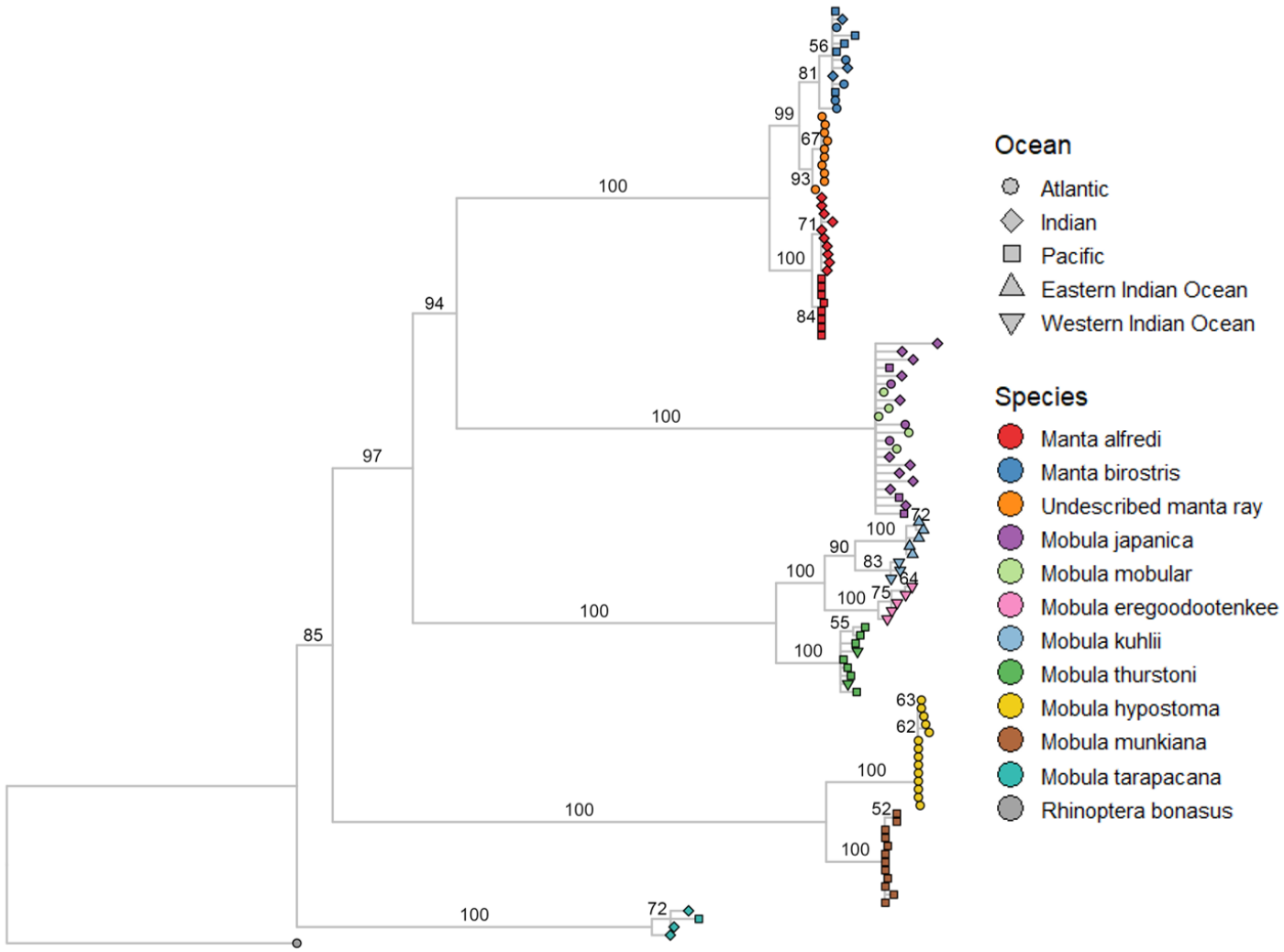
6	1733	-8422.04 (9258.92)	-8424.08 (9259.46)	Lump *Mobula hypostoma* with *Mobula thurstoni. Mobula kuhlii cf. eregoodootenkee* and *Mobula kuhlii* distinct.	To assess evidence for interaction from higher up the tree.

Supplementary Table S2.8: Details of species delimitation models tested with Bayes Factor Delimitation (BFD*) within the fourth clade identified; *Mobula hypostoma* and *Mobula munkiana*, including *Mobula tarapacana* sister clade individuals 0086, 0408, 0411 and 0847.

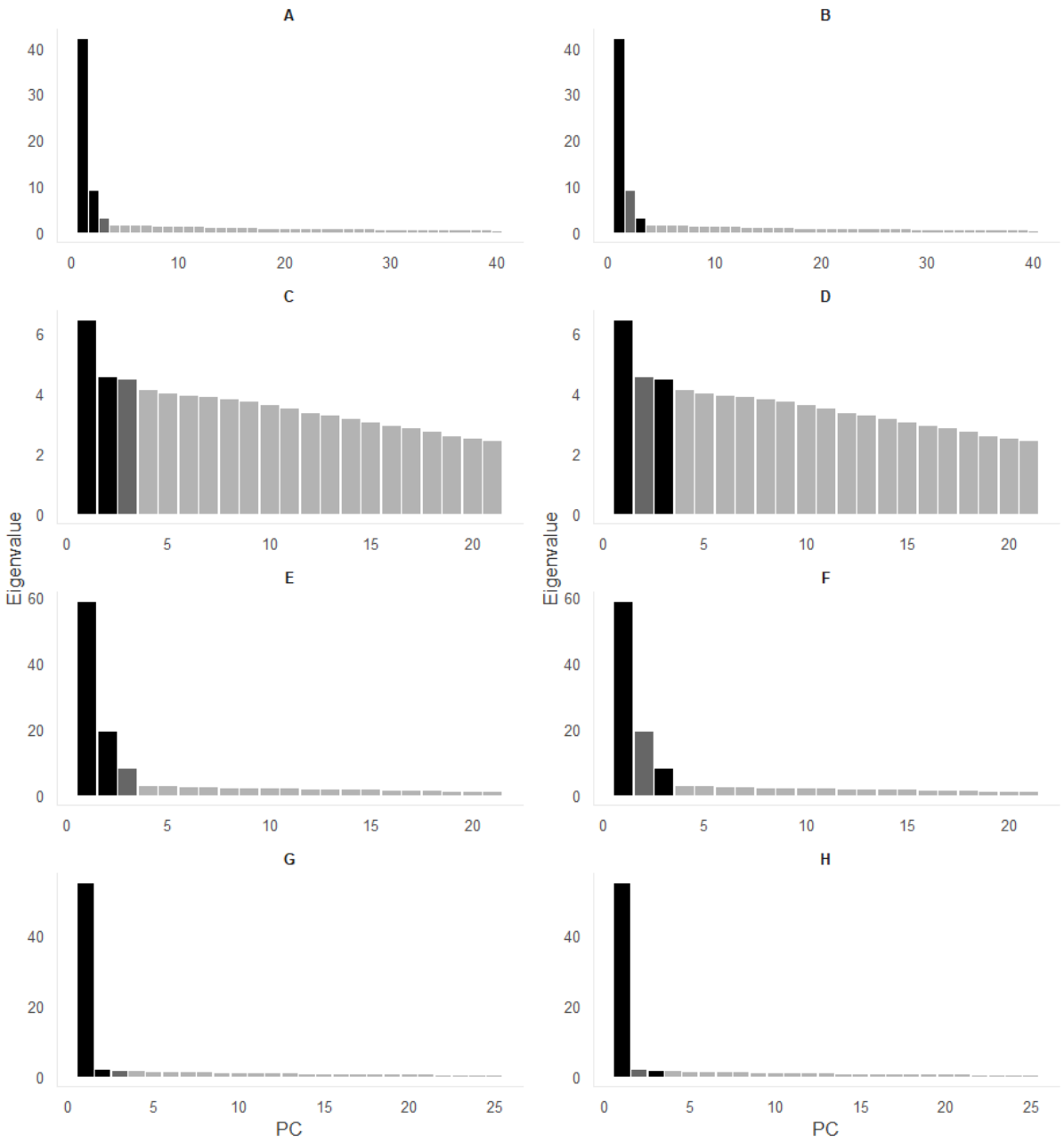
Rank	SNPs retained	MLE ($2\log_e BF$) - gamma prior	MLE ($2\log_e BF$) - default 1/X prior	Description	Rationale	Reference
1	1707	-1881.75 (Null)	-1883.71 (Null)	Null model and current arrangement - *Mobula munkiana* and *Mobula hypostoma* are distinct species.	Current taxonomic arrangement.	Notarbartolo di Sciara (1987); this study
2	1731	-2998.01 (2232.52)	-2997.63 (2227.84)	Random assignment of individuals into two species units.	To assess relative support for the other models.	
3	1731	-3005.93 (2248.36)	-3004.82 (2242.22)	Single species within this clade - *Mobula munkiana* is a junior synonym of *Mobula hypostoma.*	Suggested as a possible line of investigation in White et al. (2017).	Suggested in White et al. (2017).
4	1748	-6236.55 (8709.6)	-6238.57 (8709.72)	Lump *Mobula tarapacana* with *Mobula munkiana. Mobula hypostoma* distinct.	To assess evidence for interaction from higher up the tree.	

Supplementary Table S2.9: Individuals included in independent runs of SNAPP (Bryant et al. 2012) for species tree inference.

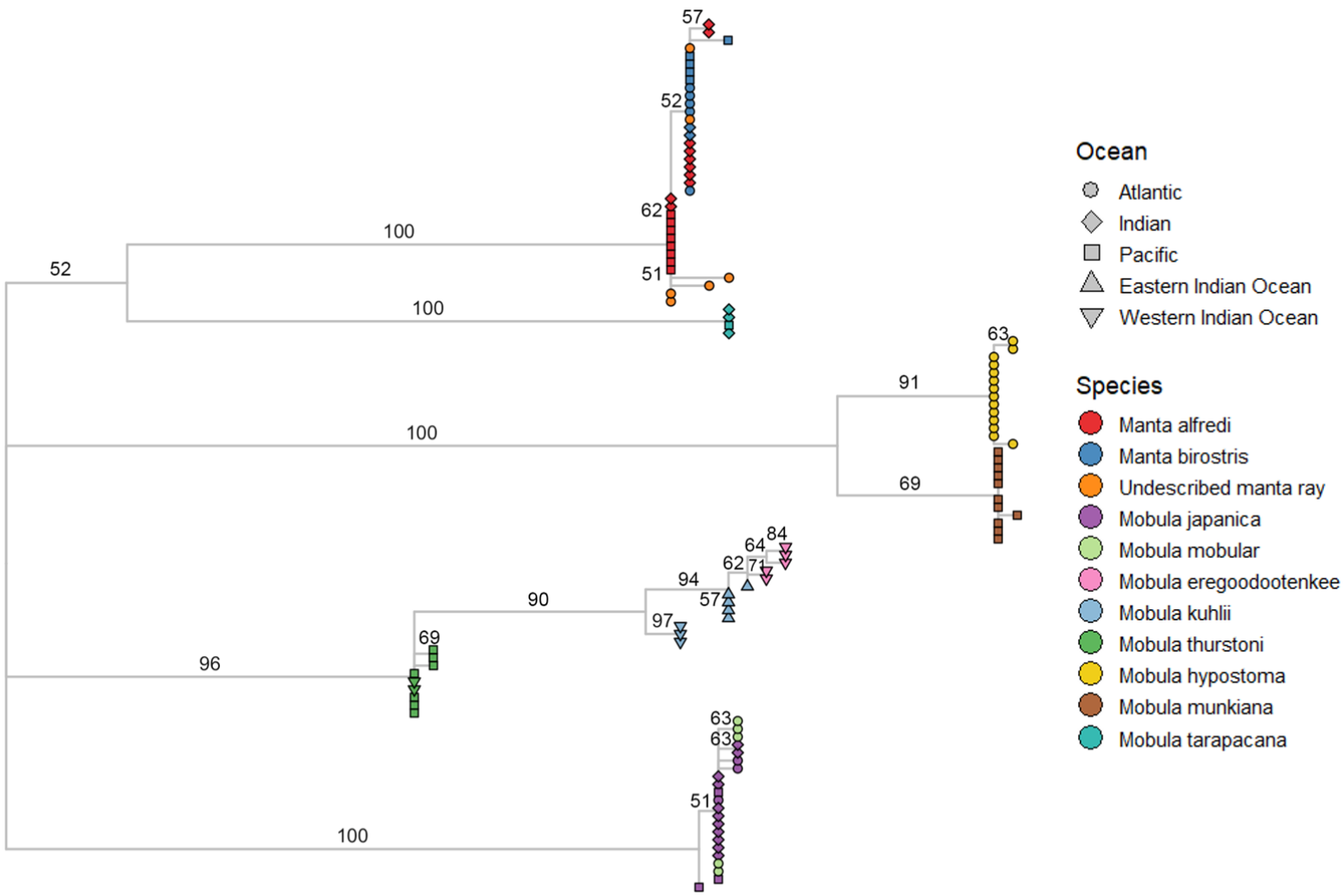
Species	Sample			
	Subset 1	Subset 2	Subset 3	Subset 4
Mobula alfredi	0135	0130	0132	0136
	0145	0140	0149	0146
	0688	0686	0687	0685
Mobula birostris	0736	1114	0732	0731
	0987	0988	0985	1110
	1168	1168	1122	1168
Mobula sp. 1	0980	1241	0981	0982
	0984	1242	1240	0983
	1327	1327	1327	1239
Mobula kuhlii cf. eregoodootenkee	0684	0684	0684	0684
	0696	0696	0697	0697
	0810	0810	0813	0813
Mobula hypostoma	0886	0873	0924	0933
	0888	0990	0938	0991
	0993	0992	0990	0993
Mobula kuhlii	0524	0524	0524	0524
	0677	0582	0677	0605
	0774	0778	0777	0776
Mobula mobular (and *Mobula mobular cf japanica*)	0112	0104	0711	0862
	0003	0771	0101	0024
	0793	0343	0219	0773
Mobula munkiana	0821	0824	0827	0833
	0844	0844	0844	0844
	1006	1019	1006	1009
Mobula tarapacana	0086	0086	0086	0086
	0847	0408	0411	0411
	0408	0847	0847	0847
Mobula thurstoni	0159	0159	0159	0159
	0712	0712	0712	0712
	0782	0787	0786	0785
No. SNPs retained	1242	1240	1253	1250



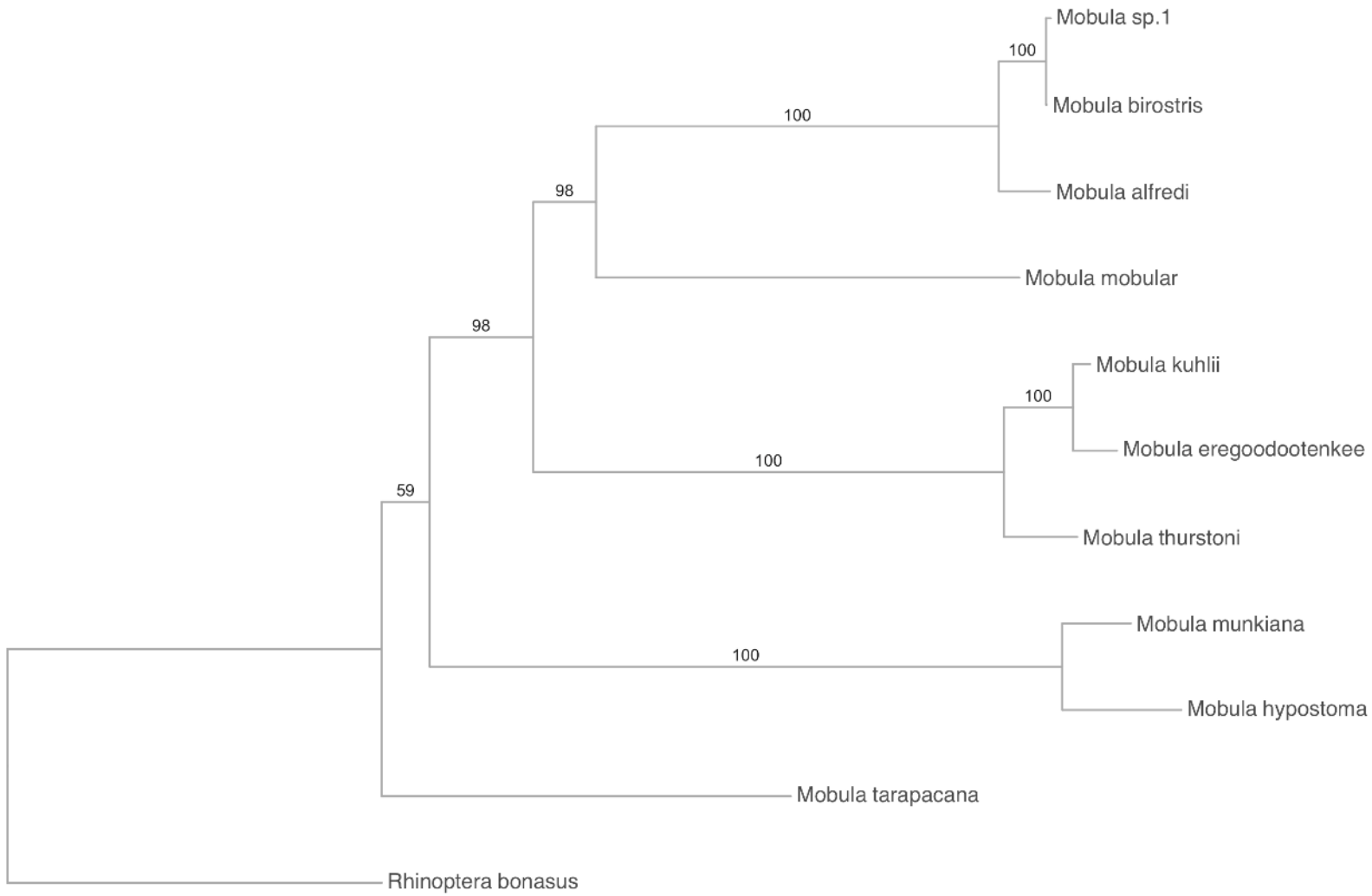
Supplementary Figure S2.1: Maximum Likelihood tree based on 1762 SNPs (dataset 'p90'). Coloured points indicate putative species, and shape indicates geographic origin of samples as specified in the key. Bootstrap values are shown on the branches and nodes with less than 50% support are collapsed. Species names are those assigned at collection, some of which are now considered invalid following White et al. (2017).



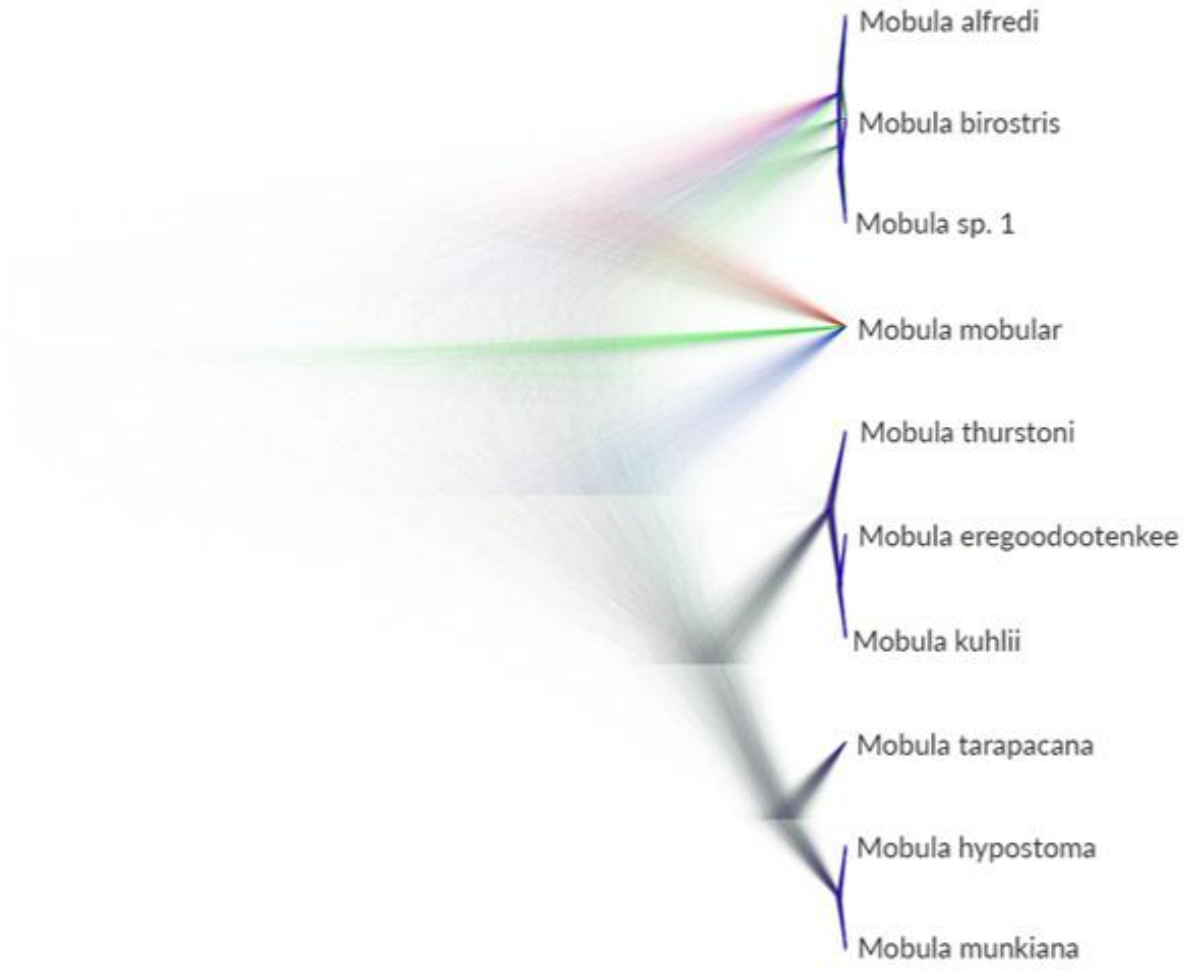
Supplementary Figure S2.2: Eigenvalue plots for each Principle Components Analysis (PCA) presented in Figure 2.3. Panel letters (A-H) correspond to the panel letters in Figure 2.3. Plotted axes are in black and retained axes in dark grey.



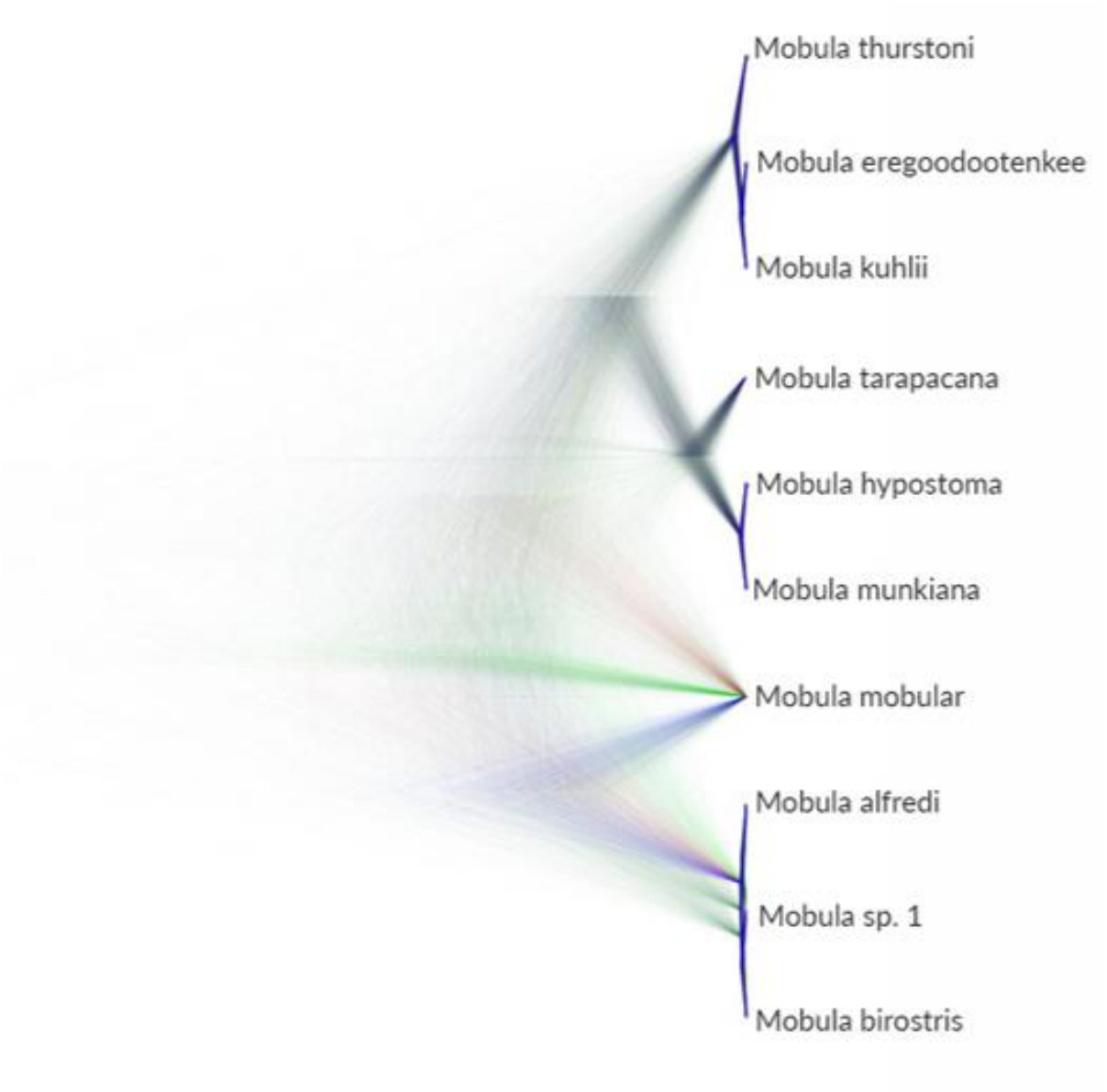
Supplementary Figure S2.3: Maximum Likelihood tree based on Cytochrome Oxidase Subunit I (COI) sequence data. Coloured points indicate putative species, and shape indicates geographic origin of samples as specified in the key. Bootstrap values are shown on the branches and nodes with less than 50% support are collapsed. Species names are those assigned to samples at collection, some of which are now considered invalid following White et al. (2017).



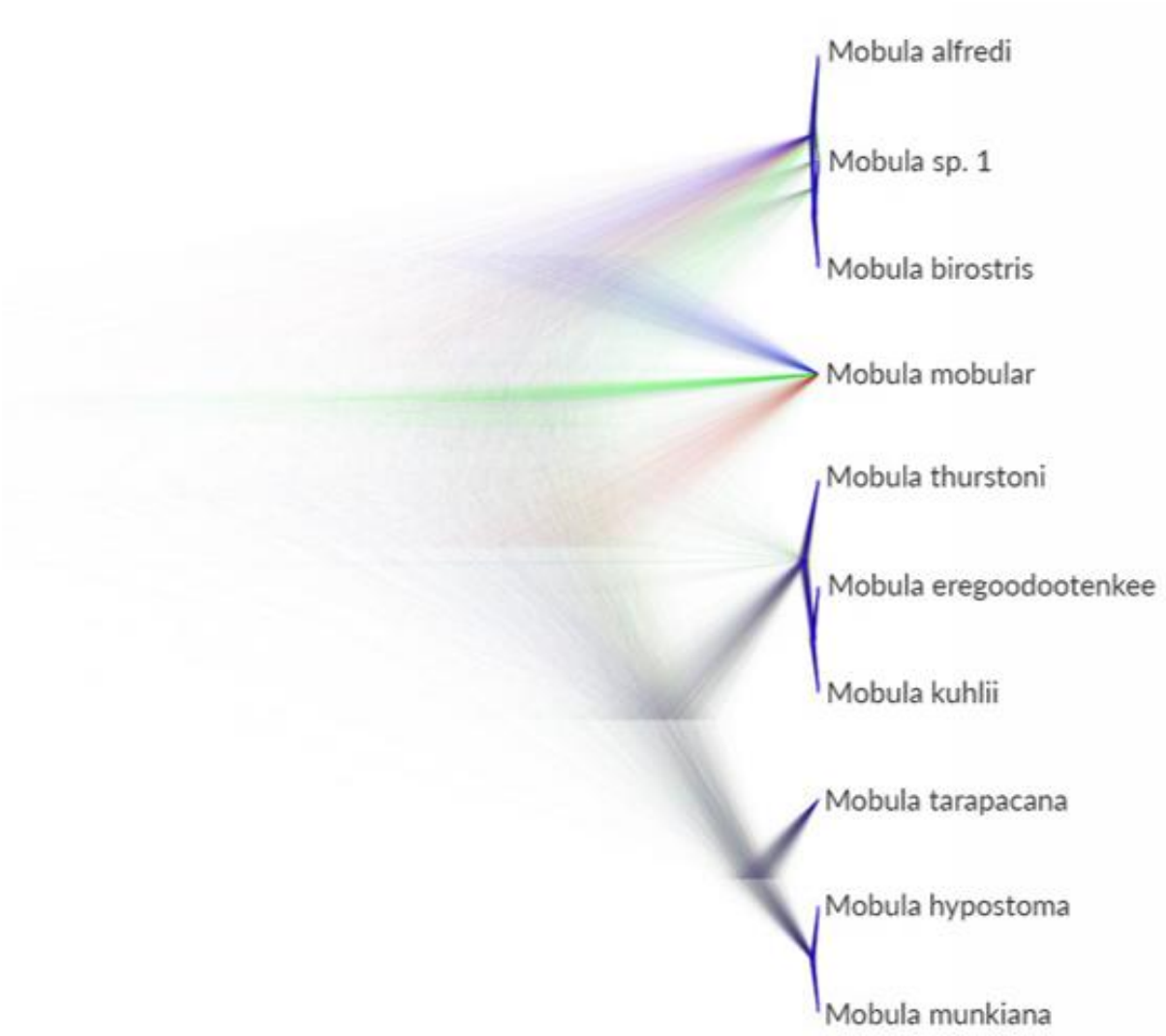
Supplementary Figure S2.4: Maximum Likelihood tree of inferred mobulid species units based on 1755 SNPs (dataset 'p90'). Bootstrap values are shown on the branches. Note that *Mobula mobular* here includes specimens identified as *Mobula mobular cf. japanica* based on geographical sampling location.



Supplementary Figure S2.5: SNP phylogeny of 30 randomly chosen individuals assigned to ten species based on 1240 SNPs (dataset 'p90', individual subset 2; Supplementary Table S2.9). Tree cloud of sampled trees produced using DENSITREE (representing samples taken every 1000 MCMC steps from 5,000,000 iterations) from SNAPP (Bryant et al. 2012) analysis to visualise the range of alternative topologies. Note that *Mobula mobular* here includes specimens identified as *Mobula mobular cf. japanica* based on geographical sampling location.



Supplementary Figure S2.6: SNP phylogeny of 30 randomly chosen individuals assigned to ten species based on 1253 SNPs (dataset 'p90', individual subset 3; Supplementary Table S2.9). Tree cloud of sampled trees produced using DENSITREE (representing samples taken every 1000 MCMC steps from 5,000,000 iterations) from SNAPP (Bryant et al. 2012) analysis to visualise the range of alternative topologies. Note that *Mobula mobular* here includes specimens identified as *Mobula mobular cf. japanica* based on geographical sampling location.



Supplementary Figure S2.7: SNP phylogeny of 30 randomly chosen individuals assigned to ten species based on 1250 SNPs (dataset 'p90', individual subset 4; Supplementary Table S2.9). Tree cloud of sampled trees produced using DENSITREE (representing samples taken every 1000 MCMC steps from 5,000,000 iterations) from SNAPP (Bryant et al. 2012) analysis to visualise the range of alternative topologies. Note that *Mobula mobular* here includes specimens identified as *Mobula mobular cf. japanica* based on geographical sampling location.

Supplementary Table S2.10: Topologies contained within the 95% Highest Posterior Density (HPD) for each subsample of individuals analysed with SNAPP (Bryant et al. 2012). Subsample 3 has 25 trees contained within the 95% HPD, and the first 20 are shown. Note that *Mobula mobular* here includes specimens identified as *Mobula mobular cf. japanica* based on geographical sampling location.

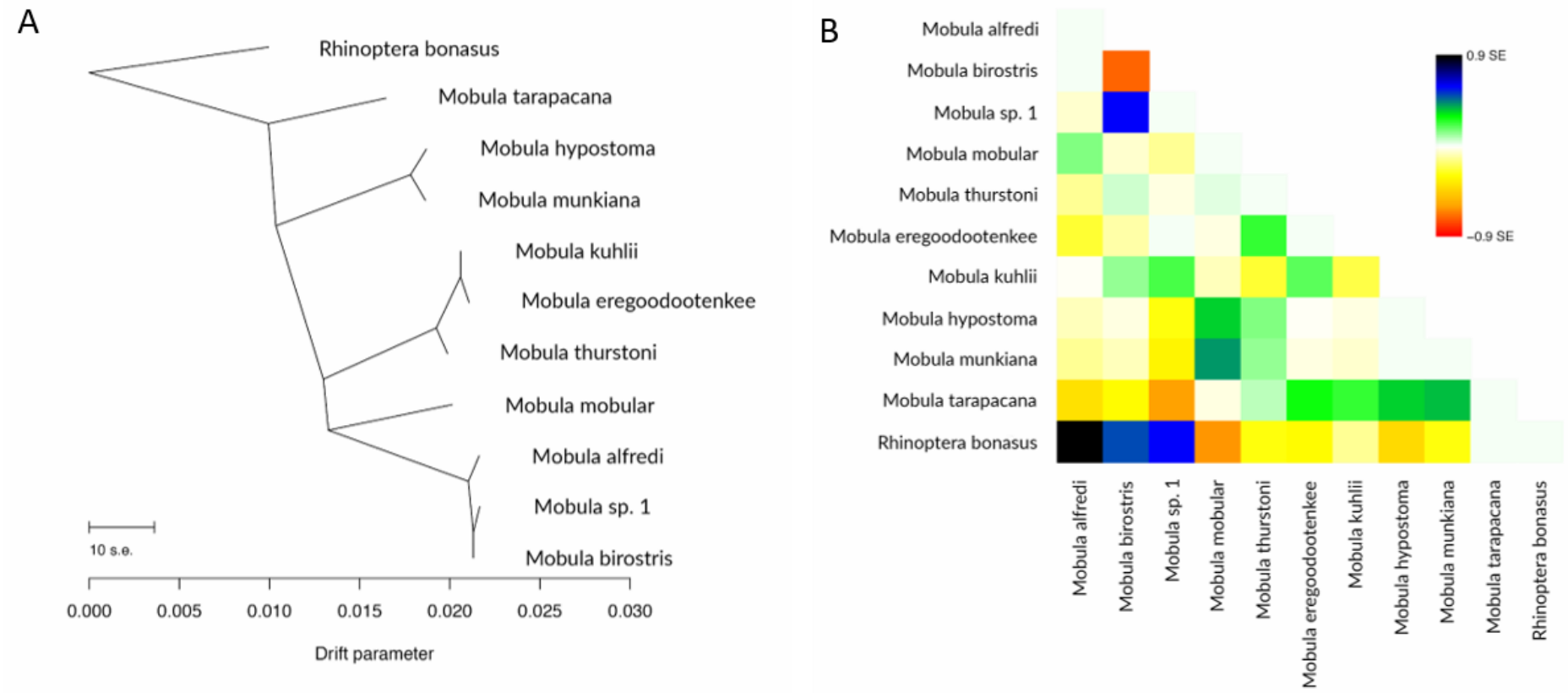
Subsample	Tree	Percentage	Tree topology
1	1	28.27%	*((Mobula mobular,(Mobula alfredi,(Mobula birostris,Mobula sp. 1))),((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))))*
	2	25.62%	*((Mobula mobular,((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee)))),(Mobula alfredi,(Mobula birostris,Mobula sp. 1)))*
	3	18.52%	*(Mobula mobular,(((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),(Mobula alfredi,(Mobula birostris,Mobula sp. 1))))*
	4	5.0%	*((Mobula mobular,((Mobula alfredi,Mobula sp. 1),Mobula birostris)),((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))))*
	5	4.92%	*((Mobula mobular,((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee)))),((Mobula alfredi,Mobula sp. 1),Mobula birostris))*
	6	3.92%	*((Mobula mobular,((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee)))),((Mobula alfredi,Mobula birostris),Mobula sp. 1))*
	7	3.50%	*((Mobula mobular,((Mobula alfredi,Mobula birostris),Mobula sp. 1)),((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))))*
	8	3.47%	*(Mobula mobular,(((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),((Mobula alfredi,Mobula sp. 1),Mobula birostris)))*
	9	2.87%	*(Mobula mobular,(((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),((Mobula alfredi,Mobula birostris),Mobula sp. 1)))*

2	1	19.55%	*(((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),(Mobula mobular,(Mobula alfredi,(Mobula birostris,Mobula sp. 1))))*
	2	19.35%	*((((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),Mobula mobular),(Mobula alfredi,(Mobula birostris,Mobula sp. 1)))*
	3	13.17%	*((((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),(Mobula alfredi,(Mobula birostris,Mobula sp. 1))),Mobula mobular)*
	4	9.27%	*((((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),Mobula mobular),((Mobula alfredi,Mobula sp. 1),Mobula birostris))*
	5	8.75%	*(((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),(Mobula mobular,((Mobula alfredi,Mobula sp. 1),Mobula birostris)))*
	6	8.20%	*((((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),Mobula mobular),((Mobula alfredi,Mobula birostris),Mobula sp. 1))*
	7	8.05%	*(((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),(Mobula mobular,((Mobula alfredi,Mobula birostris),Mobula sp. 1)))*
	8	5.97%	*((((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),((Mobula alfredi,Mobula sp. 1),Mobula birostris)),Mobula mobular)*
	9	5.05%	*((((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),((Mobula alfredi,Mobula birostris),Mobula sp. 1)),Mobula mobular)*
3	1	18.77%	*(((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),(Mobula mobular,(Mobula alfredi,(Mobula birostris,Mobula sp. 1))))*
	2	14.40%	*((((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),Mobula mobular),(Mobula alfredi,(Mobula birostris,Mobula sp. 1)))*
	3	9.70%	*((((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),(Mobula alfredi,(Mobula birostris,Mobula sp. 1))),Mobula mobular)*

4	9.25%	*(((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),(Mobula mobular,((Mobula alfredi,Mobula sp. 1),Mobula birostris)))*
5	7.72%	*(((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),(Mobula mobular,((Mobula alfredi,Mobula birostris),Mobula sp. 1)))*
6	7.0%	*((((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),Mobula mobular),((Mobula alfredi,Mobula sp. 1),Mobula birostris))*
7	6.37%	*((((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),Mobula mobular),((Mobula alfredi,Mobula birostris),Mobula sp. 1))*
8	6.20%	*((((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),((Mobula alfredi,Mobula sp. 1),Mobula birostris)),Mobula mobular)*
9	4.90%	*((((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),((Mobula alfredi,Mobula birostris),Mobula sp. 1)),Mobula mobular)*
10	1.12%	*(((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula mobular,(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee)))),(Mobula alfredi,(Mobula birostris,Mobula sp. 1)))*
11	0.92%	*(((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),Mobula mobular),((Mobula alfredi,(Mobula birostris,Mobula sp. 1)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))))*
12	0.90%	*((((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),Mobula mobular),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),(Mobula alfredi,(Mobula birostris,Mobula sp. 1)))*
13	0.90%	*(((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula alfredi,(Mobula birostris,Mobula sp. 1))),(Mobula mobular,(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))))*
14	0.72%	*(((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula mobular,(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee)))),((Mobula alfredi,Mobula sp. 1),Mobula birostris))*
15	0.72%	*((((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula alfredi,(Mobula birostris,Mobula sp. 1))),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),Mobula mobular)*

	16	0.70%	*(((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),((Mobula alfredi,Mobula sp. 1),Mobula birostris)),(Mobula mobular,(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))))*
	17	0.62%	*(((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),((Mobula alfredi,(Mobula birostris,Mobula sp. 1)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee)))),Mobula mobular)*
	18	0.57%	*((((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),Mobula mobular),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),((Mobula alfredi,Mobula sp. 1),Mobula birostris))*
	19	0.55%	*((((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),Mobula mobular),(Mobula alfredi,(Mobula birostris,Mobula sp. 1))),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee)))*
	20	0.52%	*((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),((Mobula mobular,(Mobula alfredi,(Mobula birostris,Mobula sp. 1))),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))))*
4	1	23.95%	*((Mobula mobular,(Mobula alfredi,(Mobula birostris,Mobula sp. 1))),((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))))*
	2	21.84%	*((Mobula mobular,((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee)))),(Mobula alfredi,(Mobula birostris,Mobula sp. 1)))*
	3	15.97%	*(Mobula mobular,(((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),(Mobula alfredi,(Mobula birostris,Mobula sp. 1))))*
	4	5.40%	*((Mobula mobular,((Mobula alfredi,Mobula sp. 1),Mobula birostris)),((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))))*
	5	5.30%	*((Mobula mobular,((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee)))),((Mobula alfredi,Mobula sp. 1),Mobula birostris))*
	6	4.82%	*((Mobula mobular,((Mobula alfredi,Mobula birostris),Mobula sp. 1)),((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))))*
	7	4.70%	*((Mobula mobular,((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee)))),((Mobula alfredi,Mobula birostris),Mobula sp. 1))*

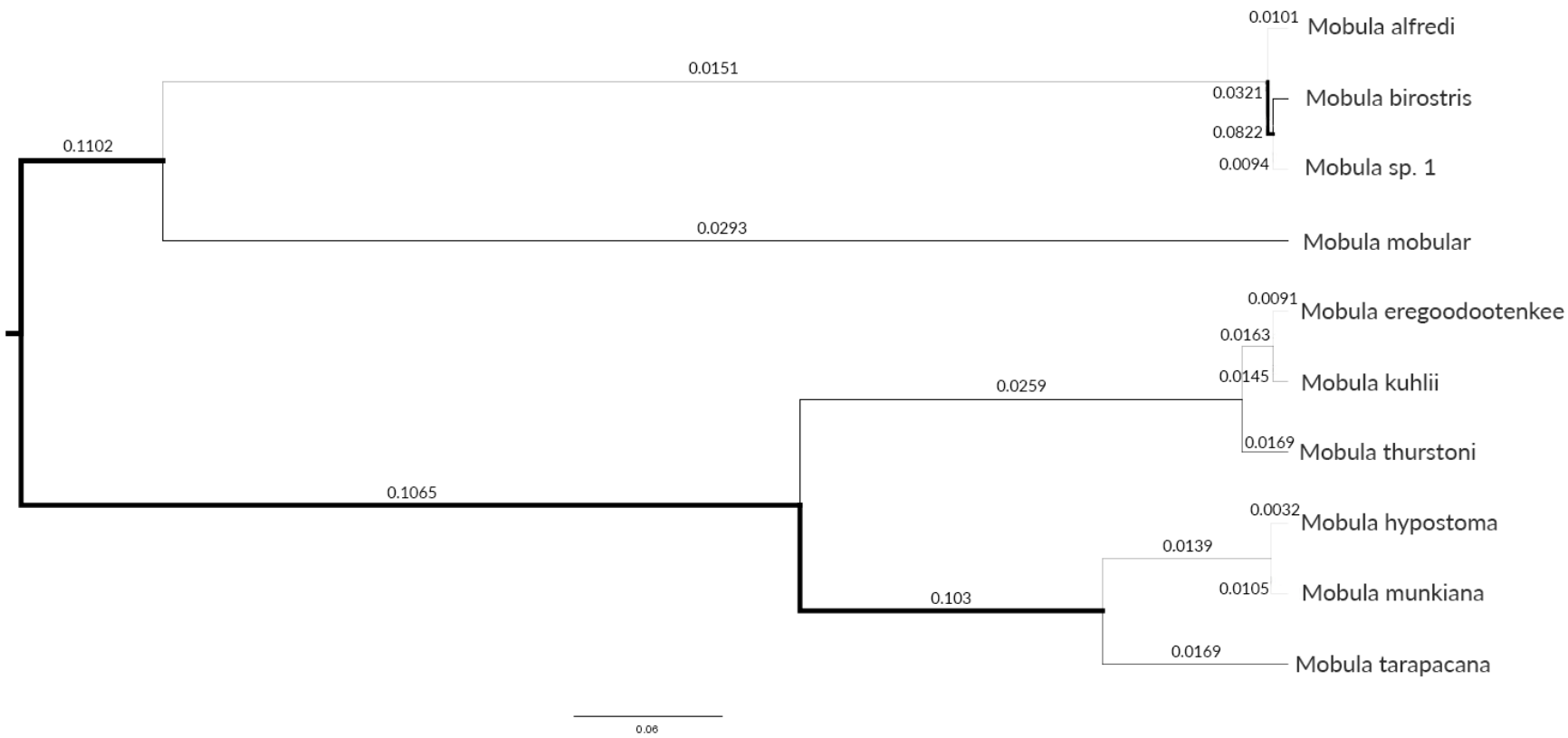
8	3.57%	*(Mobula mobular,(((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),((Mobula alfredi,Mobula sp. 1),Mobula birostris)))*
9	2.85%	*(Mobula mobular,(((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),((Mobula alfredi,Mobula birostris),Mobula sp. 1)))*
10	1.37%	*(((Mobula mobular,(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),(Mobula tarapacana,(Mobula munkiana,Mobula hypostoma))),(Mobula alfredi,(Mobula birostris,Mobula sp. 1)))*
11	1.12%	*(((Mobula mobular,(Mobula tarapacana,(Mobula munkiana,Mobula hypostoma))),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),(Mobula alfredi,(Mobula birostris,Mobula sp. 1)))*
12	0.75%	*((Mobula mobular,(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula alfredi,(Mobula birostris,Mobula sp. 1))))*
13	0.72%	*((Mobula mobular,(Mobula tarapacana,(Mobula munkiana,Mobula hypostoma))),((Mobula alfredi,(Mobula birostris,Mobula sp. 1)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))))*
14	0.70%	*(Mobula mobular,((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),((Mobula alfredi,(Mobula birostris,Mobula sp. 1)),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee)))))*
15	0.57%	*(Mobula mobular,(((Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)),(Mobula alfredi,(Mobula birostris,Mobula sp. 1))),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))))*
16	0.52%	*(((Mobula mobular,(Mobula alfredi,(Mobula birostris,Mobula sp. 1))),(Mobula tarapacana,(Mobula munkiana,Mobula hypostoma))),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee)))*
17	0.50%	*(((Mobula mobular,(Mobula alfredi,(Mobula birostris,Mobula sp. 1))),(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),(Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)))*
18	0.40%	*(((Mobula mobular,(Mobula thurstoni,(Mobula kuhlii,Mobula kuhlii cf. eregoodootenkee))),(Mobula alfredi,(Mobula birostris,Mobula sp. 1))),(Mobula tarapacana,(Mobula munkiana,Mobula hypostoma)))*



Supplementary Figure S2.8: A) Admixture graph showing relationships among inferred mobulid species units as a simple bifurcating tree, inferred using a Maximum Likelihood method in TreeMix (Pickrell & Pritchard, 2012). Horizontal branch lengths represent drift. The scale bar shows 10 times the average standard error of the values in the sample covariance matrix. This model explains 99.86% of the variance in the data. B) Residual fit of the observed versus predicted squared allele frequency difference. Note that *Mobula mobular* here includes specimens identified as *Mobula mobular cf. japanica* based on geographical sampling location.

