From rivers to ocean basins – Quantifying sex-specific connectivity in sharks

By

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“Sharks in a tornado. Sharknado. Simply stunning.”

– Sharknado –

A prime example of shark dispersal

Statements and declarations

Declaration of originality

I, Floriaan Devloo-Delva, declare that this thesis entitled, “From rivers to ocean basins – Quantifying sex-specific connectivity in sharks” contains no material that has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in this thesis and to the best of my knowledge and belief, no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does this thesis contain material that infringes copyright.

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The research associated with this this thesis abides by Australian Code for the care and use of animals for scientific purposes. The research conducted for this thesis was approved by the University of Tasmania Animal Ethics Committee (Permit No. A0016727).

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Statement of co-authorship

The following people and institutions contributed to the publication of work undertaken as part of this thesis.

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Paper 3: Accounting for kin sampling reveals genetic connectivity in Tasmanian and New Zealand School Sharks, *Galeorhinus galeus*): Located in Section 2/Chapter 1

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FDD, RMG, PMG, RBT, and PF designed the study. Samples were acquired by SIH and JDM. RMG extracted DNA from the samples. FDD analysed the data with contribution from PF. The manuscript was drafted by FDD. All authors reviewed the manuscript and gave final approval for publication. All authors agree to be accountable for all aspects of the work.

Paper 4/Chapter 3: An R-based tool for identifying sex-linked markers from restriction site-associated DNA sequencing with applications to elasmobranch conservation: Located in Section 3/Chapter 3

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Paper 5/Chapter 5: Sex-specific dispersal patterns of the threatened Northern River Shark, *Glyphis garricki*: Located in Section 4/Chapter 5

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We the undersigned agree with the above stated "proportion of work undertaken" for each of the above published peer-reviewed manuscripts contributing to this thesis:

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General abstract

From rivers to ocean basins – Quantifying sex-specific connectivity in sharks
By Floriaan Devloo-Delva (University of Tasmania)

Globally, elasmobranch populations (sharks and rays) are declining due to increasing anthropogenic and climate pressures. Genetic connectivity between elasmobranch populations is crucial to ensure their persistence and sustain the ecological integrity of ecosystems. Consequently, knowledge on connectivity is important to inform the conservation and management of threatened or commercially important species. Genetic connectivity implies gene flow among discrete populations occurring via the dispersal of individuals outside their population of origin, followed by reproduction — a process that can be biased between sexes (known as sex-biased dispersal or SBD). Male-biased dispersal (MBD) patterns have been observed for some elasmobranchs, yet the extent of SBD in this group is currently unknown.

Knowledge of SBD is often lacking due to technical limitations. Detecting SBD relies on knowledge of reproductive isolation (i.e. population structure) and the appropriate genetic and analytical tools. With improved genetic tools, SBD can be inferred directly with individual-based (e.g. population assignment testing) and population-based (e.g. spatial autocorrelation) approaches, or indirectly, with population-level metrics (e.g. comparing markers with sex-specific inheritance, termed 'mixed-marker'). However, these methods contain many prerequisites and assumptions; for example, the need for discrete populations that have reached genetic drift – gene flow equilibrium. To overcome these caveats, two novel methods have been proposed that warrant testing in elasmobranchs. To evaluate historical SBD (>1,000 generations in the past), the first method contrasts the population diversity and/or structure from genetic markers located on sex chromosomes with that from mitochondrial DNA (mtDNA) or autosomal DNA (auDNA) markers. The second approach looks at the spatial distribution of closely related individuals (i.e. close-kin) to investigate reproductive dispersal over a contemporary timescale (i.e. a single generation).

To date, 90 studies on 50 elasmobranch species have allowed inference of SBD. Most studies tested SBD using a mixed-marker approach, and specifically by comparing mtDNA to auDNA markers. Male-biased dispersal was observed in 25 of the 50 studied species, yet no distinct patterns that explained the presence of MBD emerged. Regarding the remaining species, symmetric gene flow was found across both females and males. While this could suggest equal female and male dispersal, this observation may be obscured by several confounding factors: (i) the characteristics of dispersal (e.g. rate and distance), (ii) the analysis method (e.g. power of genetic markers), and (iii) the experimental design (e.g. sample size and spatial scope). These factors are discussed in detail throughout this thesis.

This thesis uses novel genomic approaches (such as nuclear single nucleotide polymorphisms, or SNPs, and mitochondrial genomes) to provide insights into the patterns of (i) population structure, (ii) sex-chromosome systems, and (iii) SBD in elasmobranchs. My thesis focuses on three shark species that allow me to identify dispersal patterns based on life history, local ecology, population size and different seascape features: the Northern River Shark, *Glyphis garricki*; the School Shark, *Galeorhinus galeus*; and the Bull Shark, *Carcharhinus leucas*.

Specifically, I first examine the current knowledge of population structure and SBD in elasmobranchs, and the tools that are commonly used ('General Introduction'). Secondly, I investigate population structure in the three study species using genomic and close-kin methods. The School Shark and Bull Shark studies are considered separate data chapters (Chapters 1-2), while the Northern River Shark population study was published separately from my thesis, yet the results are be summarised. Thirdly, I further analyse the thousands of SNP markers to identify signals of sex-chromosome systems in my three study species and an additional 18 species with publicly available datasets (Chapters 3-4). To accomplish this, I develop a new analytical approach in the R environment to investigate the presence of sex-linked markers (SLMs). After I identify these SLMs and the spatial scale of population structure, I quantify the amount of sex-specific connectivity between populations (Chapters 5-6). Explicitly, I contrast the auDNA to mtDNA and SLMs to detect signals of long-term dispersal. Where adult sharks were available, I also look at signals of direct, contemporary (intra-generational) dispersal by assigning individuals back to their population of origin. A close-kin framework was expanded to quantify contemporary (inter-generational) philopatry and SBD.

Population structure was found at both broad (Bull Shark) and fine (Northern River Shark) spatial scales. Yet, no signals of population structure were detected for School Sharks between Tasmania and New Zealand. These results allowed me to discuss potential ecological drivers of population structure, such as biogeographical barriers, population sizes and philopatric behaviours. I also discussed how different confounding variables could obscure signals of structure (e.g. sampling bias with sex, life-stage, or family member, and inappropriate time and spatial scale of sampling).

I further demonstrated that 19 out of the 21 studied elasmobranch species contain X and Y chromosomes (overall 3,297 X-linked and 78 Y-linked markers), using the R function I developed for the 'radiator' package. The SLMs can be employed to contrast autosomal SNPs and mitochondrial genome data to investigate SBD. Given the broad taxonomic range in my results, I discussed how the XX/XY sex-chromosome system in elasmobranchs may have evolved from ancestral autosomes. This hypothesis is supported by the large number of highly conserved SLMs (n = 710) within the order of Carcharhiniformes. The Y-linked markers also allowed me to develop a rapid PCR-based test to identify

the genetic sex of White Shark (*Carcharodon carcharias*) samples, which has direct management applications.

Lastly, I found supporting evidence of MBD in the Northern River Shark and the Bull Shark; whereas the lack of population structure for the School Shark did not allow further investigation of SBD. Specifically, for the Northern River Shark, the kinship approach showed a slight bias towards male dispersal (63 % of the dispersal was attributed to males), whereas SNPs and mtDNA demonstrated strong philopatric signals and no SBD. The Bull Shark kinship results revealed a stronger signal of MBD (100 %), yet only few kin pairs were found. Therefore, this result needs to be verified with larger sample sizes. However, at an intra-ocean-basin scale, the mixed-marker approach (including X-linked markers) suggested female philopatry for the Bull Shark.

My final discussion synthesised the dispersal patterns observed from my three study species and examines the potential ecological and evolutionary drivers for these patterns. I critically compared the genetic and analytical approaches for the detection of population structure and SBD. I concluded that genomic tools have improved the resolution of population connectivity analyses, although sampling biases can have a substantial effect, and that the close-kin approach will prove a valuable tool to assess dispersal at very fine spatial and temporal scales. Overall, the potential implications of these quantitative findings for management were highlighted and future work is proposed.

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Table of content

List of figures

Figures are labelled according to Section, Chapter, and figure order within a chapter. For example, 'Figure.4.5.2' is figure 2 in Chapter 5, which is in Section 4.

List of tables

Tables are labelled according to Section, Chapter, and Table order within a chapter. For example, 'Table.4.5.2' is figure 2 in Chapter 5, which is in Section 4.

List of abbreviations

Abbreviation	Meaning	Abbreviation	Meaning
ADR	Adelaide River	**N_{au}**	Number of samples with autosomal genotypes
Aic	Corrected assignment index	**N_e**	Effective population size
AMOVA	Analysis of Molecular Variance	**N_{mt}**	Number of samples with mitochondrial sequences
A_r	Allelic richness	**N_{nu}**	Number of samples with nuclear genotypes
ARS	Arabian Sea	**nuDNA**	Nuclear DNA
auDNA	Autosomal DNA	**N_{x}♀**	Number of female samples with X chromosome genotypes
AUS	Australia	**N_{x}♂**	Number of male samples with X chromosome genotypes
BMB	Blue Mud Bay	**OKI**	Okinawa
BRZ	Brazil	**PCR**	Polymerase chain reactions
CAR	Caribbean Sea	**PNG**	Papua New Guinea
CKMR	Close-kin mark-recapture	**POP**	Parent-offspring pair
CLR	Clarence River	**R**	Relatedness
CMR	Capture-mark-recapture	**RADseq**	Restriction-site associated DNA sequencing
COR	Costa Rica	**ROR**	Roper River
DAPC	Discriminant Analysis of Principal Components	**r_{sp}**	Spatial autocorrelation
DAR	Daly River	**RUN**	Reunion
DArTcap	Diversity Arrays Technology DNA capture	**SAF**	South Africa
DArTseq	Diversity Arrays Technology sequencing	**SAR**	South Alligator River
ddRAD	Double-digest Restriction-site associated DNA	**SBD**	Sex-biased dispersal
DWC	Darwin Coastal	**SCS**	Sex-chromosome system
EAR	East Alligator River	**SEY**	Seychelles
E-ATL	Eastern Atlantic	**SNP**	Single nucleotide polymorphism
E-PAC	Eastern Pacific	**SRL**	Sri Lanka
FIJ	Fiji	**SYH**	Sydney Harbour
F_{IS}	Inbreeding coefficient	**TAI**	Thailand
F_{IS}2.5	Lower confidence interval (2.5 %) of the inbreeding coefficient	**TL**	Total length
F_{IS}97.5	Upper confidence interval (97.5 %) of the inbreeding coefficient	**TOR**	Towns River
FSP	Full-sibling pair	**TRI**	Trinity Inlet
FZR	Fitzroy River	**uH_E**	Unbiased expected heterozygosity
GOC	Gulf of California	**UNK**	Unknown
GOM	Gulf of Mexico	**URR**	Urauchi River
H	Haplotype richness	**vAIc**	variance of corrected assignment index
h	haplotype diversity	**VDG**	Van Diemen Gulf
H_O	Observed heterozygosity	**VIR**	Victoria River
HSP	Half-sibling pair	**W-ATL**	Western Atlantic
IND	Indonesia	**WER**	Wenlock River
IWP	Indo-West Pacific	**WNA**	West North Atlantic
JAP	Japan	**xDNA**	X chromosome DNA
LLR	Log likelihood ratio	**yDNA**	Y chromosome DNA
mAIc	Mean of corrected assignment index	**π_{au}**	Autosomal DNA nucleotide diversity
MBD	Male-biased dispersal	**π_{mt}**	Mitochondrial DNA nucleotide diversity
M_o	Number of monomorphic markers	**π_{nu}**	Nuclear DNA nucleotide diversity
MOZ	Mozambique	**π_{x}♀**	X chromosomes DNA nucleotide diversity for females
mtDNA	Mitochondrial DNA	**π_{x}♂**	X chromosomes DNA nucleotide diversity for males