Supplementary Table S2.11: f_3-statistics from the three-population test (Reich et al. 2009) for comparisons of different clade topologies. *Mobula alfredi, Mobula thurstoni* and *Mobula mobular* were randomly chosen to represent their respective clades.

3-taxon tree	f_3-statistic ± SE	Z-score
Mobula alfredi; Mobula mobular, Mobula thurstoni	0.092 ± 0.005	17.8885
Mobula mobular; Mobula alfredi, Mobula thurstoni	0.085 ± 0.005	17.9963
Mobula thurstoni; Mobula alfredi, Mobula mobular	0.102 ± 0.005	19.0177



Supplementary Figure S2.9: Maximum-clade-credibility (MCC) tree for inferred mobulid species units using SNAPP (individual subset 1; Supplementary Table S2.9). Branch width is proportional to theta (mutation-scaled effective population size), and theta values are shown on the branches. Note that *Mobula mobular* here includes specimens identified as *Mobula mobular cf. japanica* based on geographical sampling location.

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Chapter 3

Investigating the Genomic Signature of Speciation in Manta Rays

This Chapter has not yet been submitted for publication.

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JH, EH, GC, MdB, RO, SC and GS designed and conceived of the study and secured funding for consumables relating to laboratory work. EH, GS, AA, RB, MD, DF, KF, NF, LP, SP, AP, JS and SW were responsible for sourcing and collecting samples. **JH**, HS and JK carried out laboratory work. **JH**, EH, GC, MdB, RO, SC, HH and HS contributed to analysis of genome-wide SNP data. **JH** wrote the Chapter.

Chapter 3: Investigating the Genomic Signature of Speciation in Manta Rays

Biodiversity conservation measures focus generally on diversity within and between species and may overlook interactions between closely related species or diversity at an intermediate point along the speciation continuum. Understanding of the speciation process and dynamics of lineage divergence and convergence may help to inform conservation measures especially if closely related species occur in sympatry. Manta rays are a recently diverged group of marine megafauna of conservation concern which occur in mosaic sympatry throughout much of their range, providing an ideal opportunity to study lineage interactions early in the speciation process. In Chapter 2, evidence is presented to support the presence of a currently undescribed species of manta ray in the Gulf of Mexico, occurring in sympatry with its sister species, the oceanic manta ray. A recent study hypothesised that this divergence is associated with fluctuating sea levels which may have isolated an ancestral population in the Gulf of Mexico. Here, genome-wide SNP data is generated from the most comprehensive sampling of manta rays to our knowledge to date. Support is presented for three independently evolving lineages within the manta rays and the lineage corresponding to the undescribed species is shown to be associated with reduced ancestral effective population size, consistent with a pattern of peripatric speciation in isolation. In addition, a living hybrid between the oceanic manta ray and the as yet undescribed species is reported for the first time. Hybridisation between closely related species may provide opportunities for novel genotype combinations to come together or be an important driver of biodiversity in adaptive radiations. Conversely, hybridisation may lead to lineage collapse and 'genetic extinction' by hybridisation where hybrids are viable, or, where hybrids are inviable, such individuals represent wasted reproductive effort for the parental genotypes. Data presented here show that hybridisation is not associated with introgression in manta rays and is therefore likely to represent wasted reproductive effort in a group with slow life histories, which has additional conservation implications. This study provides an example of extremely rapid complete speciation in a marine system, and highlights concerns associated with anthropogenic climate change and accompanying sea level changes on evolution in the oceans.

3.1 Introduction

Speciation, the process by which lineages diverge to become reproductively isolated and separately evolving species, is the driving force behind the generation of biodiversity (Schluter & Pennell, 2017; Marin et al. 2018; Li & Wiens, 2019). Since reproductive isolation can be defined as a restriction or absence of gene flow attributable to a mechanism other than physical geographic separation (Seehausen et al. 2014), understanding of the speciation process and mechanisms maintaining distinct species may be enhanced with genomic data (e.g. Butlin et al. 2008; Seehausen et al. 2014; Campbell et al. 2018). Improved resolution achieved with genomic data may serve to inform conservation measures, such as species and habitat management (e.g. Hudson et al. 2013; Seabra et al. 2019), prevention of harmful interactions between closely related species (e.g. Allendorf et al. 2001; Feulner & Seehausen, 2019) and detection of cryptic species requiring independent management (e.g. Bickford et al. 2006; Razkin et al. 2016). With respect to species delimitation (see Chapter 2), challenges remain in establishing the extent of reproductive isolation in recently diverged species occurring in sympatry with opportunities for substantial gene flow (Sousa & Hey, 2013; Fitzpatrick et al. 2015; Ford et al. 2015).

Accordingly, speciation must be considered a continuous process (Sukumaran & Knowles, 2017), whereby two opposing forces, divergent selection and gene flow, may occur simultaneously (e.g. Papadopulos et al. 2011; Gagnaire et al. 2013; Nadeau et al. 2013; Whitney et al. 2018; Galtier, 2019). Speciation events may be driven by extrinsic influences, such as thermal selection (e.g. Teske et al. 2019) or sensory drive (e.g. Seehausen et al. 2008), intrinsic incompatibility, such as genome duplications (e.g. Volff, 2005) and chromosomal inversions (e.g. Noor et al. 2001; Feder & Nosil, 2009), or a combination of both factors (e.g. Christie & Strauss, 2018). Such conditions occur readily where there is a physical barrier to gene flow, i.e. in allopatry. However, in sympatry and parapatry, divergent selection must be sufficient to overcome the homogenising effect of gene flow if speciation is to progress to completion (Seehausen et al. 2014). It therefore follows that speciation events in nature may encompass multiple geographic modes of speciation, and an allopatric phase at some stage along the speciation continuum may be common (e.g. Feder et al. 2011; Quenouille et al. 2011). Nonetheless, if the speciation process is to progress past an irreversible 'tipping point',

environmental conditions driving divergence must be maintained for sufficient evolutionary time (Seehausen, 2006; Nosil et al. 2017).

Studies examining the mechanisms of speciation and factors maintaining divergence can serve to identify conservation priorities. For example, where lineages are descended from small isolated founder populations, as is the case in peripatric speciation, a lack of genetic diversity within lineages may result in species or populations that are particularly vulnerable to environmental change (Frankham, 2005; Sgrò et al. 2011; Blair et al. 2014; Hellmair & Kinziger, 2014). Furthermore, where taxa of conservation concern encompass species complexes in which speciation is recent or incomplete, interactions between lineages may have further implications for management. Such interactions are of particular concern where human activity brings closely related allopatric species into contact through deliberate or accidental introductions (Lowe et al. 2015), or where anthropogenic climate change indirectly facilitates contact through species range shifts (e.g. Davis & Shaw, 2001; Perry et al. 2005; Poloczanska et al. 2013). If sufficient reproductive isolation has already occurred, extrinsic selection and intrinsic incompatibility will reinforce lineages during secondary contact, causing hybrid offspring to exhibit lower reproductive fitness than either parental genotype (e.g. Servedio & Noor, 2003; Desvignes et al. 2019). However, hybridisation between partially diverged lineages may result in hybrids with similar or greater fitness, i.e. 'hybrid vigour' (e.g. Chen, 2013; Montanari et al. 2017), and may give rise to novel genotypes (e.g. Hedrick 2013; Oziolor et al. 2019) and/or hybrid species (e.g. Mallet, 2007; Keller et al. 2013; Ottenburghs, 2018). Hybridisation between divergent lineages may therefore lead to lineage collapse through swamping of alleles and 'extinction by hybridisation' (e.g. Seehausen, 2006; Garrick et al. 2014; Macleod et al. 2015). Furthermore, a lack of documentation and recognition of hybrids in legislation may result in insufficient measures for the protection of biodiversity (Fitzpatrick et al. 2015; Wayne & Shaffer, 2016). Where closely related species of conservation concern occur in sympatry, it is therefore prudent to assess the extent of hybridisation and introgression to establish effective conservation measures.

Historically, a perceived lack of physical barriers to gene flow in the oceans has raised difficulties in explaining the vast array of biodiversity present in the marine environment (Hauser & Carvalho, 2008; Bowen et al. 2013). However, whilst allopatric speciation is known to occur (see Hodge & Bellwood, 2016), there are now also numerous examples of speciation

occurring in the face of gene flow or along ecological boundaries in marine systems (e.g. Bowen et al. 2013; Árnason et al. 2018; Teske et al. 2019). In manta and devil rays (*Mobula* spp.), recent short bursts of speciation have been associated with periods of ocean warming and changes in upwelling intensity, which likely generated speciation with respect to feeding strategies (Poortvliet et al. 2015). Indeed, differences in gill plate morphology between closely related species, which are essential to the filter-feeding strategy of mobulid rays, may lend support to this hypothesis (Paig-Tran et al. 2013). Speciation may also have occurred due to differences in habitat preference, as hypothesised for the recent (approximately 0.5 million year old) speciation event separating the reef manta ray (*Mobula alfredi*) and oceanic manta ray (*M. birostris*) (Kashiwagi et al. 2012). The two species therefore occur in a state of mosaic sympatry across their overlapping ranges (Kashiwagi et al. 2011). There is some evidence suggestive of hybridisation between *Mobula alfredi* and *M. birostris* from genetic data (Walter et al. 2014) and observations of individuals with intermediate phenotypes for key species-specific traits (J. Hartup, pers comm). However, the extent of hybridisation between manta ray species has not been specifically studied, and questions remain regarding the degree of introgression in the group.

Given the absence of *Mobula alfredi* in the Atlantic Ocean (see Figure 3.1), a third species of manta ray is hypothesised to occur in the Caribbean and Gulf of Mexico, effectively occupying the reef-based niche that *M. alfredi* occupies elsewhere (Marshall et al. 2009; Hinojosa-Alvarez et al. 2016; Stewart et al. 2018a). In the previous Chapter, evidence is presented from genome-wide SNPs to support the presence of an undescribed species of manta ray occurring in sympatry with its sister species, *Mobula birostris,* in the Gulf of Mexico. Novel mtDNA haplotypes have previously been reported from manta rays sampled off the Yucatan peninsula, with divergence estimated to have occurred very recently, less than 100,000 years ago (Hinojosa-Alvarez et al. 2016). It is hypothesised that this divergence is associated with a global drop in sea level (see Donoghue, 2011), resulting in an elongation of the coastlines of Florida and the Yucatan Peninsula, thereby isolating a population of ancestral *Mobula birostris* in the Gulf of Mexico (Hinojosa-Alvarez et al. 2016). Since the time window for this speciation event is shorter than that estimated to be required for irreversible speciation (Seehausen, 2006) and the fact that the two species now occur in sympatry, this system provides an ideal opportunity to study the dynamics of lineage divergence and possible

subsequent convergence upon secondary contact early in the speciation process.

Here, ddRAD data is generated from the most comprehensive global sampling of manta rays to our knowledge to date and is applied to improve understanding of recent speciation dynamics within the group. Genome-wide data is used to recover manta ray species lineages and assess the extent of lineage interaction. Relative ancestral effective population sizes are reconstructed along lineages to assess support for a peripatric mode of speciation between *Mobula birostris* and *Mobula* Sp. 1, potentially associated with isolation of an ancestral population in the Gulf of Mexico.

3.2 Methods

3.2.1 Sampling

Manta ray tissue samples from 217 individuals were obtained through the established collections and projects of researchers and organisations worldwide. Where this involved taking biopsies from live animals, the procedure was approved by Bangor University's Ethics Committee. Individual manta rays were identified to species level based on characteristics described by Stevens et al. (2018b), and in the case of a currently undescribed species of manta ray referred to as *Mobula* Sp. 1, the genetic analyses detailed in Chapter 2. Reef manta ray (*Mobula alfredi*) and oceanic manta ray (*Mobula birostris*) samples were each obtained from six locations, and samples of *Mobula* Sp. 1 were obtained from two locations in the Gulf of Mexico (Figure 3.1; further details in Supplementary Table S3.1).

3.2.2 Laboratory Procedures

Genomic DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit and DNA yield measured using a Qubit 3.0 Broad Range Assay. Extracts were quality assessed on 1% agarose gels stained with SafeView. ddRAD libraries were designed based on the results of a pilot library sequenced for all available species of mobulid ray (see Supplementary Section S2.1: Method Development) and prepared using a modified version of the original protocol

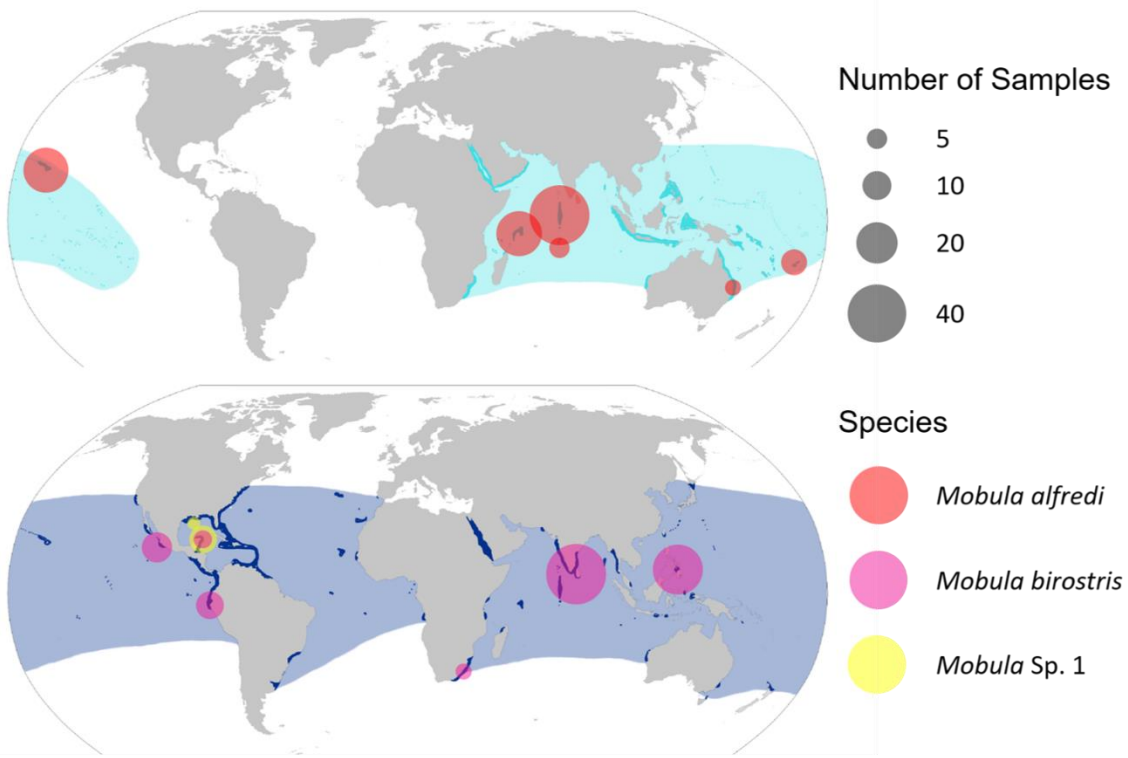


Figure 3.1: Sampling locations for manta rays across their range. Top: distribution of the reef manta ray (*Mobula alfredi*), dark areas indicate confirmed range, light areas expected range. Bottom: distribution of the oceanic manta ray (*Mobula birostris*), dark areas indicate confirmed range, light areas expected range. Samples of each species are represented by coloured circles, which are centred on the sampling location and scaled for sample size. *Mobula* Sp. 1 sampling locations are shown on the *M. birostris* distribution map. Note that individuals of *Mobula birostris* and *Mobula* Sp. 1 are both sampled at the same site off the Yucatan Peninsula. Further details are in Supplementary Table S3.1. Distribution maps have been reproduced with permission from the Manta Trust.

(Peterson et al. 2012; see Palaiokostas et al. 2015 for full protocol) with restriction enzymes *SbfI* and *SphI* (NEB). Unique P1 and P2 barcode combinations were ligated to resulting DNA fragments, which were then size-selected between 400-700bp using gel electrophoresis and PCR amplified. Libraries were sequenced by Edinburgh Genomics© on Illumina HiSeq High Output v4, 2 x 125PE read module (see Supplementary Table S3.2 for details).

3.2.3 Data Quality Control and Filtering

Following the workflow detailed in Chapter 2, data quality was assessed with FastQC (Andrews, 2010), and processed in Stacks version 1.46 (Catchen et al. 2011). The process_radtags.pl module in Stacks (Catchen et al. 2011) was used to demultiplex the data,

filter for adaptor sequences (allowing two mismatches), remove low quality sequence reads (99% probability) and discard reads with any uncalled bases. Since forward and reverse reads of the same amplicon are situated closely together in the genome and are therefore likely to violate assumptions of independence, only forward reads were retained for subsequent analyses to minimise linkage disequilibrium in the SNP data.

The denovomap.pl program in Stacks (Catchen et al. 2011) was used to assemble loci and call SNPs. The three main parameters for assembly were those generating the largest number of new polymorphic loci shared across 80% of individuals, following the method of Paris et al. (2017). Five identical reads were required to build a stack (-m 5), stacks differing by up to three nucleotides were merged into putative loci (-M 3) and putative loci across individuals differing by up to three nucleotides were written to the catalog (-n 3), giving an average coverage of 105x across samples (minimum 22x). The populations.pl program in Stacks (Catchen et al. 2011) was then used to generate a VCF file containing all SNPs present in at least 40 individuals (-p 40). To remove paralogous loci and mitigate for allele dropout (Arnold et al. 2013; Gautier et al. 2013), loci sequenced at greater than twice or less than one third the standard deviation of coverage, respectively, were identified and excluded using VCFtools (Danecek et al. 2011). The remaining loci were assessed for excess heterozygosity using VCFtools (Danecek et al. 2011), and those exhibiting a significant probability of heterozygote excess were excluded. Finally, since Stacks ignores indels (Catchen et al. 2011), SNPs in the last five nucleotide positions were assumed erroneous and excluded. The remaining loci and SNPs were written to a whitelist and filtered for a single random SNP per locus to minimise linkage using the populations.pl program in Stacks (Catchen et al. 2011). This resulted in a final SNP matrix with 4776 SNPs and 17.7% missing data. These SNPs were written to a whitelist for use in subsequent analyses (see Chapter 4).

3.2.4 Relationships among manta rays

Relationships among individuals were inferred through Maximum Likelihood phylogenetic analysis of concatenated ddRAD loci using RAxML version 8.2.11 (Stamatakis, 2014). The GTRGAMMA model of rate heterogeneity was implemented following assessment of best fit

models using both the Akaike and Bayesian information criteria in jModeltest2 (Darriba et al. 2015) and support assessed with 1,000 bootstrap replicates.

In addition, to assess how individuals cluster together, Principal Components Analysis (PCA) was performed using the R package 'adegenet' (Jombart, 2008). Three axes were retained, since only the first 3 axes explained >5% of the variance in the data. To assess whether apparent spread of data points away from major species clusters into intermediate space is the result of introgressive hybridisation, or an artefact of missing data, the PCA was repeated with only those individuals with <25% missing data (see Supplementary Table S3.1).

To evaluate genetic diversity within each of the three species of manta ray, the populations.pl program in Stacks (Catchen et al. 2011) was used to calculate expected heterozygosity (H_e), observed heterozygosity (H_o) and nucleotide diversity (π) within species. A single putative hybrid individual was excluded from these calculations since there was no justification for assigning it to either *Mobula birostris* or *Mobula* Sp. 1.

3.2.5 Inference of Hybrid Scores

The relative contributions of each genetic cluster were visualised, and hybrid scores (Q) assigned to individuals using a Bayesian clustering method implemented in STRUCTURE version 2.3.4 (Pritchard et al. 2000; Falush et al. 2003). *Mobula alfredi* individuals were excluded, since there was no evidence to suggest this species might be involved in hybridisation events from the phylogenetic and clustering analyses described above (see Section 3.3.1), retaining 3615 polymorphic SNPs in remaining individuals. STRUCTURE (Pritchard et al. 2000; Falush et al. 2003) was used to estimate the admixture proportions for *Mobula birostris* and *Mobula* Sp. 1 individuals for *K*=2 using the admixture model (Pritchard et al. 2000), with no prior information on sampling locations or species assignments. Following Miller et al. (2017), the ANCESTDIST option, which collects information on the distribution of Q-values for each individual, was enabled in STRUCTURE (Pritchard et al. 2000; Falush et al. 2003), to output the Bayesian analogue of confidence intervals for each value of Q. Five independent replicates were performed with 1,000,000 MCMC iterations and 100,000 burn-in, which were averaged in CLUMPP version 1.1.2 (Jakobsson & Rosenberg, 2007) using the Greedy algorithm with 1,000 repeats. Results were plotted using DISTRUCT version 1.1

(Rosenberg, 2004). Following Vähä & Primmer (2006) and Marie et al. (2011), individuals were identified as belonging to a single cluster if their estimated membership (Q) was ≥ 0.9 for that cluster and were considered potential hybrids if all values of Q across the full distribution was ≥ 0.25 for each of the two clusters following Senn et al. (2019).

3.2.6 Lineage Sorting and Ancestral Effective Population Size

To evaluate the extent of incomplete lineage sorting and estimate ancestral effective population size along lineages, SNAPP (Bryant et al. 2012), implemented as a plug-in to BEAST version 2.4.8 (Bouckaert et al. 2014), was used to infer phylogenetic trees whilst allowing each SNP to have its own history under the multispecies coalescent. Due to the computational capacity required to run SNAPP (Bryant et al. 2012), six individuals per species were randomly selected whilst maximising geographical coverage within species; one individual from each sampling location for *Mobula alfredi* and *M. birostris* and six random individuals from across the two sampling locations for *Mobula* Sp. 1. Random sampling of individuals with replacement was repeated a further three times, resulting in four independently subsampled alignments (Supplementary Table S3.3). A single putative hybrid individual was excluded from this analysis since there was no justification for assigning it to either *Mobula birostris* or *Mobula* Sp. 1. MCMC chains consisted of 2,000,000 iterations, sampling every 1,000 and retaining default priors on lambda and theta for each independent analysis. Convergence to stationary distributions, requiring effective sample size (ESS) values to be >200, were observed after 20% burn-in in TRACER (Rambaut et al. 2018). The distribution of trees was visualised in DensiTree version 2.2.6 (Bouckaert, 2010) and maximum clade credibility (MCC) trees drawn using TreeAnnotator version 2.4.7 (Bouckaert et al. 2014). An alternative prior combination, implementing a gamma prior on the lambda (tree height) parameter, produced highly concordant results.

3.2.7 Admixture and Introgression

Multispecies coalescent based approaches assume that any discordance of topologies among loci results from incomplete lineage sorting and do not consider introgression as a source of

discordance. To investigate the extent to which any variation is best explained by a single bifurcating tree, and identify species lineages that are poor fits to this model, TreeMix (Pickrell & Pritchard, 2012) was used to evaluate evidence for significant introgression events within the manta rays. TreeMix (Pickrell & Pritchard, 2012) was run on all individuals assigned to three species, with *Mobula alfredi* specified as the outgroup. Since specifying an outgroup in a three-population tree effectively fixes the topology, TreeMix (Pickrell & Pritchard, 2012) was run a second time, with no outgroup specified. In both cases, a single putative hybrid individual was excluded since there was no justification for assigning it to either *Mobula birostris* or *Mobula* Sp. 1.

3.3 Results

3.3.1 Manta ray diversity

The three species of manta ray were reciprocally monophyletic for the dataset of 4776 SNPs based on a Maximum Likelihood phylogenetic tree inferred with RAxML (Stamatakis, 2014; Figure 3.2). Species groups were highly supported (100% bootstrap support for monophyletic groups representing *Mobula alfredi* and *Mobula* Sp. 1, and 99% bootstrap support for *M. birostris*) and separated by long branch lengths. Two further groups are inferred within the reef manta ray (*Mobula alfredi*), corresponding to individuals sampled in the Indian and Pacific Oceans (99% and 100% bootstrap support, respectively). Furthermore, monophyletic groups are inferred for Fijian and Hawaiian *Mobula alfredi* populations (80% and 96% bootstrap support, respectively). A single individual, sampled at Flower Garden Banks National Marine Sanctuary (FGBNMS) in Texas, failed to group within any monophyletic species group, and was tentatively placed on the *Mobula birostris* lineage with 78% bootstrap support.

Principal Components Analysis (PCA) mirrored patterns shown in the phylogenetic tree. Removal of individuals with high proportions of missing data resulted in tight clusters of individuals corresponding to species assignments, separated along axes explaining large portions of variance (Figure 3.3; see Supplementary Figure S3.1 for PCA with all individuals).

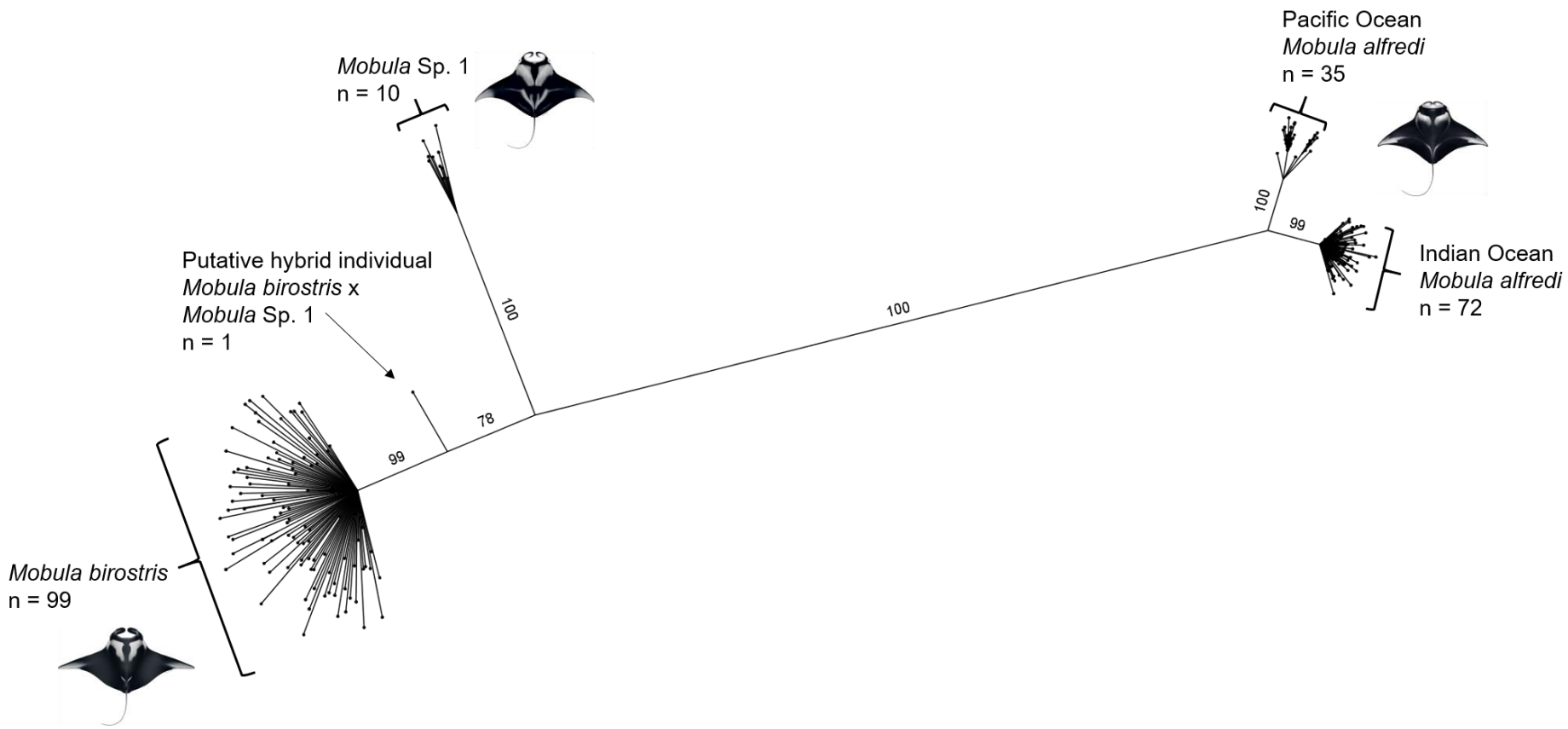


Figure 3.2: Unrooted Maximum Likelihood phylogenetic tree showing relationships among manta ray individuals based on 4776 SNPS. Species assignments and numbers of individuals are indicated. Individuals are represented by solid points on branch tips. Bootstrap values are shown on the branches and nodes with less than 50% support are collapsed. Further support was given for Fijian (80%) and Hawaiian (96%) specific groups within the *Mobula alfredi* Pacific Ocean group (not indicated). Illustrations © Marc Dando and are reproduced with permission.

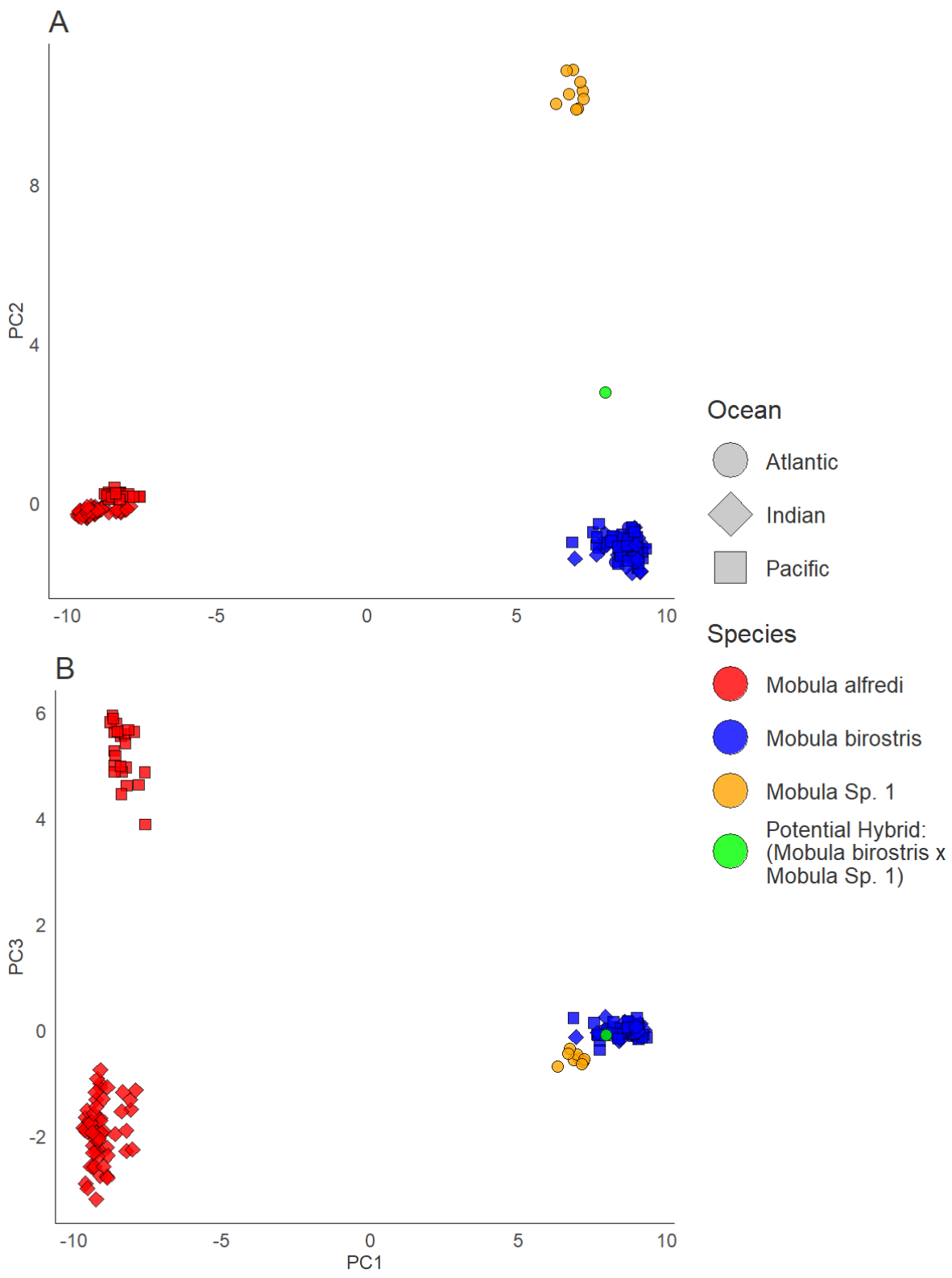


Figure 3.3: Principal Components 1-3 plotted for all manta ray individuals with less than 25% missing data (see Supplementary Table S3.1 for details). Individuals are represented by a point. Colour indicates species and shape indicates geographic origin of samples as specified in the key. The putative hybrid individual is shown in green. A) Principal Components 1 and 2, explaining 75.1% and 6.3% of the variance in the data, respectively, and B) Principal Components 1 and 3, where the latter explains 5.1% of the variance in the data. See Supplementary Figure S3.2 for full eigenvalue plot.

The three species could be clearly separated along the first two axes, whilst the third axis clearly demarcated reef manta ray (*Mobula alfredi*) individuals sampled in the Indian and Pacific Oceans. A single individual, sampled at Flower Garden Banks National Marine Sanctuary (FGBNMS) in Texas, occupied an intermediate space between *M. birostris* and *Mobula* Sp. 1.

Expected heterozygosity (H_e), observed heterozygosity (H_o), nucleotide diversity (π) and the number of polymorphic SNPs within each species (excluding the putative hybrid individual) are given in Table 3.1. *Mobula birostris* is found to be the most genetically diverse of the three species, and *Mobula alfredi* the least. Estimates of observed and expected heterozygosity (H_o and H_e, respectively), nucleotide diversity (π) and the number of polymorphic SNPs may be artificially reduced in *Mobula* Sp. 1, since the number of individuals sequenced for this species was an order of magnitude lower than the number of individuals sequenced for *M. alfredi* and *M. birostris*, resulting in less opportunity to discover rare alleles. However, both the sequencing strategy employed in this study, and the use of STACKS software (Catchen et al. 2011) reduce the effects of methodological ascertainment bias. During library preparation, individuals of all species were multiplexed together within libraries and therefore shared HiSeq lanes. This precludes the possibility of a systematic discrepancy in the number of sequencing reads returned per species. Furthermore, STACKS (Catchen et al. 2011) discovers loci *de novo* and adds all new loci and SNPs across each individual in turn to a common

Table 3.1: Genetic diversity within each species of manta ray, as measured by observed levels of heterozygosity and nucleotide diversity. The number of SNPs, out of the total of 4776, that were polymorphic within species are also given.

Species	Number of individuals	Expected heterozygosity (H_e) ± s.e	Observed Heterozygosity (H_o) ± s.e	Nucleotide Diversity (π) ± s.e	No. Polymorphic SNPs
Mobula alfredi	107	0.0257 ± 0.0013	0.0134 ± 0.0007	0.0259 ± 0.0013	1067
Mobula birostris	99	0.0592 ± 0.0017	0.0378 ± 0.0012	0.0601 ± 0.0017	3217
Mobula Sp. 1	10	0.0355 ± 0.0015	0.0297 ± 0.0014	0.0378 ± 0.0016	665

catalogue. Whilst the method presented here is therefore unlikely to have generated significant ascertainment bias, an effect caused by the discrepancy in the number of individuals typed per species cannot be ruled out. Expected heterozygosity (H_e) is reported in addition to observed heterozygosity (H_o), since this estimate of genetic diversity is less sensitive to sample size. Nonetheless, since the numbers of individuals of *Mobula alfredi* and *M. birostris* are similar, it is likely that the difference in diversity estimates and the numbers of SNPs discovered for these two species is a genuine biological occurrence.

3.3.2 Hybridisation

STRUCTURE (Pritchard et al. 2000; Falush et al. 2003) assigned all individuals previously identified as either *Mobula birostris* based on the morphological characters described in Stevens et al. (2018b), or *Mobula* Sp. 1 based on the genomic analyses detailed in Chapter 2, to one of two single clusters with Q ≥ 0.9 (Figure 3.4; Figure 3.5). However, a single individual, sampled at Flower Garden Banks National Marine Sanctuary (FGBNMS) in Texas, was identified as a potential hybrid with Q = 0.64 and Q = 0.36 for each cluster (Figure 3.4; Figure 3.5). 90% probability intervals (the Bayesian analogue of confidence intervals) for each Q-value for this individual range from 0.59-0.68 and 0.32-0.41 for each cluster, respectively

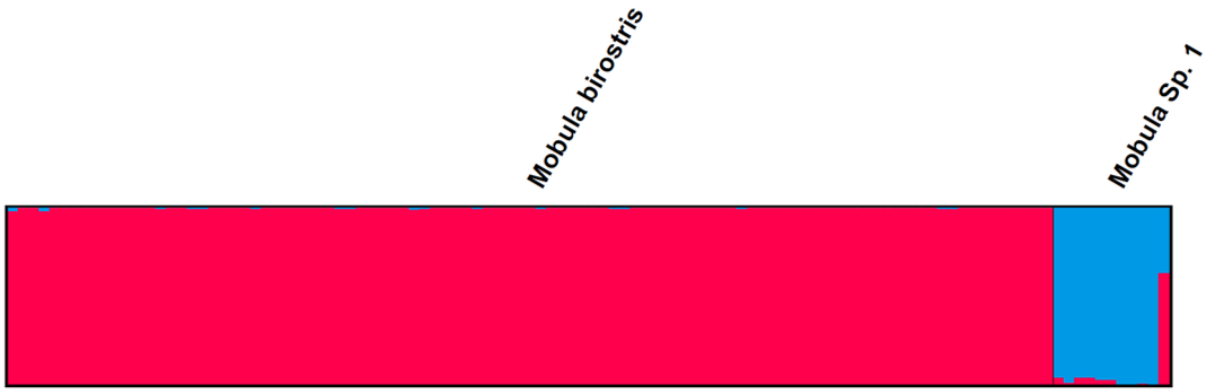


Figure 3.4: Plot showing results of Bayesian clustering analysis in STRUCTURE (Pritchard et al. 2000; Falush et al. 2003), where *K*=2 (number of clusters). Each vertical bar represents an individual, and colours represent the proportion of ancestry assigned to each cluster. Individuals are grouped by species, with the putative hybrid individual represented by the right-most vertical bar. 90% probability intervals for each individual are given in Figure 3.5.