1. SECTION 1 – General introduction, aims and thesis structure

1.1. Dispersal and connectivity

Dispersal and population structure are fundamental in the fields of behaviour, ecology, fisheries, conservation, and evolutionary biology (Clobert, Baguette, Benton, & Bullock, 2012). Dispersal is the process that determines the biological and evolutionary dynamics of spatially structured populations, resulting in population resilience, adaptive potential, and a species' persistence (Bohonak, 1999; Ronce, 2007). The dispersers have an important ecological role in sustaining the viability of populations. For example, dispersal can rescue small populations from extinction by mitigating the effects of drift and decreasing the mutational loads (Caughley, 1994; Lowe, Kovach, & Allendorf, 2017; Ralls, Sunnucks, Lacy, & Frankham, 2020). Similarly, with ongoing changes to habitats, such as climate change and habitat fragmentation, the dispersal ability of a species may be decisive for its survival (Kokko & López-Sepulcre, 2006). Overall, dispersal knowledge is paramount to improve the management of natural populations; yet, few empirical and integrated analytical studies are available to date (Duputié & Massol, 2013; Li & Kokko, 2019).

Dispersal is defined as the movement of organisms with the potential consequence of gene flow across space (Ronce, 2007). Dispersal is influenced by the physical properties of the landscape (e.g. geographic barriers or distance to available habitat) and by the mobility of the organism, such as morphological, physiological, or behavioural adaptations (Bonte et al., 2012). Two types of dispersal are recognised: natal dispersal, where immature individuals move from their natal site to the site of first reproduction, and breeding dispersal which includes any subsequent movement. The process of dispersal can be divided into three stages: the departure (or emigration), vagrant (or travelling) and settling (or immigration) stages (Ronce, 2007). Dispersal can affect the growth and vital rates of a population, termed demographic connectivity. When dispersal results in gene flow with evolutionary consequences, this is termed genetic connectivity (Lowe & Allendorf, 2010).

Population structure is a direct consequence of limited or uneven dispersal between populations, often due to physical or behavioural barriers, where a population is defined as a demographically-isolated group of individuals of the same species (Waples & Gaggiotti, 2006). In other words, an individual within the population has an equal opportunity of mating and producing offspring with all other members of that same population. Populations are often also termed 'stocks' or 'demes' in the fields of fisheries and demographic modelling. A network of connected populations is typically referred to as a 'metapopulation'.

Dispersal, and subsequently population structure, have evolved under a complex interaction between selective pressures, for instance the balance between the costs and benefits of dispersal (Bonte et al., 2012; Duputié & Massol, 2013). The costs can be categorised as energetic, time, risk, and opportunity costs, which will influence different stages of dispersal (Bonte et al., 2012). The advantages of dispersal mainly reflect individual or population-level fitness gains; for example, competition avoidance (e.g. between kin; Hamilton & May, 1977), reducing the risk of inbreeding depression (Perrin & Mazalov, 1999), or dealing with the spatiotemporal variability of resources or environments (Comins, Hamilton, & May, 1980; Hamilton & May, 1977). However, most studies show that dispersal evolves rarely under a single driver, but rather involves an interaction between drivers, where fitness benefits are not necessarily experienced directly (Waser, Nichols, & Hadfield, 2013). Many species exhibit a sex bias for dispersal rates and distances (Trochet et al., 2016). For example, one sex may continue returning to the same site or region for mating or parturition purposes (i.e. philopatry), while the other sex may undertake the majority of reproductive dispersal between sites (sex-biased dispersal, or SBD).

The evolution of SBD has been driven by sex-specific differences in selective pressures. The most investigated driver is the unequal competition between females and males for resources or mates (Dobson, 1982; Greenwood, 1980; Hamilton, 1967). For instance, the sex with a high investment in reproduction may choose to stay in familiar habitat to defend local resources (e.g. nursery sites or food), and consequently ensure offspring survival. On the other hand, the sex with less energetic input may undertake 'mate-searching' dispersal to escape local competition for mates, and hence increase its reproductive output (Greenwood, 1980). Nonetheless, these drivers do not explain why individuals disperse if competition exists in other sites. The theory of kin competition avoidance suggests that SBD has evolved to reduce competition between parents and their offspring or between siblings, thus indirectly increasing the overall fitness of individuals in the population (Brom, Massot, Legendre, & Laloi, 2016; Comins et al., 1980; Hamilton & May, 1977). Sexual differences in dispersal could also be driven by inbreeding avoidance (Perrin & Mazalov, 1999, 2000). Both kin competition and inbreeding avoidance are more likely to drive natal dispersal rather than breeding dispersal (Lawson Handley & Perrin, 2007).

The differences in genetic architecture between females and males, due to the presence of sex chromosomes, has also been suggested as a driver for SBD (Brom, Massot, & Laloi, 2018). The sex-specific inheritance of sex chromosomes could favour enhanced social cooperation in the homogametic sex (XX or ZZ) if altruistic genes are located on the homogametic chromosomes (Whitney, 1976). Specifically, higher social cohesiveness between full sibling females (for XX) or males (for ZZ) may lead to direct and indirect benefits from philopatry for this sex (Haig, 2000; Kawecki, 1991). More recently, Brom et al. (2018) investigated the possibility of dispersal genes located on the

heterogametic sex chromosome (Y or W). Because heterogametic full sibling relatedness depends on the Y/W chromosome (R = 1 for Y/W and R = 1/2 for X/Z) while homogametic full sibling relatedness depends on the X/Z chromosome (R = 3/4 for X/Z), and Y(W)-linked genes are three times less numerous than the X(Z)-linked genes, selective pressures (such as kin competition avoidance) will have a higher impact on the heterogametic sex, which can lead to increased dispersal for this sex.

Generally, the selective pressures that drive the evolution of SBD are often associated with mating systems, parental care, sexual dimorphism, and territoriality (Trochet et al., 2016). Typically, mammals have been shown to exhibit male-biased dispersal (MBD; Dobson, 2013; Greenwood, 1980), while female-biased dispersal (FBD) appears predominant in birds (Clarke, Sæther, & Røskaft, 1997; Greenwood, 1980). This observation led Greenwood (1980) to postulate that a female-defence polygyny would drive the evolution of MBD and a resource-defence monogamy could result in FBD (Lawson Handley & Perrin, 2007). Other covariates that should be considered are demographic conditions (such as population density or age structure) and environmental factors, for example any sex-specific spatiotemporal stochasticity of fitness between patches (Gros, Poethke, & Hovestadt, 2009; Henry, Coulon, & Travis, 2016). On the other hand, the cost of dispersal, the advantage of habitat familiarity, and the benefits from kin-cooperation will discourage dispersal and select for more philopatric behaviours (Bonte et al., 2012; Lawson Handley & Perrin, 2007). Thus, philopatry may be more favourable if the fitness costs of staying in the same location, such as kin competition or inbreeding depression, are lower than the dispersal costs (e.g. finding suitable habitat, or the energetic cost to develop dispersal phenotypes).

The exact costs and benefits of dispersal will differ between species and geographical regions. For instance, species can exhibit different population densities, sex-ratios, body sizes, temperature tolerances, or habitat preferences (Clobert et al., 2012; Trochet et al., 2016) or geographical differences such as habitat patchiness, spatiotemporal habitat disturbances, or biogeographical barriers (Bohonak, 1999; Bonte et al., 2012). These factors can obscure the investigation of patterns and drivers of dispersal and it is critical that studies focus on the correct experimental design, considering these confounding factors, and the appropriate analytical methods to better assess SBD and philopatry.

1.2. Methods to study dispersal and sex-biased dispersal

Several approaches exist to investigate the intra- and inter-generational connectivity of populations. The analytical approaches to measure dispersal and SBD can be categorised into two groups: 1) direct methods, where the actual movement — which may lead to gene flow — is observed, and 2) indirect methods, which measure the consequences of gene flow (Gagnaire et al., 2015). Various tools exist to

estimate dispersal rates, including tagging and genetic analysis. The use of genetic markers (such as allozymes, mitochondrial DNA or mtDNA, microsatellites, and single nucleotide polymorphisms or SNP) is the most common approach, as it has proven powerful in measuring connectivity at large spatiotemporal scales (Hedgecock, Barber, & Edmands, 2007; Koenig, Van Vuren, & Hooge, 1996).

Direct methods measure dispersal based on the ability to identify immigrant individuals from geographically or genetically distinct groups. Conventionally, this is done with capture-mark-recapture (CMR) studies, where individuals tagged in one population can be recaptured in another (e.g. Hutchings & Gerber, 2002). However, this method is bound by many logistic difficulties (see Cayuela et al., 2018); for example, the spatial restriction of a study area may discourage the study of long-distance migration (Koenig et al., 1996). Electronic tags have the ability to follow animals in real-time, yet their cost often imposes small sample sizes. On the other hand, genetic tags (e.g. from bi-parentally inherited autosomal DNA, or auDNA) have the power to assign post-dispersal individuals to their population of origin and hence detect direct first-generation (F_0) immigrants (Berry, Tocher, & Sarre, 2004; Manel, Gaggiotti, & Waples, 2005). However, this method can be complicated by the existence of unsampled source populations or high genetic connectivity (Berry et al., 2004; Lawson, van Dorp, & Falush, 2018).

Direct methods like individual-based assignment tests can reveal 'instantaneous' SBD by investigating sex-ratios of direct immigrants (Favre, Balloux, Goudet, & Perrin, 1997; Mossman & Waser, 1999) or return migrations based on genetically-recaptured individuals at the parturition site (e.g. Feldheim et al., 2014). Other statistical tests that measure SBD at a population-level with recombining auDNA include contrasting fixation indices (F_{ST}), inbreeding coefficients (F_{IS}), the mean and variances of the corrected assignment index (mAIc and vAIc), relatedness (R) and spatial autocorrelation (r_{sp}) between sexes (Banks & Peakall, 2012; Goudet, Perrin, & Waser, 2002; Paetkau, Slade, Burden, & Estoup, 2004; Prugnolle & de Meeus, 2002). In the more dispersive sex, average F_{ST}, mAIc, r, and R are expected to be lower and F_{IS} and vAIc will be higher due to a mixture of resident and immigrant individuals (Goudet et al., 2002). Due to the recombination of the auDNA, any sex-specific signal will be lost in the second generation. Consequently, these methods do not necessarily indicate genetic contribution from migrants to the local gene pool (i.e. effective or reproductive dispersal). If migrants differ from local individuals in terms of life history, behaviour, or are maladapted to the local environment (Bonte et al., 2012), these immigrants will have no or minimal influence the local gene pool and any potential rescue effect to small inbred populations may be lost (Frankham et al., 2017). Similarly, if the offspring from migrants are maladapted (i.e. outbreeding depression), they may have negligible effect on population persistence (Rhymer & Simberloff, 1996). Nonetheless, direct methods can often provide valuable quantitative estimates of dispersal and SBD.