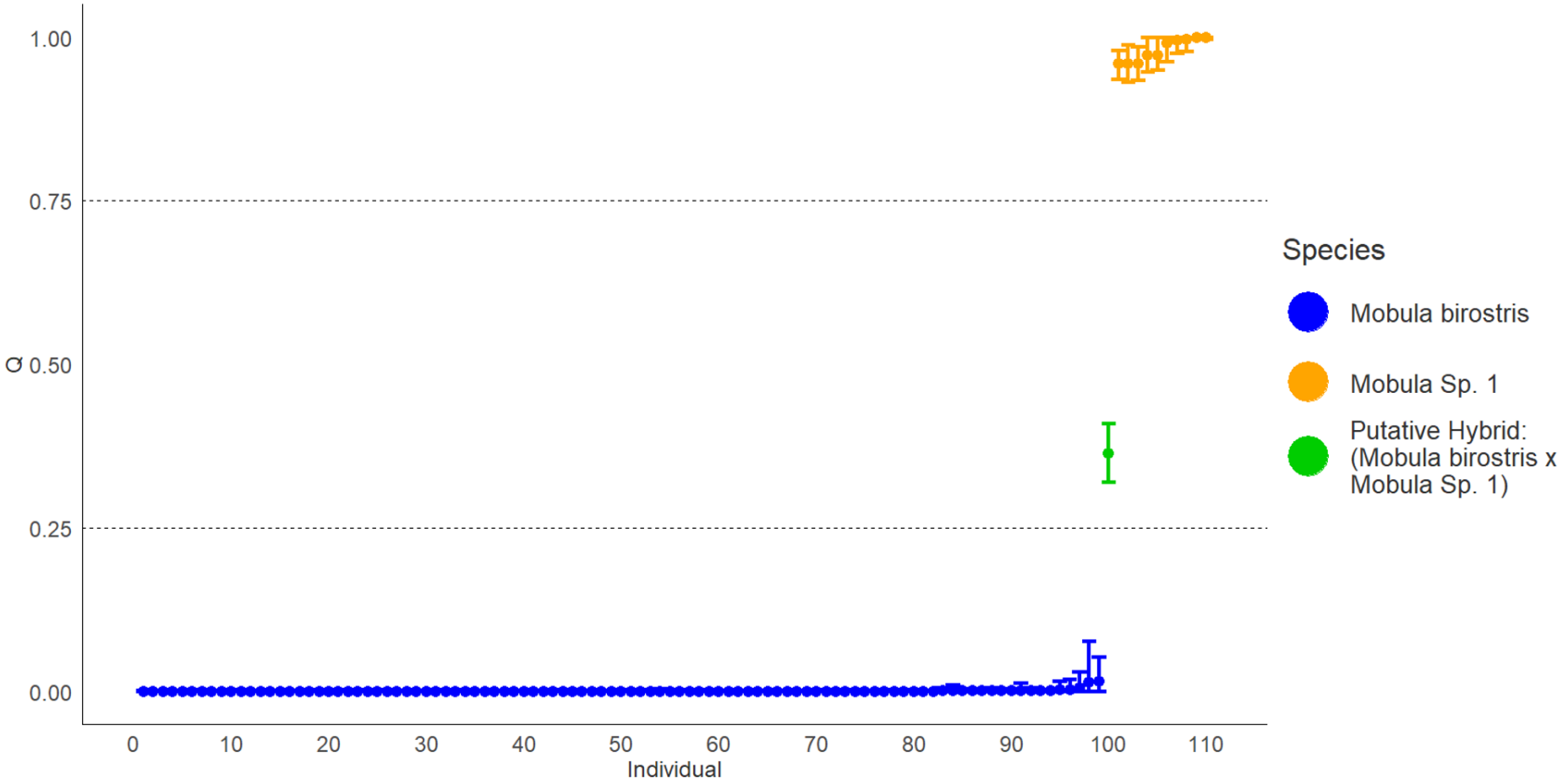


Figure 3.5: Q-values ± 90% probability intervals to the *Mobula* Sp. 1 cluster for each individual analysed in STRUCTURE (Pritchard et al. 2000; Falush et al. 2003). Dotted lines are shown at Q-values of 0.25 and 0.75, since these represent the threshold used by Senn et al. (2019) to define a putative hybrid.

(Figure 3.5), meaning that this individual meets the criteria to be considered a potential hybrid as defined by Senn et al. (2019). Some evidence of shared substructure between species was also apparent. However, given that STRUCTURE (Pritchard et al. 2000; Falush et al. 2003) provides no formal tests for admixture, this may be explained if shared ancestral genotypes are retained in both species, and accordingly, results are given for a formal test for admixture in Section 3.3.4.

3.3.3 Lineage Sorting and Demographic History

Consensus species trees estimated under the multispecies coalescent produced highly consistent topologies and relative theta estimates across independent runs, indicating no major effect of subsampling individuals on species relationships or patterns of ancestral effective population size inferred with SNAPP (Bryant et al. 2012). Across all four subsampled alignments, a single tree topology made up the 95% highest posterior density (HPD) and all nodes were therefore supported with a posterior probability of 1.0. All trees were of the form ((*Mobula alfredi)*; *Mobula birostris*, *Mobula* Sp. 1)), indicating that *Mobula* Sp. 1 is sister to *Mobula birostris,* with no evidence of incomplete lineage sorting within the manta rays. Estimates of theta (mutation scaled ancestral effective population size) were largest for the *Mobula birostris* lineage and for the shared ancestral lineage with *Mobula* Sp. 1. However, theta values were substantially smaller in the *Mobula* Sp. 1 and *M. alfredi* lineages (Figure 3.6 and Supplementary Figures S3.4-S3.6).

3.3.4 Admixture and Introgression

TreeMix (Pickrell & Pritchard, 2012) inferred admixture graphs similar to the trees produced with RAxML (Stamatakis, 2014), (Figure 3.7; Supplementary Figure S3.7). Regardless of whether an outgroup was specified, these graphs explained 100% of the variance in the data, with associated residuals plots indicating that placement of all species is unaffected by admixture and that the fit could therefore not be improved by allowing for migration between lineages.

Figure 3.6: Maximum clade credibility (MCC) tree for manta ray species inferred using SNAPP (Bryant et al. 2012), (individual subset 1; see Supplementary Table S3.3). Branch width is proportional to theta (ancestral effective population size), and theta values are shown on the branches. Posterior probabilities are shown on the nodes.

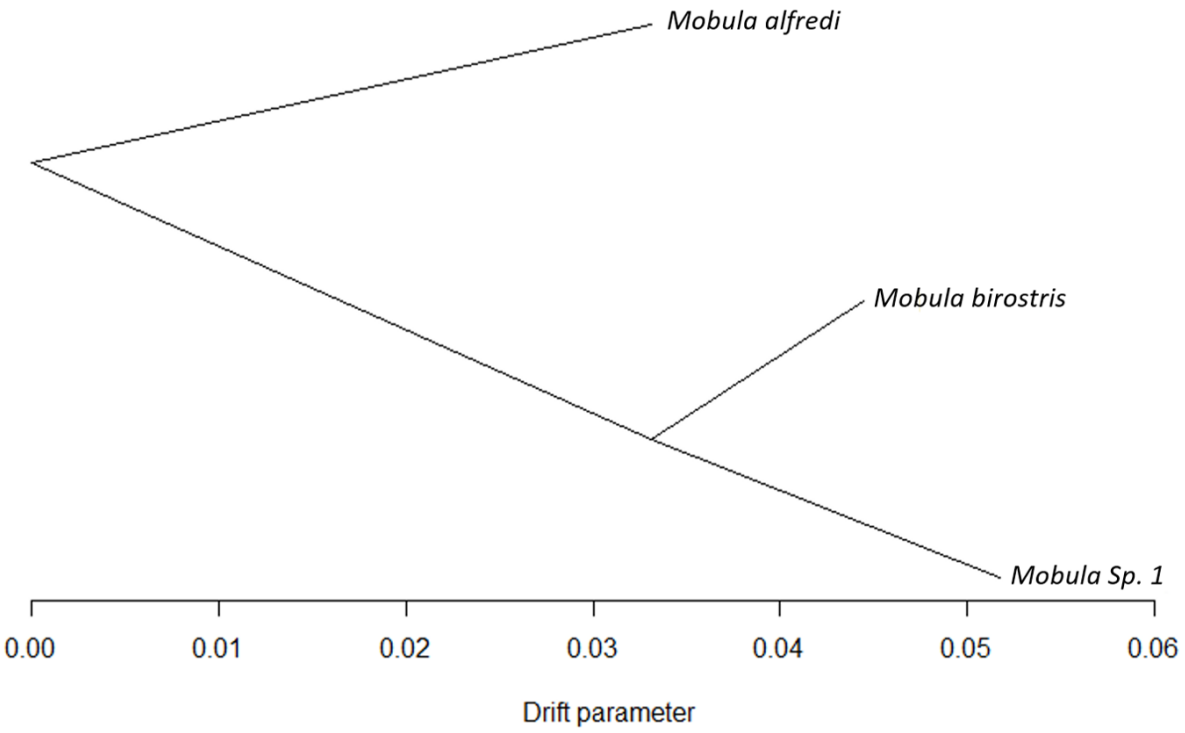


Figure 3.7: Admixture graph showing relationships among manta ray species as a simple bifurcating tree, inferred using a Maximum Likelihood method in TreeMix (Pickrell & Pritchard, 2012). *Mobula alfredi* was specified as the outgroup. Horizontal branch lengths represent drift. This model explains 100% of the variance in the data indicating the fit could not be improved by adding migration edges.

3.4 Discussion

Genome-wide SNP data generated from samples collected across the known ranges of our species of interest generate compelling support for the reproductive isolation of three fully sorted manta ray lineages, indicating that there is an undescribed species of manta ray, referred to as *Mobula* Sp. 1, present in sympatry with *Mobula birostris* in the Gulf of Mexico. Such findings further validate the evidence provided in Chapter 2. Future work will necessarily involve formal description of this species and determining the extent of its range. Our data show a restriction in ancestral effective population size of the *Mobula* Sp. 1 lineage after divergence from the shared node with *Mobula birostris*, consistent with this species being descended from a small founder population. This has conservation implications with respect to genetic diversity of the extant species (Blair et al. 2014), where low genetic diversity has been associated with increased vulnerability to environmental change (e.g. Frankham, 2005; Sgrò et al. 2011; Hellmair & Kinziger, 2014). In addition, we show evidence for the first record of a living *Mobula birostris* x *Mobula* Sp. 1 hybrid, whilst also demonstrating that such hybridisation is not introgressive, suggestive of low reproductive fitness of hybrid individuals.

In contrast to previous studies (see Seehausen, 2006), our findings show that irreversible speciation can occur over very short evolutionary timescales, even in a group with long generation times (Dulvy et al. 2014; Croll et al. 2016). *Mobula alfredi* is estimated to have diverged as recently as 0.5 million years ago (Kashiwagi et al. 2012) and the divergence between *Mobula* Sp. 1 and *M. birostris* is estimated to be less than 100,000 years old (Hinojosa-Alvarez et al. 2016). It is known that low effective population sizes can accelerate lineage sorting under a coalescence model (Tian & Kubatko, 2017), and consistent with this we find low ancestral effective population sizes for *Mobula alfredi* and *Mobula* Sp. 1 (see Figure 3.6), which may have accelerated lineage sorting in this group.

Rapid ecological speciation may be facilitated by differences in mate choice, breeding behaviour, habitat preference or feeding morphology (e.g. Momigliano et al. 2017; Lemoine et al. 2019), and indeed the speciation process is expected to be boosted by either strong selection on a single trait, or selection on multiple traits (Nosil et al. 2009). It is hypothesised that *Mobula alfredi* and *M. birostris* diverged due to differences in habitat preference, given the contemporary mosaic sympatry exhibited by the species (Kashiwagi et al. 2011; Kashiwagi

et al. 2012). It is possible that a similar mechanism maintains the divergence between *Mobula birostris* and *Mobula* Sp. 1, since the former is typically associated with pelagic habitats (Couturier et al. 2012; Stewart et al. 2016), compared to the more coastal habitat preference of the latter (Hinojosa-Alvarez et al. 2016).

Dynamics of lineage divergence and convergence associated with global temperature oscillations and accompanying sea level change is concerning where human-mediated climate change disrupts and exacerbates such processes (e.g. Poloczanska et al. 2013; Taylor et al. 2015). Global temperature fluctuations have been shown to play a role in divergence and introgression in elasmobranch megafauna (e.g. Walter et al. 2017), and appear to be an important factor driving the evolution of mobulid diversity (Poortvliet et al. 2015). It has previously been hypothesised that the divergence between *Mobula birostris* and *Mobula* Sp. 1 was driven by an extrinsic barrier to gene flow associated with changes in sea level (Hinojosa-Alvarez et al. 2016). Consistent with this, we find a restriction in ancestral effective population size along the lineage corresponding to *Mobula* Sp. 1 associated with the divergence event (Figure 3.6), suggesting that this species is descended from a small founder population. In addition, a similar pattern is shown for the reef manta ray, *Mobula alfredi*, although this may be an artefact of existing population structure and possible mode of colonisation in this species (see Chapter 4 for details). Nonetheless, peripatric speciation can lead to a lack of genetic diversity in rare species (e.g. Blair et al. 2014), and result in a reduction of 'choosiness' in derived females, increasing the likelihood of the production of hybrids (Odeen & Florin, 2002), both scenarios with subsequent conservation implications. Indeed, previous studies of speciation dynamics among manta rays report evidence of introgression from *Mobula birostris* into *Mobula alfredi* (Kashiwagi et al. 2012) and *Mobula* Sp. 1 (Hinojosa-Alvarez et al. 2016).

Whilst our data shows the reproductive isolation of three manta ray lineages, for the first time, we find evidence of ongoing hybridisation between *Mobula* Sp. 1 and *M. birostris* from a single individual sampled at Flower Garden Banks National Marine Sanctuary (FGBNMS) in Texas (Figure 3.4; Figure 3.5). In contrast, we were unable to recover any evidence of ongoing hybridisation involving *Mobula alfredi*, despite previous reports (Walter et al. 2014). Whilst our study represents the most comprehensive sampling of manta rays to date, we were unable to analyse samples collected in suspected *Mobula alfredi* x *Mobula birostris* hybrid

zones, such as the Red Sea (Walter et al. 2014) and Guam (J. Hartup, pers comm). Consequently, assessing the extent of hybridisation and introgression at these locations with genome-wide data would be a priority next step.

Despite such hybridisation events within the manta rays, we find no evidence of gene flow among lineages. Data are in contrast to the two previous studies of manta ray speciation and divergence, which report patterns consistent with an isolation-with-migration model, each based on a single nuclear and mitochondrial marker (Kashiwagi et al. 2012; Hinojosa-Alvarez et al. 2016). Recent studies involving empirical and simulated data have shown that introgression may be erroneously supported by isolation-with-migration models where small datasets with low divergence are used as the basis for such inference (Cruickshank & Hahn, 2014; Hey et al. 2015). Our genome-wide SNP data finds some evidence of shared substructure between *Mobula birostris* and *Mobula* Sp. 1 (Figure 3.4). However, since our formal tests for admixture produce a model that could not be improved upon by allowing migration between lineages (Figure 3.7), such shared substructure is likely the result of retained ancestral variation in these sister-species rather than introgression.

We can therefore conclude that selection overcomes the homogenising effect of gene flow in manta rays on secondary contact, since we see no breakdown of patterns of divergence between the three species. Such patterns of hybridisation without associated introgression are suggestive of strong selection against hybrids (Servedio & Noor, 2003; Desvignes et al. 2019). The Texan hybrid sample analysed here was one of two taken from Flower Garden Banks National Marine Sanctuary (FGBNMS), a known nursery area (Stewart et al. 2018a), and accordingly, this individual was a juvenile at the time of sampling (J. Stewart, pers obs). Unfortunately, researchers in the field were unable to take photographs of this individual at the time of sampling, although it was noted at the time that this individual could not be easily visually identified as either *Mobula birostris* or *Mobula* Sp. 1 (J. Stewart, pers comm). It is unknown whether there is a difference in mortality rates among individuals of mixed ancestry compared with individuals that can confidently be assigned to a single species. Future work should involve more comprehensive sampling of this site, in order to establish the extent of hybridisation and backcrossing. Nonetheless, hybridisation without introgression suggests that these three manta ray lineages can unequivocally be considered separate species, and that any hybrids are likely to be inviable. Such assertions have conservation implications

where hybrids represent unsuccessful reproductive effort in a group already vulnerable to human activity as a result of very low reproductive rates (Dulvy et al. 2014; Croll et al. 2016). Future work should focus on establishing the prevalence of interspecific mate choice and hybrid births, allowing management measures to account for these rates through life history analysis.

Here, genome-wide SNP data supports the characterisation of three species of manta ray, including an undescribed species in the Gulf of Mexico, which is likely descended from a small, isolated founder population. These findings have implications for the conservation of this charismatic and economically important group of marine megafauna where hybrids likely represent unsuccessful reproductive effort in a vulnerable group of species. Our data indicate that natural temperature oscillations and fluctuations in sea level may be an important mechanism driving the evolution of biodiversity in the oceans, and that reproductive isolation and lineage sorting can occur extremely rapidly in evolutionary time. However, the rate at which human activity is causing such changes is concerning and may lead to maladaptive interactions, such as the production of inviable hybrids, or extinction by hybridisation between closely related species where climate change disrupts ocean processes.

Chapter 3: Supplementary Materials

Chapter 3: Supplementary Materials

S3.1 Supplementary Figures and Tables

Supplementary Table S3.1: Manta ray sample Information. CITES information provided in footnote*.

Sample Codes	Species	Site	Location	Contacts	Previous Publications
0130, 0131, 0132, 0133‡, 0134, 0135, 0136, 0137, 0138, 0139, 1307, 1312, 1313, 1314, 1315‡, 1318, 1319, 1320, 1321, 1322, 1323, 1324, 1325, 1326	*Mobula alfredi*	D'Arros, Amirante Islands	Seychelles	L. Peel, G. Stevens	
0140, 0141, 0142, 0143, 0144, 0146, 0147, 0148	*Mobula alfredi*	Barefoot Channel, Yasawas Islands	Fiji	S. Pollett, D. Bowling, H. Pacey	
0685, 0686	*Mobula alfredi*	Egmont	British Indian Ocean Territory (BIOT), Chagos Archipelago	D. Fernando	
0687, 0688	*Mobula alfredi*	Diego Garcia	British Indian Ocean Territory (BIOT), Chagos Archipelago	D. Fernando	

0689‡	*Mobula alfredi*	Ile Anglaise, Salomon Atoll	British Indian Ocean Territory (BIOT), Chagos Archipelago	D. Fernando	
1256, 1257‡, 1258, 1259, 1261, 1262, 1263, 1264, 1265, 1267, 1269, 1270, 1272, 1273, 1274, 1275, 1276, 1300, 1301, 1302, 1303‡	*Mobula alfredi*	Baa Atoll	Maldives	G. Stevens, N. Froman, T. Sawers	
1260, 1271, 1288, 1289, 1290, 1291, 1292, 1293, 1294, 1295‡, 1297‡, 1298‡	*Mobula alfredi*	Raa Atoll	Maldives	G. Stevens, N. Froman, T. Sawers	
1277‡, 1278‡, 1279, 1281‡, 1282, 1283, 1284‡, 1285, 1286	*Mobula alfredi*	Laamu Atoll	Maldives	G. Stevens, N. Froman, T. Sawers	
1329‡, 1331, 1332‡, 1333, 1334‡, 1335, 1336‡, 1337‡, 1340, 1341, 1342, 1346, 1347‡, 1348, 1350‡, 1352‡, 1353, 1354‡, 1355, 1356	*Mobula alfredi*	Kona Island	Hawaii	M. Deakos, J. Whitney	
1358, 1359‡, 1360‡, 1364‡	*Mobula alfredi*	Maui	Hawaii	M. Deakos, J. Whitney	
1376, 1378, 1379	*Mobula alfredi*		Australia, East Coast	A. Armstrong	Armstrong et al. (2019)
0718, 0719‡, 0721‡, 0724, 0726, 0727, 0730‡, 0732‡, 0733, 0734, 0737, 0738‡, 0742, 0745‡, 0747, 0748, 0749, 0751, 1072, 1073, 1074, 1075, 1076, 1077, 1078, 1079, 1080, 1081	*Mobula birostris*	Negombo	Sri Lanka	D. Fernando	Poortvliet et al. (2015); Stewart et al. (2016); Stewart et al. (2017)

0720, 0722, 0723, 0728, 0729, 0731, 0743‡, 0750, 0752, 1065, 1066, 1071‡, 1083‡, 1084, 1085	*Mobula birostris*	Mirissa	Sri Lanka	D. Fernando	Poortvliet et al. (2015); Stewart et al. (2016); Stewart et al. (2017)
1054, 1056, 1057, 1058, 1060, 1061, 1062, 1063, 1064	*Mobula birostris*		Peru	K. Forsberg, J. Stewart	Stewart et al. (2017)
0985‡, 0986, 0987, 0988	*Mobula birostris*	Yucatan Northern tip	Mexico Caribbean	R. Bonfil	
1102, 1103, 1104‡, 1105, 1108, 1109, 1112, 1113, 1114‡, 1115, 1117‡, 1119, 1121, 1122, 1123, 1124, 1126‡, 1127, 1128, 1129, 1131, 1132, 1133‡, 1134, 1135‡, 1136, 1137, 1138, 1139	*Mobula birostris*	Jagna, Bohol landing site	Philippines	A. Ponzo	Stewart et al. (2017)
1152‡, 1153‡, 1154, 1156‡, 1163, 1164, 1166, 1167, 1168	*Mobula birostris*	Bahia de Banderas, Jalisco	Mexico Pacific	J. Stewart	Stewart et al. (2016)
1172‡, 1173‡	*Mobula birostris*	Revillagigedo Islands	Mexico Pacific	J. Stewart	Stewart et al. (2016)
1181, 1182, 1183	*Mobula birostris*	Durban	South Africa	S. Wintner	
0980, 0981, 0982, 0983, 0984, 1239, 1240‡, 1241, 1242	*Mobula* Sp. 1	Yucatan Northern tip	Mexico Caribbean	R. Bonfil	Hinojosa-Alvarez et al. (2016)
1327, 1328†	*Mobula* Sp. 1	Flower Garden Banks National Marine Sanctuary	Texas, USA	J. Stewart	

* Where relevant, samples are associated with CITES permit numbers 4980, 551058/01, 10/0004/2014/01, 531703/01, 16-MV/0009/E9, 550069/01, PWS2017-AU-000256, 553090/01, MX80544, 542913/01 and 548594/01 or were exchanged between institutions under CITES scientific exemption codes GB015 and US150(A).

† Likely F1 hybrid (*Mobula birostris* x *Mobula sp. 1*).

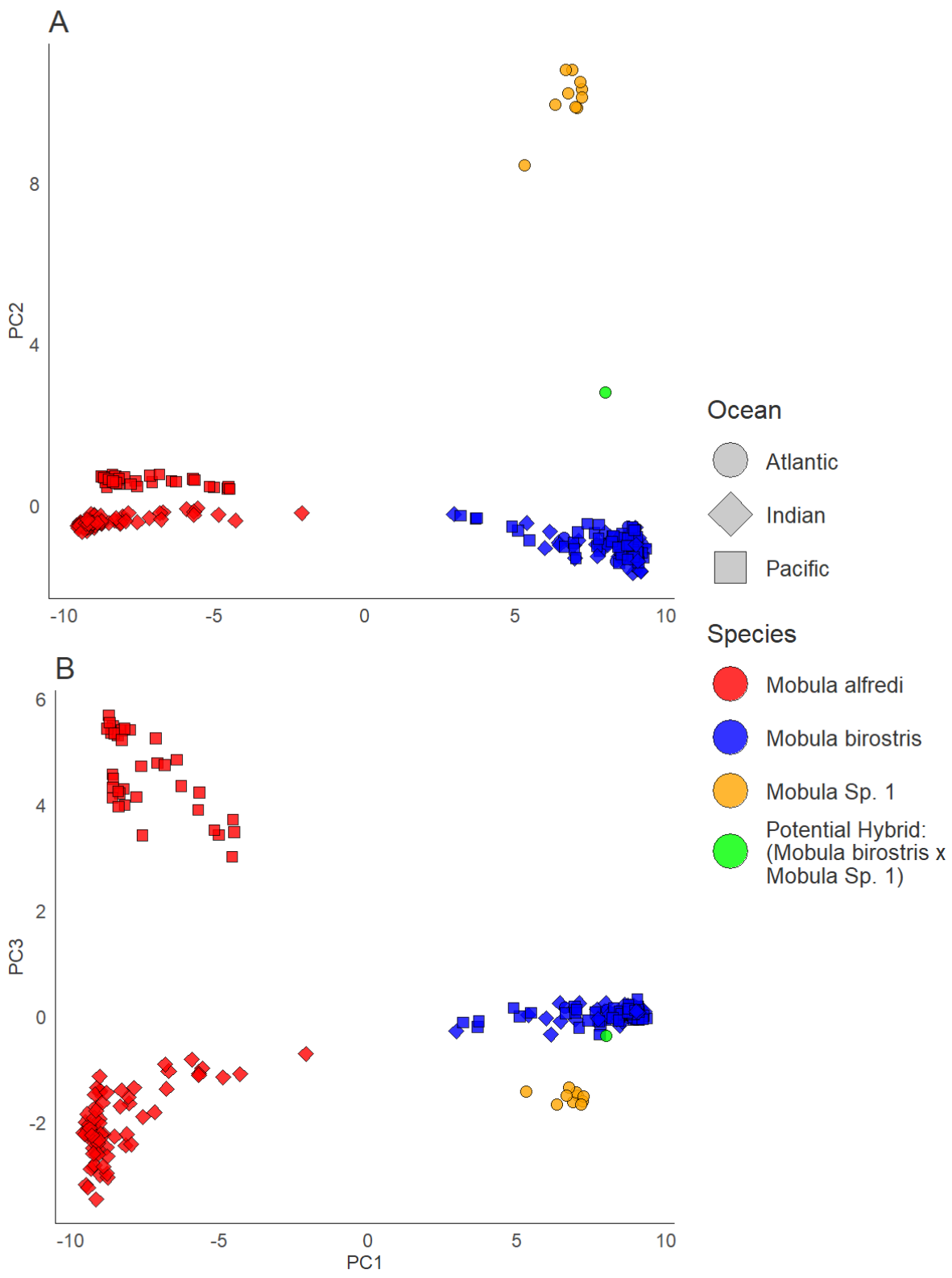
‡ Individual excluded from PCA due to having >25% missing data.

Supplementary Table S3.2: Sequencing information for ddRAD libraries used in the study presented in Chapter 3. Each library consisted of 96 individual samples multiplexed together and was sequenced in a single lane of Illumina HiSeq.

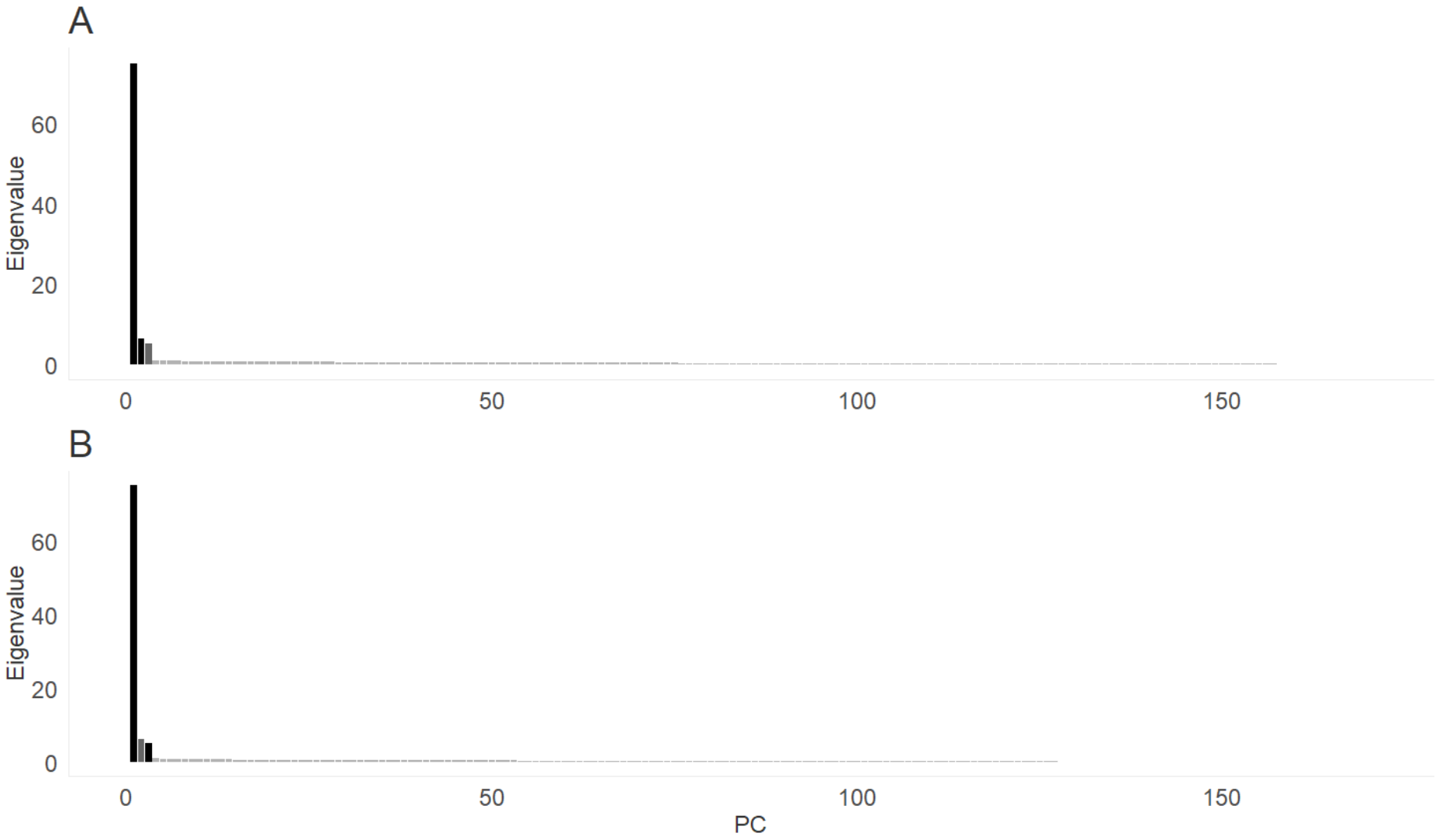
Library	Number of Reads (forward and reverse)
3	179,487,028
4	186,549,846
5	231,797,146

Supplementary Table S3.3: Individuals included in independent runs of SNAPP (Bryant et al. 2012) for manta ray species tree inference.

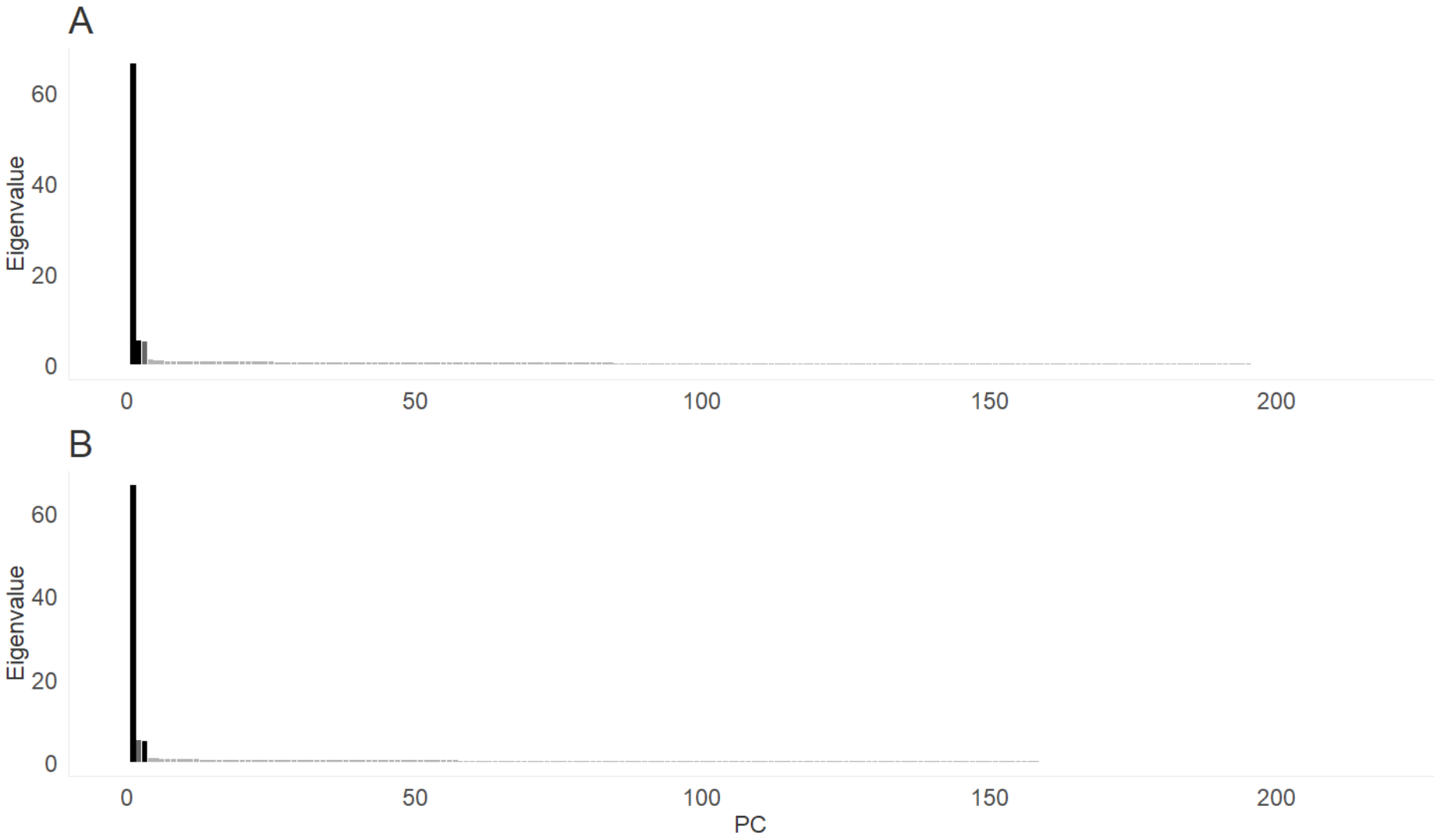
Species	Sample			
	Subset 1	Subset 2	Subset 3	Subset 4
Mobula alfredi	1326	1323	1313	0136
	0144	0148	0142	0146
	0689	0687	0689	0686
	1271	1282	1279	1265
	1347	1355	1350	1358
	1378	1378	1378	1376
Mobula birostris	0734	1072	1066	0732
	0987	0987	0985	0987
	1054	1063	1061	1054
	1129	1137	1113	1137
	1156	1163	1166	1168
	1181	1181	1183	1182
Mobula Sp. 1	1240	1240	0983	0984
	0983	0980	1242	1327
	1241	1239	1241	0983
	1239	1241	0982	1242
	0984	1327	0981	1240
	1327	0984	1327	0982
No. SNPs retained	1649	1761	1709	1722



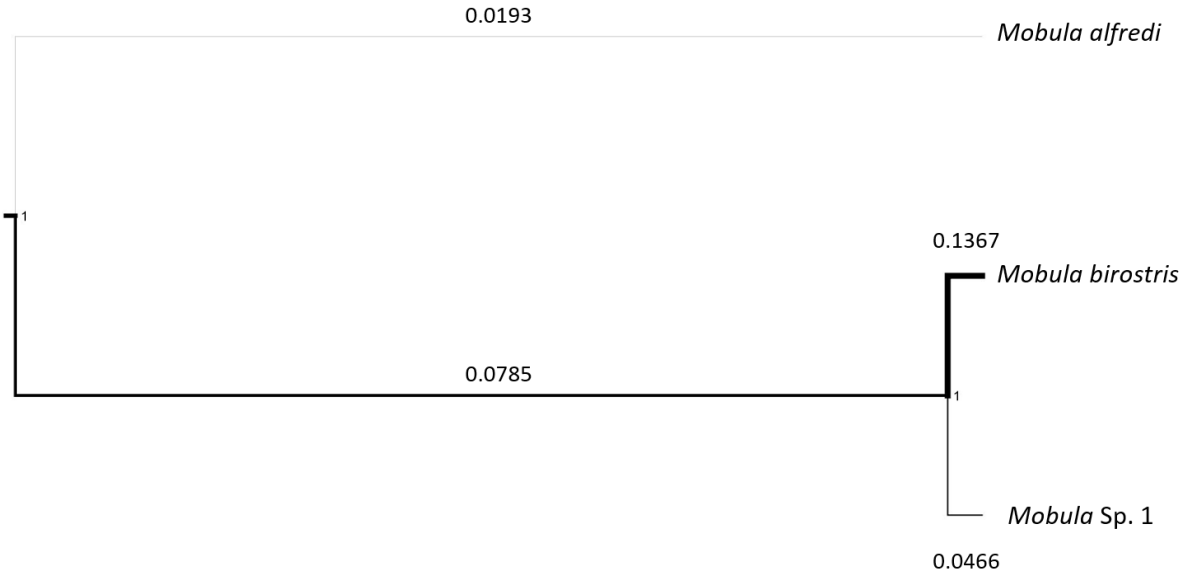
Supplementary Figure S3.1: Principal Components 1-3 plotted for all manta ray individuals. Individuals are represented by a point. Colour indicates species and shape indicates geographic origin of samples as specified in the key. The putative hybrid individual is shown in green. A) Principal Components 1 and 2, explaining 66.6% and 5.3% of the variance in the data, respectively, and B) Principal Components 1 and 3, where the latter explains 5.3% of the variance in the data. See Supplementary Figure S3.3 for full eigenvalue plot.



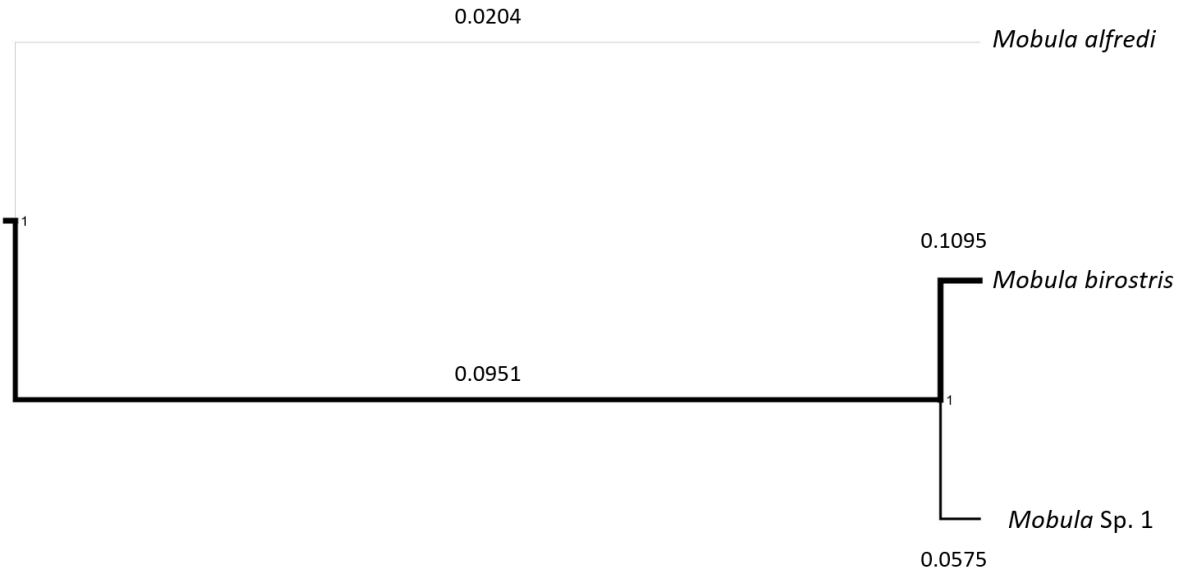
Supplementary Figure S3.2: Eigenvalue plots for Principle Components Analysis (PCA) where individuals with >25% missing data were excluded. Panel letters (A-B) correspond to the panel letters in Figure 3.3. Plotted axes are in black and retained axes in dark grey.



Supplementary Figure S3.3: Eigenvalue plots for Principle Components Analysis (PCA) including all manta ray individuals. Panel letters (A-B) correspond to the panel letters in Supplementary Figure S3.1. Plotted axes are in black and retained axes in dark grey.

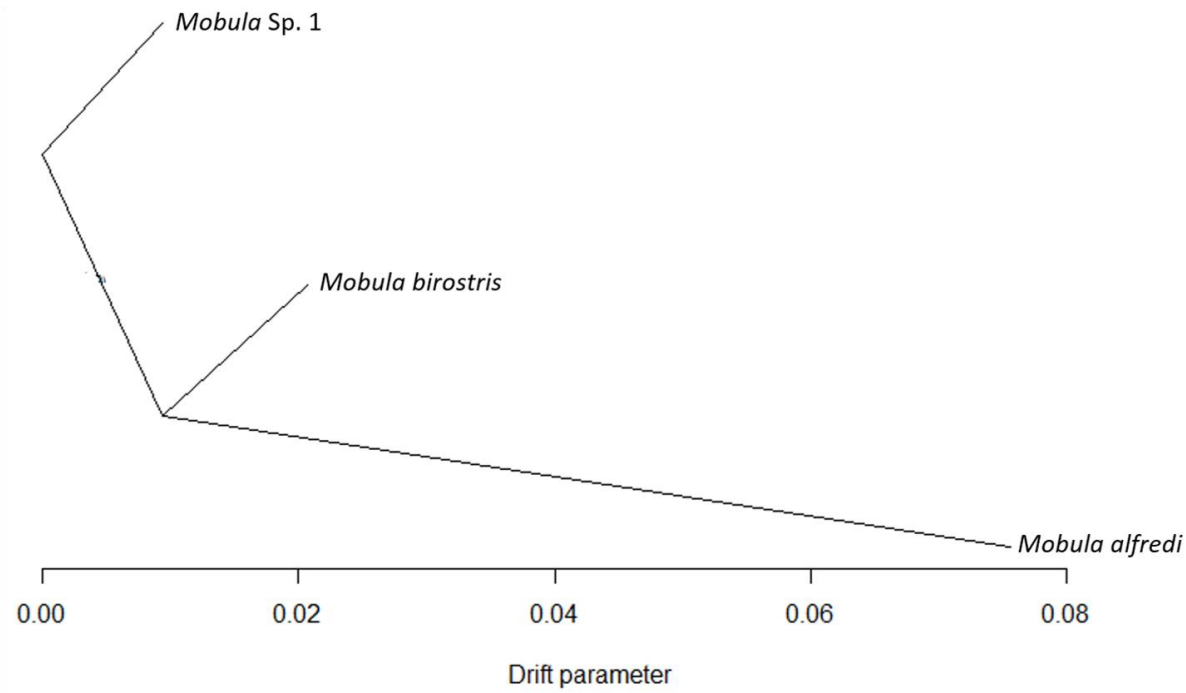


Supplementary Figure S3.4: Maximum clade credibility (MCC) tree for manta ray species inferred using SNAPP (Bryant et al. 2012), (individual subset 2; see Supplementary Table S3.3). Branch width is proportional to theta (ancestral effective population size), and theta values are shown on the branches. Posterior probabilities are shown on the nodes.