Indirect methods investigate the consequences of dispersal through differences in allele frequencies or coalescent reconstruction. Allele frequencies are commonly compared using Wright's F_{ST} (Wright, 1931) and its derivatives (e.g. Weir & Cockerham, 1984). Using the Island Model, the number of migrants per generation (Nm) can be estimated by $Nm = (1-F_{ST})/(4F_{ST})$, although the model assumptions, such as mutation–migration–drift equilibrium, are rarely met (Guillot, 2011; Whitlock & McCauley, 1999). Coalescent theory (Kingman, 1982) allows likelihood-based approaches to estimate effective dispersal based on genealogical and mutational history of genes (Broquet & Petit, 2009; Cayuela et al., 2018). Coalescent methods still rely on independence of loci, selective neutrality, and migration–drift equilibrium. It often takes thousands of generations to reach equilibrium; thus, indirect methods do not reflect contemporary connectivity. Due to the decoupling of demographic and genetic processes, contemporary migration may have stopped, while historical connectivity can still be detected (Fig. 1.2.1; Schregel, Kopatz, Eiken, Swenson, & Hagen, 2017; Waples & Gaggiotti, 2006). However, direct methods can be assessed to circumvent this bias.

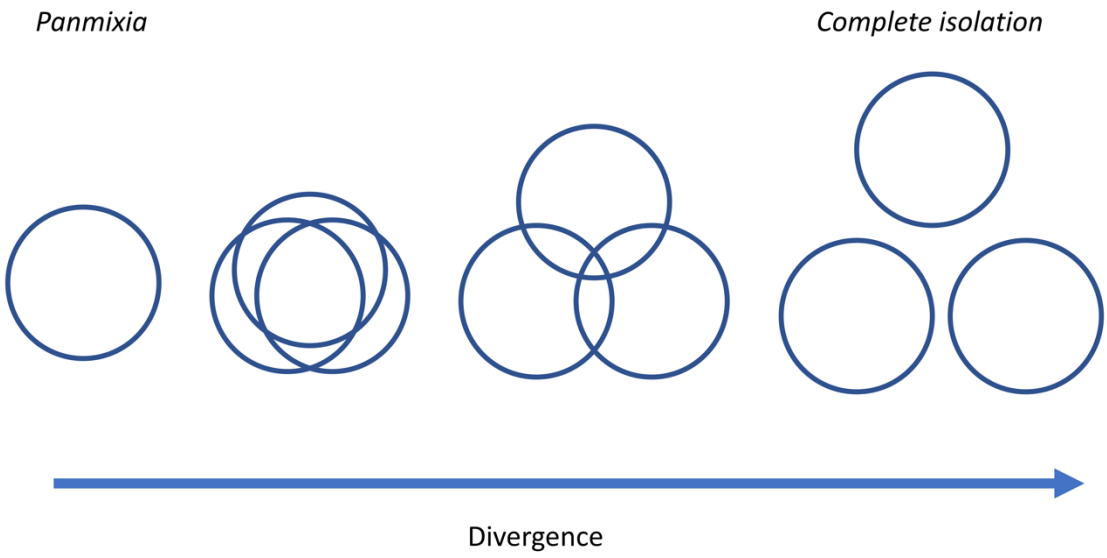

Figure 1.2.1: A conceptual figure that illustrates the continuous process of population divergence; adapted from Waples and Gaggiotti (2006). Most F_{ST}-based approaches would have difficulty in differentiating total panmixia from a 'strong connectivity' scenario if insufficient time has passed for each group to acquire private alleles.

Sex-biased gene flow (also termed effective SBD) can be identified indirectly by contrasting genetic differentiation (e.g. F_{ST}) from markers that are not equally inherited between sexes — most commonly mtDNA vs auDNA (Prugnolle & de Meeus, 2002; Wilson Sayres, 2018). This population-level 'mixed-marker' approach has been applied to a range of vertebrate species, such as turtles (e.g. Karl, Bowen, & Avise, 1992), whales (e.g. Lyrholm, Leimar, Johanneson, & Gyllensten, 1999), bats (e.g. Petit, Balloux, & Goudet, 2001), and ducks (e.g. Peters, Bolender, & Pearce, 2012). While the comparative marker-F_{ST} approach to investigating SBD is common, it contains a number of caveats. Firstly, mtDNA is

expected to show stronger population differentiation than auDNA in situations when SBD is lacking (Ballard & Whitlock, 2004). Since mtDNA is haploid and maternally inherited, it experiences one-quarter the effective population size (N_e) compared to auDNA, causing it to be more sensitive to the effects of genetic drift, selection, and demographic processes such as population size changes (Lawson Handley & Perrin, 2007; Webster & Sayres, 2016; Wilson Sayres, 2018). For example, populations that have undergone recent spatial or demographic expansion will exhibit a 'mitonuclear discordance', where mitochondrial population divergence was one order of magnitude higher than the nuclear markers (e.g. Larmuseau, Raeymaekers, Hellemans, Van Houdt, & Volckaert, 2010; Toews & Brelsford, 2012). Similarly, sex-specific differences in N_e can be driven by sex-biased demographic forces, other than dispersal; for example, unequal sex ratios, reproductive skew between sexes (high variance in fitness), non-random mating (monogamy or polygamy), or differences in generation time and mating cycle (Karl, 2008; Wilson Sayres, 2018). Further, the mixed-marker approach uses F_{ST} values calculated under an ideal Island Model (Wright, 1931), which, as described above, makes several biologically unrealistic assumptions (Guillot, 2011; Whitlock & McCauley, 1999). Other marker comparisons, such as mtDNA vs Y chromosome DNA (yDNA) or auDNA vs X-chromosome DNA (xDNA), have been proposed and applied to reduce these biases (Petit, Balloux, & Excoffier, 2002; Seielstad, Minch, & Cavalli-Sforza, 1998; Wilkins & Marlowe, 2006; Yannic, Basset, Buchi, Hausser, & Broquet, 2012). Because mtDNA and yDNA are non-recombining and uniparentally inherited, they each form a single, linked marker and are more sensitive to evolutionary forces, such as selection (Wilson Sayres, 2018). On the other hand, auDNA and xDNA show recombination, which leads to unlinked markers that are less affected by selection (Schaffner, 2004). This multi-locus data will show higher statistical power to detect demographic differences between females and males (Wilkins & Marlowe, 2006).

The direct and indirect approaches to estimate connectivity and SBD (described above) are at opposite ends of the temporal spectrum (Fig. 1.2.2). Some software such as BayesAss can detect second-generation migrants in moderate to highly-differentiated populations (F_{ST}>0.05; Faubet, Waples, & Gaggiotti, 2007). Nonetheless, only a few studies have estimated dispersal based on second-generation migrants because it requires low migration rates (m<0.1) and negligible drift over the past two generations (Broquet & Petit, 2009; Dutta et al., 2013; Nykänen et al., 2018). Recently, a new method has been proposed based on close-kin mark-recapture (CKMR) framework to look at reproductive dispersal within one to two generations (Bravington, Skaug, & Anderson, 2016; Feutry et al., 2017). Instantaneous dispersal (without evidence of reproduction) can be observed directly by analysing the spatiotemporal distribution of parent-offspring pairs (POP) or full-sibling pairs (FSP; e.g. Harrison et al., 2012; Kanno, Vokoun, & Letcher, 2011; Mourier & Planes, 2013; Salles et al., 2016). Reproductive dispersal can be inferred by identifying cross-cohort half-sibling pairs (HSPs) that are

distributed across natal sites. Under the assumption that HSPs have not moved from their natal site, this indicates that one of the parents dispersed between breeding seasons and subsequently produced offspring. The mtDNA can provide a likelihood for whether the HSPs are maternally or paternally related, and hence if the mother or father moved between populations (Feutry et al., 2017). These kinship-based methods have the advantage of quantifying dispersal even at high dispersal rates (FST<0.05, m>0.1), but on the other hand, they require exhaustive sampling (Conn, Bravington, Baylis, & Ver Hoef, 2020; Palsbøll, Zachariah Peery, & Berube, 2010).

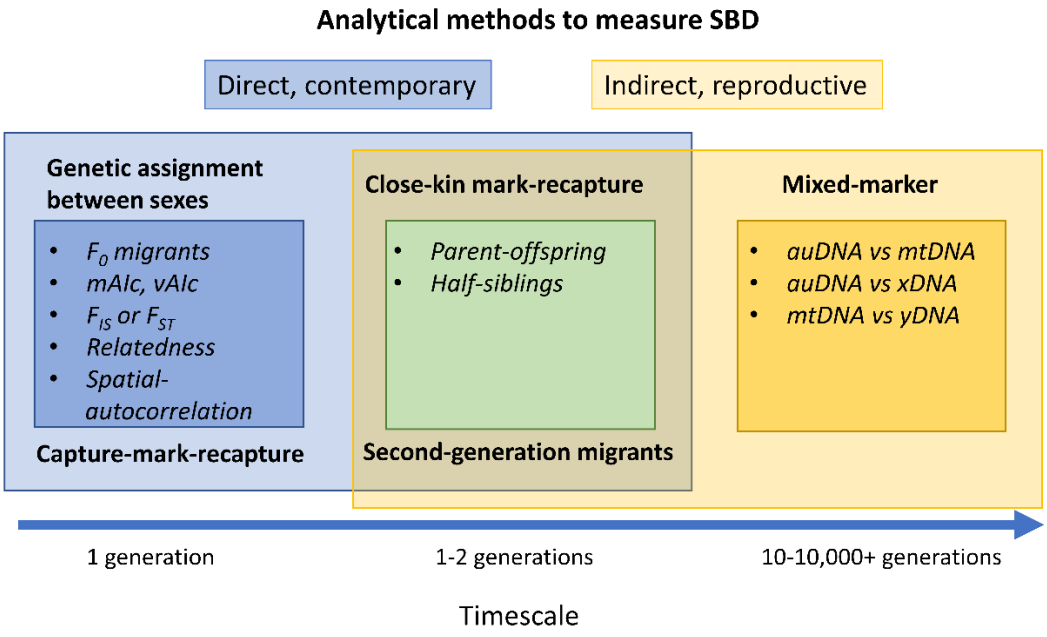

Figure 1.2.2.: Analytical methods to study sex-biased dispersal which provide information at different timescales (from intragenerational to thousands of generations). The term capture-mark-recapture includes the electronic recaptures (i.e. pings) from satellite or acoustic tags.