Supplementary Figure S3.5: Maximum clade credibility (MCC) tree for manta ray species inferred using SNAPP (Bryant et al. 2012), (individual subset 3; see Supplementary Table S3.3). Branch width is proportional to theta (ancestral effective population size), and theta values are shown on the branches. Posterior probabilities are shown on the nodes.

Supplementary Figure S3.6: Maximum clade credibility (MCC) tree for manta ray species inferred using SNAPP (Bryant et al. 2012), (individual subset 4; see Supplementary Table S3.3). Branch width is proportional to theta (ancestral effective population size), and theta values are shown on the branches. Posterior probabilities are shown on the nodes.



Supplementary Figure S3.7: Admixture graph showing relationships among manta ray species as a simple bifurcating tree, inferred using a Maximum Likelihood method in TreeMix (Pickrell & Pritchard, 2012). No outgroup was specified. Horizontal branch lengths represent drift. This model explains 100% of the variance in the data, where the associated residuals plot (not shown) indicates that placement of species is unaffected by admixture i.e. the fit could not be improved by adding migration edges.

Chapter 4

Evaluating the Population Genetic Structure of the Reef Manta Ray, *Mobula alfredi* and the Oceanic Manta Ray, *Mobula birostris*

This Chapter has not yet been submitted for publication.

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JH, EH, GC, MdB, RO, SC and GS designed and conceived of the study and secured funding for consumables relating to laboratory work. EH, GS, AA, RB, MD, DF, KF, NF, LP, SP, AP, JS and SW were responsible for sourcing and collecting samples. **JH**, HS and JK carried out laboratory work. **JH**, EH, GC, MdB, RO, SC, HH and HS contributed to analysis of genome-wide SNP data. **JH** wrote the Chapter.

Chapter 4: Evaluating the Population Genetic Structure of the Reef Manta Ray, *Mobula alfredi* and the Oceanic Manta Ray, *Mobula birostris*

Knowledge of population genetic structure in species of conservation concern can help to inform effective and appropriate management strategies. Highly structured populations may be effectively managed at local or regional scales, whereas large, migratory or broadly distributed populations will require international cooperation for effective conservation. High-throughput sequencing approaches now allow evaluation of vast numbers of both selectively neutral and putatively adaptive loci. As such, genomic approaches are particularly applicable to fisheries management, where in order to determine the impact of fishing on a species it is necessary to understand population structure and connectivity. Reef and oceanic manta rays (Mobula alfredi and Mobula birostris, respectively) exhibit contrasting habitat preferences across their overlapping ranges and are threatened by targeted and bycatch fisheries supplying the international trade in their gill plates. Targeted exploitation has been met with national and international legislation to protect these species; however, the status of manta rays is not matched by knowledge of their population genetic structure, gene flow and connectivity. Here, we use genome-wide SNP data representing among the most comprehensive global sampling of manta rays to date, to evaluate population genetic structure in these species. We find highly contrasting patterns, where reef manta rays show a high degree of population structure among sampling locations, indicative of limited gene flow, compared with genetic panmixia on a global scale for oceanic manta rays. Our data may offer insights regarding colonisation of new habitats in reef manta rays, where declining genetic diversity eastwards across the Pacific Ocean may be suggestive of successive founder events. Global genetic panmixia in oceanic manta rays may relate to past demographic processes, or differential dispersal among life stages. Our study highlights the importance of evaluating population structure and adaptive divergence individually for related species of conservation concern, rather than relying on an assumption that closely related species display similar patterns.

4.1 Introduction

Population genetic structure describes the extent and distribution of genetic diversity within species, and the preservation of genetic diversity is one of the key goals of conservation research. As such, genome-wide data provides opportunities for establishing population boundaries (e.g. Gagnaire et al. 2015; Williams et al. 2015; Silliman, 2019), quantifying genetic diversity and effective population sizes within populations (e.g. Allendorf et al. 2010; Funk et al. 2012; Hoelzel et al. 2019), evaluating the extent of adaptive variation (e.g. Nielsen et al. 2009; Stapley et al. 2010; Flanagan et al. 2018) and applying results to conservation and management (Shafer et al. 2015). Furthermore, such information can be applied to conservation policy and law enforcement through traceability tools and recommendations (Ogden et al. 2009; Nielsen et al. 2012; Ogden & Linacre, 2015).

In recent years, the declining cost and increasing accessibility of high-throughput sequencing approaches have allowed studies to evaluate vast numbers of both neutral and putatively adaptive markers (Allendorf et al. 2010; Metzker, 2010; Stapley et al. 2010; Zhang et al. 2011; Gagnaire et al. 2015). Neutral markers provide useful insights into evolutionary processes such as gene flow and genetic drift (Allendorf et al. 2010; Yoder et al. 2018), allowing the delineation of demographically independent populations characterised by limited gene flow. Such inferences can be used to delineate conservation and management units (Funk et al. 2012), thereby underpinning an empirical framework to develop regulatory policy. In addition, genetic diversity within populations may be evaluated in order to assess resilience to environmental change and extinction risk (Frankham, 2005; Sgrò, et al. 2011; Hellmair & Kinziger, 2014). Highly structured populations may be successfully managed at regional or local scales at which conservation and management strategies are usually implemented and enforced (Mace, 2004). In contrast, large, migratory, or broadly distributed populations may require international cooperation for effective conservation, such as that implemented under the Convention on the International Trade in Endangered Species of Wild Fauna and Flora (CITES) or the Convention on the Conservation of Migratory Species of Wild Animals (CMS). In addition, inference of migration rates between populations or management units can help to inform the design of conservation measures that may include consideration of migratory corridors (e.g. Kaczensky et al. 2011; Linnell et al. 2016) and predict where populations are

likely to have significant adaptive differences (Funk et al. 2012). In comparison, putatively adaptive outlier loci allow quantification of adaptive divergence and identification of locally and/or differentially adapted populations (e.g. Nielsen et al. 2009; Stapley et al. 2010; Bekkevold et al. 2016; Sarkar et al. 2019). Studies characterising adaptive differentiation between populations have sparked interest in using locally adaptive variation to inform conservation (Flanagan et al. 2018). However, concerns have been raised regarding conservation strategies designed to preserve small genomic regions and/or a handful of traits, especially where these strategies may be based on limited data (Kardos & Shafer, 2018).

In order to determine the impact of fishing on a species it is necessary to understand population structure and connectivity, where isolated populations typically have higher vulnerability to population declines than those with increased connectivity and gene flow (Hellmair & Kinziger, 2014). Genomic approaches are therefore particularly applicable to fisheries management. Numerous examples of population structure in marine fish (e.g. Henriques et al. 2017; Westgaard et al. 2017; Lehnert et al. 2019) coupled with reduced effective population sizes in overexploited populations (Hauser et al. 2002) have now overturned the traditional assumption of inexhaustible panmictic populations of commercially important fish species (Hauser & Carvalho, 2008). Furthermore, genomic methods have allowed evaluation of adaptive differentiation between fish populations (e.g. Nielsen et al. 2009; Bekkevold et al. 2016) and identified localised genomic regions responsible for ecotype divergence (e.g. Hemmer-Hansen et al. 2013; Malinsky et al. 2015; Larson et al. 2017).

Fisheries have been associated with a decline in genetic diversity (Pinsky & Palumbi, 2014), leaving overexploited populations more vulnerable to environmental change (Sgrò et al. 2011; Hellmair & Kinziger, 2014). Partitioning stocks along political boundaries is therefore inappropriate for effective management of marine fisheries, and genomic data is useful for establishing biologically meaningful management units with restricted gene flow (Carvalho & Hauser, 1994; Reiss et al. 2009; Kerr et al. 2017; Mullins et al. 2018). Taken together, an understanding of population boundaries and the extent and patterns of migration, gene flow and connectivity can yield valuable insights into the levels of genetic diversity and adaptive divergence within and among populations under fishing pressure and enhance the detection and study of management units.

Manta and devil rays (*Mobula* spp.) are circumglobally distributed megafauna of high conservation priority (Lawson et al. 2017), carrying substantial economic value through tourism (O'Malley et al. 2013). However, international trade in manta and devil ray gill plates has led to the expansion of unsustainable fisheries, many of which are not regulated or monitored (Couturier et al. 2012; O'Malley et al. 2017). Compounded by the effects of losses through bycatch (Croll et al. 2016), such consumptive exploitation is likely to be met by population declines exacerbated by slow life history traits, hindering recovery from fishing impacts (Dulvy et al. 2014). Indeed, concerning declines in mobulid species have been reported in fisheries around the world (e.g. Lewis et al. 2015; see CITES CoP17 proposal 44, 2016).

Fishing pressure faced by manta and devil ray species is being met with the implementation of national and international laws to prevent further declines, such as the recent listings of all species on CITES Appendix II and CMS Appendices I and II. However, the status of mobulid rays is not matched by knowledge of population structure, gene flow, genetic diversity and adaptive variation. Furthermore, a lack of representative samples has hindered or prevented studies aiming to fill these knowledge gaps (Stewart et al. 2018b). To date, the majority of mobulid studies have targeted local populations. Whilst such projects can produce impact on a local scale, their capacity for assessing the global state of these species and broad patterns of the extent and distribution of genetic diversity is limited.

The reef manta ray (*Mobula alfredi*) and the oceanic manta ray (*M. birostris*) are thought to have diverged recently due to differences in habitat preference (Kashiwagi et al. 2012). As the names suggest, the reef manta ray is more commonly associated with shallow, inshore, reef-based habitats, in contrast to the offshore pelagic habitats usually favoured by the oceanic manta ray (Marshall et al. 2009; Kashiwagi et al. 2011; Couturier et al. 2012; Stevens, 2016; Stewart et al. 2016). As such, reef manta rays are expected to show a high degree of population structure, given the fragmented nature of reef-based habitats in the oceans, whereas highly connected pelagic habitats may offer more opportunities for gene flow in the oceanic manta ray. However, tagging and photo identification studies are indicative of a high degree of residency and spatial structuring in both species (e.g. Jaine et al. 2014; Stewart et al. 2016; Couturier et al. 2018), although more long-range movements between sites are not unknown (Couturier et al. 2011; Germanov & Marshall, 2014). Furthermore, there is some

evidence of fine-scale population structure from genomic data in the oceanic manta ray (Stewart et al. 2016). However, few studies have explicitly examined population structure and gene flow in manta rays, and uncertainty in assessments of population boundaries, dispersal and gene flow compromises the ability to design effective conservation and management strategies for these species (Stewart et al. 2018b).

Chapters 2 and 3 present an assessment of mobulid diversity at the species level, and at an intermediate point along the speciation continuum, respectively. Here, genome-wide SNP data from the most comprehensive global sampling of manta rays to our knowledge to date is examined, and is applied to assess population structure and genetic diversity in the reef manta ray, *Mobula alfredi*, and the oceanic manta ray, *Mobula birostris*, which show contrasting habitat preferences, with potential for differing levels of connectivity.

4.2 Methods

4.2.1 SNP data

In Chapter 3, a ddRAD approach was used to produce a quality-controlled dataset of 4776 SNP markers for three species of manta ray (see sections 3.2.2 and 3.2.3 for details of laboratory procedures and data processing, respectively). Here, this same set of SNPs is used to evaluate population genetic structure within the reef manta ray (*Mobula alfredi*) and the oceanic manta ray (*M. birostris*), since these two species were the most extensively sampled and show contrasting habitat preferences, with potential for differing levels of connectivity. Specifically, data are evaluated representing 107 *Mobula alfredi* individuals and 99 *M. birostris* individuals, each sampled at six independent locations (see Chapter 3, Figure 3.1; further details given in Chapter 3, Supplementary Table S3.1). Of the original 4776 SNPs discovered in Chapter 3, 1067 and 3217 were retained as polymorphic within *Mobula alfredi* and *M. birostris,* respectively.

Many model-based methods for inferring population genetic structure, such as STRUCTURE implemented herein, assume that loci are inherited independently, i.e. not in linkage disequilibrium (LD) (Pritchard et al. 2000; Falush et al. 2003). Whilst the SNP datasets above

were quality controlled to minimise linkage, retaining only forward sequence reads and filtering for a single random SNP per locus (see Section 3.2.3 for details), a formal test for LD between loci was additionally performed in a pairwise manner using the R package 'genepop' (Rousset, 2008). The exact test for genotypic association between each pair of 1067 SNPs was performed for *Mobula alfredi,* using default values for the length of the dememorization step of the Markov Chain algorithm (10,000), the number of batches (100) and the number of iterations per batch (5000). However, due to computational constraints, it was not possible to compute all pairwise comparisons for the larger *Mobula birostris* dataset of 3217 SNPs. The dataset was therefore randomly subsampled with replacement, resulting in 10 smaller subsets of 1500 SNPs from the original 3217, and pairwise tests for genotypic association were performed as above for each. Resulting p-values were corrected for multiple comparisons using the Bonferroni method.

Each of the analyses below was carried out three times, with different subsets of these polymorphic SNPs. Selectively neutral loci, traditionally used in population genetics, may be limited in their capacity to infer genetic differentiation in marine species with high dispersal and large population sizes (see Gagnaire et al. 2015). Since the aim of this study is to evaluate population structure in two species of manta ray and apply results to conservation and management, the framework for delineating conservation units published by Funk et al. (2012) is followed, first carrying out analyses using all polymorphic loci, inclusive of any outlier loci. These datasets are referred to as the 'all SNPs' datasets within each species.

However, many model-based methods, such as STRUCTURE used in the analyses herein, assume that loci are selectively neutral (Pritchard et al. 2000; Falush et al. 2003). To identify loci that may violate the assumption of neutrality, BAYESCAN software version 2.1 (Foll & Gaggiotti, 2008) was run for 100,000 iterations using default model parameters within each species, and setting populations that corresponded to sampling locations. In brief, BAYESCAN works by comparing two models, one with and one without selection, by decomposing F_{ST} coefficients into population-specific and locus-specific components using a logistic regression (Foll & Gaggiotti, 2008). Posterior odds (log(PO)) are used to identify those loci where the model with selection is required to explain the observed pattern of diversity in the data, providing a means of identifying loci with significant departure from neutrality (Foll & Gaggiotti, 2008). BAYESCAN (Foll & Gaggiotti, 2008) identified 6 and 1 SNPs as potential

outliers in *Mobula alfredi* and *M. birostris,* respectively, with a False Discovery Rate (FDR) of 5% (Supplementary Figures S4.1 and S4.2). Since the aim of this study was to establish management units using neutral loci following the framework in Funk et al. (2012), rather than quantify adaptive differentiation *per se*, a conservative approach was taken, removing all SNPs within the 90th percentile of log(PO) for each species, before using the remaining SNPs to run the analyses below a second time. These datasets are referred to as the 'neutral SNPs' datasets within each species.

Finally, a recent study showed that inference of population genetic structure may be affected by minor allele frequency thresholds, and specifically recommends that singletons (loci with a minor allele count of 1) be excluded from model-based analyses (Linck & Battey, 2019). In line with this recommendation, singletons were excluded from the 'all SNPs' dataset within each species using VCFtools (Danecek et al. 2011), retaining 535 and 1868 SNPs in *Mobula alfredi* and *M. birostris,* respectively, which also underwent the analyses below. These datasets are referred to as the 'no singletons' datasets within each species.

4.2.2 Inference of Population Genetic Structure

To assess how individuals cluster together, Principal Components Analyses (PCA) were performed for each of the three datasets within each species using the R package 'adegenet' (Jombart, 2008). For *Mobula alfredi*, three axes were evaluated and retained, cumulatively explaining between 9% and 13% of the variance in the data, depending on which SNP dataset was used. All other axes explained <1% of the variance in the data, and were therefore not examined (see Supplementary Figures S4.3A and S4.4-S4.5). For *Mobula birostris*, the variance explained by each axis was minimal (maximum 1.5%) and eigenvalues highly similar (see Supplementary Figures S4.3B and S4.6-S4.7). Five axes, cumulatively explaining >5% of the variance in the data, were therefore retained in order to more thoroughly evaluate population structure in this species. Seven *Mobula birostris* individuals, sampled in Sri Lanka and the Philippines, dominated differentiation along the first two axes, and so the analyses were repeated with these individuals removed to better visualise population structure among the remaining individuals. In addition, since data representing samples collected from *Mobula alfredi* individuals at three locations within the Maldives (Baa, Raa and Laamu atolls) and two

locations within Hawaii (Maui and Kona islands) were available (see Chapter 3, Supplementary Table S3.1 for details), PCAs were also run exclusively on individuals sampled within these locations in order to evaluate fine-scale population structure. In both cases, the variance explained by each axis was minimal (maximum 1%) and eigenvalues highly similar (Supplementary Figure S4.8), so two axes were retained.

Population genetic structure within species was further investigated using a model-based Bayesian clustering method implemented in STRUCTURE version 2.3.4 (Pritchard et al. 2000; Falush et al. 2003). STRUCTURE was run independently for each SNP dataset within each species, initially without any prior information on sampling location, and implementing the admixture model for K=1-10 (Pritchard et al. 2000). Five independent replicates were performed with 1,000,000 MCMC iterations and 100,000 burn-in. Replicates were entered into STUCTURE HARVESTER version 0.6.94 (Earl & vonHoldt, 2012) to identify the most likely value of K using the ΔK method (Evanno et al. 2005). Outputs for both species for K=2 were averaged in CLUMPP version 1.1.2 (Jakobsson & Rosenberg, 2007) using the Greedy algorithm with 1,000 repeats and results plotted using DISTRUCT version 1.1 (Rosenberg, 2004). Since K=2 clearly distinguished *Mobula alfredi* individuals sampled in the Indian and Pacific Oceans, STRUCTURE (Pritchard et al. 2000; Falush et al. 2003) was re-run (K=1-6) for *M. alfredi* individuals sampled in each Ocean independently and evaluated and visualised using the procedure above. Finally, where data are weakly structured, providing STRUCTURE (Pritchard et al. 2000; Falush et al. 2003) with prior information on sampling location may help to identify weak population structure (Hubisz et al. 2009). Since the above implementation of STRUCTURE (Pritchard et al. 2000; Falush et al. 2003) was unable to recover any population structure in *Mobula birostris*, the procedure above was repeated using the 'locprior' model (Hubisz et al. 2009), allowing prior information on sampling locations to inform the model for this species.

F_{ST} estimates were obtained per locus and over all loci, and pairwise F_{ST} values (Weir & Cockerham, 1984) were calculated between sampling locations for each species and dataset using the R package 'hierfstat' (Goudet & Jombart, 2015). Confidence intervals (99%) were obtained by performing 1000 bootstrap replicates over loci of pairwise F_{ST}, allowing for significant difference from zero to be established for F_{ST} values.

4.2.3 Genetic diversity

To evaluate genetic diversity within inferred populations (for *Mobula alfredi*) and within sampling locations (for *M. birostris*), the populations.pl program in Stacks (Catchen et al. 2011), was used to calculate the expected heterozygosity (H_e), observed heterozygosity (H_o) and nucleotide diversity (π) within populations. This approach was applied to both species for consistency despite no evidence that sampling locality is associated with genetic population structure in *Mobula birostris*.

4.3 Results

4.3.1 Linkage Disequilibrium

After Bonferroni correction, $p < 0.01$ for 0.71% of pairwise comparisons for *Mobula alfredi*, indicating that 0.71% of SNP pairs may be linked and therefore violate the assumption of independence. For *Mobula birostris*, $p < 0.01$ for an average of 0.00012% of pairwise comparisons across 10 random subsets of 1500 SNPs (results for individual subsets are given in Supplementary Table S4.1). In both cases, levels of LD within the SNP datasets analysed here were very low.

4.3.2 Population Structure

The datasets representing two species of manta ray studied here produced highly contrasting patterns of population structure. Principal Components Analysis (PCA) showed reef manta ray, (*Mobula alfredi)*, individuals clustered within sampling location (Figure 4.1A). Consistent with patterns observed in Chapters 2 and 3 for this species, the first Principal Component, hereafter PC1, clearly separated individuals sampled in the Indian and Pacific Oceans and explained 9.7% of the variance in the data when the analysis was applied to the 'all SNPs' dataset (Supplementary Figure S4.3A). Plotting additional PCs clearly separated out sampling locations within each ocean (see Figure 4.1A and Supplementary Figure S4.9). These patterns were highly consistent across the 'neutral SNPs' and 'no singletons' datasets (see

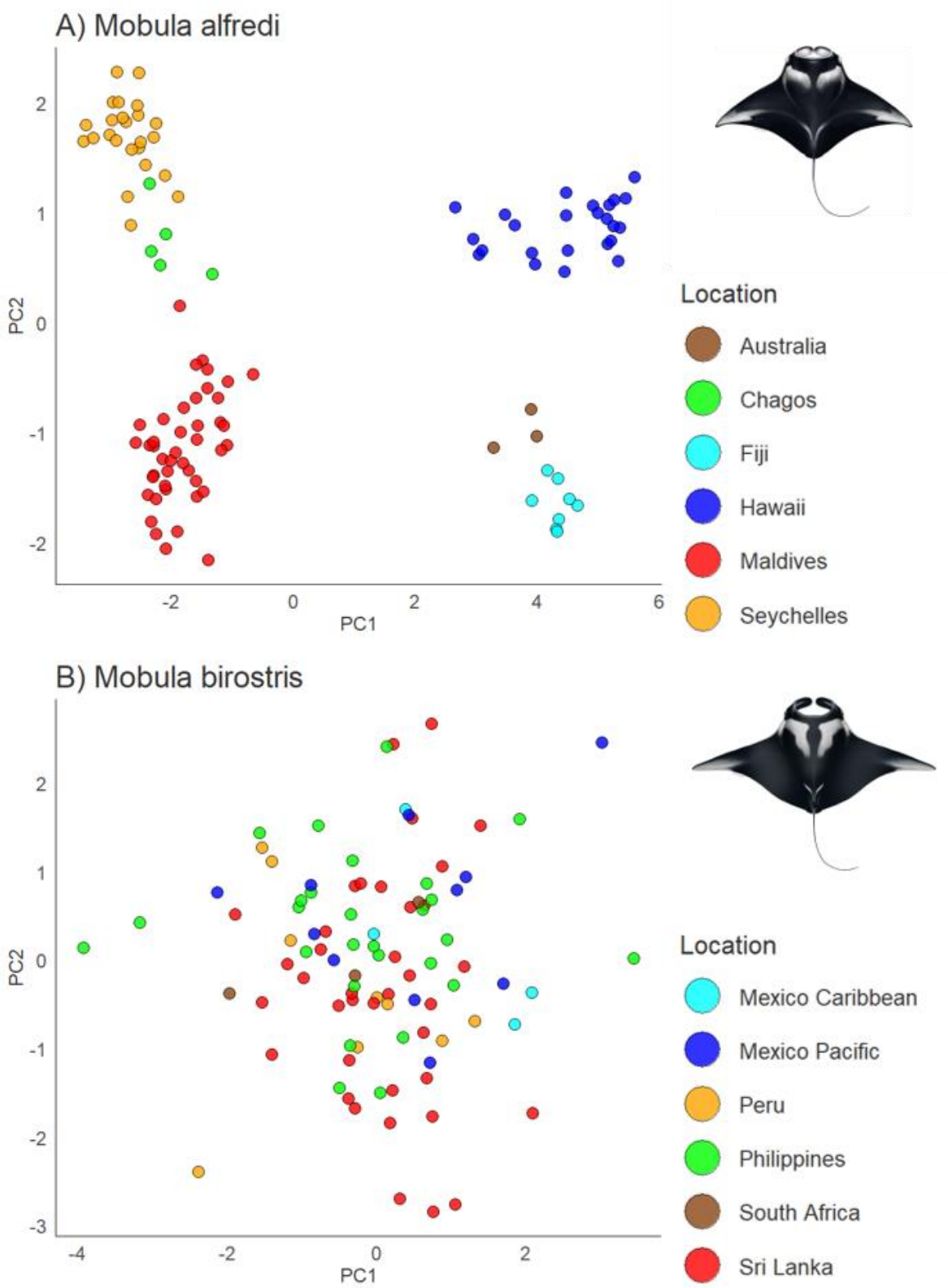


Figure 4.1: Principal Components 1 and 2 plotted for A) *Mobula alfredi,* and B) *Mobula birostris* 'all SNPs' datasets. Individuals are represented by a point, where colour indicates sampling location as specified in the respective keys. PC1 and PC2 explain 9.7% and 1.7% of the variance in the data respectively for *Mobula alfredi*, and 1.4% and 1.3% in *M. birostris*. See Supplementary Figure S4.3 for full eigenvalue plots. Illustrations © Marc Dando and are reproduced with permission.

Supplementary Figures S4.4 and S4.5 for eigenvalue plots and S4.10 and S4.11 for PCA plots). Our data was unable to recover any further structure within *Mobula alfredi* through PCA among sites within the Maldives and Hawaii (Figure 4.2).

Principal Components Analysis (PCA) was unable to recover differentiation among sampling locations in the oceanic manta ray, *Mobula birostris* (Figure 4.1B), where the first Principal Component, hereafter PC, explains <1.4% of the variance in the data (Supplementary Figure S4.3B). Plotting additional PCs failed to find evidence of any population structure in this species (Supplementary Figure S4.12) and observations were highly consistent across the 'neutral SNPs' and 'no singletons' datasets (see Supplementary Figures S4.6 and S4.7 for eigenvalue plots and S4.13 and S4.14 for PCA plots). Whilst a handful of individuals failed to cluster well with most individuals, these patterns were not consistent across sampling locations (Supplementary Figure S4.15; see Supplementary Figure S4.16 for eigenvalue plot). The cause of these outlier genotypes is not clear, since missing data for these individuals is consistent with the average levels of missing data across the dataset. However, these individuals do possess a small number of rare alleles.

Plots of ΔK and LnP(K) generated in STRUCTURE HARVESTER (Earl & vonHoldt, 2012) indicated two as the most likely number of genetic clusters present in the 'all SNPs' dataset for *Mobula alfredi* (Supplementary Figure S4.17A-B) and these broadly describe individuals sampled in the Indian and Pacific Oceans (Figure 4.3A). Extracting subsets of the data to look for further division within oceans identified clusters broadly describing the Seychelles and the Maldives populations within the Indian Ocean at K=2 (Figure 4.3B). Individuals sampled in Chagos were assigned to mixed clusters at both K=2 and K=3 (Figure 4.3B-C). Similarly, clusters describing populations in Fiji and Hawaii were identified at K=2 in the Pacific Ocean (Figure 4.3D) and individuals sampled in Australia were assigned to mixed clusters at K=2 and K=3 (Figure 4.3D-E). These patterns were highly consistent with the 'neutral SNPs' dataset (see Supplementary Figures S4.18 and S4.19 for STRUCTURE plots and STRUCTURE HARVESTER output, respectively). However, removing singletons from the alignment (i.e. using the 'no singletons' dataset) produced a much clearer signal of population structure corresponding to sampling locations in *Mobula alfredi* (see Supplementary Figures S4.20 and S4.21 for STRUCTURE plots and STRUCTURE HARVESTER output, respectively), consistent with Linck & Battey (2019).

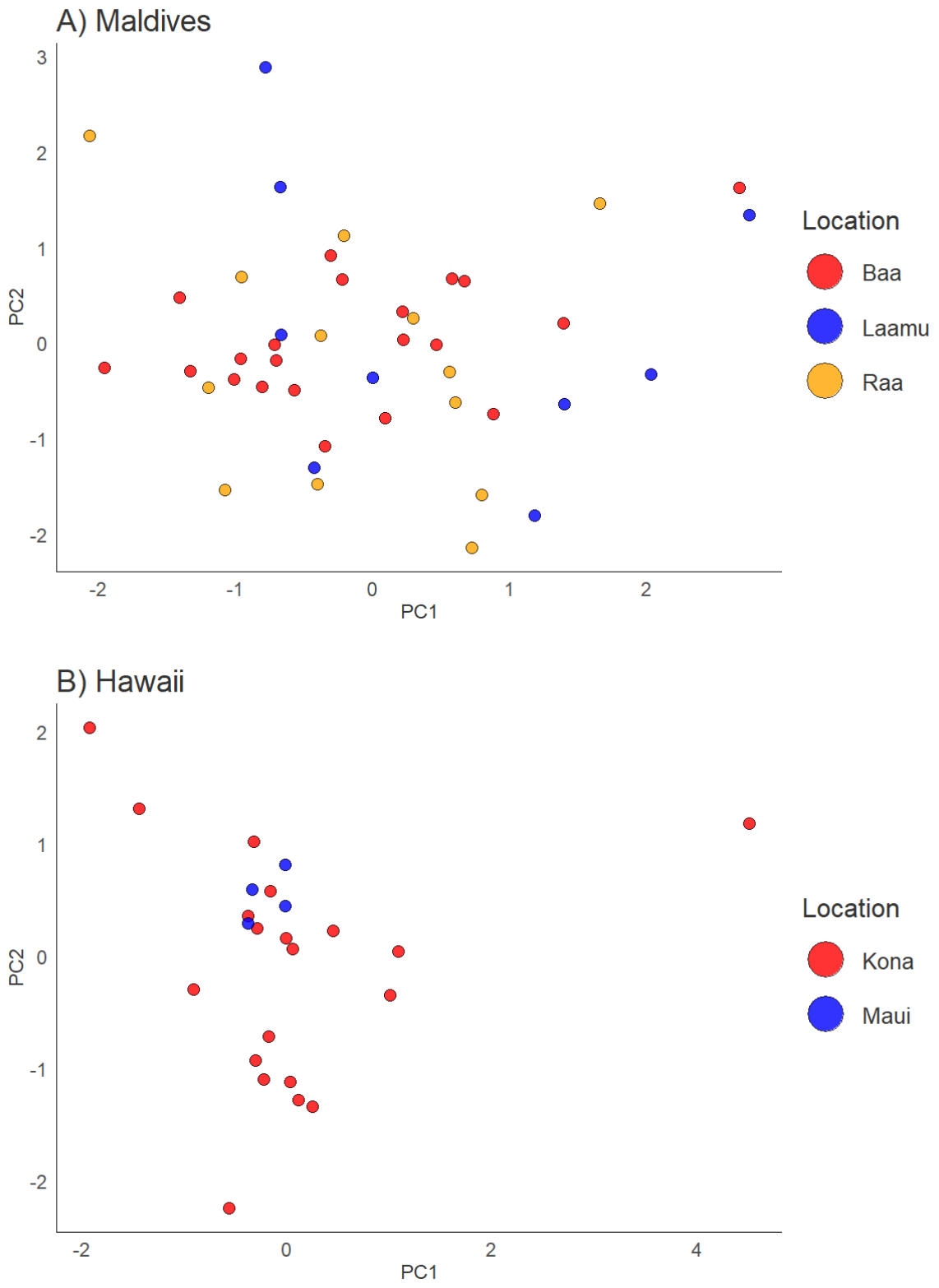


Figure 4.2: Principal Components 1 and 2 plotted for *Mobula alfredi* individuals sampled in multiple locations across A) the Maldives and B) Hawaii, using the 'all SNPs' dataset. Individuals are represented by a point, where colour indicates sampling site as specified in the respective keys. PC1 and PC2 explain 1.2% and 1.1% of the variance in the data respectively in the Maldivian population, and 1.3% and 0.9% respectively in the Hawaiian population. See Supplementary Figure S4.8 for full eigenvalue plots.

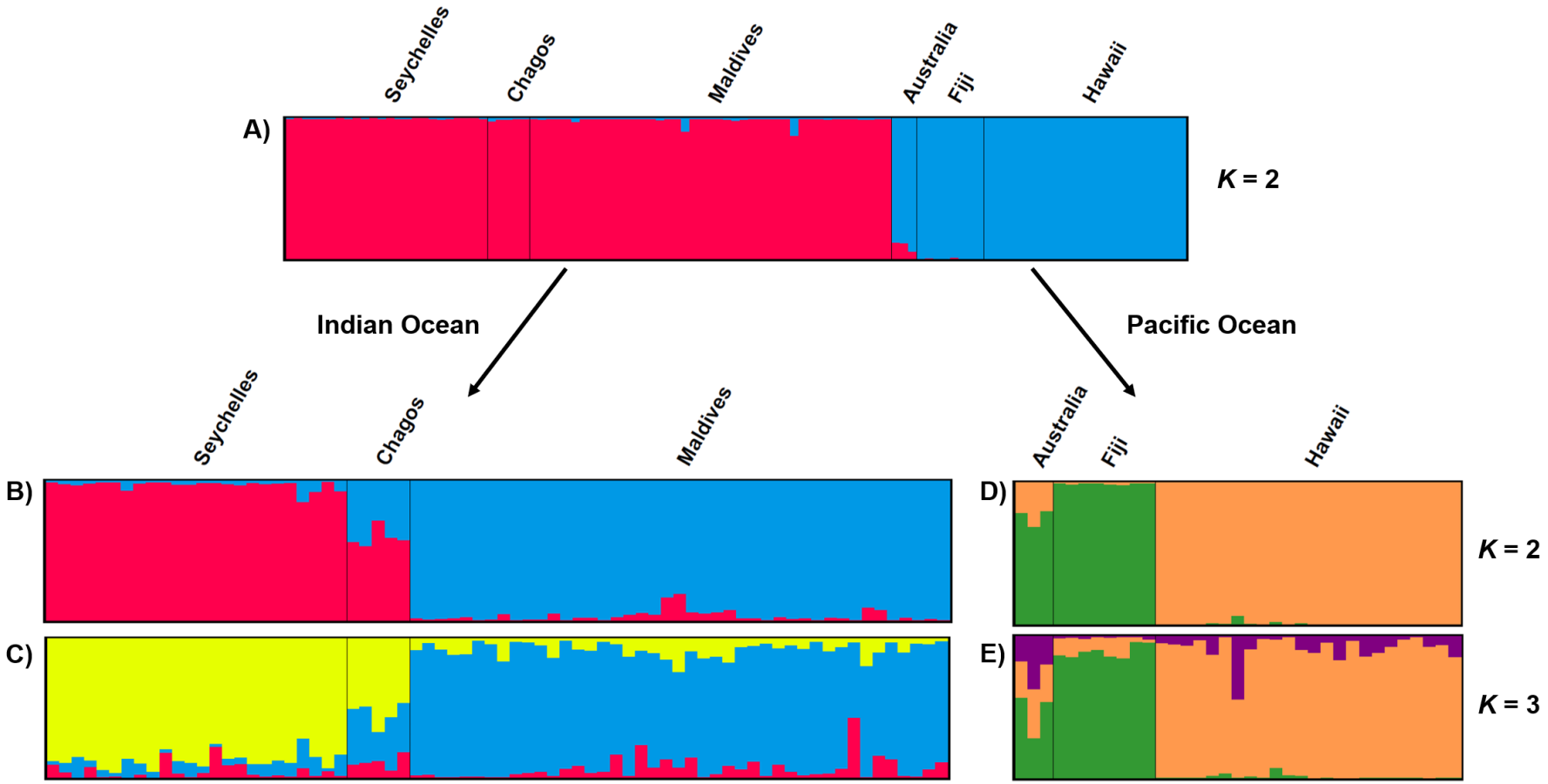


Figure 4.3: Plot showing results of Bayesian clustering analysis with no prior information on sampling locations implemented in STRUCTURE using the admixture model for *Mobula alfredi* 'all SNPs' dataset. A) All individuals analysed together at *K*=2. The data was then split into inferred clusters, corresponding to individuals sampled in the Indian and Pacific oceans, and plotted for *K*=2 and *K*=3: B) Indian Ocean *K*=2, C) Indian Ocean *K*=3, D) Pacific Ocean *K*=2, E) Pacific Ocean *K*=3. Each vertical bar represents an individual, and colours represent the proportion of ancestry assigned to each cluster for each individual. Individuals are grouped by sampling location. STRUCTURE HARVESTER output, identifying optimal values of *K*, are given in Supplementary Figure S4.17.

In contrast, plots of Δ*K* and LnP(*K*) generated in STRUCTURE HARVESTER (Earl & vonHoldt, 2012) indicated a single cluster as the most likely number of genetic clusters present in the 'all SNPs' dataset for *Mobula birostris* (Supplementary Figure S4.22). Plotting *K*=2 shows no population structure within this species (Figure 4.4). Allowing STRUCTURE to use prior information on sampling locations (Pritchard et al. 2000; Hubisz et al. 2009) failed to improve resolution (Figure 4.5; Supplementary Figure S4.23) and results were highly consistent using the 'neutral SNPs' dataset (see Supplementary Figures S4.24 and S4.25 for STRUCTURE plots and STRUCTURE HARVESTER output, respectively). In contrast to an equivalent analysis in *Mobula alfredi*, excluding singletons from the *M. birostris* dataset following Linck and Battey (2019) failed to improve inference of population structure (see Supplementary Figures S4.26 and S4.27 for STRUCTURE plots and STRUCTURE HARVESTER output, respectively), even where sampling locations were provided as prior information (see Supplementary Figures S4.28 and S4.29 for STRUCTURE plots and STRUCTURE HARVESTER output, respectively).

Pairwise F_{ST} values between sampling locations, and global intra-specific F_{ST} values calculated among loci based on the 'all SNPs' datasets within species are given in Tables 4.1 and 4.2 for *Mobula alfredi* and *M. birostris,* respectively. Locus-specific F_{ST} values are plotted in Supplementary Figure S4.30 for *Mobula alfredi*, and Supplementary Figure S4.31 for *M. birostris*. Results were broadly consistent when calculated based on the 'neutral SNPs' datasets within each species (Supplementary Tables S4.2 and S4.3 and Supplementary Figures S4.32 and S4.33), and with 'no singletons' datasets within each species (Supplementary Tables S4.4 and S4.5 and Supplementary Figures S4.34 and S4.35).

4.3.3 Genetic Diversity within Populations

Expected heterozygosity (H_e), observed heterozygosity (H_o), nucleotide diversity (π) and the number of polymorphic SNPs within each sampling location are given in Tables 4.3 and 4.4 for *Mobula alfredi* and *M. birostris,* respectively. Results were broadly consistent when calculated based on the 'neutral SNPs' datasets (Supplementary Tables S4.6 and S4.7), and with the 'no singletons' datasets (Supplementary Tables S4.8 and S4.9). Estimates of observed and expected heterozygosity (H_o and H_e, respectively), nucleotide diversity (π) and the number of polymorphic SNPs may be artificially reduced in some sampling locations, due to large

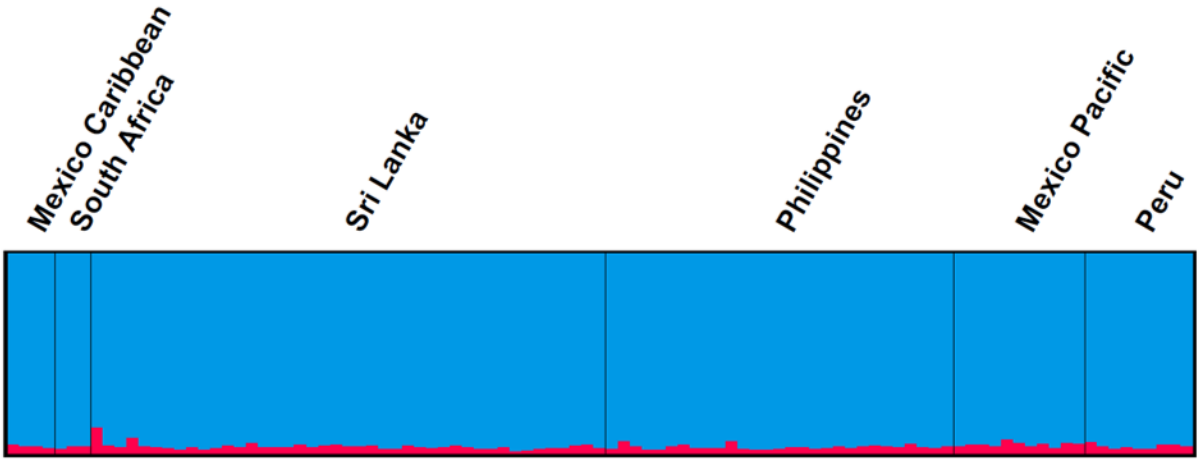


Figure 4.4: Plot showing results of Bayesian clustering analysis with no prior information on sampling locations implemented in STRUCTURE using the admixture model at *K*=2 for *Mobula birostris,* 'all SNPs' dataset. Plot shown is for *K*=2, since the output from STRUCTURE HARVESTER indicated an optimal value of *K*=1 (see Supplementary Figure S4.22). Each vertical bar represents an individual, and colours represent the proportion of ancestry assigned to each cluster for each individual. Individuals are grouped by sampling location.