1.3. Dispersal and population structure in chondrichthyans

Over a third (37 %) of the 1,261 chondrichthyan fishes (sharks, rays, and chimaeras) are globally threatened with extinction (Dulvy et al., 2021; Ebert, Wintner, & Kyne, 2021; IUCN, 2021). The main threats include fisheries (large-scale industrial, small-scale artisanal, or recreational) and habitat modification (anthropogenic or environmental). Coastal and euryhaline species are particularly susceptible to these threats, given their proximity to human populations (Grant, Kyne, Simpfendorfer, White, & Chin, 2019; Knip, Heupel, & Simpfendorfer, 2010). The low growth rates and late age-at-maturity of chondrichthyans can make them vulnerable to these threats (Dulvy et al., 2014; Musick, 1999; Pacoureau et al., 2021). Management of chondrichthyans includes both the assessment of these threats and other biological parameters (e.g. population size or intrinsic population growth rates) at the appropriate spatial scale, as well as regulating catch rates or preserving certain habitats (e.g.

nursery areas) from human impact. Consequently, evidence of (sex-specific) demographic or genetic connectivity, or the lack thereof, is essential to aid management decisions. For example, if females are philopatric to a nursery area, but males disperse further, the removal of sharks in this area will lead to a local depletion of females (which ultimately controls recruitment) that is unlikely to recover rapidly from immigration (Hueter, Heupel, Heist, & Keeney, 2005).

Unlike teleost fishes — which have both passive and active larval dispersal — elasmobranchs (sharks and rays) actively disperse after reaching sexual maturity, making them fascinating study organisms to improve our knowledge on dispersal (Chapman, Feldheim, Papastamatiou, & Hueter, 2015; Frisk, Jordaan, & Miller, 2014). To date, many studies have focused on elasmobranch movement using approaches such as active acoustic tracking (e.g. George, Martins, Heupel, & Simpfendorfer, 2019), passive acoustic telemetry (e.g. Hoenner et al., 2018), satellite telemetry (e.g. Stevens, Bradford, & West, 2010), CMR (e.g. Francis, 2010), and genetic methods (see review Green, Simpfendorfer, & Devloo-Delva, in press). While tagging and telemetry studies provide valuable information regarding habitat utilisation, physiological response, and mortality rates (Espinoza, Cappo, Heupel, Tobin, & Simpfendorfer, 2014; Knip, Heupel, & Simpfendorfer, 2012; Pillans, Fry, Steven, & Patterson, 2020), my thesis focuses on the genetic methods as they measure reproductive movements, and consequently hold importance for the persistence of populations and species.

Green et al. (in press) identified 157 studies to date that have investigated population structure in chondrichthyans using either genetic, life-history, microchemistry, parasite, telemetry, or a combination of these approaches. The majority (80 %) of those studies have used genetic methods and only six used a multi-method approach that mostly combined life-history, genetic, and telemetry data (e.g. Gardner & Ward, 1998; Jorgensen et al., 2010). The analysis of mtDNA regions, such as NADH dehydrogenase 2 and control region, is most popular in population genetic studies (Green et al., in press; Heist, 2012). Mitochondrial DNA tends to mutate faster than auDNA (Ballard & Whitlock, 2004) and is also expected to show stronger differentiation compared to auDNA due to the effect of genetic drift on this haploid, maternally-inherited marker (Avise et al., 1987). Microsatellites have also proven useful to study dispersal and population structure in elasmobranchs (e.g. Daly-Engel et al., 2012; Phillips, Chaplin, Peverell, & Morgan, 2017; Pirog et al., 2019). Due to a large amount of variation, microsatellites are valuable for studying structure at fine temporal or spatial scales, but the high rates of allelic homoplasy tend to cause underestimation of genetic differentiation at larger scales (Balloux, Brunner, Lugon-Moulin, Hausser, & Goudet, 2000; Green et al., 2019). Increased statistical power can be obtained from thousands of SNPs (Layton et al., 2020; Morin, Martien, & Taylor, 2009). Therefore, SNPs are becoming more prevalent in elasmobranch research (Dimens, Willis, Grubbs, & Portnoy, 2019; Manuzzi, Zane, Muñoz-Merida, Griffiths, & Veríssimo, 2018; Momigliano et al., 2017). Many of

the studies exploring chondrichthyan population structure used both mtDNA and auDNA (Green et al., in press), which may allow the study of sex-specific demographic events on contemporary (most recent generations; e.g. 1–3 generations) and evolutionary (influenced by mutation-drift; e.g. >1,000 generations) timescales (Prugnolle & de Meeus, 2002).

General dispersal patterns (rate, distance, and sex-bias) in elasmobranchs are challenging to distinguish due to confounding differences in life-history and biogeography between species, as well as methodological constraints; however, some trends emerge (see Heist, 2012). Large pelagic sharks, such as the Shortfin Mako, *Isurus oxyrhincus*; Blue Shark, *Prionace glauca*; Whale Shark, *Rhincodon typus*; and White Shark, *Carcharodon carcharias*, show little genetic divergence across their range (Bailleul et al., 2018; Castro et al., 2007; Jorgensen et al., 2010; Nikolic et al., 2020; Schrey & Heist, 2003; Vignaud et al., 2014). Coastal or insular elasmobranchs, such as the Scalloped Hammerhead, *Sphyrna lewini*; lemon sharks, *Negaprion* spp.; and Galapagos Shark, *C. galapagensis*; have shown genetic connectivity along continuous coastlines, but minimal dispersal across known biogeographic barriers (Daly-Engel et al., 2012; Pazmiño et al., 2018; Schultz et al., 2008). The major marine biogeographic barriers (due to distance, land bridge, or oceanographic features) include the Isthmus of Panama, the Mid Atlantic Barrier, the Benguela Upwelling, the Indo-Pacific Barrier, and the East Pacific Barrier (Dudgeon et al., 2012; Rocha, Craig, & Bowen, 2007). Within the Indo-West Pacific (IWP) region, Pember, Chaplin, Loneragan, and Braccini (2020) reviewed the population structure of 20 whaler shark species (family Carcharhinidae) and found that small to medium-bodied (<250 cm total length) and reef-associated sharks (e.g. Whitetip Reef Shark, *Triaenodon obesus*) exhibit strong genetic population structure, driven by open ocean distance (Whitney, Robbins, Schultz, Bowen, & Holland, 2012). For instance, the Blacktip Reef Shark, *C. melanopterus*, had limited dispersal across long stretches (>100 km) of deep (>1,000 m) water (Maisano Delser et al., 2019; Vignaud, Clua, Mourier, Maynard, & Planes, 2013). Other biogeographic barriers in the IWP, such as deep-water trenches, or historical sea-level changes forming land bridges or current shifts, have strongly affected smaller species with limited coastal distributions and low vagility (such as the Zebra Shark, *Stegostoma tigrinum*; or epaulette sharks, *Hemiscyllium* spp.) at small spatial distances (e.g. Dudgeon, Broderick, & Ovenden, 2009; Dudgeon et al., 2020). Similarly, euryhaline species (e.g. the Largetooth Sawfish, *Pristis pristis*), which are associated with coastal and riverine habitats, have shown discrete population structure due to patchiness of suitable nursery habitat (Feutry, Kyne, et al., 2015; Phillips et al., 2017).

Dispersal distance is commonly measured with tagging or telemetry studies. For example, White Sharks can move more than 20,000 km (Bonfil et al., 2005), while reef-associated species (e.g. Blacktip Reef and Whitetip Reef Shark) only move up to 20–130 km (Heupel, Papastamatiou, Espinoza, Green, & Simpfendorfer, 2019; Whitney, Pyle, Holland, & Barcz, 2012). Nonetheless, very little is known about

reproductive dispersal distance, since the lack of genetic divergence does not necessarily indicate contemporary genetic connectivity (Waples & Gaggiotti, 2006). Individual-assignment methods have identified several putative second-generation migrants in the Blacknose Shark, *Carcharhinus acronotus*; Thornback Ray, *Raja clavata*; and the Basking Shark, *Cetorhinus maximus*; suggesting reproductive movement of tens to hundreds of kilometres for these species (Dimens et al., 2019; Lieber et al., 2020). Similarly, Feutry et al. (2017) were able to identify parental movement, followed by reproduction, of the Speartooth Shark, *Glyphis glyphis*, between two river systems nearly 150 km apart.

1.4. PAPER 1 – Philopatry and sex-biased dispersal in elasmobranchs

Phillips, N. M.; Devloo-Delva, F.; McCall, C. & Daly-Engel, T. S. (2021). You can go your own way: reviewing the genetic evidence for sex-biased dispersal in elasmobranchs. *Reviews in Fish Biology and Fisheries*: Provided in Appendix A

The most recent reviews of philopatry and sex-biased dispersal in elasmobranchs are represented by Chapman et al. (2015), Flowers et al. (2016) and a paper I have co-authored (Phillips, Devloo-Delva, McCall, & Daly-Engel, 2021). Chapman et al. (2015) and Flowers et al. (2016) covered 174 publications on philopatry of 75 species (until December 2015), and Phillips et al. (2021) examined 90 publications on sex-biased dispersal (SBD) in 50 species (until June 2020). Thus far, only 33 elasmobranch species showed evidence of female reproductive philopatry, while 26 species demonstrated signals of SBD, with all but one male-biased. The most common approach in these studies was to analyse F_{ST} values from genetic markers that had sex-specific inheritance patterns (only maternally inherited or mixed-marker approach). For instance, female reproductive philopatry was assumed if mtDNA indicated significant difference between sampling locations (all 33 species; Chapman et al., 2015; Flowers et al., 2016). Female philopatry was present in most investigated elasmobranch species, from large-bodied pelagic (e.g. White Shark) to medium-sized coastal (e.g. hammerhead sharks) and small demersal (e.g. Red Stingray, *Dasyatis akajei*) species (Blower, Pandolfi, Bruce, Gomez-Cabrera, & Ovenden, 2012; Chapman, Pinhal, & Shivji, 2009; Li, Chen, et al., 2015). Although philopatric behaviour is mostly identified on a regional level (~100 km), females from two species (Largetooth Sawfish and Speartooth Shark) showed a strong mtDNA population signal, indicating philopatry to their site of birth (i.e. natal philopatry; Feutry et al., 2017; Feutry, Kyne, et al., 2015). Other studies have demonstrated natal philopatry in the Lemon Shark, *N. brevirostris* and Blacktip Reef Shark using genetic identification of recaptures or parent-offspring relationships (Feldheim et al., 2014; Mourier & Planes, 2013). Contrary to the overwhelming evidence of philopatry globally, certain species such as the sharpnose sharks, *Rhizoprionodon* spp., have not shown any mitochondrial philopatric signal (Davis, Suárez-Moo, & Daly-Engel, 2019; Heist, Musick, & Graves, 1996; Ovenden et al., 2011).

If auDNA showed less genetic divergence than mtDNA between sampling locations, it was assumed that males must be facilitating greater gene flow (24 of the 50 species; Phillips et al., 2021), or vice versa if auDNA exhibited greater structuring than mtDNA (one species, *Carcharhinus galapagensis*; Pazmiño, Maes, Simpfendorfer, Salinas-de-León, & van Herwerden, 2017). However, as discussed in Section 1.2, differences in F_{ST} may arise irrespective of SBD and elasmobranchs exhibit life-history traits and reproductive strategies that may bias inferences of SBD from comparative F_{ST} approaches; for example, different sex-ratios between populations. Further, skewed female and male reproductive success can result from parthenogenesis (reviewed in Ovenden, Dudgeon, Feutry, Feldheim, & Maes, 2018), polyandry (e.g. Green, Appleyard, White, Tracey, & Ovenden, 2017; Lyons, Chabot, Mull, Holder, & Lowe, 2017), intrauterine cannibalism (e.g. Gilmore, Putz, & Dodrill, 2005), and different breeding cycles between females and males (e.g. Papastamatiou et al., 2013; Walker, Punt, Taylor, & Brown, 1999). However, the effect and magnitude of these traits on SBD inference have not been studied in many elasmobranchs. To overcome these drawbacks, individual-level assignment methods have been applied to 16 elasmobranch species (e.g. Corrigan, Huveneers, Stow, & Beheregaray, 2016; Corrigan et al., 2018; Day, Clark, Williamson, Brown, & Gillings, 2019), yet only provided evidence of SBD for six species — all male-biased (e.g. Roycroft, Le Port, & Lavery, 2019; Sandoval-Castillo & Beheregaray, 2015).

Interestingly, SBD was not detected in half of the investigated elasmobranch species. Potential explanations include, driving forces for male philopatry (e.g. mate accessibility), equal female and male dispersal, or unsuitable experimental design, analytical, or genetic approaches. Large mobile pelagic elasmobranchs are usually scarcely distributed in the oceans and mating opportunities are few. The patchiness of suitable nursery habitat could present a consistent aggregation of females that are accessible for mating after parturition. Similarly, if male-biased dispersal (MBD) is driven by kin competition or inbreeding avoidance, individuals from a species with regional philopatry, opposed to natal philopatry, may have less pressure to disperse (Perrin & Mazalov, 2000). On the other hand, increased dispersal, regardless of the sex, can evolve under a scenario of stochastic habitat perturbations (Hamilton & May, 1977). Further, many of the studies had opportunistic sampling, yielding low or uneven sample sizes (e.g. $n<20$) and only half of the studies on SBD reported the age class within their samples. Klein, Bester-van der Merwe, Dicken, Mmonwa, and Teske (2019) demonstrated the importance of sampling non-dispersive juvenile sharks to identify significant population structure at fine spatial scales. The experimental design is closely linked to the analytical method used since juvenile samples are important for a mixed-marker approach, but adults should be sampled for direct migrant detection (Lawson Handley & Perrin, 2007). Lastly, the power of the genetic data may be an equally important factor explaining the lack of SBD in certain species. A total of 33

studies used few microsatellite loci (i.e. <10) to compare against small mtDNA fragments (<1,000 bp); potentially yielding lower power to detect statistical differences between sampling locations. These issues with the experimental design, analytical method and power of the genetic data could lead to misinterpretation of results and inappropriate conclusions.

1.5. Thesis objectives and structure

1.5.1 HYPOTHESIS AND OBJECTIVES

Sex-biased dispersal is driven by the differing needs for female and male fitness. Theory would predict MBD in elasmobranchs since i) males increase their fitness by mating with multiple females and consequently have strong competition for mates, ii) females invest significantly more energy into reproduction and available resources, or nursery habitat is heterogeneous and females benefit from habitat familiarity, iii) populations are often small and inbreeding should be avoided, and iv) males are the heterogametic sex.

Of the ~1260 chondrichthyan species, only 50 have been studied for the presence of SBD, with a major bias towards the order Carcharhiniformes (ground sharks). Additionally, most studies are subjected to unrealistic assumptions and sampling limitations. Although knowledge of dispersal is critical for managing natural populations, few elasmobranch studies have attempted to quantify the number of dispersers between breeding groups. With recent technical advances, we are now able to tackle these issues with higher precision and accuracy. In my thesis, I develop a workflow to reduce bias and assumptions, as well as move towards more quantitative estimates. This includes improved investigation of population structure (Section 2), identification of sex-linked markers (SLMs; Section 3), and the use of multiple methods to identify SBD (Section 4). Finally, I conclude my thesis with a discussion of the patterns and potential drivers for dispersal and SBD, as well as a guide to improve experimental design (Section 5).

1.5.2 APPROACH AND THESIS STRUCTURE

In this thesis, I apply new analytical and genomic methods for investigating dispersal and SBD, to then evaluate patterns and drivers in a range of elasmobranch species (Fig. 1.5.1). This thesis follows a methodological central theme: to estimate SBD with genetic tools, I first need to identify the populations between which individuals disperse and secondly discover informative markers with unique inheritance patterns (such as X- or Y-linked markers) to contrast against mtDNA and auDNA, respectively. Further, by investigating multiple species with different population densities, life-history traits, ecologies, and biogeographic contexts, I enhance the likelihood of detecting factors that are driving dispersal. Currently, very little is known of the interplay between connectivity at a

demographic and genetic level (Lowe & Allendorf, 2010; Lowe et al., 2017), and only through a multi-method approach can we improve our knowledge on dispersal at multiple time scales.

Section 2: Population structure of three shark species

First, the genetic, or reproductive, population structure of three shark species is investigated before dispersal and genetic connectivity can be quantified. This section focuses on three shark species that allow for different spatial resolutions, from small scale (few hundreds of km) to medium scale (thousands of km) to large scale (global): the Northern River Shark, *Glyphis garricki*; the School Shark, *Galeorhinus galeus* (Chapter 1); and, the Bull Shark, *Carcharhinus leucas* (Chapter 2); respectively. Genomic tools, such as the mitochondrial genome (mitogenome) and nuclear SNPs, are used to improve resolution, compared to single-gene or microsatellite analyses, and overcome potential methodological and sampling biases. Overall, this section demonstrates the multiple aspects of dispersal at different scales, relative to the biological characteristics of the species and seascape features.

Section 3: Sex-linked markers and sex-chromosome systems in elasmobranchs

Second and most innovating, I describe how the genomic data from population genetic studies can be used to obtain markers that are putatively located on sex chromosomes. I develop an R-based analytical tool to identify sex-linked markers (SLMs; Chapter 3). The patterns these SLMs exhibit (i.e. presence-absence, heterozygosity, and read depth) provide information on the sex-chromosome system within the examined species. By detecting SLMs in 19 elasmobranch species, supplemented with available information on elasmobranch sex chromosomes, I evaluate the most parsimonious pathways for the evolution of sex-chromosome systems in elasmobranchs (Chapter 4). This new knowledge has a range of applications, from the genetic assignment of sex from tissue samples, to using X- and Y-linked markers for population genetic analyses. These applications are explored in this thesis and potential improvements and future studies are suggested.

Section 4: Sex-linked markers and sex-chromosome systems in elasmobranchs

Third, the identification of the spatial scale of population structure and SLMs provide a starting point to quantify SBD in my study species using a multi-method approach (mixed-marker, assignment testing, and CKMR). Explicitly, I contrast autosomal DNA to mitochondrial DNA and SLMs to detect signals of long-term dispersal. Where adult sharks were available, I also look at signals of direct contemporary (intra-generational) dispersal by assigning individuals back to their population of origin. A close-kin framework was expanded to quantify contemporary inter-generational philopatry and SBD

from juveniles that were sampled from their natal sites. The strengths and weaknesses of these methods are discussed.

Section 5: General Discussion

In the General Discussion, I synthesise the observed dispersal patterns from my three study species considering their local ecology, and examine the potential drivers of dispersal. I critically compare the genetic and analytical approaches to the detection of population structure and SBD. I highlight several important considerations and solutions to the confounding factors that influence dispersal studies. Finally, I emphasise the key insights and advances that emerged from my thesis and I argue for the relevance and utility of including this information in management decisions.

Note: I use the word "I" in Sections 1 and 5 (Introduction and Discussion) but the word "we" in Sections 2 through 4 which are collaborative works. The main body of the thesis (Chapters 1–6 in Sections 2–4) is presented as stand-alone papers developed for publication. Accordingly, it is inevitable that there is overlap in the 'Introduction' and, to a lesser extent, 'Discussion' of these chapters. My contribution to each chapter is summarised in Table 1.5.1.

Table 1.5.1: My thesis comprises six research-based data chapters (Chapter 1 to Chapter 6) and two co-authored peer-reviewed publications (Paper 1 and Paper 2). My contributions to Chapters 2 and 6, concerning the Bull Shark, are combined.

	Contribution of the PhD student (%)						
Task	Paper 1	Paper 2	Ch. 1	Ch. 2&6	Ch. 3	Ch. 4	Ch. 5
Concept and design	25	0	80	90	80	80	80
Acquisition of data/samples	0	0	0	50	0	80	50
Data analysis	NA	50	100	100	100	100	100
Preparation of figs & tables	100	50	100	100	100	100	100
Drafting of the manuscript	33	20	100	100	100	100	100