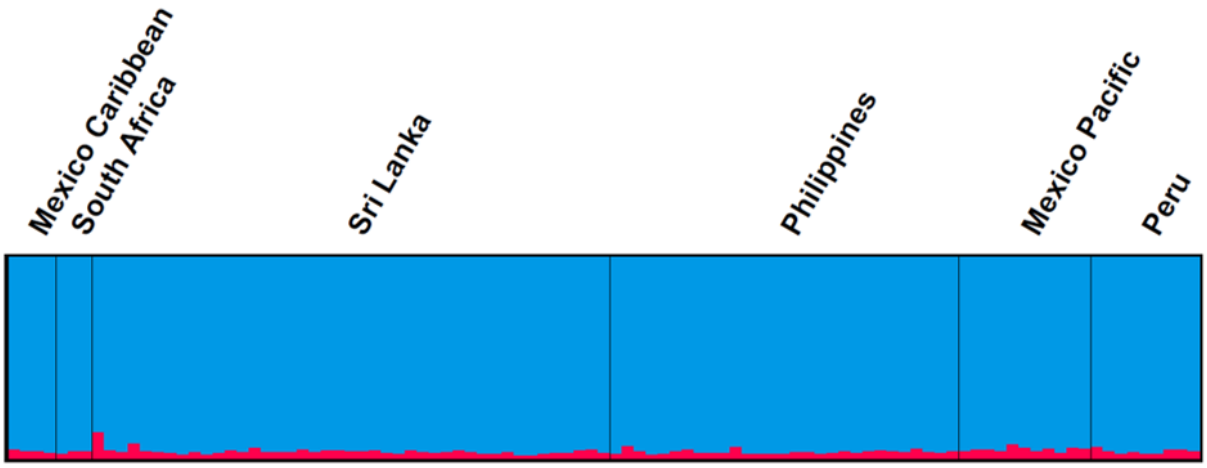


Figure 4.5: Plot showing results of Bayesian clustering analysis implemented in STRUCTURE using the admixture model at *K*=2 for *Mobula birostris,* 'all SNPs' dataset with prior information on sampling locations provided ('locprior' model). Plot shown is for *K*=2, since the output from STRUCTURE HARVESTER indicated an optimal value of *K*=1 (see Supplementary Figure S4.23). Each vertical bar represents an individual, and colours represent the proportion of ancestry assigned to each cluster for each individual. Individuals are grouped by sampling location.

discrepancies in the number of individuals sampled between sampling locations, resulting in less opportunity to discover rare alleles. However, both the sequencing strategy employed in this study (see Section 3.2.2), and the use of STACKS software (Catchen et al. 2011; see Section 3.2.3) reduce the effects of methodological ascertainment bias. During library preparation, individuals of both species and from all sampling locations were multiplexed together within libraries and therefore shared HiSeq lanes. This precludes the possibility of a systematic discrepancy in the number of sequencing reads returned per species and sampling location. Furthermore, STACKS (Catchen et al. 2011) discovers loci *de novo* and adds all new loci and SNPs across each individual in turn to a common catalogue. Whilst the method presented here is therefore unlikely to have generated significant ascertainment bias, an effect caused by the discrepancy in the number of individuals typed per species and sampling location cannot be ruled out. Expected heterozygosity (H_e) is reported in addition to observed heterozygosity (H_o), since this estimate of genetic diversity is less sensitive to sample size. Nonetheless, since the numbers of individuals of *Mobula alfredi* sampled from the Seychelles and Hawaii are similar, it is likely that the difference in diversity estimates for these two populations is a genuine biological occurrence.

Table 4.1: Pairwise F_{ST} values ± 99% Confidence Intervals (Weir & Cockerham, 1984) between *Mobula alfredi* sampling locations, calculated based on the 'all SNPs' dataset. Values that are significantly different from zero are marked with *. Global average intra-specific F_{ST} is also given. See Supplementary Figure S4.30 for locus specific estimates of F_{ST}.

	Seychelles	Chagos	Maldives	Australia	Fiji
Seychelles					
Chagos	0.061* ± 0.019				
Maldives	0.054* ± 0.010	0.012 ± 0.015			
Australia	0.267* ± 0.043	0.136* ± 0.043	0.110* ± 0.030		
Fiji	0.288* ± 0.040	0.266* ± 0.041	0.186* ± 0.026	0.181* ± 0.044	
Hawaii	0.286* ± 0.028	0.178* ± 0.030	0.185* ± 0.024	0.054* ± 0.027	0.184* ± 0.020
Global average intra-specific F_{ST} = 0.144					

Table 4.2: Pairwise F_{ST} values ± 99% Confidence Intervals (Weir & Cockerham, 1984) between *Mobula birostris* sampling locations, calculated based on the 'all SNPs' dataset. Values that are significantly different from zero are marked with *. Global average intra-specific F_{ST} is also given. See Supplementary Figure S4.31 for locus specific estimates of F_{ST}. Negative F_{ST} values can be interpreted as zero i.e. there is no differentiation, and therefore do not have a confidence interval.

	Mexico Caribbean	South Africa	Sri Lanka	Philippines	Mexico Pacific
Mexico Caribbean					
South Africa	-0.033				
Sri Lanka	-0.017	-0.049			
Philippines	-0.029	-0.049	-0.003		
Mexico Pacific	-0.025	-0.004	0.047* ± 0.006	0.020* ± 0.004	
Peru	-0.008	-0.031	-0.006	-0.006	0.043* ± 0.008
Global average intra-specific F_{ST} = -0.006					

Table 4.3: Genetic diversity within *Mobula alfredi* populations, calculated using the 'all SNPs' dataset. Expected heterozygosity (H_e), observed heterozygosity (H_o), nucleotide diversity (π) and the number of polymorphic SNPs within each population are given.

Population	Number of individuals	Expected Heterozygosity (H_e) ± s.e	Observed Heterozygosity (H_o) ± s.e	Nucleotide Diversity (π) ± s.e	No. SNPs
Seychelles	24	0.0838 ± 0.0045	0.0644 ± 0.0038	0.0863 ± 0.0047	427
Chagos	5	0.0709 ± 0.0046	0.0641 ± 0.0048	0.0803 ± 0.0052	222
Maldives	43	0.0929 ± 0.0047	0.0670 ± 0.0037	0.0945 ± 0.0048	550
Australia	3	0.0595 ± 0.0044	0.0570 ± 0.0049	0.0736 ± 0.0055	164
Fiji	8	0.0553 ± 0.0042	0.0507 ± 0.0044	0.0603 ± 0.0046	181
Hawaii	24	0.0457 ± 0.0034	0.0326 ± 0.0028	0.0471 ± 0.0035	298

Table 4.4: Genetic diversity within *Mobula birostris* sampling locations, calculated using the 'all SNPs' dataset. Expected heterozygosity (H_e), observed heterozygosity (H_o), nucleotide diversity (π) and the number of polymorphic SNPs within each population are given.

Population	Number of individuals	Expected Heterozygosity (H_e) ± s.e	Observed Heterozygosity (H_o) ± s.e	Nucleotide Diversity (π) ± s.e	No. SNPs
Mexico Caribbean	4	0.0639 ± 0.0025	0.0550 ± 0.0025	0.0757 ± 0.0030	604
South Africa	3	0.0599 ± 0.0025	0.0626 ± 0.0030	0.0735 ± 0.0030	527
Sri Lanka	43	0.0851 ± 0.0023	0.0579 ± 0.0017	0.0871 ± 0.0024	2102
Philippines	29	0.0789 ± 0.0023	0.0514 ± 0.0017	0.0810 ± 0.0024	1632
Mexico Pacific	11	0.0787 ± 0.0024	0.0492 ± 0.0018	0.0844 ± 0.0026	1082
Peru	9	0.0748 ± 0.0024	0.0566 ± 0.0021	0.0802 ± 0.0026	961

4.4 Discussion

Here, genome-wide SNP data elucidate highly contrasting patterns of population structure in two closely related species of conservation concern. For the first time, we show evidence of a high degree of structuring in the reef manta ray, *Mobula alfredi,* indicative of limited gene flow among sampling locations, and suggesting that this species may benefit from local or regional approaches to conservation and management. However, we were unable to recover fine-scale population structure between sites in the Maldives up to 350km apart, indicating a high degree of connectivity along island chains, with associated implications for spatial management strategies such as Marine Protected Areas (MPAs). Consistent with findings from with Chapters 2 and 3, populations in the Pacific and Indian Oceans are very clearly differentiated. Genetic diversity within populations is reduced in the Pacific Ocean compared with the Indian Ocean, and appears to decline eastwards in this species, with conservation implications where reduced genetic diversity is correlated with a diminished resilience to selection pressures such as environmental change (e.g. Frankham, 2005; Sgrò et al. 2011; Hellmair & Kinziger, 2014). In direct contrast, we were unable to recover any evidence of

population structure on a global scale in the oceanic manta ray, *Mobula birostris,* indicating a requirement for international cooperation for effective conservation and management.

Patterns of population structure and clustering may be erroneously detected where a continuous population exhibiting isolation by distance (IBD) is discretely sampled (Audzijonyte & Vrijenhoek, 2010; Meirmans, 2015). Our analyses did not include specific tests for IBD, and as such a pattern of isolation by distance driving population structure in the reef manta ray (*Mobula alfredi*) cannot be explicitly ruled out. However, since reef manta rays are not continuously distributed, but heavily associated with highly fragmented reef based habitats interspersed by large areas of open ocean (Marshall et al. 2009; Couturier et al. 2012), it is likely that clusters inferred with our data represent discrete populations. Nonetheless, the sampling coverage utilised in this study is by no means exhaustive, and future sampling efforts should ideally focus on currently unrepresented populations.

A recent sighting of a pregnant reef manta ray (*Mobula alfredi*) at Cocos Island in the Eastern Pacific, 6000km from the nearest known population of this species, has sparked discussion in the community regarding how this species may colonise new habitats (Arauz et al. 2019). Rare long-range migrations of pregnant individuals has previously been proposed as a possible mechanism of colonisation among reef-based elasmobranch species (López-Garro et al. 2012). Such a mechanism would be expected to result in reduced genetic diversity in recently colonised sites, i.e. 'founder effects'. Across the Pacific Ocean, the data presented in this study show a pattern of declining genetic diversity eastwards, which may be consistent with successive recent colonisations by small founder populations. It would be interesting to contrast with other sites across the Pacific Ocean to see if this pattern holds. Nonetheless, our results suggest that Pacific populations of *Mobula alfredi* are comparatively isolated and may be more vulnerable to environmental change and fishing pressures.

Previous tagging studies and stable isotope analyses have indicated a high degree of residency in the oceanic manta ray, *Mobula birostris* across several sites (Dewar et al. 2008; Graham et al. 2012; Stewart et al. 2016). In addition, the only previous population genetic study for this species finds evidence for modest population structure between three sampling locations based on genome-wide SNPs, albeit with a large degree of shared substructure (Stewart et al. 2016). In direct contrast, we were unable to recover any structure for this species with genome-wide SNP data (Figure 4.1b; Figure 4.4; Figure 4.5), suggestive of substantial gene

flow between sampling locations. This discrepancy is most likely due to a difference in the SNP filtering process of Stewart et al. (2016) compared with the current study. Here, a lack of structure is inferred from neutral SNPs passing our quality control filters (see Section 3.2.3 for details) and is further corroborated with a similar analysis inclusive of putative outlier loci (Figure 4.4; Figure 4.5; Supplementary Figure S4.26; Supplementary Figure S4.28). However, the authors of the previous study inferred structure based on SNPs in the 90th percentile of F_{ST} among populations that were predefined by sampling locality (Stewart et al. 2016). F_{ST} outliers provide the basis for commonly used methods to identify putatively adaptive loci (e.g. Foll & Gaggiotti, 2008) and as such these SNPs may violate the assumption of neutrality required by model-based methods of inferring population structure (Pritchard et al. 2000). SNP markers utilised by Stewart et al. (2016) will therefore likely be uninformative with respect to characterising the extent of gene flow at neutral loci, but may indicate a degree of local adaptation in oceanic manta rays.

Apparent global genetic panmixia of a spatially structured species is inherently paradoxical, since true homogeneity in a panmictic population requires random mating and associated gene flow. Broad scale genetic homogeneity is not unheard of in elasmobranch species (e.g. Hoelzel et al. 2006; Ovenden et al. 2011) and may be explained by differences in dispersal among the sexes where studies examine uniparentally inherited markers (e.g. Daly-Engel et al. 2012), or genetic time lags associated with demographic changes and transitional phases termed the 'population grey zone' (e.g. Bailleul et al. 2018). Future work may therefore involve simulation studies as in Bailleul et al. (2018) to determine migration rates and effective population sizes required to produce the lack of structure shown here in *Mobula birostris*, which could potentially be achieved with relatively rare migrations where there is large effective population size. Indeed, the analyses in Chapter 3 demonstrate a high degree of genetic diversity in *Mobula birostris* compared with *Mobula alfredi* and *Mobula* Sp. 1, possibly suggestive of large population size.

Long-range migrations have been documented in large elasmobranch species despite very limited sample sizes in tagging studies (e.g. Dewar et al. 2018), suggesting that similar movements are not occurring in populations of oceanic manta rays examined with larger numbers of acoustic and satellite tags (Dewar et al. 2008; Graham et al. 2012; Stewart et al. 2016). However, very little is known about the dispersal ability of juvenile oceanic manta rays,

and studies examining spatial dynamics in the species have focussed exclusively on adults (Stewart et al. 2018b). Indeed, a recent study has shown long-range movements of juvenile white sharks (Bruce et al. 2019), suggesting that juvenile stages may represent an important mode of gene flow. Furthermore, absence of juveniles at known mobulid aggregation sites is suggestive of size segregation (Deakos, 2010; McCauley et al. 2014), and may be associated with age-related population structure (Klein et al. 2019). Indeed, several prior studies have demonstrated the importance of considering life history stage in the sampling design in studies aiming to investigate subtle population structure in marine fish (e.g. Hutchinson et al. 2001; Hauser & Carvalho, 2008; Bozano et al. 2015; Meirmans, 2015). As such, future work in this area is likely to focus on spatial dynamics across life history stages in oceanic manta rays. Determining mechanisms of migration and gene flow will be pivotal to implementing informed and appropriate management for the oceanic manta ray, both to ensure that sensitive life stages are not at risk, but also to avoid disrupting a mode of gene flow, which may be important for long-term resilience of populations.

Contrasting patterns of population structure and gene flow have previously been recorded in closely related marine species (Holland et al. 2017), and may be associated with differences in habitat preference, such as depth (Strugnell et al. 2017). Here, we show highly contrasting patterns of population structure in closely related species of manta ray occurring in mosaic sympatry associated with differences in habitat preference. Localised genomic regions have previously been associated with migratory and stationary ecotypes of marine fish in the early stages of divergence (e.g. Hemmer-Hansen et al. 2013), and a similar mechanism may have been important in driving divergence in manta rays. In addition, quantifying adaptive differentiation between distinct populations and sampling locations will be an intuitive next step towards defining optimal conservation units and identifying locally adapted populations within these species (Nielsen et al. 2009; Funk et al. 2012; Sarkar et al. 2019). Furthermore, detailed tests for outlier loci may provide sufficient power for assignment tests and traceability due to typically high levels of genetic differentiation (e.g. Nielsen et al. 2012; Bekkevold et al. 2015; Villacorta-Rath et al. 2016; Jorde et al. 2018).

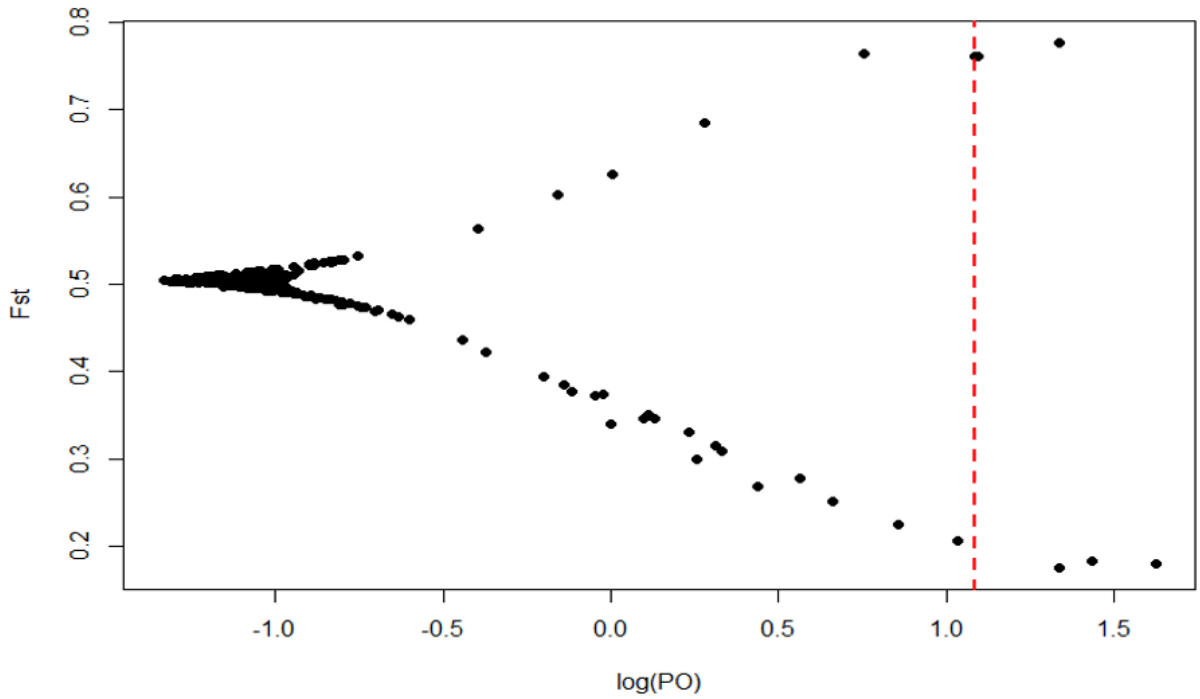
Here, genome-wide SNP data demonstrate contrasting patterns of population structure and gene flow in two closely related marine species, the reef manta ray (*Mobula alfredi*) and the oceanic manta ray (*Mobula birostris*). These findings have implications for conservation of

these species threatened by targeted and bycatch fishing pressure and international trade in wildlife products. Highly structured populations such as those exhibited by the reef manta ray, *Mobula alfredi*, will likely benefit from protection and management at the national level. Alternatively, global genetic panmixia in the oceanic manta ray, *Mobula birostris*, indicates that international cooperation may be required to effectively manage this species, and could be used in conjunction with targeted local approaches to manage resident adult populations. Our study highlights the importance of evaluating population structure and adaptive divergence individually for related species of conservation concern, rather than relying on an assumption that closely related species display similar patterns.

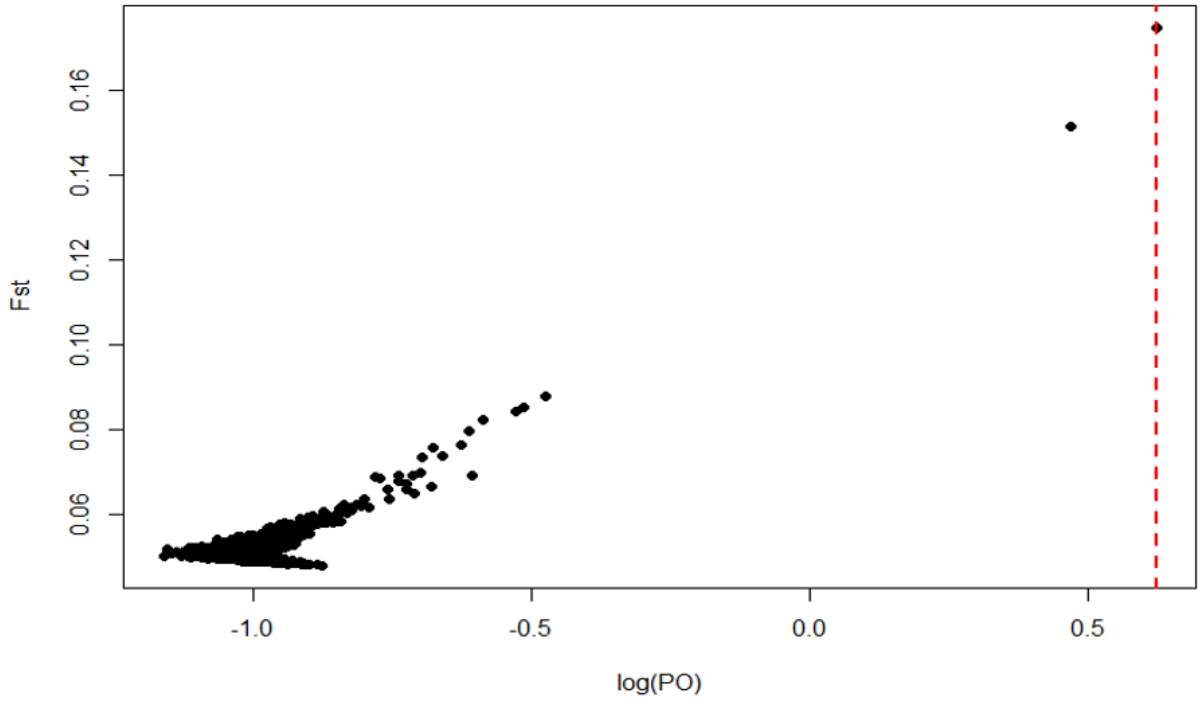
Chapter 4: Supplementary Materials

Chapter 4: Supplementary Materials

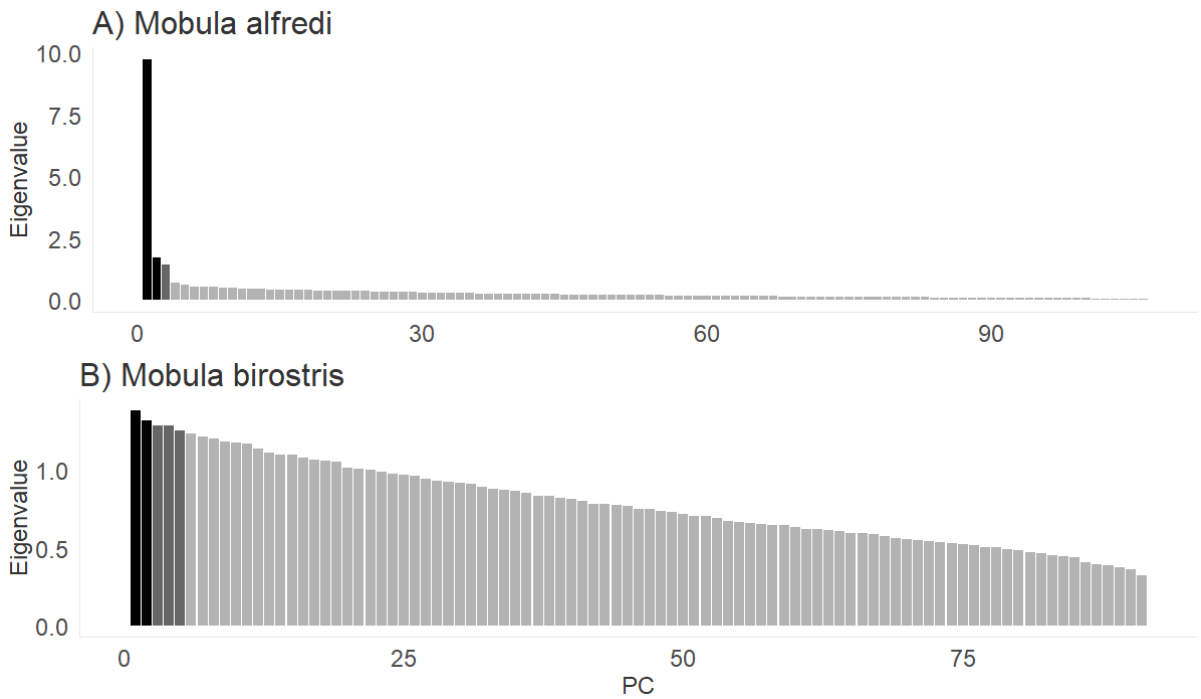
S4.1 Supplementary Figures and Tables



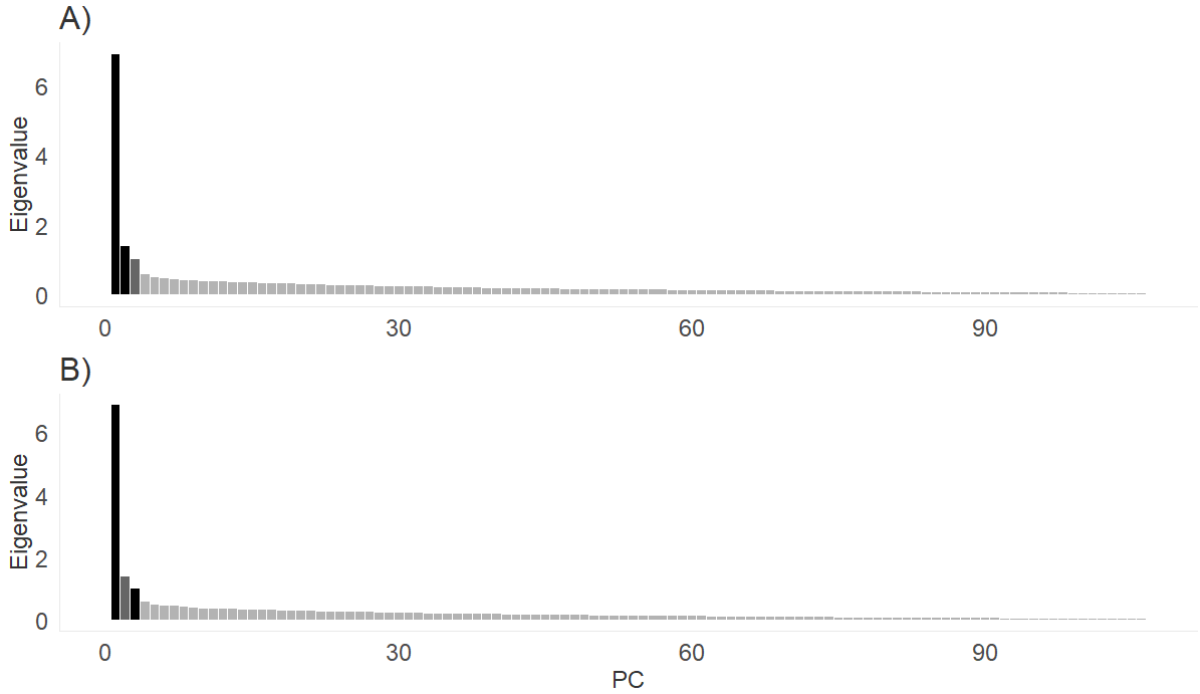
Supplementary Figure S4.1: Bayescan 2.1 (Foll & Gagiotti, 2008) plot of 1067 SNPs ('all SNPs' dataset) in 107 individuals of the reef manta ray, *Mobula alfredi,* sampled among 6 sampling locations across the range of this species. Estimates of F_{ST} for each SNP are plotted against the logarithm of the posterior odds. SNPs to the right of the red dashed line (indicating a False Discovery Rate of 5%) are candidate markers for signatures of genomic selection.



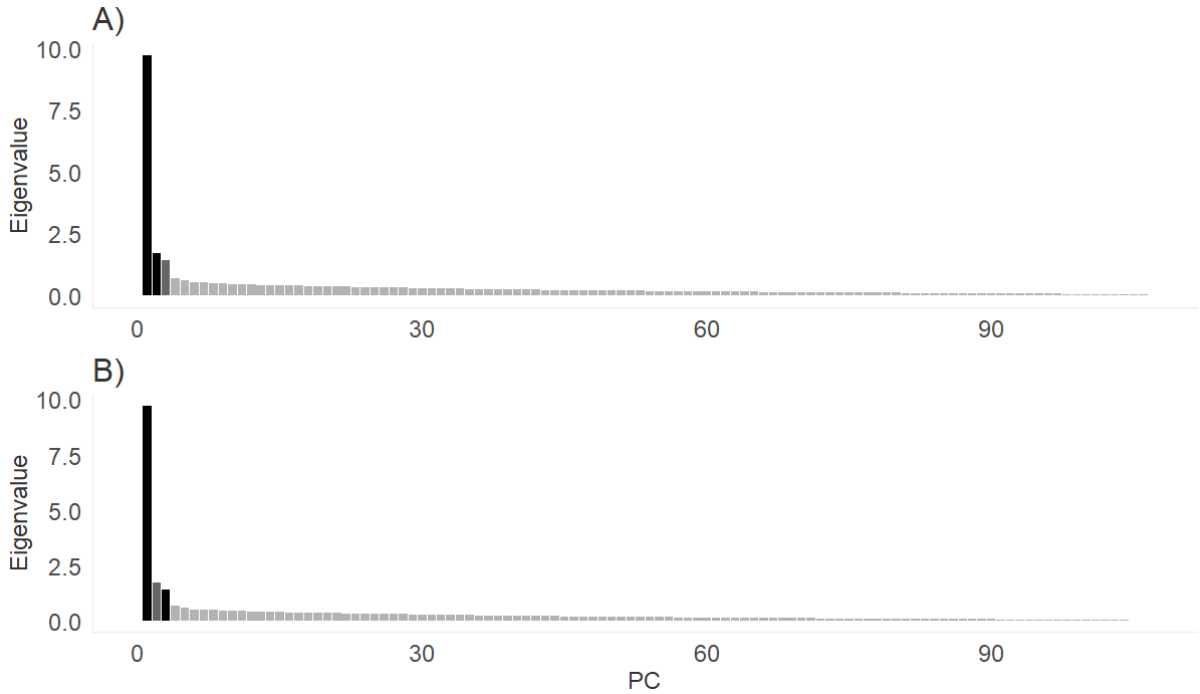
Supplementary Figure S4.2: Bayescan 2.1 (Foll & Gagiotti, 2008) plot of 3217 SNPs ('all SNPs' dataset) in 99 individuals of the oceanic manta ray, *Mobula birostris,* sampled among 6 sampling locations across the range of this species. Estimates of F_{ST} for each SNP are plotted against the logarithm of the posterior odds. SNPs to the right of the red dashed line (indicating a False Discovery Rate of 5%) are candidate markers for signatures of genomic selection.



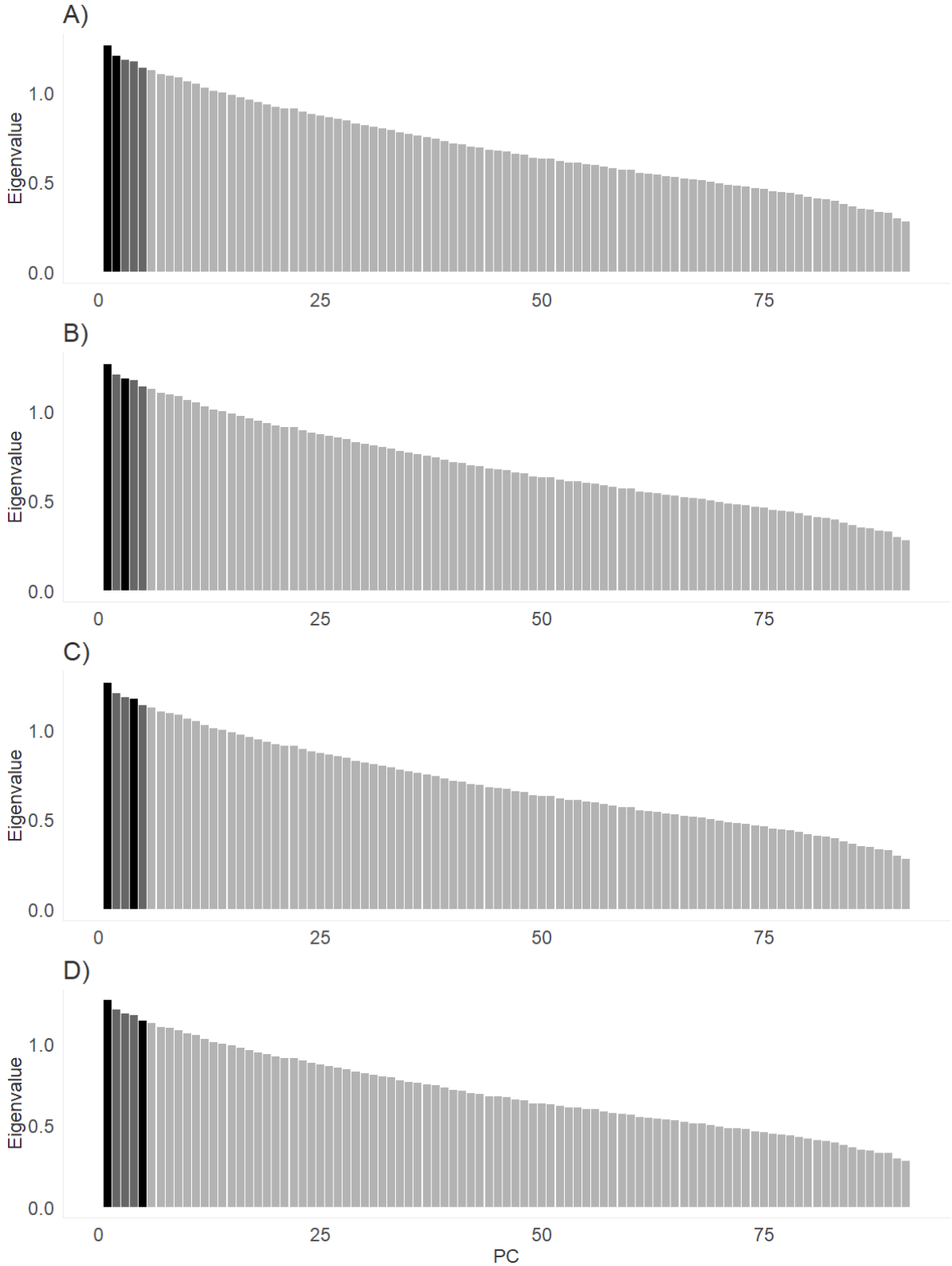
Supplementary Figure S4.3: Eigenvalue plots for Principle Components Analyses (PCA) for A) *Mobula alfredi* and B) *Mobula birostris,* using the 'all SNPs' datasets. Panel letters (A-B) correspond to the panel letters in Figure 4.1. Axes plotted in Figure 4.1 are in black and retained axes are in dark grey.



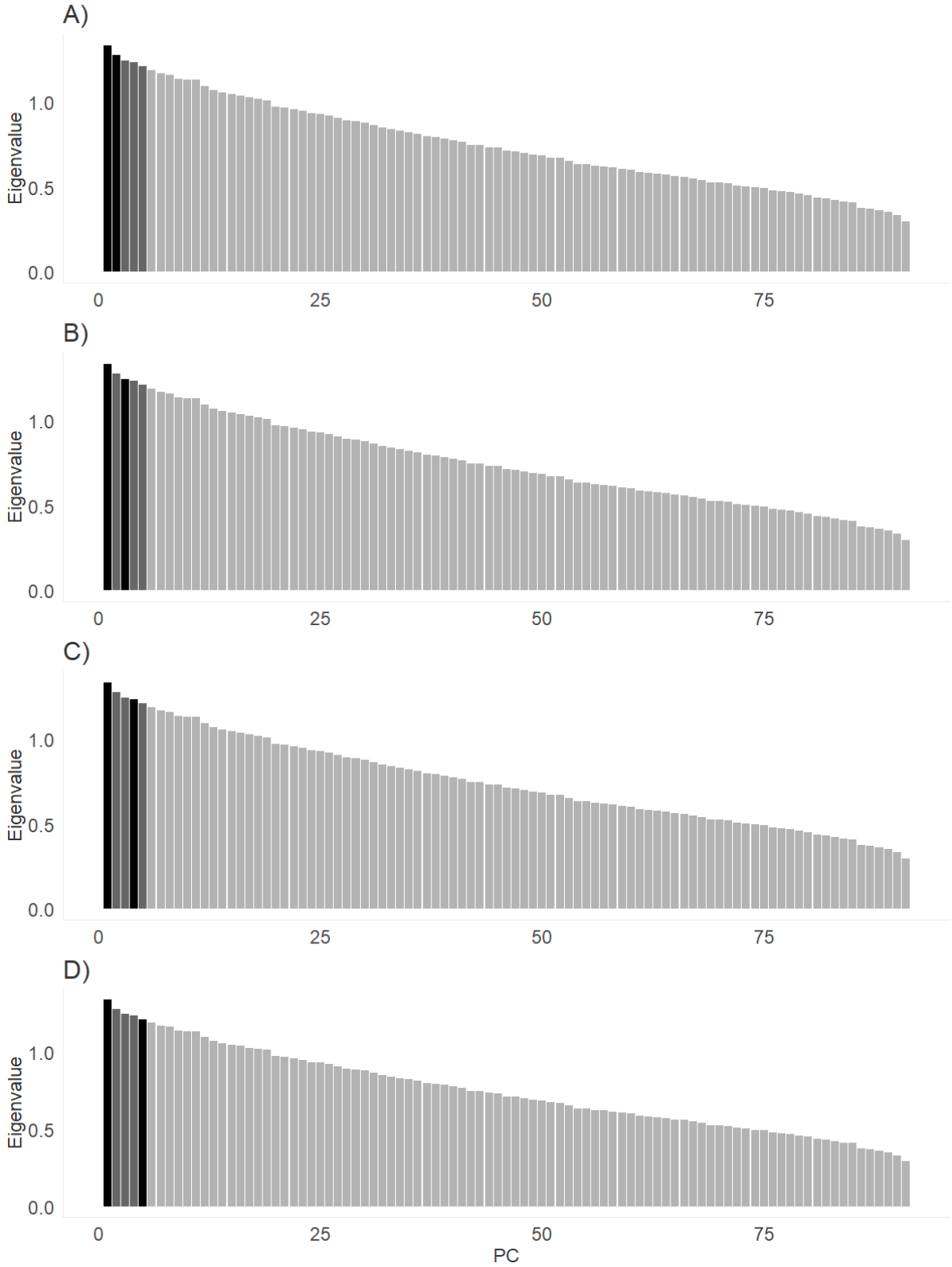
Supplementary Figure S4.4: Eigenvalue plots for Principle Components Analyses (PCA) for *Mobula alfredi* using the 'neutral SNPs' dataset. Panel letters (A-B) correspond to the panel letters in Supplementary Figure S4.10. Axes plotted in Supplementary Figure S4.10 are in black and retained axes are in dark grey.



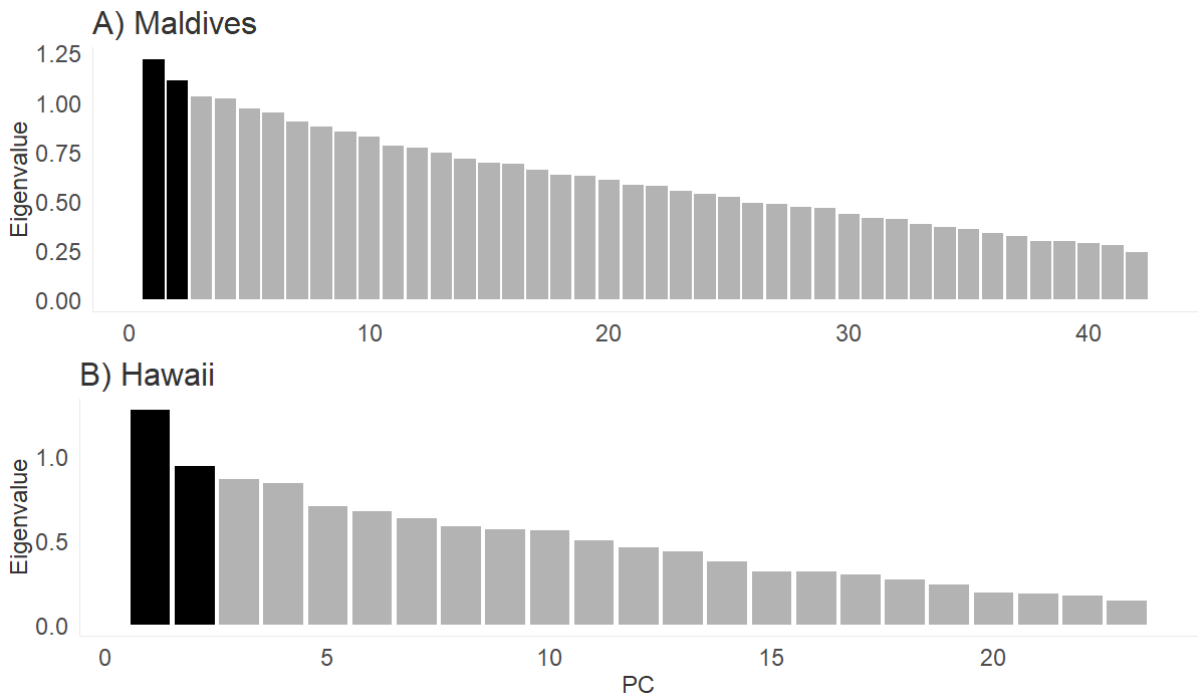
Supplementary Figure S4.5: Eigenvalue plots for Principle Components Analyses (PCA) for *Mobula alfredi* using the 'no singletons' dataset. Panel letters (A-B) correspond to the panel letters in Supplementary Figure S4.11. Axes plotted in Supplementary Figure S4.11 are in black and retained axes are in dark grey.



Supplementary Figure S4.6: Eigenvalue plots for Principle Components Analyses (PCA) for *Mobula birostris* using the 'neutral SNPs' dataset. Panel letters (A-D) correspond to the panel letters in Supplementary Figure S4.13. Axes plotted in Supplementary Figure S4.13 are in black and retained axes are in dark grey.



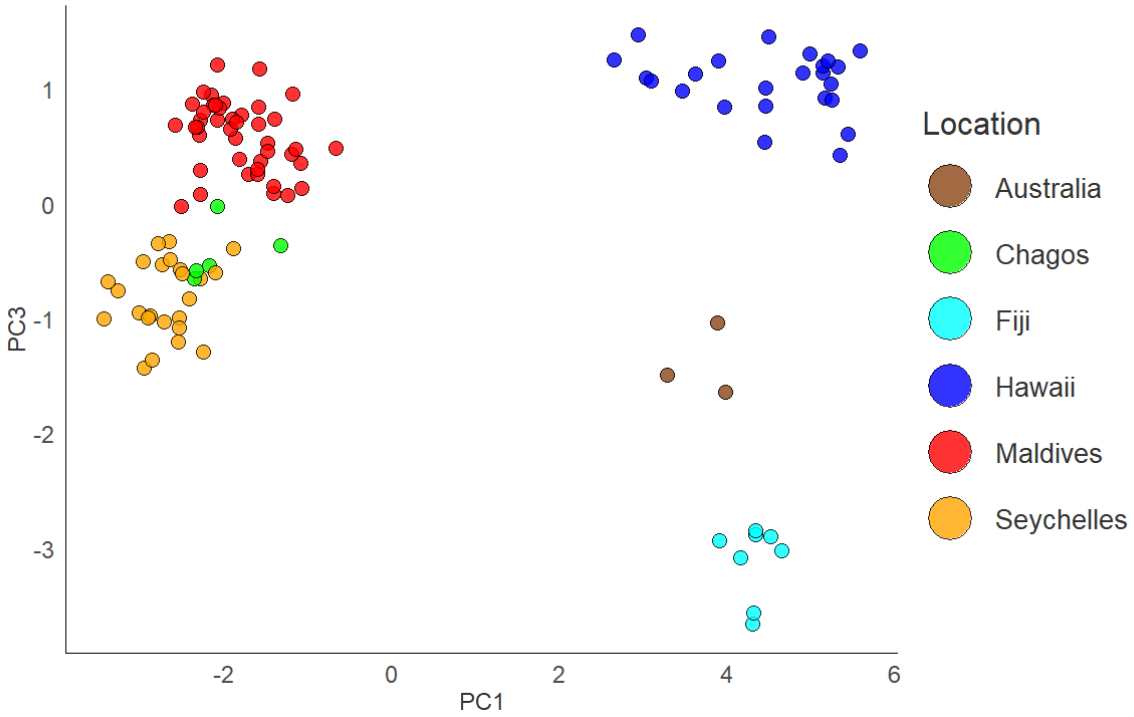
Supplementary Figure S4.7: Eigenvalue plots for Principle Components Analyses (PCA) for *Mobula birostris* using the 'no singletons' dataset. Panel letters (A-D) correspond to the panel letters in Supplementary Figure S4.14. Axes plotted in Supplementary Figure S4.14 are in black and retained axes are in dark grey.