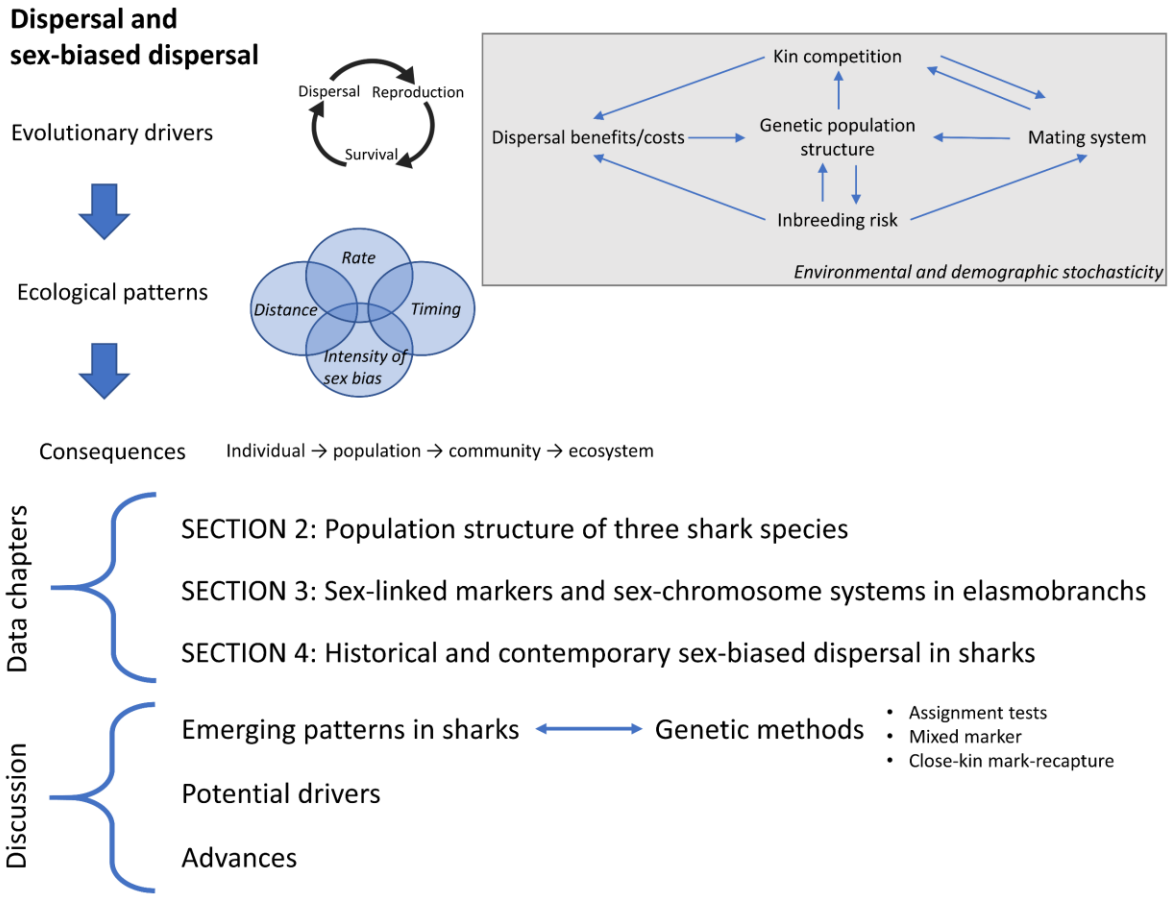

Figure 1.5.1: This conceptual structure of the thesis describes how dispersal has evolved through a multitude of evolutionary forces, shaping the patterns of dispersal that can be observed in elasmobranchs today. A lack of dispersal may have consequences to the individual (e.g. decreased fitness), the population or species (risk of local or global extinction) and the community or ecosystem (loss of functional diversity and ecosystem functioning). My thesis, specifically, focuses on a limited number of species to identify 1) population structure, 2) sex-linked markers and 3) sex-biased dispersal based on the results from Sections 1 and 2. The General Discussion places these results in context of current knowledge of dispersal and SBD in elasmobranchs.

2. SECTION 2 – Population structure of three shark species

Summary

- This section forms a baseline to estimate dispersal. Given the methodological constraints, a clear population structure signal needs to be present to quantify dispersal. A population is formed when insufficient connectivity is present between groups to counteract the process of genetic drift. Over time these groups will evolve distinct allele frequencies from each other. The rate of this process (drift) depends on the life history, ecology, and environment. At a molecular level, the rate of change is also dependent on the mutation rate of the genes. For these reasons, I have selected three species that show different biological and ecological characteristics, yet allow for both fine- and broad-scale assessment of population structure. I use genomic markers (SNPs and mitogenomes) to cover a range of different mutation rates.
- The Northern River Shark, *Glyphis garricki*, is a euryhaline species, known to have small population sizes and a limited distribution, and can grow up to 250 cm. A total of 468 individuals (mostly juveniles) were sampled from 11 riverine and coastal locations in five regions of northern Australia and Papua New Guinea, covering the entire known geographic range of the species. Population structure, historical demography and kinship were investigated using ~1,700 SNPs, genotyped with a DNA capture approach (DArTcap). Results showed that each geographical region forms a distinct population (F_{ST} = 0.09–0.40) with limited gene flow (Nm = 0.37–2.5) between them. Kinship results showed that one region (Van Diemen Gulf, Northern Territory, Australia) exhibited signals of a metapopulation with variable levels of connectivity between the rivers within this region.
- The School Shark, *Galeorhinus galeus*, is a demersal shark species that can grow up to 160 cm, with a global distribution. Despite its demersal ecology, the School Shark is considered to have large dispersal abilities as illustrated by its long-distance migration (up to 4,000 km). Eighty-eight neonate sharks were analysed using ~6,500 SNPs from a genome-scan (DArTseq) to identify population structure at a relatively small spatial scale (between Tasmania, Australia and New Zealand). Results showed that a biased sampling towards kin (e.g. full siblings) leads to a significant genetic divergence (F_{ST} = 0.0023, p = 0.00). Yet, when these kin were removed, the F_{ST} value was not significant (F_{ST} = 0.0003, p = 0.12); thus indicating the importance of sampling bias when using genomic markers.
- The Bull Shark, *Carcharhinus leucas*, is also a euryhaline species with a body size up to 400 cm, but in contrast to *G. garricki,* this shark typically exhibits much larger population sizes and a circum-global distribution. Nearly 1,000 sharks from across the globe were genotyped using

the DArTcap approach (~3,400 nuclear SNPs) and sequenced for their full mitochondrial genomes. The nuclear DNA results revealed reproductive isolation at an ocean-basin scale (eastern Pacific, western Atlantic, eastern Atlantic, Indo-West Pacific) with signals of separate island populations (Japan and Fiji). The mitochondrial genomes demonstrated population genetic structure at a finer spatial scale (at the country or oceanographic area level). Even finer-scale structure was discovered using a close-kin approach, showing philopatric behaviour at a river or estuary level. Lastly, a diagnostic SNP panel could be designed to identify species, provenance, kinship, and sex from tissues samples, which has direct monitoring applications.

PAPER 2 – Population structure in Northern River Sharks from northern Australia and Papua New Guinea

Feutry, P.; Devloo-Delva, F.; Tran Lu Y, A.; Mona, S.; Gunasekera, R. M.; Johnson, G.; Pillans, R. D.; Jaccoud, D.; Kilian, A.; Morgan, D. L.; Saunders, T.; Bax, N. J. & Kyne, P. M. (2020). **One panel to rule them all: DArTcap genotyping for population structure, historical demography, and kinship analyses, and its application to a threatened shark.** *Molecular Ecology Resources*, *20*(6), 1470-1485. Doi 10.1111/1755-0998.13204. Results are paraphrased given their importance for Chapters 4 and 5.

The Northern River Shark, *Glyphis garricki* Compagno, White, & Last 2008, was first recorded in the Adelaide River, Northern Territory, Australia in 1989 (Taniuchi, Shimizu, Sano, Baba, & Last, 1991). Currently, it is found in several macrotidal tropical rivers with high turbidity throughout northern Australia and southern Papua New Guinea (Kyne, Heupel, White, & Simpfendorfer, 2021; Pillans, Stevens, Kyne, & Salini, 2009; White et al., 2018). These rivers are known pupping and nursery areas, yet the location of mating aggregations is currently unknown (Pillans et al., 2009). It is classified as a euryhaline species since adults are mainly found in high salinity waters; suggesting the possibility that breeding occurs in coastal marine environments.

The Northern River Shark presumably exhibits small population sizes throughout its distribution (Bravington et al., 2019). In addition, *G. garricki* is subjected to a number of riverine and marine threats, mainly commercial and recreational fishing, and habitat modification (Fields et al., 2018; Grant et al., 2019; Kyne & Feutry, 2017; White et al., 2018). The small population sizes combined with the threats it faces may have serious impacts on the continued persistence of this species. Consequently, this species is listed as Vulnerable by the IUCN Red List of Threatened Species (Kyne, Rigby, et al., 2021) and as Endangered on Australia's national environmental legislation.

Its congener the Speartooth Shark (*Glyphis glyphis* Müller & Henle 1839) has shown strong population genetic structure (Feutry et al., 2017). High fixation indices (Φ_{ST} and F_{ST}) were found between three river systems in the Northern Territory and Queensland, which was supported by the lack of close-kin movement between these rivers (only 18 out of 217 kin pairs). This leads to the hypothesis that its sister species would exhibit similar strong population structure.

To test this hypothesis, 468 tissue samples were taken from 11 riverine and coastal locations in five regions: King Sound, Cambridge Gulf (West Cambridge Gulf and Ord River), Daly River, Van Diemen Gulf (Adelaide River, Sampan Creek, Wildman River, West Alligator River, South Alligator River, East Alligator River), and Papua New Guinea. Further, a novel DNA capture method (DArTcap) was developed. Briefly, 93 samples, with a minimum of eight per location, were genotyped using a genome-scan approach (DArTcap). This yielded 3,530 SNPs, of which 2,007 were selected for biotinylated RNA bait development and 1,709 passed the bait-development quality control.

Subsequently, a total of 468 *G. garricki* samples were genotyped, which yielded 461 sharks and 1,729 SNPs after data filtering (Appendix B2).

Population structure results revealed a strong population genetic structure, where each of the five geographic regions forms a small ($N_e \approx 100–500$) isolated population with low connectivity ($Nm < 2.5$). Specifically, the pairwise F_{ST} values ranged from 0.09 to 0.40 between regions, while rivers within each region had F_{ST} values that were one to two orders of magnitude lower. A similar pattern was observed from the clustering analyses (ADMIXTURE and discriminant analysis of principal components; DAPC). One hundred and sixty-eight close-kin pairs were identified within the Van Diemen Gulf. Results suggested the presence of subpopulations within this region since most kin pairs were found within the same river, but 25 pairs were also found between rivers.

The strong nuclear population structure can be explained by philopatric behaviours from both sexes to their respective region. This philopatric behaviour may be driven by the low availability of the niche habitat that juvenile *G. garricki* requires (high turbidity and low salinity; Pillans et al., 2009). Within the Van Diemen Gulf, the kinship results suggested a higher dispersal rate between rivers. These may warrant further investigation of sex-biased dispersal.

2.1. CHAPTER 1 – Population structure in School Sharks from Tasmania and New Zealand

Devloo-Delva, F.; Maes, G. E.; Hernández, S. I.; Mcallister, J. D.; Gunasekera, R. M.; Grewe, P. M.; Thomson, R. B. & Feutry, P. (2019). **Accounting for kin sampling reveals genetic connectivity in Tasmanian and New Zealand School Sharks, *Galeorhinus galeus*.** *Ecology and evolution*, *9*, 4465-4472

2.1.1. INTRODUCTION

Amongst marine organisms, sharks are of the highest conservation concern; 25 % of all chondrichthyan species are currently at risk of extinction (Dulvy et al., 2014). These species are particularly vulnerable to targeted or by-catch fisheries, partly due to late maturity and small litter size (Kyne, Bax, & Dulvy, 2015). School Sharks (*Galeorhinus galeus* Linnaeus 1758) have been intensively fished throughout Australian waters since the 1920s for their oily livers and later on for their meat (Olsen, 1954). By the 1950s there was concern that overfishing had depleted the stock of this species with low biological productivity (i.e. 15-43 pups every 2 years; AFMA, 2015; Olsen, 1984), causing a shift towards targeting the faster reproducing Gummy Shark (*Mustelus antarcticus* Günther 1870) (Walker, 1999). However, School Shark catch continued and the stock is currently estimated to lie between 8 and 17 % of the pristine level (Thomson, 2012; Thomson & Punt, 2009). Consequently, the School Shark has been listed as Conservation Dependent under the Environment Protection and Biodiversity Conservations Act (EPBC Act, 1999). Globally, the species is recorded as Critically Endangered on the IUCN Red List (Walker et al., 2020) and has recently been designated as a priority for conservation (Dulvy et al., 2017).