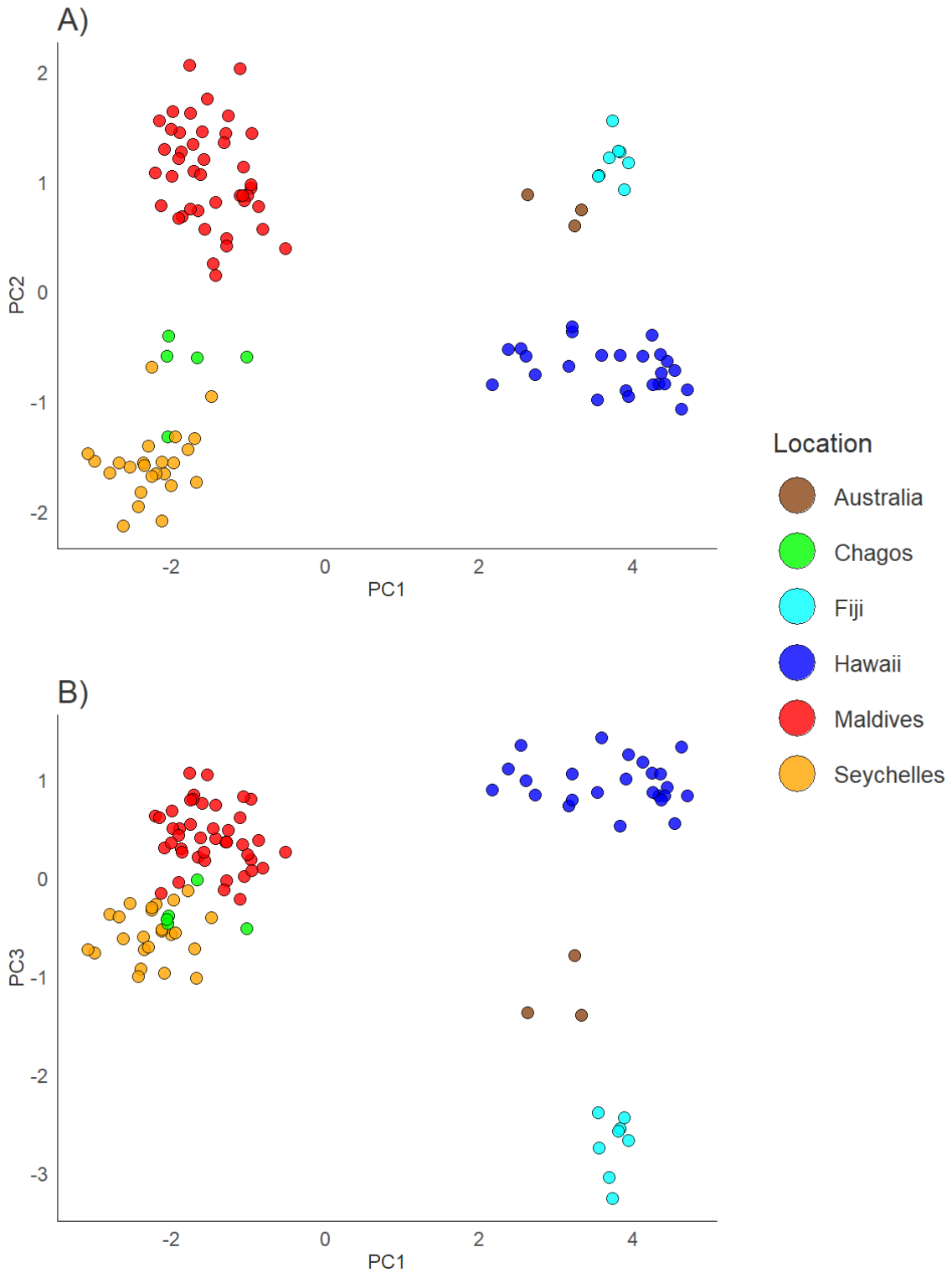
Supplementary Figure S4.8: Eigenvalue plots for Principle Components Analyses (PCA) for *Mobula alfredi* individuals sampled across sites in A) the Maldives and B) Hawaii, using the 'all SNPs' dataset. Panel letters (A-B) correspond to the panel letters in Figure 4.2. Axes plotted in Figure 4.2 are in black.

Supplementary Table S4.1: Percentage of pairwise comparisons where $p < 0.01$ after Bonferroni correction following an exact test for genotypic linkage disequilibrium in the *Mobula birostris* 'all SNPs' dataset. 10 subsets of 1500 SNPs were randomly subsampled from the original set of 3217 SNPs.

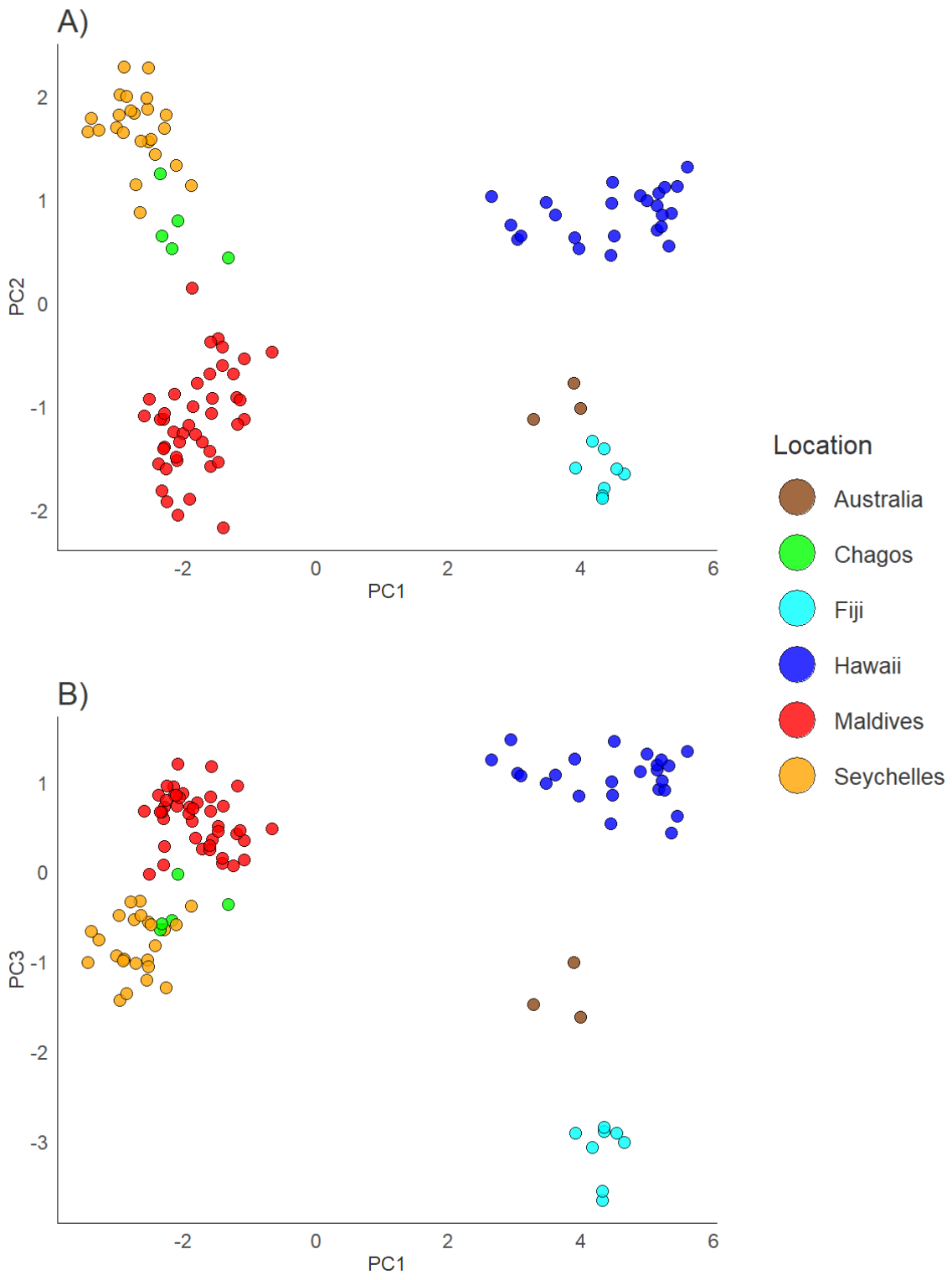
Subset	Percentage of pairwise comparisons where $p < 0.01$ following Bonferroni correction
1	0.00027%
2	0.00009%
3	0.00000%
4	0.00009%
5	0.00000%
6	0.00009%
7	0.00009%
8	0.00018%
9	0.00027%
10	0.00018%
Average across subsets	0.00012%



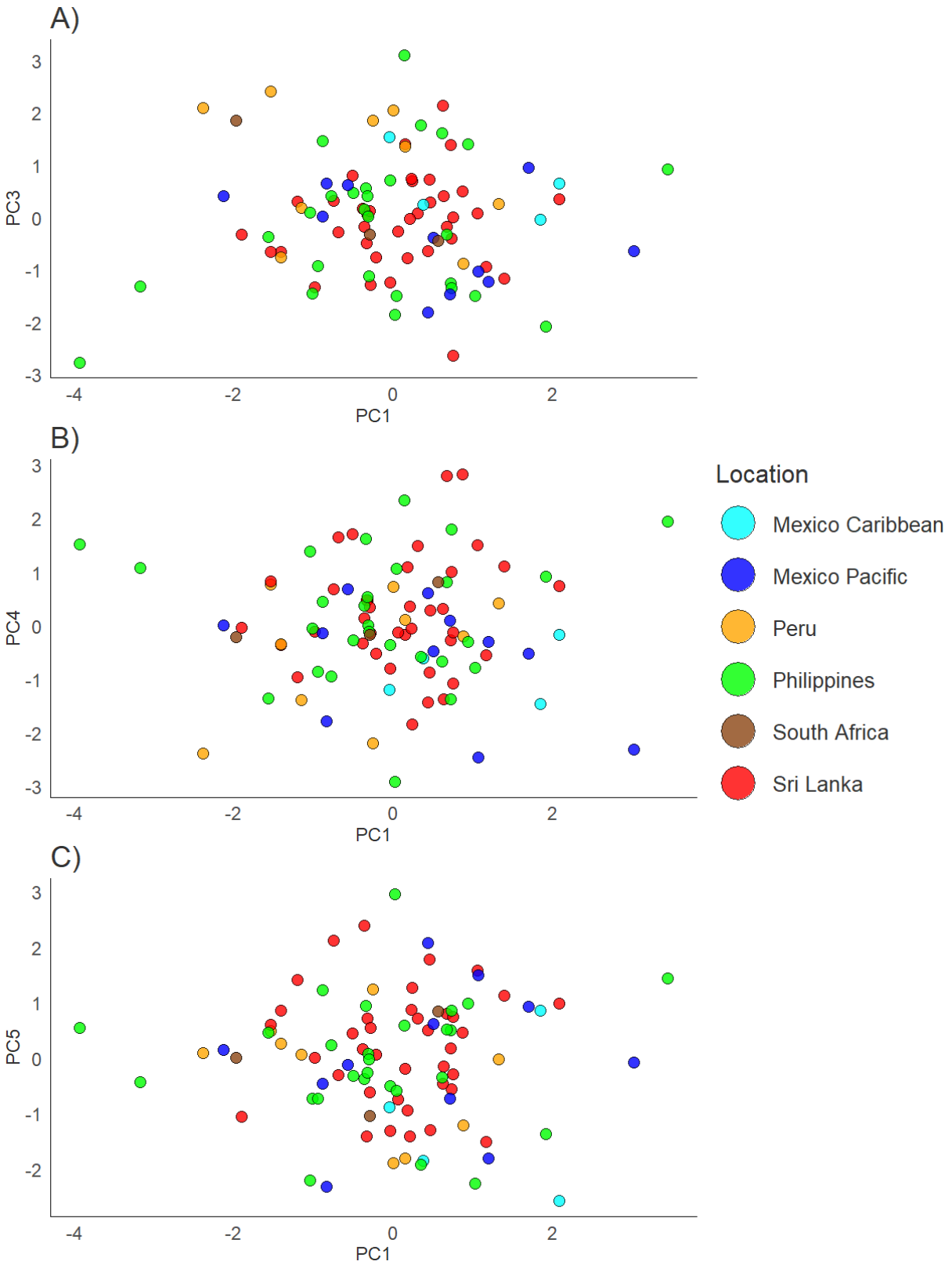
Supplementary Figure S4.9: Principal Components 1 and 3 plotted for *Mobula alfredi* 'all SNPs' dataset. Individuals are represented by a point, where colour indicates sampling location as specified in the key. PC1 and PC3 explain 9.7% and 1.4% of the variance in the data, respectively. See Supplementary Figure S4.3A for full eigenvalue plot.



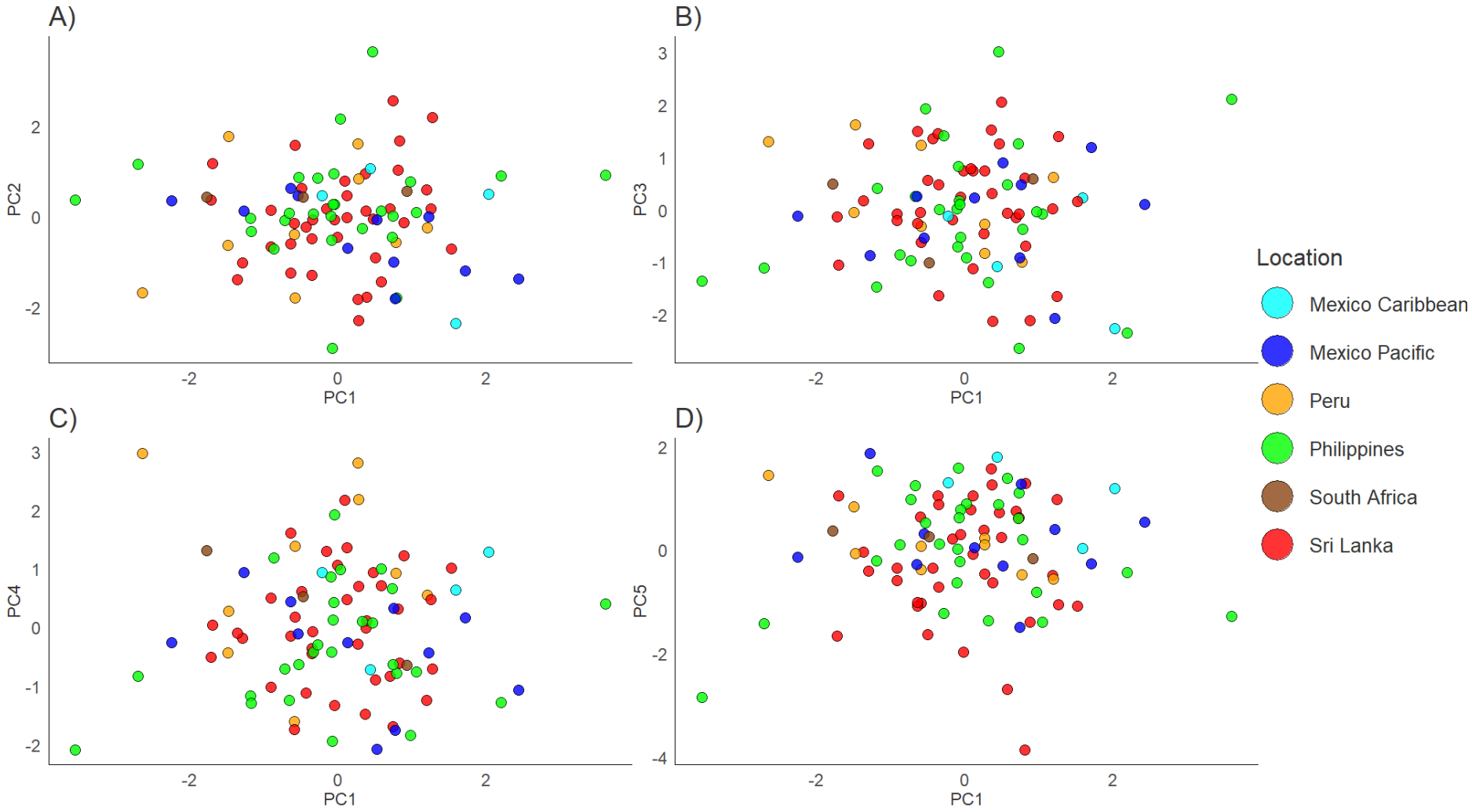
Supplementary Figure S4.10: Principal Components A) 1 and 2, and B) 1 and 3 plotted for *Mobula alfredi* 'neutral SNPs' dataset. Individuals are represented by a point, where colour indicates sampling location as specified in the key. PC1, PC2 and PC3 explain 6.9%, 1.4% and 1.0% of the variance in the data, respectively. See Supplementary Figure S4.4 for full eigenvalue plot.



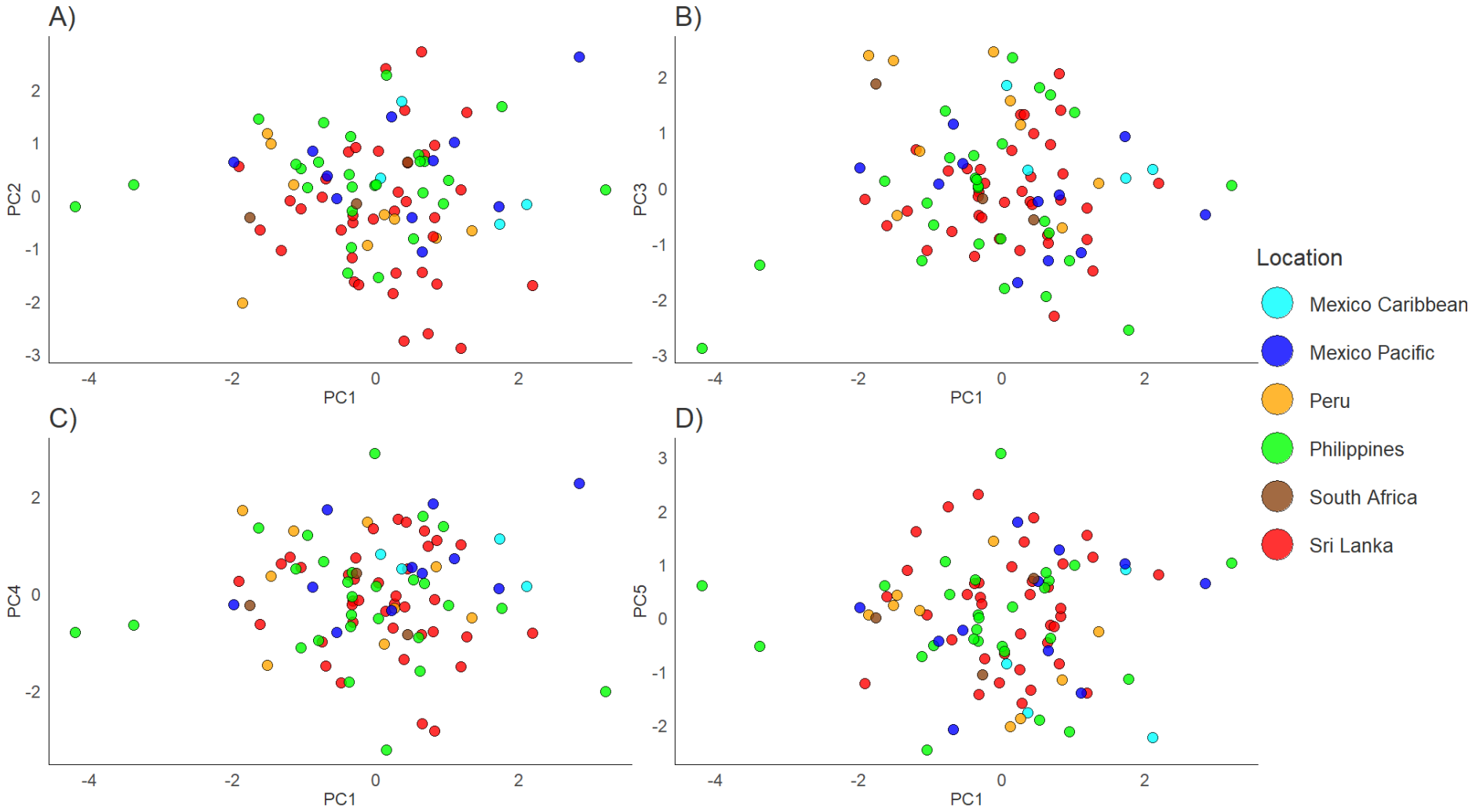
Supplementary Figure S4.11: Principal Components A) 1 and 2, and B) 1 and 3 plotted for *Mobula alfredi* 'no singletons' dataset. Individuals are represented by a point, where colour indicates sampling location as specified in the key. PC1, PC2 and PC3 explain 9.7%, 1.7% and 1.4% of the variance in the data, respectively. See Supplementary Figure S4.5 for full eigenvalue plot.



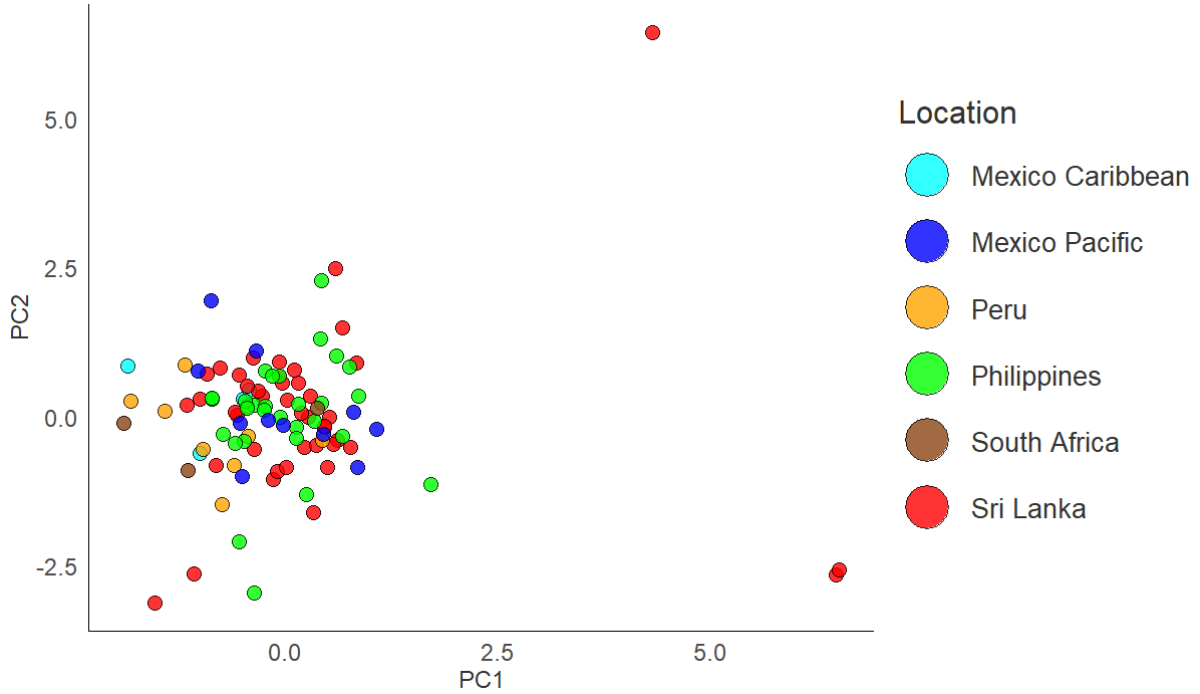
Supplementary Figure S4.12: Principal Components A) 1 and 3, B) 1 and 4 and C) 1 and 5 plotted for *Mobula birostris* 'all SNPs' dataset, but with seven anomalous individuals removed (see Supplementary Figure S4.15 for PCA with all individuals). Individuals are represented by a point, where colour indicates sampling location as specified in the key. PC3, PC4 and PC5 each explain 1.3% of the variance in the data. See Supplementary Figure S4.3B for full eigenvalue plot.



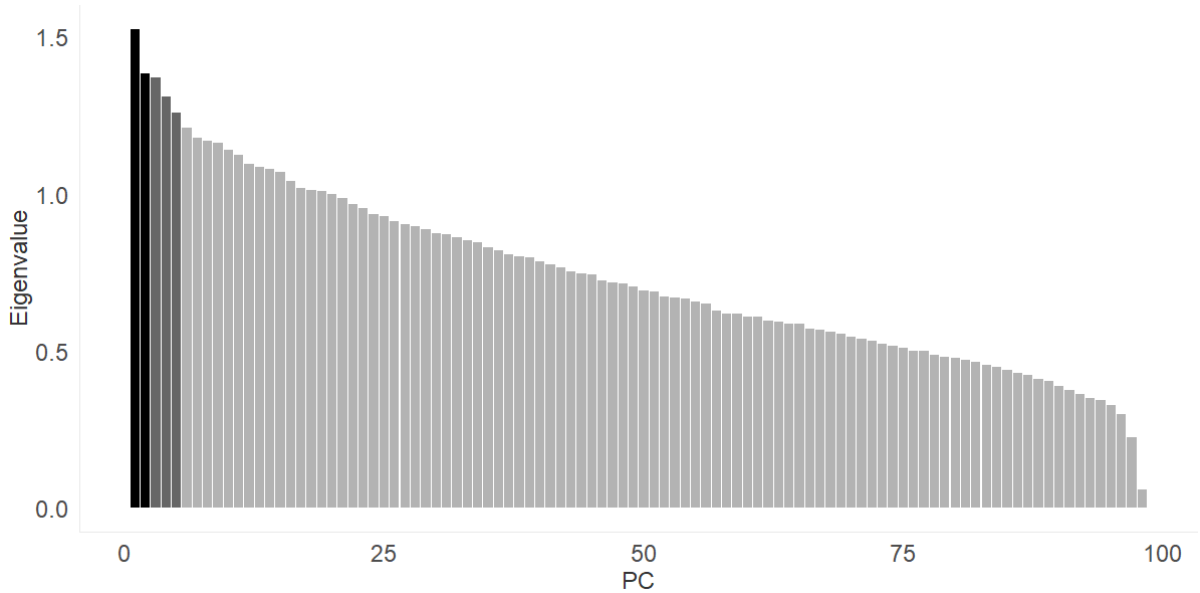
Supplementary Figure S4.13: Principal Components A) 1 and 2, B) 1 and 3, C) 1 and 4 and D) 1 and 5 plotted for *Mobula birostris* 'neutral SNPs' dataset, but with seven anomalous individuals removed. Individuals are represented by a point, where colour indicates sampling location as specified in the key. PC1 explains 1.3% of the variance in the data, PC2, PC3 and PC4 each explain 1.2% of the variance in the data, and PC5 explains 1.1% of the variance in the data. See Supplementary Figure S4.6 for full eigenvalue plot.



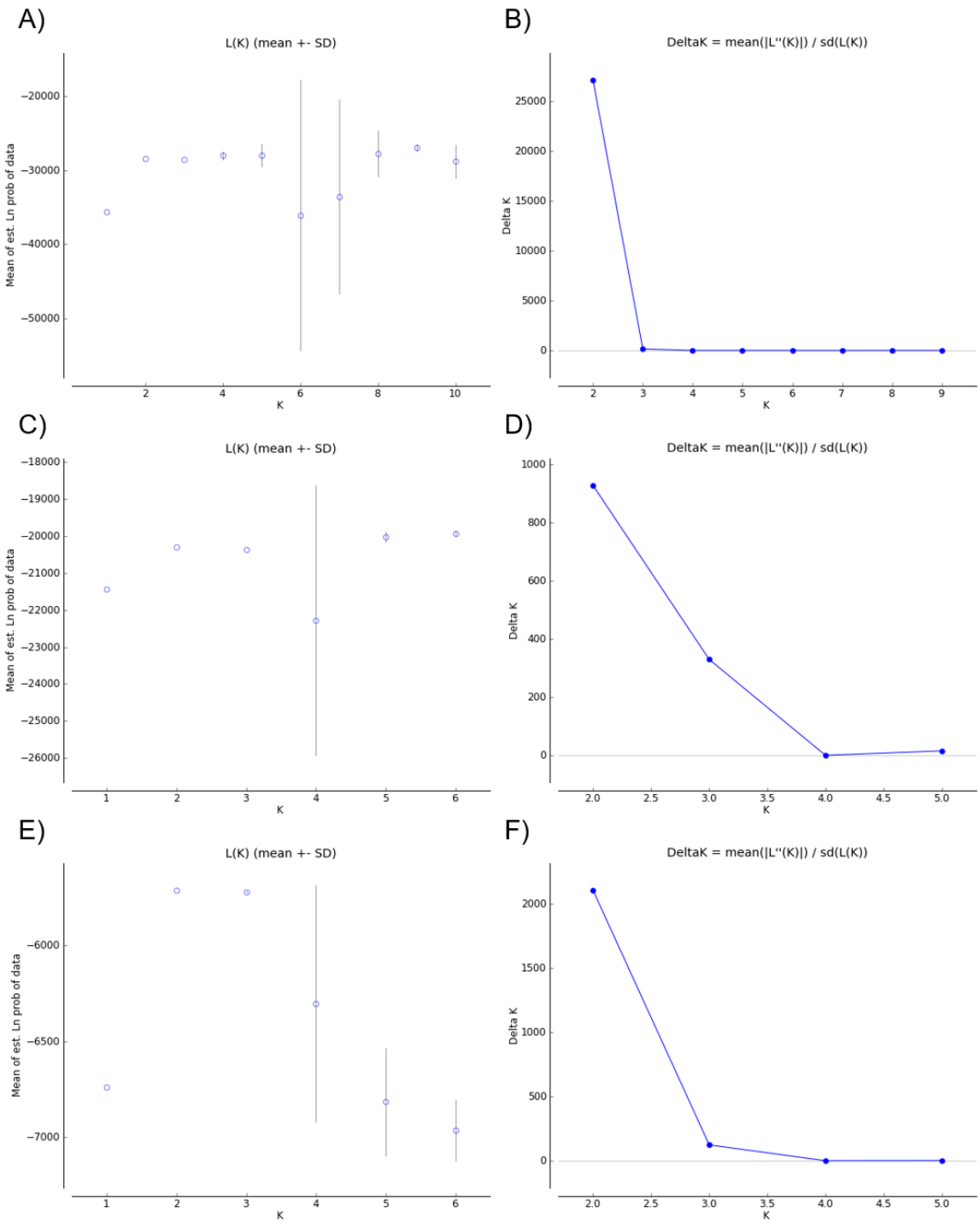
Supplementary Figure S4.14: Principal Components A) 1 and 2, B) 1 and 3, C) 1 and 4 and D) 1 and 5 plotted for *Mobula birostris* 'no singletons' dataset, but with seven anomalous individuals removed. Individuals are represented by a point, where colour indicates sampling location as specified in the key. PC1 and PC2 each explain 1.3% of the variance in the data, and PC3, PC4 and PC5 each explain 1.2% of the variance in the data. See Supplementary Figure S4.7 for full eigenvalue plot.



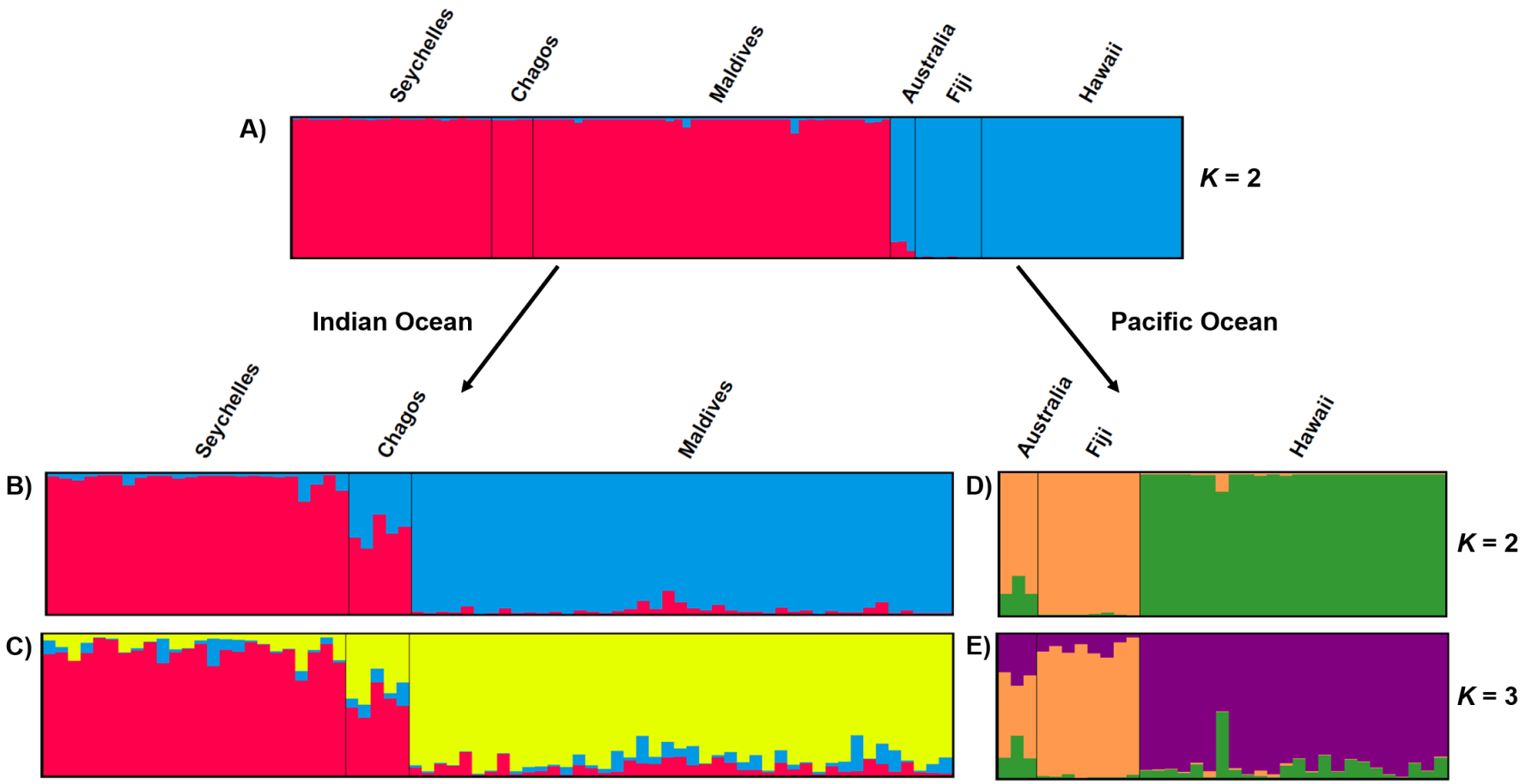
Supplementary Figure S4.15: Principal Components 1 and 2 plotted for *Mobula birostris* 'all SNPs' dataset with all individuals. Individuals are represented by a point, where colour indicates sampling location as specified in the key. The cause of the outlier genotypes is not clear, since missing data for these individuals is consistent with the average levels of missing data across the dataset. However, these individuals do possess a small number of rare alleles. PC1 and PC2 explain 1.5% and 1.4% of the variance in the data, respectively. See Supplementary Figure S4.16 for full eigenvalue plot.



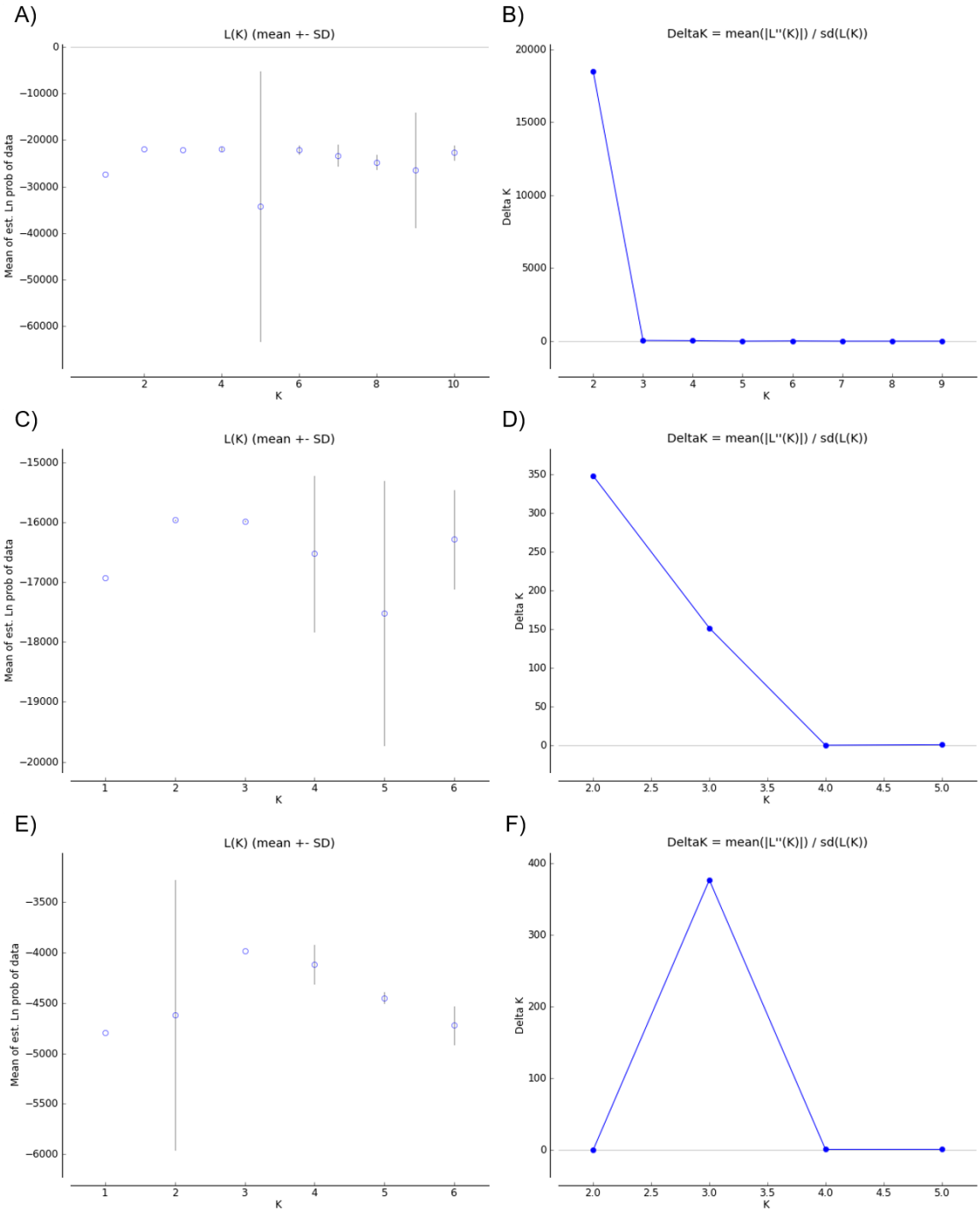
Supplementary Figure S4.16: Eigenvalue plots for Principle Components Analyses (PCA) for all *Mobula birostris* individuals using the 'all SNPs' dataset. Axes plotted in Supplementary Figure S4.15 are in black and retained axes are in dark grey.



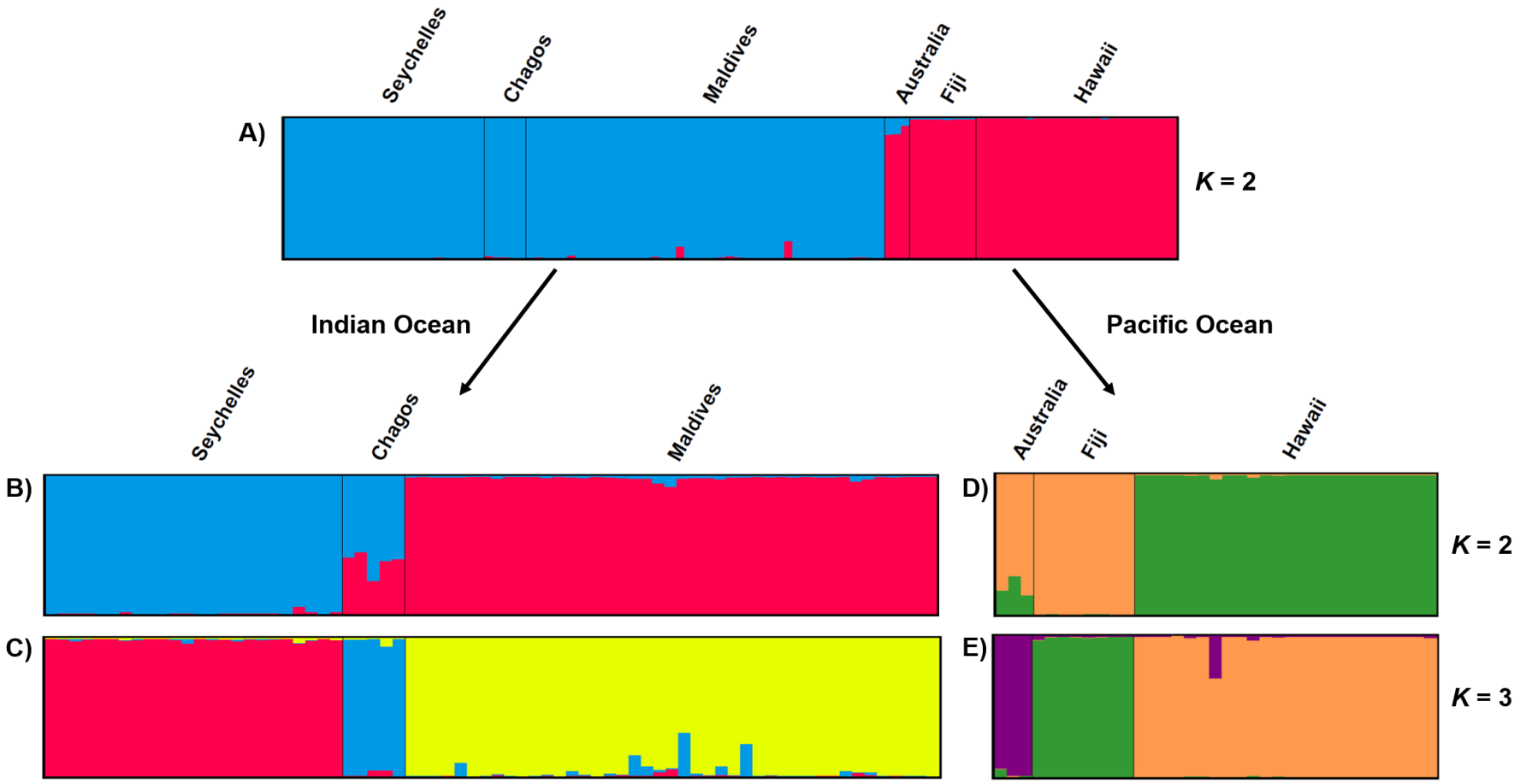
Supplementary Figure S4.17: Output from STRUCTURE HARVESTER for *Mobula alfredi* 'all SNPs' dataset. A) LnP(*K*) plotted against value of *K* across all *Mobula alfredi* individuals, B) Δ*K* plotted against value of *K* across all *Mobula alfredi* individuals, C) LnP(*K*) plotted against value of *K* across Indian Ocean *Mobula alfredi* individuals, D) Δ*K* plotted against value of *K* across Indian Ocean *Mobula alfredi* individuals, E) LnP(*K*) plotted against value of *K* across Pacific Ocean *Mobula alfredi* individuals and F) Δ*K* plotted against value of *K* across Pacific Ocean *Mobula alfredi* individuals. See Figure 4.3 for associated STRUCTURE plots.



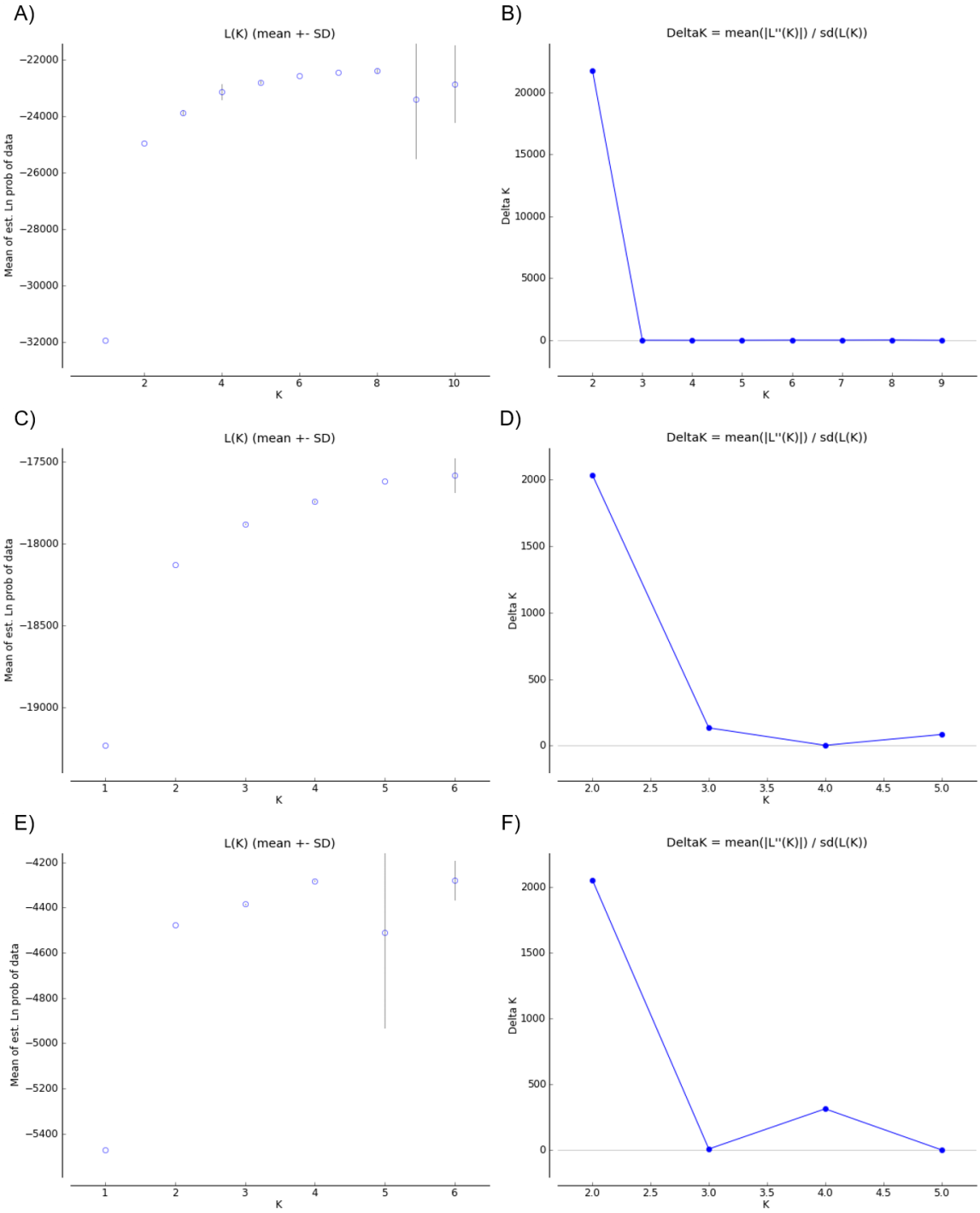
Supplementary Figure S4.18: Plot showing results of Bayesian clustering analysis with no prior information on sampling locations implemented in STRUCTURE using the admixture model for *Mobula alfredi* 'neutral SNPs' dataset. A) all individuals analysed together at *K*=2. The data was then split into inferred clusters, corresponding to individuals sampled in the Indian and Pacific oceans, and plotted for *K*=2 and *K*=3: B) Indian Ocean *K*=2, C) Indian Ocean *K*=3, D) Pacific Ocean *K*=2, E) Pacific Ocean *K*=3. Each vertical bar represents an individual, and colours represent the proportion of ancestry assigned to each cluster for each individual. Individuals are grouped by sampling location. STRUCTURE HARVESTER output, identifying optimal values of *K*, are given in Supplementary Figure S4.19.



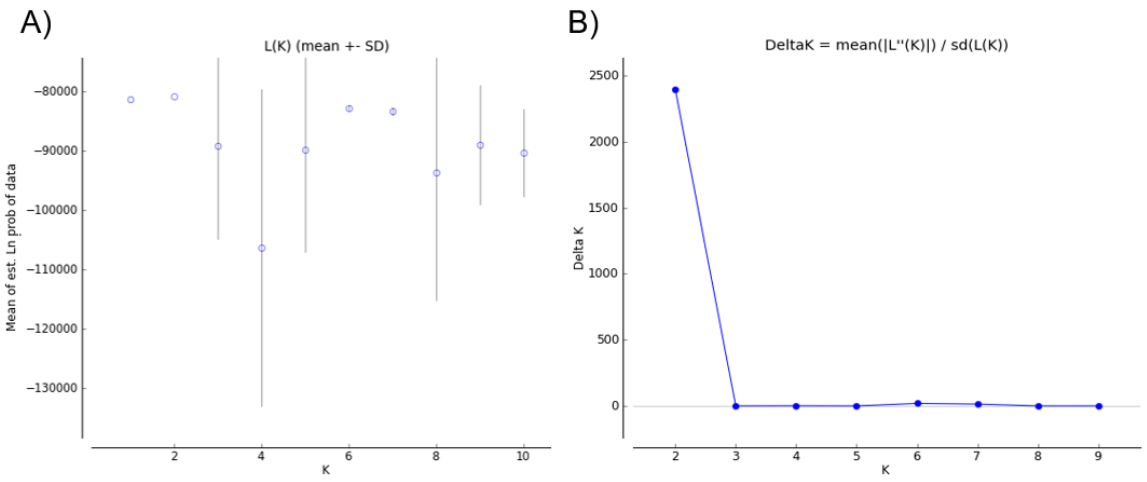
Supplementary Figure S4.19: Output from STRUCTURE HARVESTER for *Mobula alfredi* 'neutral SNPs' dataset. A) LnP(*K*) plotted against value of *K* across all *Mobula alfredi* individuals, B) Δ*K* plotted against value of *K* across all *Mobula alfredi* individuals, C) LnP(*K*) plotted against value of *K* across Indian Ocean *Mobula alfredi* individuals, D) Δ*K* plotted against value of *K* across Indian Ocean *Mobula alfredi* individuals, E) LnP(*K*) plotted against value of *K* across Pacific Ocean *Mobula alfredi* individuals and F) Δ*K* plotted against value of *K* across Pacific Ocean *Mobula alfredi* individuals. See Supplementary Figure S4.18 for associated STRUCTURE plots.



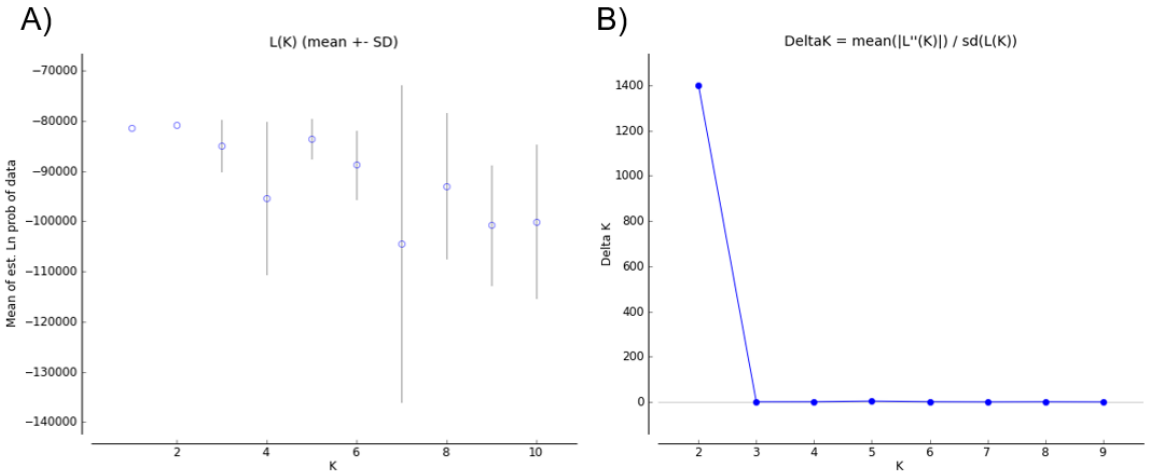
Supplementary Figure S4.20 Plot showing results of Bayesian clustering analysis with no prior information on sampling locations implemented in STRUCTURE using the admixture model for *Mobula alfredi* 'no singletons' dataset. A) All individuals analysed together at *K*=2. The data was then split into inferred clusters, corresponding to individuals sampled in the Indian and Pacific oceans, and plotted for *K*=2 and *K*=3: B) Indian Ocean *K*=2, C) Indian Ocean *K*=3, D) Pacific Ocean *K*=2, E) Pacific Ocean *K*=3. Each vertical bar represents an individual, and colours represent the proportion of ancestry assigned to each cluster for each individual. Individuals are grouped by sampling location. STRUCTURE HARVESTER output, identifying optimal values of *K*, are given in Supplementary Figure S4.21.



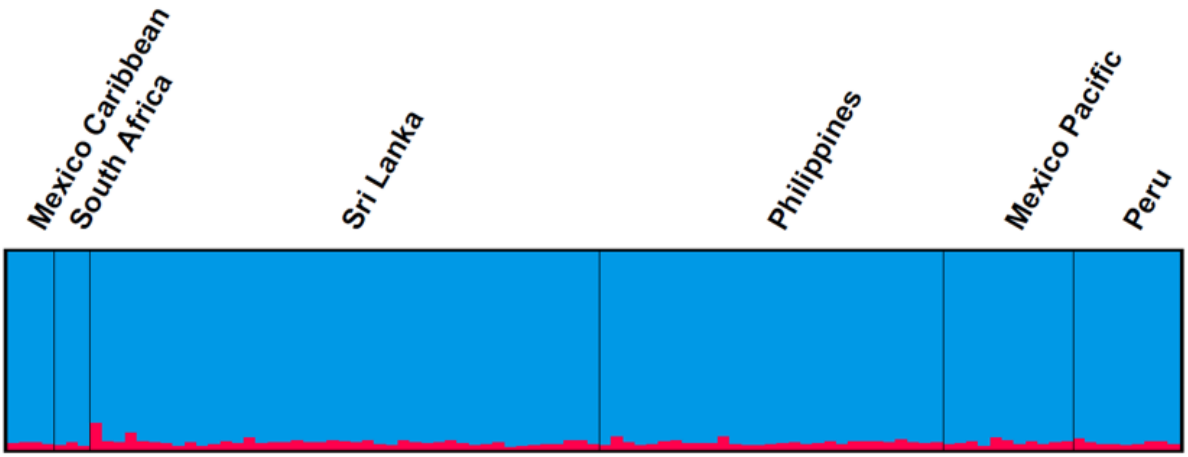
Supplementary Figure S4.21: Output from STRUCTURE HARVESTER for *Mobula alfredi* 'no singletons' dataset. A) LnP(*K*) plotted against value of *K* across all *Mobula alfredi* individuals, B) Δ*K* plotted against value of *K* across all *Mobula alfredi* individuals, C) LnP(*K*) plotted against value of *K* across Indian Ocean *Mobula alfredi* individuals, D) Δ*K* plotted against value of *K* across Indian Ocean *Mobula alfredi* individuals, E) LnP(*K*) plotted against value of *K* across Pacific Ocean *Mobula alfredi* individuals and F) Δ*K* plotted against value of *K* across Pacific Ocean *Mobula alfredi* individuals. See Supplementary Figure S4.20 for associated STRUCTURE plots.



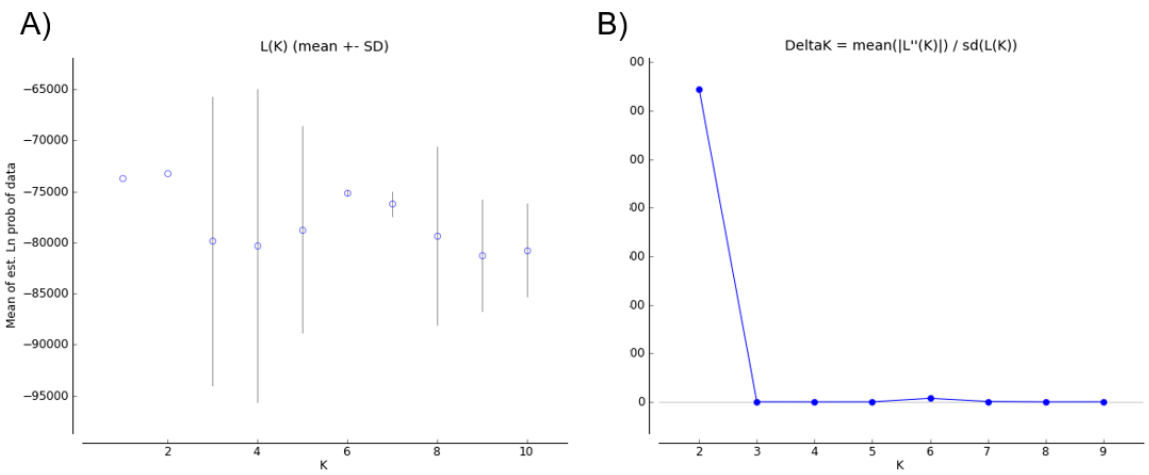
Supplementary Figure S4.22: Output from STRUCTURE HARVESTER for *Mobula birostris* 'all SNPs' dataset. A) LnP(*K*) plotted against value of *K,* and B) Δ*K* plotted against value of *K.* See Figure 4.4 for associated STRUCTURE plot.



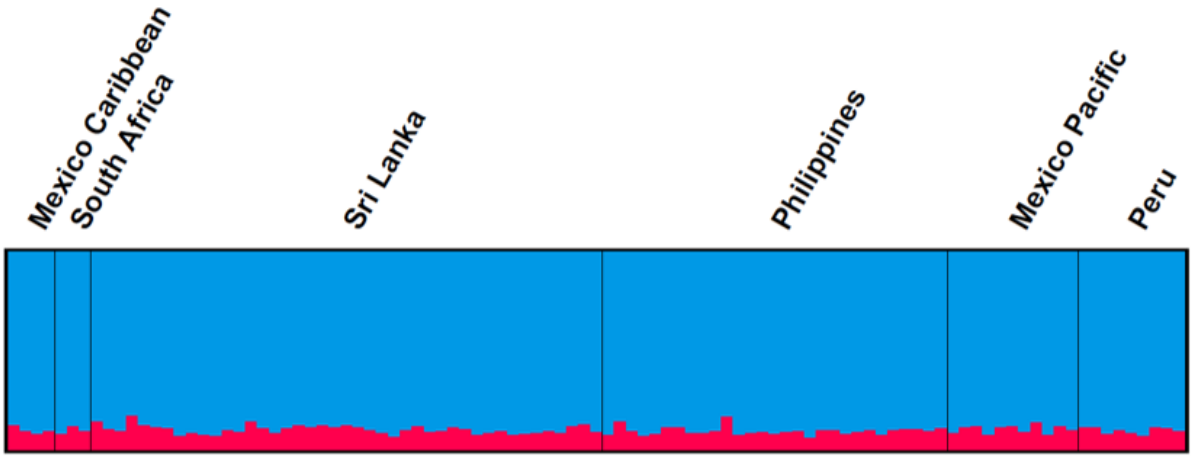
Supplementary Figure S4.23: Output from STRUCTURE HARVESTER for *Mobula birostris* 'all SNPs' dataset where prior information was provided on sampling location ('locprior model'). A) LnP(*K*) plotted against value of *K,* and B) Δ*K* plotted against value of *K.* See Figure 4.5 for associated STRUCTURE plot.



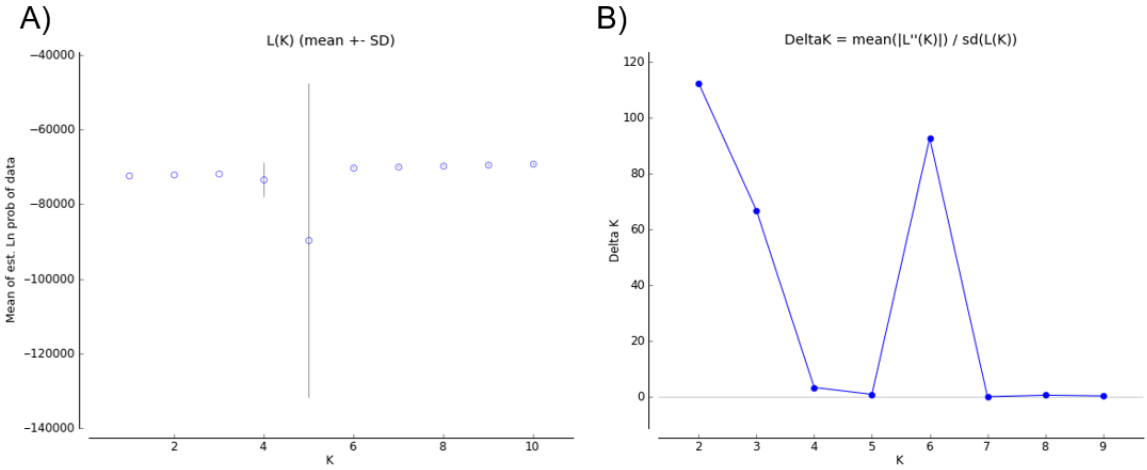
Supplementary Figure S4.24: Plot showing results of Bayesian clustering analysis with no prior information on sampling locations implemented in STRUCTURE using the admixture model for *Mobula birostris* 'neutral SNPs' dataset. Plot shown is for *K*=2, since the output from STRUCTURE HARVESTER indicated an optimal value of *K*=1 (see Supplementary Figure S4.25). Each vertical bar represents an individual, and colours represent the proportion of ancestry assigned to each cluster for each individual. Individuals are grouped by sampling location.



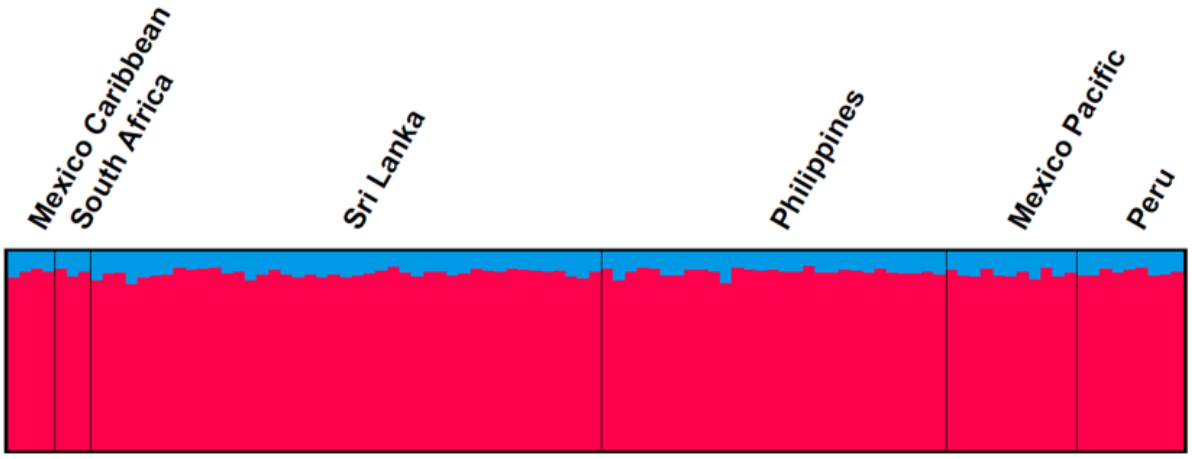
Supplementary Figure S4.25: Output from STRUCTURE HARVESTER for *Mobula birostris* 'neutral SNPs' dataset. A) LnP(*K*) plotted against value of *K,* and B) Δ*K* plotted against value of *K.* See Supplementary Figure S4.24 for associated STRUCTURE plot.



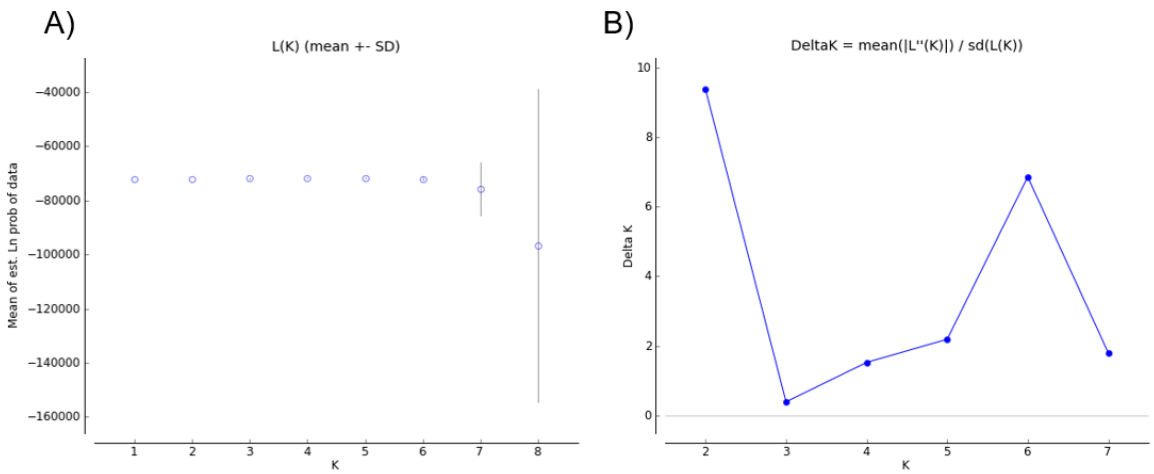
Supplementary Figure S4.26: Plot showing results of Bayesian clustering analysis with no prior information on sampling locations implemented in STRUCTURE using the admixture model for *Mobula birostris* 'no singletons' dataset. Plot shown is for *K*=2, since the output from STRUCTURE HARVESTER indicated an optimal value of *K*=1 (see Supplementary Figure S4.27). Each vertical bar represents an individual, and colours represent the proportion of ancestry assigned to each cluster for each individual. Individuals are grouped by sampling location.



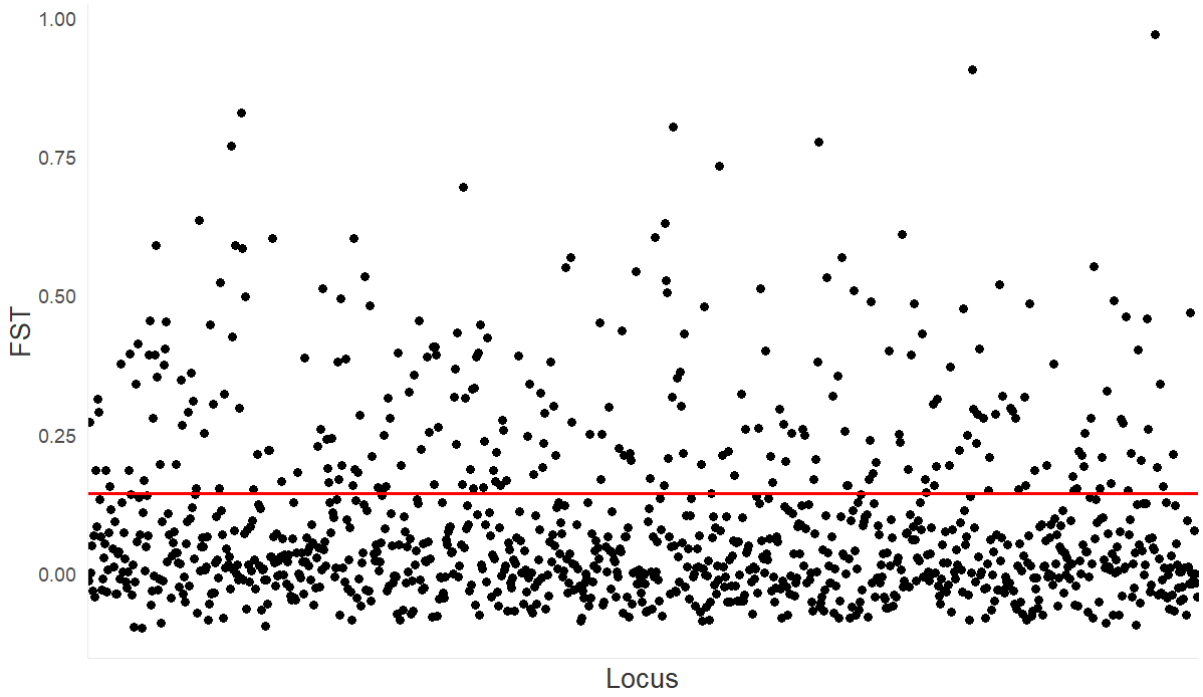
Supplementary Figure S4.27: Output from STRUCTURE HARVESTER for *Mobula birostris* 'no singletons' dataset. A) LnP(*K*) plotted against value of *K,* and *B*) Δ*K* plotted against value of *K.* See Supplementary Figure S4.26 for associated STRUCTURE plot.



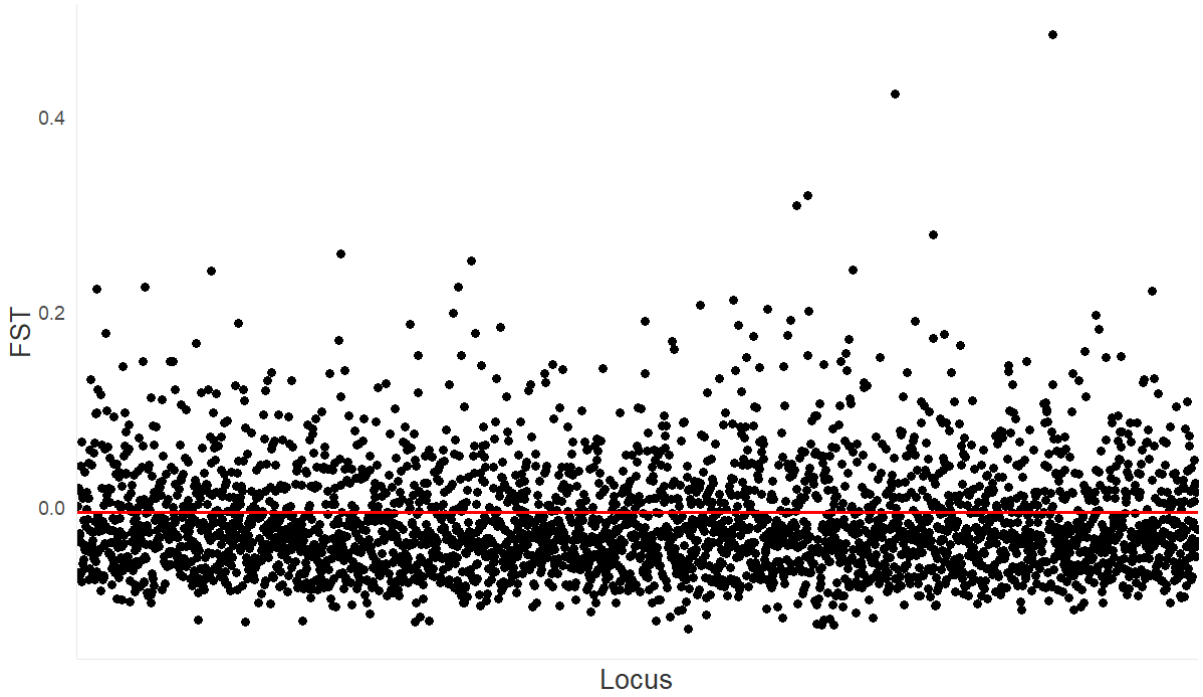
Supplementary Figure S4.28: Plot showing results of Bayesian clustering analysis implemented in STRUCTURE using the admixture model for *Mobula birostris* 'no singletons' dataset with prior information on sampling locations provided ('locprior' model). Plot shown is for *K*=2, since the output from STRUCTURE HARVESTER indicated an optimal value of *K*=1 (see Supplementary Figure S4.29). Each vertical bar represents an individual, and colours represent the proportion of ancestry assigned to each cluster for each individual. Individuals are grouped by sampling location.



Supplementary Figure S4.29: Output from STRUCTURE HARVESTER for *Mobula birostris* 'no singletons' dataset where prior information was provided on sampling location ('locprior' model). A) LnP(*K*) plotted against value of *K,* and B) Δ*K* plotted against value of *K.* See Supplementary Figure S4.28 for associated STRUCTURE plot.



Supplementary Figure S4.30: Plot showing locus-specific estimates of F_{ST} for the *Mobula alfredi* 'all SNPs' dataset with individuals assigned to populations corresponding to sampling location. The global average intra-specific F_{ST} = 0.144 and is shown as a red line.



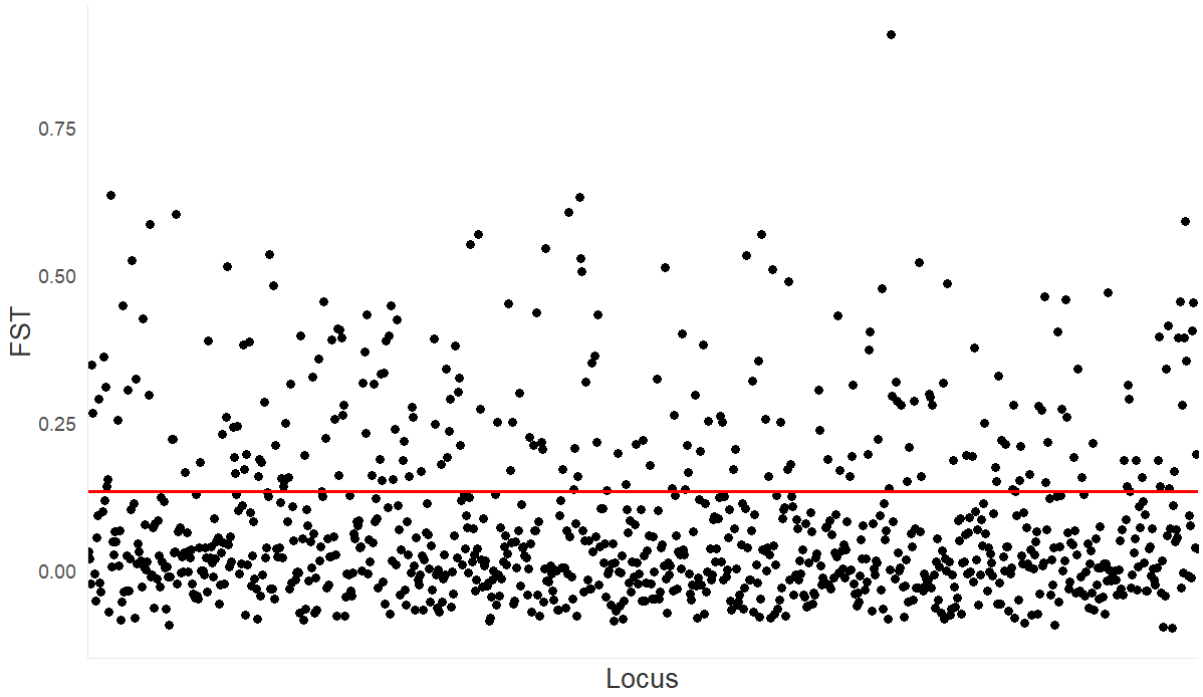
Supplementary Figure S4.31 Plot showing locus-specific estimates of F_{ST} for the *Mobula birostris* 'all SNPs' dataset with individuals assigned to populations corresponding to sampling location. The global average intra-specific F_{ST} = -0.006 and is shown as a red line.

Supplementary Table S4.2: Pairwise F_{ST} values ± 99% Confidence Intervals (Weir & Cockerham, 1984) between *Mobula alfredi* sampling locations, calculated based on the 'neutral SNPs' dataset. Values that are significantly different from zero are marked with *. Global average intra-specific F_{ST} is also given. See Supplementary Figure S4.32 for locus specific estimates of F_{ST}.

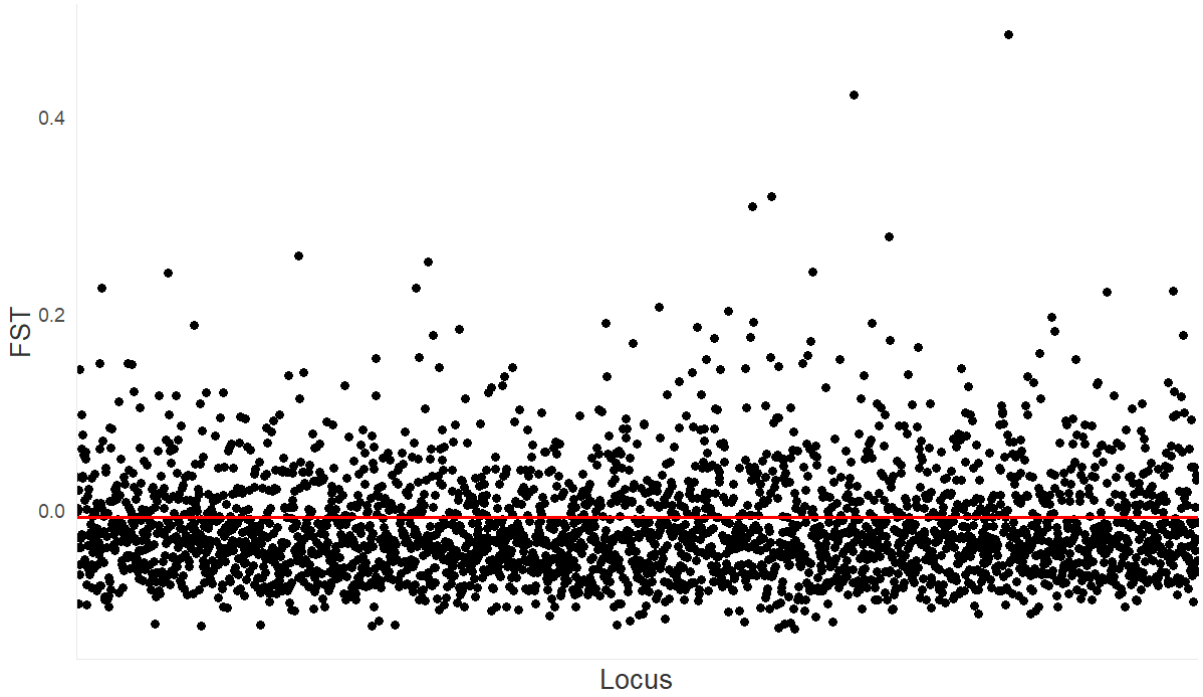
	Seychelles	Chagos	Maldives	Australia	Fiji
Seychelles					
Chagos	0.061* ± 0.019				
Maldives	0.051* ± 0.011	0.008 ± 0.017			
Australia	0.245* ± 0.043	0.120* ± 0.040	0.087* ± 0.030		
Fiji	0.266* ± 0.041	0.253* ± 0.041	0.169* ± 0.026	0.189* ± 0.050	
Hawaii	0.271* ± 0.027	0.164* ± 0.030	0.169* ± 0.025	0.048* ± 0.025	0.183* ± 0.018
Global average intra-specific F_{ST} = 0.134					

Supplementary Table S4.3: Pairwise F_{ST} values ± 99% Confidence Intervals (Weir & Cockerham, 1984) between *Mobula birostris* sampling locations, calculated based on the 'neutral SNPs' dataset. Values that are significantly different from zero are marked with *. Global average intra-specific F_{ST} is also given. See Supplementary Figure S4.33 for locus specific estimates of F_{ST}. Negative F_{ST} values can be interpreted as zero i.e. there is no differentiation, and therefore do not have a confidence interval.

	Mexico Caribbean	South Africa	Sri Lanka	Philippines	Mexico Pacific
Mexico Caribbean					
South Africa	-0.037				
Sri Lanka	-0.017	-0.054			
Philippines	-0.029	-0.053	-0.003		
Mexico Pacific	-0.028	-0.008	0.044* ± 0.006	0.017* ± 0.004	
Peru	-0.007	-0.036	-0.007	-0.006	0.042* ± 0.008
Global average intra-specific F_{ST} = -0.008					



Supplementary Figure S4.32: Plot showing locus-specific estimates of F_{ST} for the *Mobula alfredi* 'neutral SNPs' dataset with individuals assigned to populations corresponding to sampling location. The global average intra-specific F_{ST} = 0.134 and is shown as a red line.



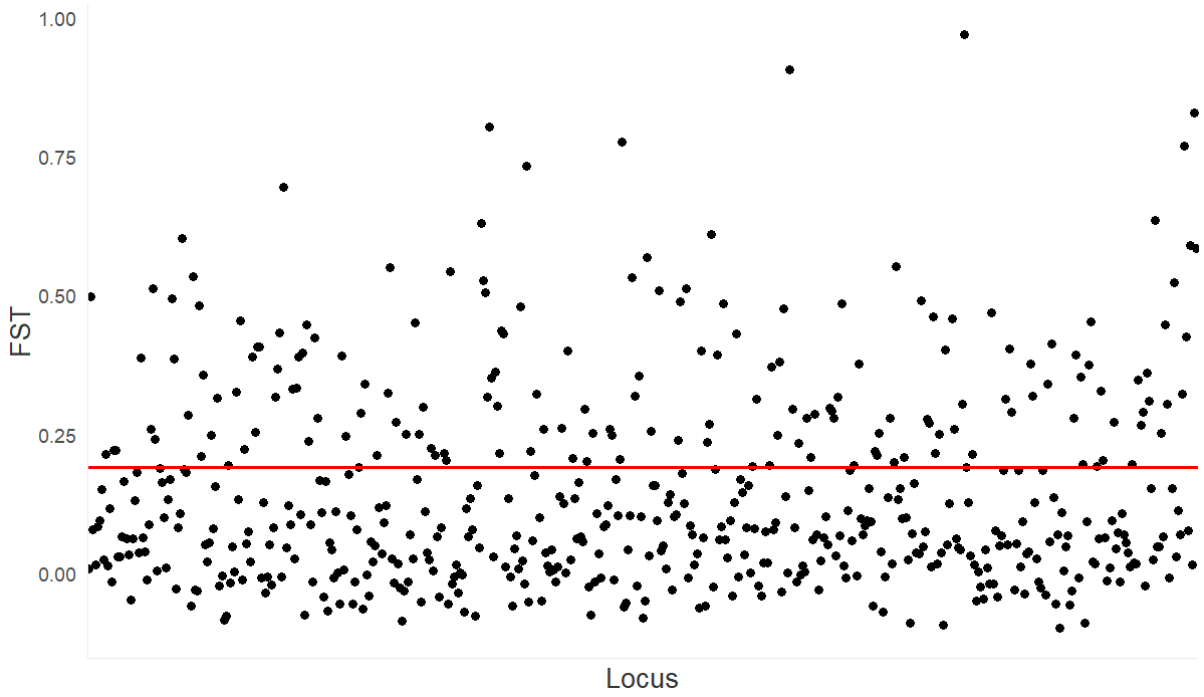
Supplementary Figure S4.33: Plot showing locus-specific estimates of F_{ST} for the *Mobula birostris* 'neutral SNPs' dataset with individuals assigned to populations corresponding to sampling location. The global average intra-specific F_{ST} = -0.008 and is shown as a red line.

Supplementary Table S4.4: Pairwise F_{ST} values ± 99% Confidence Intervals (Weir & Cockerham, 1984) between *Mobula alfredi* sampling locations, calculated based on the 'no singletons' dataset. Values that are significantly different from zero are marked with *. Global average intra-specific F_{ST} is also given. See Supplementary Figure S4.34 for locus specific estimates of F_{ST}.