Management of highly migratory species, such as the School Shark, presents difficulties given that international agreements may be needed to properly manage shared stocks (Fowler, 2014). Consequently, straddling stocks are sometimes managed on a less appropriate national scale. Such a problem may exist for School Sharks, which are managed independently in Australia and New Zealand (Francis, 2010), despite tagging and genetics studies that have questioned the assumption of separate stocks. Individuals are reported crossing the Tasman Sea and migrating up to 4,500 km (Coutin, Bruce, & Paul, 1992; Francis, 2010; Hurst, Baglet, McGregor, & Francis, 1999; McMillan, Huveneers, Semmens, & Gillanders, 2019). Nevertheless, such tagging studies do not provide any information about successful reproduction of migrants. It should be noted that the level of gene flow required to overcome genetic separation is much lower than that required to assume complete mixing and, hence, joint-stock management (Begg & Waldman, 1999).

A lack of apparent genetic structure between these Australian and New Zealand sharks has been reported using allozyme, mitochondrial DNA (mtDNA), and microsatellites (Hernández et al., 2015; Ward & Gardner, 1997), thus questioning the existence of impervious reproductive boundaries in this

region. However, a more recent study, with the mitochondrial and similar nuclear microsatellite markers, found a clear separation in the microsatellite data between Tasmania and New Zealand (Bester-van der Merwe et al., 2017). Single nucleotide polymorphisms (SNPs) have been shown to outperform microsatellites in population discrimination due to their random spread across the genome, lower ascertainment bias, higher accuracy and resolution, reproducibility, and comparability (Andrews, Good, Miller, Luikart, & Hohenlohe, 2016; Fischer et al., 2017; Muñoz et al., 2017; Seeb et al., 2011). Single nucleotide polymorphisms allow for a relatively cheap and easy way to obtain a full genome scan (Andrews et al., 2016). The large number of markers permits the inference of kinship with high certainty, investigation of population structure at higher resolution (Feutry et al., 2017), and accurate calculation of genetic diversity (as argued by Domingues, Hilsdorf, & Gadig, 2018).

In highly migratory species, sampling adults can introduce bias due to dispersal of individuals after birth, and hence decreases the signal to noise ratio (Waples, 1998). This realized dispersal is much lower in neonate and juvenile School Sharks (Olsen, 1954) and studying them should improve the power to detect fine-scale structure. However, sampling juveniles results in a higher risk of generating a false signal of genetic structure through the 'Allendorf–Phelps effect' (Allendorf & Phelps, 1981; Waples, 1998), due to biased sampling toward family members. Additionally, the presence of family members within a sample set has been reported to artificially increase the number of distinct genetic pools detected by clustering algorithms commonly used in population structure studies (Anderson & Dunham, 2008). Both biases have been previously reported in sharks (Feutry et al., 2017).

This study aims at testing the hypothesis of a single panmictic population of School Shark between Tasmanian and New Zealand waters using novel genomic markers, while accounting for the 'Allendorf–Phelps effect'. To investigate this, we genotyped neonates and juveniles from Tasmania and New Zealand. This work provides basic knowledge for the management of this commercially important species and contributes to the discussion around sampling design and data analysis when investigating the genetic structure of highly migratory species.

2.1.2. MATERIAL AND METHODS

Sample collection

Eighty-eight School Sharks were collected between 2009 and 2013 using long lines and gillnets from Tasmania (TAS, n = 47) and New Zealand (NZ, n = 41; Fig. 2.1.1). Sampling sites in both countries were known nursery areas and only neonates and juveniles (total length < 60 cm) were caught. Individuals smaller than 70 cm (i.e. 0-2 years old) are considered to have limited dispersal (Olsen, 1954). Muscle tissues or fin clips were collected and stored in ethanol. A modified version of the CTAB protocol (Doyle & Doyle, 1987; Grewe et al., 1993) was used to extract total genomic DNA.

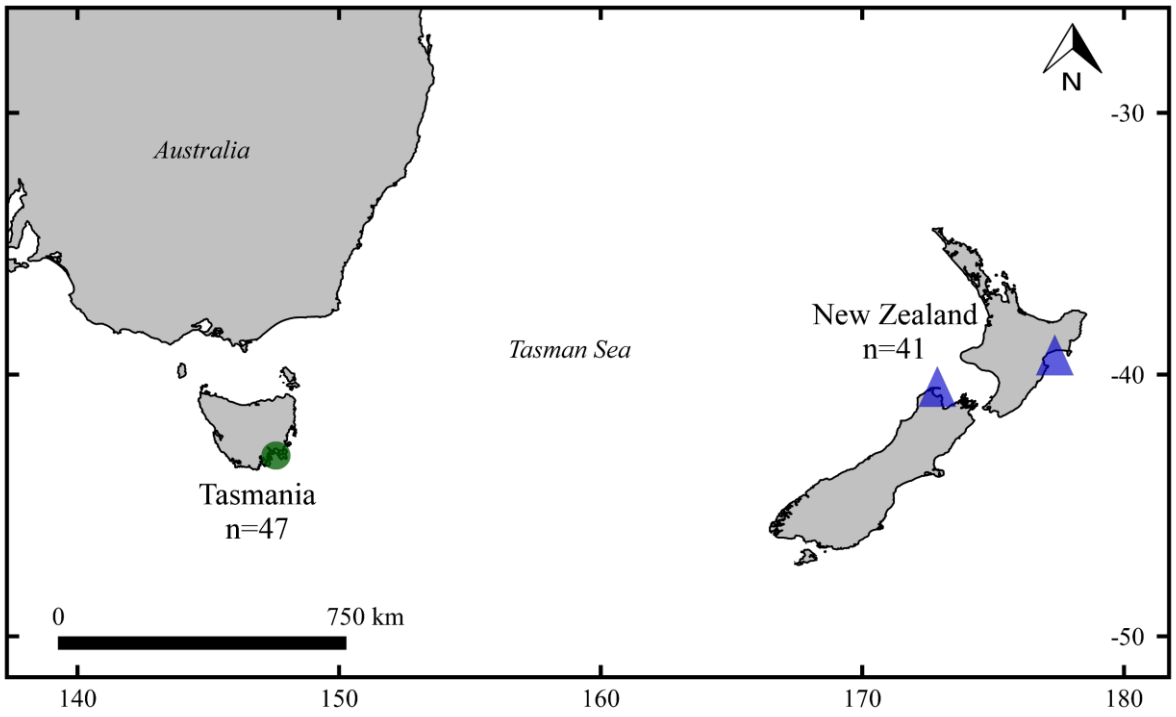

Figure 2.1.1: Sampling map for neonate School Sharks from Tasmania and New Zealand. Green circle represents Pittwater and Norfolk Bay. Blue triangles represent Golden Bay (West, n=33) and Napier (East, n=8).

SNP genotyping and filtering

Single nucleotide polymorphism genotyping was carried out by Diversity Array Technologies (DArT, Canberra, Australia) using the DArTseq protocol, a method of complexity reduction sequencing. The DArTseq protocol used in this study was identical to the one previously described by Grewe et al. (2015). The DArTseq output consisted of 69 bp fragments containing one or more SNPs. Seventeen samples were genotyped twice to assess genotyping reproducibility.

Quality filtering was performed in R v3.5.1 (R Core Team, 2020; Appendix C2), using the dartR v1.1.6 (Gruber, Unmack, Berry, & Georges, 2018) and the Adegenet v2.1.1 (Jombart & Ahmed, 2011) packages. Low call rate (proportion of scored loci for an individual) and high heterozygosity may indicate bad DNA quality or sample contamination, respectively. Therefore, individuals with a call rate below 95 % and/or heterozygosity above 20 % were removed from the dataset prior to proceeding to the next SNP filtering step. Single nucleotide polymorphisms with a call rate (proportion of scored individuals for a locus) lower than 95 %, a genotyping reproducibility below 98 %, or a minor allele frequency lower than 5 % were removed (Table 2.1.1). After which, loci with an average read depth <15 and >90 sequences per locus were filtered out to remove unreliable or paralogous loci, respectively. Monomorphic loci (i.e. fixed for all individuals) were deleted since they contain no discriminating information. Outlier analysis was performed with OutFLANK v0.2 (Whitlock & Lotterhos, 2015) at a q-value of 0.01 and significant outliers were removed in order to only retain neutral markers

in HWE. All the cut-off values used in these filtering steps were defined after plotting the data to observe the loci/individuals' distributions (see Appendix C2).

Moreover, two datasets (with and without siblings) were created to test the effect of non-random sampling of siblings (Table 2.1.1). Sibship (full and half sibling relationships) among all individuals was checked with Colony2 v2.0.6.1 (Jones & Wang, 2010) using the initially filtered dataset (see Appendix C3 for the analysis parameters). To build the second dataset, only one individual per sibling group was kept prior to re-filtering all SNPs (following similar filtering steps).

Population diversity and structure analyses

Genetic diversity (H_O, F_{IS}, and A_r), fixation (F_{ST}) and allelic differentiation (Jost's D or D_{est}) indices were calculated with diveRsity v1.9.90 (Keenan, McGinnity, Cross, Crozier, & Prodöhl, 2013), StaMPP v1.5.1 (Pembleton, Cogan, & Forster, 2013; Weir & Cockerham, 1984) and mmod v1.3.3 (Jost, 2008; Winter, 2012) packages, respectively, applying a bootstrap of 10,000. Population structuring was assessed with a Discriminant Analysis of Principal Components (DAPC, Adegenet v2.1.1; Jombart & Ahmed, 2011) and STRUCTURE v2.3.4 (Pritchard, Stephens, & Donnelly, 2000). With DAPC, the optimal number of clusters (K) was determined by the lowest Bayesian Information Criterion (BIC) and a successive K-means algorithm was used to group the sharks according to the determined number of clusters (Schwarz, 1978). The optimal number of principal components retained for the DAPC analysis was selected through cross-validation with a 10 % hold-out set and 10,000 replicates. The admixture model of STRUCTURE was applied with correlated allele frequencies for 100,000 burn-in and 500,000 replicate runs. The program was set to assess structure between one to nine putative populations with 20 iterations for each group. The optimal K was assessed based on the mean estimated natural logarithm of the probability (lnP). Except for the STRUCTURE analyses, all data filtering and analyses were performed and visualized using the R software.

2.1.3. RESULTS

Data filtering

An average of 2,028,777 sequences per sample were obtained and the DArTsoft14 pipeline identified 31,550 SNPs. One individual from TAS with an excess of heterozygous loci compared to other sharks, probably due to cross-contamination, was removed from the data. For these remaining 87 sharks, a total of 6,760 neutral SNPs passed all the filtering steps. Sibship analysis of this dataset revealed seven full sibling groups (but no half siblings) among the TAS neonates. One individual from each of the seven full sibling groups was retained (11 removed) to avoid biased clustering of family members. This resulted in a total of 76 neonate and juvenile sharks. After all filtering steps, 6,587 neutral SNPs were available for analysis.

Table 2.1.1: Quality-filtering steps for loci and sharks.