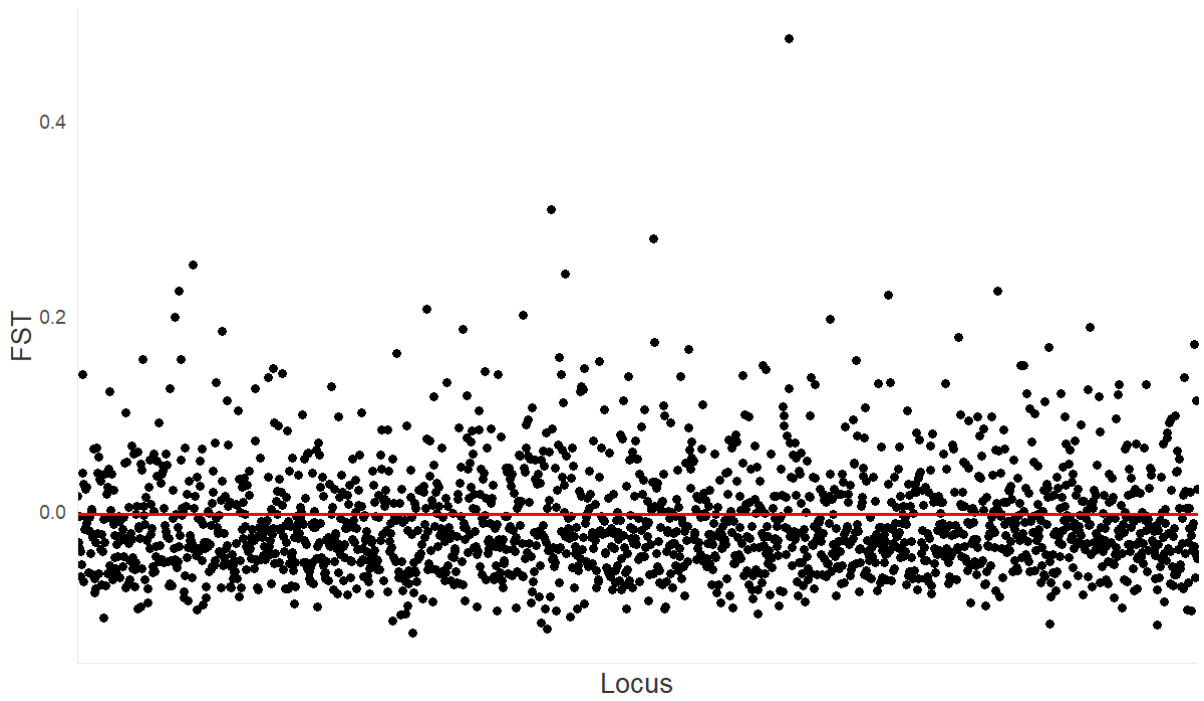
	Seychelles	Chagos	Maldives	Australia	Fiji
Seychelles					
Chagos	0.071* ± 0.024				
Maldives	0.065* ± 0.014	0.040* ± 0.022			
Australia	0.319* ± 0.048	0.211* ± 0.053	0.181* ± 0.036		
Fiji	0.349* ± 0.049	0.331* ± 0.049	0.248* ± 0.034	0.184* ± 0.053	
Hawaii	0.342* ± 0.036	0.261* ± 0.043	0.246* ± 0.032	0.089* ± 0.041	0.208* ± 0.031
Global average intra-specific F_{ST} = 0.191					

Supplementary Table S4.5: Pairwise F_{ST} values ± 99% Confidence Intervals (Weir & Cockerham, 1984) between *Mobula birostris* sampling locations, calculated based on the 'no singletons' dataset. Values that are significantly different from zero are marked with *. Global average intra-specific F_{ST} is also given. See Supplementary Figure S4.35 for locus specific estimates of F_{ST}. Negative F_{ST} values can be interpreted as zero i.e. there is no differentiation, and therefore do not have a confidence interval.

	Mexico Caribbean	South Africa	Sri Lanka	Philippines	Mexico Pacific
Mexico Caribbean					
South Africa	-0.020				
Sri Lanka	-0.008	-0.038			
Philippines	-0.019	-0.039	-0.001		
Mexico Pacific	-0.018	-0.008	0.039* ± 0.006	0.016* ± 0.005	
Peru	-0.002	-0.021	-0.004	-0.003	0.038* ± 0.009
Global average intra-specific F_{ST} = -0.003					



Supplementary Figure S4.34 Plot showing locus-specific estimates of F_{ST} for the *Mobula alfredi* 'no singletons' dataset with individuals assigned to populations corresponding to sampling location. The global average intra-specific F_{ST} = 0.191 and is shown as a red line.



Supplementary Figure S4.35: Plot showing locus-specific estimates of F_{ST} for the *Mobula birostris* 'no singletons' dataset with individuals assigned to populations corresponding to sampling location. The global average intra-specific F_{ST} = -0.003 and is shown as a red line.

Supplementary Table S4.6: Genetic diversity within *Mobula alfredi* populations, calculated using the 'neutral SNPs' dataset. Expected heterozygosity (H_e), observed heterozygosity (H_o), nucleotide diversity (π) and the number of polymorphic SNPs within each population are given.

Population	Number of individuals	Expected Heterozygosity (H_e) ± s.e	Observed Heterozygosity ± s.e	Nucleotide Diversity (π) ± s.e	No. SNPs
Seychelles	24	0.0737 ± 0.0045	0.0561 ± 0.0038	0.0760 ± 0.0047	354
Chagos	5	0.0612 ± 0.0045	0.0543 ± 0.0047	0.0694 ± 0.0052	174
Maldives	43	0.0798 ± 0.0048	0.0565 ± 0.0036	0.0812 ± 0.0049	462
Australia	3	0.0454 ± 0.0041	0.0426 ± 0.0044	0.0564 ± 0.0051	116
Fiji	8	0.0409 ± 0.0038	0.0364 ± 0.0038	0.0448 ± 0.0042	128
Hawaii	24	0.0336 ± 0.0029	0.0235 ± 0.0023	0.0346 ± 0.0030	243

Supplementary Table S4.7: Genetic diversity within *Mobula birostris* sampling locations, calculated using the 'neutral SNPs' dataset. Expected heterozygosity (H_e), observed heterozygosity (H_o), nucleotide diversity (π) and the number of polymorphic SNPs within each population are given.

Population	Number of individuals	Expected Heterozygosity (H_e) ± s.e	Observed Heterozygosity ± s.e	Nucleotide Diversity (π) ± s.e	No. SNPs
Mexico Caribbean	4	0.0632 ± 0.0026	0.0547 ± 0.0026	0.0749 ± 0.0031	543
South Africa	3	0.0581 ± 0.0026	0.0613 ± 0.0031	0.0712 ± 0.0031	464
Sri Lanka	43	0.0852 ± 0.0024	0.0590 ± 0.0018	0.0872 ± 0.0025	1966
Philippines	29	0.0806 ± 0.0024	0.0529 ± 0.0018	0.0828 ± 0.0025	1536
Mexico Pacific	11	0.0751 ± 0.0025	0.0485 ± 0.0018	0.0805 ± 0.0027	942
Peru	9	0.0746 ± 0.0025	0.0581 ± 0.0023	0.0799 ± 0.0027	875

Supplementary Table S4.8: Genetic diversity within *Mobula alfredi* populations, calculated using the 'no singletons' dataset. Expected heterozygosity (H_e), observed heterozygosity (H_o), nucleotide diversity (π) and the number of polymorphic SNPs within each population are given.

Population	Number of individuals	Expected Heterozygosity (H_e) ± s.e	Observed Heterozygosity ± s.e	Nucleotide Diversity (π) ± s.e	No. SNPs
Seychelles	24	0.1572 ± 0.0078	0.1193 ± 0.0068	0.1618 ± 0.0080	331
Chagos	5	0.1315 ± 0.0081	0.1165 ± 0.0087	0.1490 ± 0.0092	196
Maldives	43	0.1755 ± 0.0080	0.1237 ± 0.0065	0.1786 ± 0.0081	363
Australia	3	0.1055 ± 0.0080	0.0968 ± 0.0090	0.1306 ± 0.0100	137
Fiji	8	0.1040 ± 0.0076	0.0932 ± 0.0080	0.1125 ± 0.0083	155
Hawaii	24	0.0764 ± 0.0065	0.0495 ± 0.0054	0.0788 ± 0.010	148

Supplementary Table S4.9: Genetic diversity within *Mobula birostris* sampling locations, calculated using the 'no singletons' dataset. Expected heterozygosity (H_e), observed heterozygosity (H_o), nucleotide diversity (π) and the number of polymorphic SNPs within each population are given.

Population	Number of individuals	Expected Heterozygosity (H_e) ± s.e	Observed Heterozygosity ± s.e	Nucleotide Diversity (π) ± s.e	No. SNPs
Mexico Caribbean	4	0.1024 ± 0.0039	0.0854 ± 0.0041	0.1212 ± 0.0047	537
South Africa	3	0.0990 ± 0.0040	0.1024 ± 0.0048	0.1215 ± 0.0049	493
Sri Lanka	43	0.1381 ± 0.0035	0.0907 ± 0.0027	0.1411 ± 0.0036	1534
Philippines	29	0.1283 ± 0.0035	0.0804 ± 0.0026	0.1315 ± 0.0036	1291
Mexico Pacific	11	0.1241 ± 0.0038	0.0723 ± 0.0028	0.1332 ± 0.0041	876
Peru	9	0.1208 ± 0.0038	0.0887 ± 0.0034	0.1295 ± 0.0041	828

Chapter 5

General Discussion

Elements of this Chapter are discussed in a published paper:

Stewart JD, Jaine FRA, Armstrong AJ, Armstrong AO, Bennett MB, Burgess KB, Couturier LIE, Croll DA, Cronin MR, Deakos MH, Dudgeon CL, Fernando D, Froman N, Germanov ES, Hall MA, Hinojosa-Alvarez S, **Hosegood JE**, Kashiwagi T, Laglbauer BJL, Lezama-Ochoa N, Marshall AD, McGregor F, Notarbartolo di Sciara, G, Palacios MD, Peel LR, Richardson AJ, Rubin RD, Townsend KA, Venables SK and Stevens GMW. 2018. Research Priorities to Support Effective Manta and Devil Ray Conservation. Frontiers in Marine Science **5**:314. https://doi.org/10.3389/fmars.2018.00314.

See Appendix I: Published Work for copy.

The PhD candidate is a co-author on this paper and contributed by leading Section 1: Taxonomy and Diversity, which was written in collaboration with Mike Bennett, Tom Kashiwagi, Silvia Hinojosa-Alvarez and Giuseppe Notarbartolo di Sciara, and contributing Section 5.4: Fisheries Impacts on Genetic Diversity, included within Section 5: Fisheries and Bycatch, which was led by Daniel Fernando.

Chapter 5: General Discussion

5.1 Thesis Highlights

When I was interviewed for this PhD project in early 2014, a search on Web of Science for studies utilising genetic data to improve understanding of manta and devil rays yielded just two papers published in peer-reviewed scientific journals. Accordingly, mobulid species diversity was recorded almost exclusively based on morphological data, and knowledge of population structure, gene flow and adaptive differentiation was lacking entirely. Whilst a single published study provided some details regarding the recent speciation between the two described species of manta ray (Kashiwagi et al. 2012), this was based on a single nuclear gene and two mitochondrial genes. At the same time, concerns were being raised regarding the alarming rate at which manta and devil ray species are exploited in targeted and bycatch fisheries, mostly in order to satiate international demand for their gill plates, and knowledge gaps were a common theme in discussions regarding designing and implementing effective management and conservation measures.

In the years since that initial literature search, the interest in utilising molecular data to inform conservation of manta and devil rays has expanded considerably within the community. At the species level, a dated molecular phylogeny has been published for the Mobulidae using nuclear and mitogenome data (Poortvliet et al. 2015), and a recent taxonomic revision makes use of genomic data (White et al. 2017). Genetic data have also been used to demonstrate diversity previously undocumented with morphological data (Hinojosa-Alvarez et al. 2016) and provide evidence of hybridisation within the group (Walter et al. 2014). Finally, at the population level, studies are now utilising genetic and genomic data to evaluate population structure in manta and devil rays (Poortvliet, 2015; Stewart et al. 2016). However, a recurrent theme within these studies is a lack of representative samples of manta and devil rays geographically and taxonomically, which preclude detailed inferences regarding species boundaries and therefore taxonomy, speciation, population structure, gene flow and adaptation within these species of conservation concern.

To address these knowledge gaps and contribute to current understanding of the extent and distribution of diversity within mobulid rays, the current project set out to produce genomic data to inform conservation and management of these charismatic and economically important species. By combining resources and bringing together available samples of manta and devil rays, the project has examined the largest and most comprehensive set of samples across representative species, with input from organisations and researchers worldwide, to achieve its aims and objectives.

By generating genome-wide SNP data for all available species across a broad geographical range the current project provides a substantial contribution to current knowledge pertaining to manta and devil rays. Since biodiversity conservation is enacted through global conventions acting at the species level, Chapter 2 aimed to delimit mobulid species for conservation, estimate the species tree for the group, and investigate the extent of incomplete lineage sorting and ancient admixture. By explicitly testing alternative species delimitation hypotheses using a coalescent based approach applied to genome-wide SNP data for 115 individuals of mobulid ray representing all currently recognised species, and demonstrating reciprocal monophyly among individual lineages, the authors identify a number of mismatches between species recognised in current taxonomy and biologically relevant species units optimal for conservation. Specifically, we show robust evidence for an undescribed species of manta ray in the Gulf of Mexico, occurring in sympatry with the oceanic manta ray, *Mobula birostris,* with evidence suggestive of hybridisation between the two species. Furthermore, we show that the shorthorned pygmy devil ray, *Mobula kuhlii* and the longhorned pygmy devil ray, *Mobula kuhlii cf. eregoodootenkee*, which were recently synonymised in a taxonomic review (White et al. 2017) are actually distinct and reproductively isolated. Accordingly, we recommend that such units coincide with enforceable protection implemented through international conventions via recognition as distinct species. In addition, we uncover substantial geographically mediated population structure in the reef manta ray, *Mobula alfredi* and the shorthorned pygmy devil ray, *M. kuhlii,* indicating potential for future traceability work determining regional location of catch. Bayesian phylogenetic reconstruction identifies a novel phylogenetic placement for the sicklefin devil ray, *Mobula tarapacana,* and shows substantial incomplete lineage sorting, with implications for the formerly recognised genus *Manta*. The incomplete lineage sorting is likely derived from

standing variation in extinct ancestral populations and may drive taxonomic uncertainty in extant species.

Having investigated the extent of mobulid diversity at the species level, including the identification of an undescribed species of manta ray in the Gulf of Mexico and possible hybridisation with *Mobula birostris,* Chapter 3 further explored this speciation event, which had previously been hypothesised to be associated with sea level changes isolating an ancestral population of *M. birostris* in the Gulf of Mexico (Hinojosa-Alvarez et al. 2016). The hypothesis of ongoing hybridisation between manta ray species is explicitly tested, and the extent of introgression among lineages investigated. Genome-wide SNP data representing 217 individuals of all three species of manta ray (*Mobula alfredi*, *M. birostris* and *Mobula* Sp. 1) identified three independently evolving lineages, giving further support for *Mobula* Sp. 1 as an undescribed species, and provides an example of extremely rapid speciation in a marine system (Momigliano et al. 2017; Lemoine et al. 2019). By reconstructing ancestral effective population sizes along lineages, the authors disclosed a decline in genetic diversity associated with the lineage corresponding to *Mobula* Sp. 1, consistent with a pattern of peripatric mechanism of speciation in isolation (Blair et al. 2014). Hybrid ancestry (*Mobula birostris* x *Mobula* Sp. 1) is confirmed for a single juvenile individual sampled at Flower Garden Banks National Marine Sanctuary (FGBNMS). However, tests for introgression show no evidence of gene flow among lineages, indicating that hybrids may be inviable. As such, hybridisation events in manta rays are likely to represent unsuccessful reproductive effort, and is especially significant in these taxa notoriously identified as having increased vulnerability to environmental change and overexploitation due to low fecundity and extended gestation periods (Dulvy et al. 2014; Croll et al. 2016).

Finally, having evaluated mobulid diversity at the species level, and at an intermediate point along the speciation continuum, Chapter 4 focussed on characterising population genetic structure for the reef manta ray, *Mobula alfredi*, and the oceanic manta ray, *M. birostris*. These two species had the widest coverage and representative sample sizes for population level analysis. Whilst the reef manta ray occupies highly fragmented reef-based habitats, the oceanic manta ray is typically associated with offshore, pelagic habitats, potentially with greater opportunities for gene flow. However, previous studies have suggested a high degree of residency and spatial segregation of adult populations in both species (Dewar et al. 2008;

Jaine et al. 2014; Stewart et al. 2016; Couturier et al. 2018). Genome-wide SNP data for 107 *Mobula alfredi* individuals and 99 *M. birostris* individuals, each sampled at 6 independent sites across the overlapping ranges of these species, revealed highly contrasting patterns of population structure. As expected, the reef manta ray shows a high degree of structuring, indicative of limited gene flow between sampling locations. Patterns of declining genetic diversity eastwards within populations across the Pacific Ocean may be suggestive of successive colonisation events (Ramachandran et al. 2005; Deshpande et al. 2009; Pierce et al. 2014). In direct contrast, our data show a pattern of global genetic panmixia in the oceanic manta ray, an apparently resident species (Stewart et al. 2016). This juxtaposition may be explained by past demographic processes (e.g. Bailleul et al. 2018), or via differential dispersal among life history stages (e.g. Hutchinson et al. 2001; Daly-Engel et al. 2012). Nonetheless, this study highlights the importance of independently evaluating population genetic structure when designing conservation strategies for closely related species.

Having presented some highlights of the research carried out in this thesis, the wider significance of certain aspects of the work are considered. Specifically, species as units for conservation are critically evaluated, and support is presented for inclusion of marine species on the Convention on the International Trade in Endangered Species of Wild Fauna and Flora (CITES), which although controversial, is justified and the case strengthened with molecular data. In addition, the paradox of global genetic panmixia in an apparently resident species, the oceanic manta ray (*Mobula birostris*) is discussed, together with the potential for development of traceability tools for manta and devil rays using genomic data generated herein. Finally, suggestions and priorities for future research are presented in the context of advancing the long-term conservation and management of this charismatic group of marine megafauna.

5.2 Species as Units for Conservation Policy: CITES Case Study

Biodiversity conservation is enacted through global conventions and regulatory frameworks implemented through legislation acting at the species level (Vincent et al. 2014; Donaldson et al. 2016). Organisations include the International Union for Conservation of Nature (IUCN),

which assesses the threat status of species, the Convention on the International Trade in Endangered Species of Wild Fauna and Flora (CITES), which aims to ensure that trade in wildlife products does not threaten the survival of species, and the Convention on the Conservation of Migratory Species of Wild Animals (CMS), which coordinates efforts to protect and conserve migratory species. It is therefore imperative that species boundaries are judiciously defined within a robust taxonomic framework, since management plans will be most effective when applied to biologically relevant units. However, defining such units remains challenging where species represent an essentially man-made concept, with no universally accepted definition (De Queiroz, 2007; Frankham et al. 2012). Indeed, interactions between species lineages in nature are not uncommon (e.g. Sousa & Hey, 2013; Ford et al. 2015) and challenges remain where speciation events are incomplete or ongoing (e.g. Nosil et al. 2009) or where secondary contact results in the formation of hybrid zones (e.g. Garrick et al. 2014; Fitzpatrick et al. 2015; Macleod et al. 2015). Nonetheless, genomic data can be used to delimit species units on the basis of reproductive isolation, as defined by a restriction or absence of gene flow between independently evolving lineages (Frankham et al. 2012; Seehausen et al. 2014), and effectively catalogue biodiversity for conservation purposes.

Signed in 1973, CITES aims to ensure that international trade does not contribute to the extinction of commercially exploited species, including marine fish. Trade among the 183 signatory countries, referred to as 'Parties', is regulated via listing of species on one of three Appendices. Appendix I includes species threated with extinction, and as such commercial trade is broadly prohibited (1003 species at the time of writing). Appendix II lists species not necessarily threatened with extinction, but for which trade must be controlled and regulated for their sustainable future (34,596 species at the time of writing). Finally, Appendix III represents species for which individual countries have asked for assistance from the other Parties to control trade (202 species at the time of writing). Appendix I and II species may be traded subject to the issue of import, export and re-export permits as appropriate from the relevant Management Authority appointed by each Party. Changes to species listings on CITES Appendices generally occur at the Conference of the Parties (CoP), which takes place approximately every three years. Any Party may submit a proposal to add a species to an Appendix, remove a species, or move a species between Appendices, and proposals are discussed during the CoP. Changes to Appendices I and II require two-thirds majority support

from Parties, which is established by a vote at the CoP.

Despite this well-defined regulatory framework, the inclusion of marine fish species on CITES remains a controversial topic. For example, there are concerns that CITES listings and any associated export quotas will simply lead to increased discards of marine species caught as bycatch, rather than effectively reducing pressure on wild populations. However, it has also been argued that listing of species threatened with bycatch on Appendix II might lead indirectly to better regulation, since trade in Appendix II specimens requires proof that such specimens were legally obtained (see Vincent et al. 2014). Furthermore, in many cases, countries will move towards national protection for threatened species following listing on CITES (see Friedman et al. 2018). Nonetheless, some Parties see CITES as a possible source of interference in fisheries management, arguing that national or regional level management is more appropriate. However, this represents a common source of confusion, where CITES is focussed specifically on trade, rather than fisheries management (see Vincent et al. 2014). Fishers have been known to protest against proposals to list marine fish species, and the first few shark proposals were initially rejected, before being subsequently adopted at a later CoP. Initial discussions largely centred around issues regarding country of origin for marine fish, which may not be clear cut in cases of fishing in international waters, or where the flag nation of the fishing vessel responsible differs from the jurisdiction of the waters in which it is fishing or of the nation in which it lands its catch (Vincent et al. 2014). Within the CITES framework however, fishing in international waters is referred to as 'Introduction from the Sea' and is the responsibility of flag and port states.

Further objections have been raised where the traditional view that marine fish populations are widespread, unstructured and essentially inexhaustible unfortunately remains (Hauser & Carvalho, 2008), and knowledge gaps regarding the status of populations, fisheries and trade can make listing of marine species on CITES difficult to justify. Recommendations from the Food and Agriculture Organisation (FAO) have suggested accounting for resilience and productivity of populations when considering CITES listing (Cochrane, 2015), however, in many cases, such considerations are precluded by a lack of scientific data. Furthermore, problems identifying parts in trade to species and regional level, both due to taxonomic uncertainties and a lack of resources for reliable species identification are commonly cited as barriers to enforcement of regulations (Cochrane, 2015). Indeed, despite CITES listings, parts

and derivatives of various species of marine fish are still traded illegally (e.g. Zeng et al. 2016; Cardeñosa et al. 2018).

Molecular data can be effectively used to target these knowledge gaps that may prevent proposed listings gaining sufficient support from CITES Parties, or appropriate enforcement of regulations. Utility of genomic data has been demonstrated in species delimitation (e.g. Leache et al. 2014; Herrera & Shank, 2016), traceability of parts in trade, including distinguishing between listed and unlisted species (e.g. Ogden, 2008; Nielsen et al. 2012; Bekkevold et al. 2015; Ogden & Linacre, 2015; Jorde et al. 2018), and evaluating genetic diversity and resilience of species and populations proposed for inclusion on the CITES appendices (e.g. Frankham, 2005; Sgrò et al. 2011; Hellmair & Kinziger, 2014).

Manta and devil rays were initially proposed for inclusion on CITES Appendix II due to exploitation of natural populations for their gill plates (Couturier et al. 2012; Lawson et al. 2017; O'Malley et al. 2017) and morphological similarities between species (see Stevens et al. 2018b). A proposal to include the formerly recognised genus *Manta* on Appendix II was adopted at CoP16 in 2013, and devil rays (*Mobula* spp.) followed at CoP17 in 2016. On both occasions, the case was made for listing all species within genera due to morphological similarity of gill plates among species (see CITES CoP17 Proposal 44, 2016). However, whilst trade is now regulated under CITES, monitoring and permitting remains hindered by taxonomic uncertainty and morphological similarities among species.

In the phylogenomics study described in Chapter 2, genome-wide SNP data is presented, providing evidence to support ten species within the CITES-listed Mobulidae: *Mobula alfredi, Mobula birostris, Mobula mobular, Mobula thurstoni, Mobula kuhlii, Mobula eregoodootenkee, Mobula hypostoma, Mobula munkiana, Mobula tarapacana* and a currently undescribed species of manta ray, *Mobula* Sp. 1, in the Gulf of Mexico. Whist *Mobula hypostoma cf. rochebrunei* was not represented within the dataset, concerns are highlighted regarding the conservation status of this formerly recognised species, and the basis upon which it was synonymised with *Mobula hypostoma* (White et al. 2017). Policymakers, particularly the large conventions such as the CITES and CMS, and the relevant specialist group within the IUCN, are emphatically urged to evaluate these as separate units in their assessments and when implementing conservation policy. Indeed, this study was recently used to help inform the development of a mobulid species ID guide and key (Stevens

et al. 2018b), which is being used by fisheries workers and enforcement agencies tasked with monitoring and enforcement of conservation regulations. Furthermore, in addition to findings presented in Chapter 2, data presented in Chapter 4 show strong geographic population genetic structure in the reef manta ray, *Mobula alfredi,* indicating potential for future work to determine regional location of catch and/or traded parts (see Ogden, 2008; Ogden & Linacre, 2015). Whilst the authors find no population structure in the oceanic manta ray, *Mobula birostris*, a previous study was able to show some structure in this species based on loci with high F_{ST} (Stewart et al. 2016), indicating potential to use outlier loci for regional traceability (e.g. Nielsen et al. 2012; Bekkevold et al. 2015; Jorde et al. 2018; see Section 5.4 for further discussion).

The data generated through the current project thus provide a substantial contribution towards effective monitoring and enforcement of CITES regulations for manta and devil rays and demonstrates the utility of genomic data in supporting legislation designed to preserve biodiversity. Clear paths forward are identified with regards to evaluating adaptive differentiation between populations and outlier loci for regional traceability of parts under CITES.

5.3 Paradox: How can global genetic panmixia occur in a resident species?

Coupling an understanding of population genetic structure with knowledge of spatial ecology and population dynamics can yield valuable insights for conservation and management of species and populations (Hellmair & Kinziger, 2014). In Chapter 4, evidence is presented for genetic panmixia on a global scale in the oceanic manta ray, *Mobula birostris*. These results are inherently paradoxical, since true homogeneous panmixia requires random mating and associated gene flow, and tagging and stable isotope data suggest that *Mobula birostris* is highly resident with no recorded long-range migrations (Dewar et al. 2008; Stewart et al. 2016). It is worth noting that in the study presented in Chapter 4, samples representing the oceanic manta ray, *Mobula birostris*, were processed together, both in the laboratory and bioinformatically, with samples representing the reef manta ray, *Mobula alfredi,* which shows highly contrasting pattern of strong population differentiation among sampling locations,

thereby providing confidence in these results. Here, I present five scenarios that could produce this pattern of global genetic panmixia in the oceanic manta ray, *Mobula birostris*. I discuss these hypotheses in the context of current knowledge and identify areas where further work could assist in resolving this paradox.

First, and probably the simplest, is a hypothesis I call the 'oceanic nomad hypothesis'. Under this scenario, individuals are highly migratory, moving incredibly large distances over the course of a lifetime. Such a scenario would allow panmixia to occur on the basis of random mating between nomadic individuals. However, this hypothesis is not supported by tagging data from multiple locations, which has failed to produce any evidence of nomadic behaviour in *Mobula birostris* of either sex (Dewar et al. 2008; Graham et al. 2012; Stewart et al. 2016). Whilst some pelagic sharks are known to alternate between resident and travelling behavioural states (Francis et al. 2019), broad scale movements have still been detected with a smaller investment in terms of tagging effort than has been deployed for the oceanic manta ray. Furthermore, differentiation of stable isotopes has been shown between individuals sampled among locations (Stewart et al. 2016), indicating longer-term residency than can be shown with satellite tags and acoustic telemetry. Nonetheless, seasonal appearances of oceanic manta rays at remote locations such as the Azores (A. Sobral, pers comm) raises the question of how far individuals will travel to visit such locations. Indeed, resident, transient and migratory ecotypes are documented within marine species (e.g. Hemmer-Hansen et al. 2013; Foote & Morin, 2016), and a similar mechanism could be responsible for genetic panmixia at neutral loci in the oceanic manta ray. Current tagging studies have targeted locations where oceanic manta rays may be more resident, precisely because suitable encounters are reliable. Future efforts should focus on sites where seasonal or rare sightings of this species are the norm, such as the Azores. In addition, if such tagging studies were to produce evidence of resident and migratory ecotypes, evaluation of outlier loci would be a logical next step to establish a possible genetic basis (e.g. Hemmer-Hansen et al. 2013; Bekkevold et al. 2016).

Second, such panmixia could be produced under a scenario where individuals travel to a single location to mate, similar to the spawning aggregations that have been documented for other species of marine fish (e.g. Asch & Erisman, 2018; Rowell et al. 2019). I call this the 'global breeding ground hypothesis'. In this scenario, it might be expected that pregnant females

migrate to such a breeding ground, primarily to give birth. Since parturition is expected to be followed immediately by copulation (Uchida et al. 2008; Stevens, 2016; Stevens et al. 2018a), in this scenario, it might be expected that males aggregate to take advantage of newly receptive females. However, this hypothesis suffers from the same shortcomings as the 'oceanic nomad hypothesis', where studies to date have failed to produce evidence of any long-range movements in the oceanic manta ray (Dewar et al. 2008; Graham et al. 2012; Stewart et al. 2016). Nonetheless, such studies generally do not include data for pregnant females, and future work could focus on this life history stage. However, both juveniles and mating behaviour are occasionally observed at multiple locations (see Stewart et al. 2018a-b), and support for this hypothesis is therefore distinctly lacking.

However, absence of a single global breeding ground does not preclude gene flow being mediated by rare long-distance dispersal of pregnant females among discrete populations. I call this third scenario the 'pregnant female dispersal hypothesis'. A recent sighting of a pregnant reef manta ray, *Mobula alfredi*, in the Eastern Pacific, 6000km from the nearest known population of this species, has sparked discussion regarding colonisation of new habitats (Arauz et al. 2019). Similar to the oceanic manta ray, *Mobula birostris*, tagging and photographic identification studies targeting reef manta rays indicate residency (Deakos et al. 2011; Marshall et al. 2011; Jaine et al. 2014; Couturier et al. 2018), suggesting that if long-range movements do occur, they are rare enough to avoid detection. Nonetheless, female-biased dispersal is known as a mechanism of inbreeding avoidance in nature (e.g. van Hooff et al. 2005; Vigilant et al. 2015). Indeed, female manta rays are known to be more mobile than the males (Stevens, 2016) and given that female manta rays are thought to mate immediately following parturition (Uchida et al. 2008; Stevens 2016; Stevens et al. 2018a), dispersal of pregnant females as a means of inbreeding avoidance could be a possible mechanism of gene flow in the oceanic manta ray.

Fourth, gene flow may also be mediated by juvenile dispersal. Under this 'juvenile dispersal hypothesis', juvenile oceanic manta rays are highly mobile, dispersing widely before settling and becoming resident as adults. Long-distance movements have previously been documented in juveniles of several species of shark (e.g. Bruce et al. 2019; Francis et al. 2019). However, juvenile stages remain underrepresented in mobulid ray research (see Stewart et al. 2018b). Whilst the locations of important juvenile habitats remain largely unknown, they

are thought to be spatially segregated from adult populations (e.g. Deakos, 2010; Marshall & Bennett, 2010; Stewart et al. 2018a). To date, tagging, stable isotope and genetic studies have focussed exclusively on adults, and this sampling bias may obscure dispersal during juvenile stages. Indeed, juvenile dispersal may lead to the panmixia among neutral loci described in Chapter 4, whilst also providing an explanation for modest population structure indicated by loci potentially evolving in a non-neutral manner (Stewart et al. 2016). If we assume that loci with high $F_{ST,}$ as used in Stewart et al. (2016) are candidates for loci under selection, population structure at these loci may be detectable in a scenario of juvenile dispersal if populations are sampled after selection has acted. Furthermore, age-related population structure has previously been shown in several shark species (e.g. Klein et al. 2019) and targeting young-of-the-year individuals may help to elucidate population structure before it is obscured by dispersal. Accordingly, a carefully designed sampling strategy may reveal more complex dynamics in the oceanic manta ray (e.g. Hutchinson et al. 2001; Hauser & Carvalho, 2008; Bozano et al. 2015; Meirmans, 2015).

Finally, global genetic panmixia does not necessarily need to be associated with gene flow. Instead, panmixia may be explained by a 'population grey zone hypothesis', where past demographic processes, coupled with large effective population size, produce a genetic time-lag effect preventing the detection of demographically independent populations with genetic data (see Bailleul et al. 2018 for example). Future work may therefore involve simulation studies as in Bailleul et al. (2018) to determine migration rates and effective population sizes required to produce the lack of structure observed here in *Mobula birostris*, which could potentially be achieved with relatively rare migrations where there is large effective population size. Nonetheless, data presented in Chapter 3 have shown that divergence of such a magnitude as to be considered a speciation event has occurred incredibly rapidly in manta rays. It therefore seems likely that global panmixia is maintained either by ongoing gene flow, or very large effective population size.

Global genetic panmixia in the oceanic manta ray, *Mobula birostris*, indicates that international cooperation may be required to effectively manage this species, and could be used in conjunction with targeted local approaches designed to manage resident adult populations (Stewart et al. 2016). Furthermore, establishing the extent and mechanism of gene flow among populations will help to inform the design of management strategies, both

to ensure that sensitive life stages are not unduly at risk, but also to avoid disrupting a mode of gene flow which maybe important for long-term resilience of populations (Sgrò et al. 2011; Hellmair & Kinziger, 2014).

5.4 Towards development of traceability tools for manta and devil rays

International trade in manta and devil ray gill plates has led to the expansion of unsustainable fisheries, many of which are not regulated or monitored (Couturier et al. 2012; O'Malley et al. 2017). The increasing fishing pressure faced by mobulid species is being met with the implementation of national and international laws to prevent further decline, for example the 2013 listing of the formerly recognised genus *Manta* on CITES Appendix II, and more recently, the equivalent listing of the genus *Mobula* in 2016. In addition to taxonomic uncertainties, morphological similarities between species of mobulid rays presents a major challenge for fisheries monitoring and enforcement of regulations (Stevens et al. 2018b). Wildlife DNA forensic techniques can be effective in identifying a part to species and regional level (e.g. Ogden, 2011; Ogden & Linacre, 2015), and reduced DNA sequencing costs have increased the use of genetic markers in conservation to address such problems (e.g. Metzker, 2010; Funk et al. 2012; Reuter et al. 2015; Shafer et al. 2015; Bleidorn, 2016).

The current project aims to support the listing of all species of mobulid ray on CITES Appendix II by producing data that can be used to develop traceability tools to facilitate species and regional level identification, monitor trade and assist enforcement of regulations. This is only possible in the context of a clear taxonomic framework, where recognised species match meaningful biological units. The current project has contributed considerably towards achieving such a framework for mobulid rays (see Chapter 2), where phylogenomics and species delimitation based on genome-wide SNP data identified an undescribed species of manta ray and found two recently synonymised species to be distinct and reproductively isolated. Furthermore, species and population level genomic datasets produced during this project may be used for future work to develop traceability tools for mobulid rays.

In terms of such work to develop traceability tools, an appropriate first step could be to

evaluate traditional barcoding genes, such as COI sequenced in Chapter 2, for fixed differences at the species level. Whilst this locus alone will not differentiate between all species of mobulid ray (see Chapter 2 Supplementary Figure S2.3), it will likely resolve some individual species (e.g. *Mobula tarapacana*) or identify two or three candidate species, providing a means to immediately narrow the search criteria. In addition, mitochondrial markers such as COI are very cheap to sequence and will readily amplify from poor or degraded samples (Hebert et al. 2003). For these reasons, it is likely to be a useful first marker to examine when presented with an unknown sample (e.g. Helyar et al. 2014). The species level SNP dataset produced herein, representing all available species of mobulid ray, provides further resolution. Mining this dataset for phylogenetically informative SNPs encompassing fixed differences among lineages (see Ogden et al. 2009), and targeting loci driving divergence among species (e.g. Malinsky et al. 2015) will increase robustness of tools since such differences will likely also apply to unknown samples from populations not represented in the existing dataset. Together, these approaches will allow flexibility in a range of circumstances and provide a cost-effective means for enforcement and monitoring agencies to identify species.

In addition, genome-wide SNP data presented herein shows strong population genetic structure in the reef manta ray, *Mobula alfredi* (Chapters 2 and 4), and the shorthorned pygmy devil ray, *Mobula kuhlii* (Chapter 2), providing the first step towards assigning unknown samples to regional location of origin in these species. Furthermore, despite a lack of population structure in the oceanic manta ray, *Mobula birostris,* a previous study has inferred structure based on SNPs in the 90th percentile of F_{ST} among populations that were predefined by sampling locality (Stewart et al. 2016). As such, applying rigorous tests for potentially adaptive outliers to the globally representative set of SNPs presented here will be useful for assigning an unknown sample to region of origin in mobulid rays. The approach involves scanning genome-wide data for markers with highly elevated divergence among populations that do not conform to expectations under a neutral model (e.g. Nielsen et al. 2012). Such markers may be associated with locally adapted genes and can be considered candidates for identifying region of origin in widespread marine fish experiencing divergent ecological conditions. Indeed, this approach has been successfully used for regional traceability of marine fish products, even in cases where population structure is weak at

neutral loci (e.g. Nielsen et al. 2012; Bekkevold et al. 2015; Villacorta-Rath et al. 2016; Jorde et al. 2018).

Finally, through the course of this project, an exceptional global collection of mobulid tissue samples has been compiled, representing efforts and contributions of researchers and organisations worldwide. Unfortunately, the number of samples received exceeds the number for which it was possible to generate genomic data. However, this resource provides opportunities for conducting assignment tests using traceability tools under development on samples of known species and geographical origin, but which were not represented in the genomic datasets described herein. Such validation represents an important aspect of developing robust traceability tools for wildlife conservation and law enforcement (e.g. Nielsen et al. 2012; Villacorta-Rath et al. 2016).

5.5 Priorities for Future Research

Priorities for future research facilitating mobulid conservation were the subject of a recent review, where remaining knowledge gaps collectively identified by researchers in the field of mobulid conservation are discussed in detail (Stewart et al. 2018b; see Appendix I: Published Work). Whilst the current PhD project has provided a substantial contribution to current knowledge with respect to phylogenomics, species diversity, speciation, demographic history and population structure in manta and devil rays, it has also identified other priority questions and directions for future research.

Immediate priorities include formal description of *Mobula* Sp. 1, shown here to be distinct from *Mobula birostris*, and determining the extent of its range, which will be necessary to assess the conservation status of this species and implement appropriate management measures. In addition, comprehensive sampling of manta rays in the Gulf of Mexico can create opportunities for investigating the extent and dynamics of hybridisation between *Mobula* Sp. 1 and *M. birostris* (see Garrick et al. 2014; Macleod et al. 2015; Montanari et al. 2017), as well as investigating patterns of population genetic structure of this currently undescribed species.

Mechanisms underlying the lack of population genetic structure on a global scale in the

oceanic manta ray, *Mobula birostris*, are not currently understood. In Section 5.3, I discuss some hypotheses that may help to explain the observed pattern of global genetic panmixia in this apparently resident species and identify avenues for further research to test these hypotheses. Priorities include understanding spatial ecology and dispersal among life history stages, such as juveniles (e.g. Bruce et al. 2019; Francis et al. 2019) and pregnant females (see Arauz et al. 2019), since knowledge of differential dispersal and structure of such sensitive life history stages will invariably inform conservation measures. In addition, simulation studies similar to that described in Bailleul et al. (2018) will help to disentangle gene flow from past demographic processes as possible mechanisms maintaining these patterns.

Chapter 4 presents the most comprehensive study of population genetic structure in mobulid rays, both in terms of numbers of individuals, and numbers of populations represented, conducted to date. However, this study focusses on manta rays, and at the time of writing, there are no published studies examining patterns of population genetic structure in the remaining devil rays, despite the existence of active fisheries (e.g. Zeeberg et al. 2006; Couturier et al. 2012; Acebes & Tull, 2016). This lack of studies is primarily the result of difficulties obtaining genetic samples, and it is for this reason that researchers can benefit from collaboration with other academics and with stakeholders for effective sample sharing, as demonstrated throughout this PhD project.

With respect to specific populations of interest, such as those that are exploited on a commercial scale, a first step would be to establish whether the stock is distinct from other populations of the same species (Carvalho & Hauser, 1994). Multi-locus approaches are typically applied to samples of representative coverage within and among populations to secure robustness of estimates of differentiation. Analysis of neutral and putatively adaptive loci can help to elucidate weak population structure, and delineate conservation units (Funk et al. 2012; Gagnaire et al. 2015). Longer term, studies of general patterns of effective population sizes and description of demographic histories of mobulid populations associated with fisheries can establish valuable baseline reference points. I suggest evaluating genetic diversity across a gradient of fishing pressure to establish if and where fishing has led to a loss of genetic diversity and population bottlenecks (e.g. Hauser et al. 2002; Pinsky & Palumbi, 2014).

The extent of local adaptation in populations should also be investigated via analysis of

putative outlier loci to generate recommendations for prioritising populations for conservation (e.g. Hemmer-Hansen et al. 2013; Bekkevold et al. 2016; Flanagan et al. 2018). Ideally, such information is used to prioritise management action for the most vulnerable populations and establish quotas. In addition, scanning genome-wide data for markers with elevated divergence among populations is useful in developing traceability tools assigning an unknown sample back to region of origin, and can be invaluable for enforcement of conservation regulations for species exploited in trade (e.g. Nielsen et al. 2012; Bekkevold et al. 2015; Villacorta-Rath et al. 2016; Jorde et al. 2018).

5.6 Concluding Remarks

This PhD project represents a substantial contribution to current knowledge regarding the extent and distribution of diversity in manta and devil rays; a group of conservation concern. The work demonstrates the utility of genomic data in increasing understanding of species and population level diversity, speciation and population genetic structure, thereby bridging knowledge gaps that preclude the implementation of informed and appropriate management strategies. It comes at a time when increased awareness and uptake of genetic and genomic principles both in fisheries management (e.g. Kerr et al. 2017; Mullins et al. 2018) and in conservation more generally (e.g. Cammen et al. 2016; Hoelzel et al. 2019; Sandström et al. 2019) are allowing management measures the best chance of success in achieving long-term sustainability in natural populations.

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Appendix I

Published Work

Stewart JD, Jaine FRA, Armstrong AJ, Armstrong AO, Bennett MB, Burgess KB, Couturier LIE, Croll DA, Cronin MR, Deakos MH, Dudgeon CL, Fernando D, Froman N, Germanov ES, Hall MA, Hinojosa-Alvarez S, **Hosegood JE**, Kashiwagi T, Laglbauer BJL, Lezama-Ochoa N, Marshall AD, McGregor F, Notarbartolo di Sciara, G, Palacios MD, Peel LR, Richardson AJ, Rubin RD, Townsend KA, Venables SK and Stevens GMW. 2018. Research Priorities to Support Effective Manta and Devil Ray Conservation. Frontiers in Marine Science **5**:314. https://doi.org/10.3389/fmars.2018.00314.

The PhD candidate is a co-author on this paper and contributed by leading Section 1: Taxonomy and Diversity, which was written in collaboration with Mike Bennett, Tom Kashiwagi, Silvia Hinojosa-Alvarez and Giuseppe Notarbartolo di Sciara, and contributing Section 5.4: Fisheries Impacts on Genetic Diversity, included within Section 5: Fisheries and Bycatch, which was led by Daniel Fernando.