	With full siblings		Without full siblings	
	Loci	Sharks	Loci	Sharks
Start	31,550	88	31,550	77
Multiple loci on the same sequence	24,504	88	24,504	77
Monomorphic loci	21,275	88	20,951	77
Locus call rate ≥ 0.95 & Shark call rate ≥ 0.95	13,931	88	13,579	77
Shark heterozygosity ≥ 0.20	13,931	87	13,579	76
Monomorphic loci	13,918	87	13,555	76
Average reproducibility ≤ 0.98	13,581	87	13,237	76
Coverage ≤ 15 reads	13,439	87	13,103	76
Coverage ≥ 90 reads	13,363	87	1,3031	76
Minor allele frequency ≤ 0.05	6,768	87	6,603	76
Locus observed heterozygosity ≥ 0.6	6,763	87	6,594	76
Outlier loci	6,760	87	6,587	76

With full sibs

Genetic diversity indices were similar for sharks from TAS and NZ. (Table 2.1.2). The fixation and differentiation indices for the neutral SNPs indicated a significant genetic difference between TAS and NZ (F_{ST} = 0.0023, CI_{95} = [0.0017, 0.0028], p = 0.0000; D_{est} = 0.0014 ± 0.0002, CI_{95} = [0.0010, 0.0017]). However, this signal was not visible from the DAPC plot, where the BIC indicated that eight groups seemed to be the optimal solution (Fig. 2.1.2A). Five of those eight groups were comprised of full siblings and no differentiation between TAS and NZ could be found (Fig. 2.1.2B). The sibling-driven clustering was not as obvious in the STRUCTURE as in the DAPC results; with a similar likelihood for K = 1, 2, 5 or 7 (Appendix C3).

Without full sibs

Neutral genetic diversity did not differ from the dataset with full siblings, and did not show any differences between TAS and NZ (Table 2.1.2). Pairwise F_{ST} became non-significant (F_{ST} = 0.0003, CI_{95} = [-0.0002, 0.0009], p = 0.1163; D_{est} = 0.0006 ± 0.0002, CI_{95} = [0.000, 0.001]) and based on the BIC of the DAPC and the mean lnP of the STRUCTURE analysis, one population seemed to be the best clustering solution (Fig. 2.1.3A, Appendix C3). This result is supported by the lack of visible structure in the DAPC (Fig. 2.1.3A) and STRUCTURE plots (Appendix C3).

Table 2.1.2: Genetic diversity of 87 (6,760 SNPs) and 76 (6,587 SNPs) sharks, respectively. N, sample size; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient with 95 % confidence interval in brackets; A_r, allelic richness.

	With full siblings			Without full siblings		
	Overall	**TAS**	**NZ**	**Overall**	**TAS**	**NZ**
N	87	46	41	76	35	41
H_o	0.263	0.264	0.262	0.265	0.265	0.264
H_E	0.285	0.285	0.284	0.285	0.284	0.285
F_{IS}	0.068[0.049, 0.062]	0.070[0.050, 0.063]	0.069[0.047, 0.061]	0.066[0.043, 0.059]	0.065[0.040, 0.057]	0.067[0.046, 0.060]
A_r	1.995	1.995	1.994	1.992	1.990	1.993

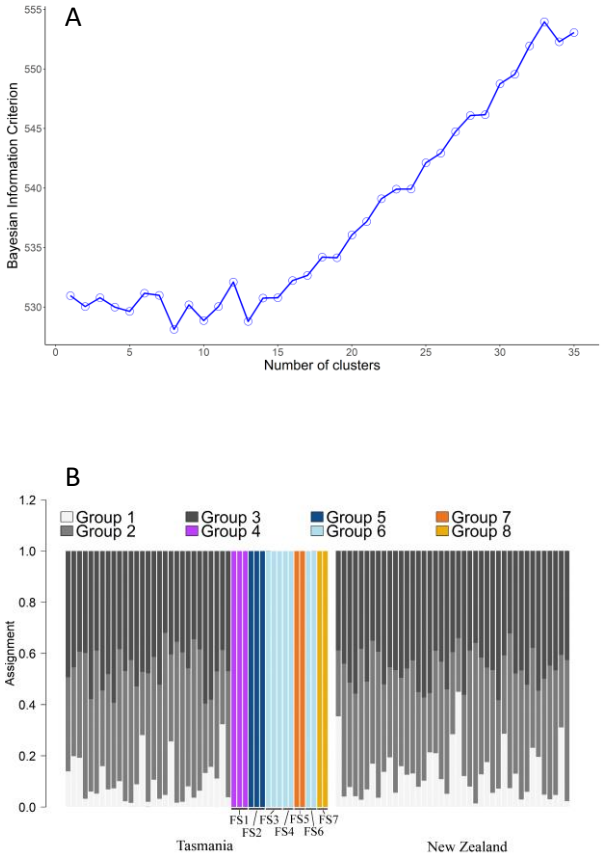

Figure 2.1.2: A) Optimal number of cluster selection, based on Bayesian Information Criterion with 29 PCs. B) DAPC assignment plot between Tasmania and New Zealand (full siblings included), based on seven PCs.

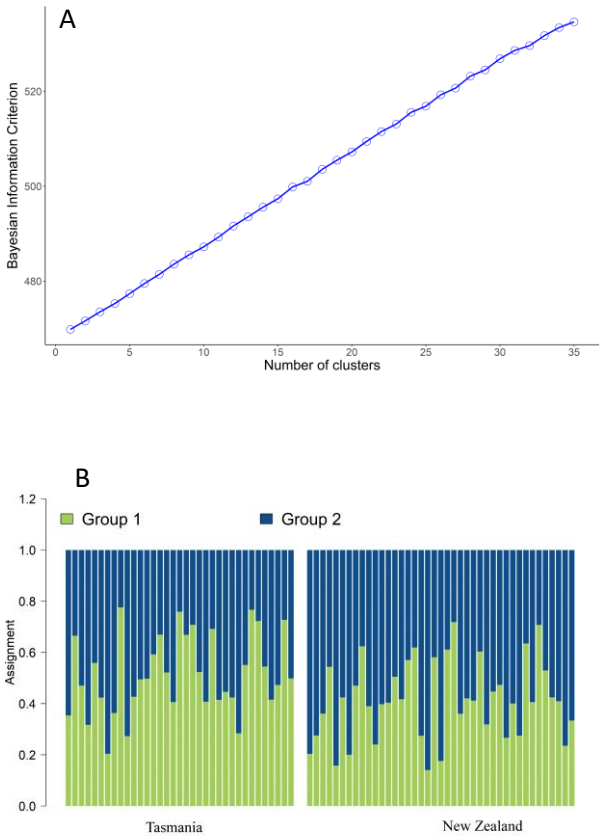

Figure 2.1.3: A) Optimal number of cluster selection, based on Bayesian Information Criterion with 25 PC's. B) DAPC assignment plot between Tasmania and New Zealand (full siblings excluded), based on 35 PCs.

2.1.4. DISCUSSION

Population structure with or without siblings?

The conclusions drawn from this study greatly depend on which dataset is interpreted (with or without full siblings). By removing full sibling groups from the dataset, the F_{ST} value decreased by one order of magnitude and the optimal number of clusters decreased from eight to one (Fig. 2.1.2A & 2.1.3A). If the sibling groups are left in the dataset, there is a risk of misinterpreting population structure for what is actually family structure. However, Waples and Anderson (2017) demonstrated that the trending common practice, consisting of purging groups of siblings prior to population genetic analyses, can introduce a bias if the presence of these groups is not a sampling artefact but rather the result of a small localized population. Removing the right amount of closely related individuals is theoretically feasible, but requires knowledge of (at least) the effective population size. Unfortunately, family structure also creates a bias when estimating this quantity (Waples & Anderson, 2017), which makes it a circular issue. In this study, all full siblings were sampled from the same nursery within the same year, with a maximum of four months between captures (with majority caught less than a month apart), which indicates that their presence is a sampling artefact. Another indicator of a family sampling bias is the absence of half siblings. If the presence of such a high proportion of full siblings in Tasmania was due to a small and localized population, and given that males are not believed to be monogamous and that females are expected to reproduce more than once across the sampling period (Walker, 2005), one would have expected to detect half siblings too. More likely, the presence of full sibs in this dataset reflects a higher probability of sampling litter mates (individuals having the same mother and born at the same place and time). The relatively small surface area of the Pittwater nursery likely increased the chance to sample litter mates. Due to the inter-dependence between effective population size, population structure and family structure, we suggest repetitive sampling over time can help interpret population structure in the presence of family members.

Population structure compared to previous studies

Interestingly, our findings contradict nuclear DNA results from a recent study by Bester-van der Merwe et al. (2017) that Tasmania and New Zealand form distinct populations. Potential sibling- or sex-biased sampling could explain the observed nuclear signal of structure (Allendorf & Phelps, 1981; Benestan et al., 2017; Feutry et al., 2017; Waples, 1998). School Sharks are known to school by size and sex (Francis, 2010; Olsen, 1984). The nine Tasmanian and 20 New Zealand individuals from Bester-van der Merwe et al. (2017) were obtained to identify biased sampling. We were unable to test the sex-biased sampling hypothesis, because of missing sex information, but we re-analyzed the 19 microsatellites in COLONY2. Eight pairs of individuals had a probability over 75 % of being either full or half siblings; settings and results are presented in Appendix C3. Due to the low sample size and missing alleles, a

reliable estimate of allele frequencies could not be made and these results must be interpreted with caution. In addition, a recent publication from McMillan et al. (2019) described partial migratory behavior of Australian School Sharks, where some females appeared to be resident. Consequently, the possibility of a small and localized population in Tasmania cannot be excluded.

This study builds on the many telemetry and genetic studies that have investigated movement and connectivity of School Sharks within Oceania (e.g. Bester-van der Merwe et al., 2017; Coutin et al., 1992; Hernández et al., 2015; Hurst et al., 1999; McAllister, Barnett, Lyle, & Semmens, 2015; McMillan et al., 2019; Olsen, 1954; Ward & Gardner, 1997). Based on current results, the null hypothesis of a single panmictic population cannot be rejected. Both F_{ST} and D_{est}, as well as diversity and clustering analyses, did not detect differentiation between TAS and NZ neonates and juveniles. This is supported by the large dispersal abilities of School Sharks (Coutin et al., 1992; Hurst et al., 1999; McAllister et al., 2015; McMillan et al., 2019; Olsen, 1954). Genetic diversity was similar between both sampling regions, but lower compared to previous studies (He = 0.5-0.75; Bester-van der Merwe et al., 2017; Domingues et al., 2018; Hernández et al., 2015). This discrepancy with other studies can be explained by the choice of genetic markers. This study presents the first genomic study of School Sharks and in theory allows a more accurate calculation of genetic diversity (Fischer et al., 2017). Overall, our diversity measures correspond to other genomic studies in sharks from different geographic locations (Feutry et al., 2017; Maisano Delser et al., 2019; Pazmiño et al., 2018). Furthermore, Ward and Gardner (1997) found weak evidence of genetic differentiation; however, this was based on a single allozyme and mitochondrial DNA markers. Hernández et al. (2015) showed the presence of a single genetic population in Oceania, using mtDNA and microsatellites. With the increased power of genome-wide SNPs, we found similar results. The observed signal could also be attributed to other explanations that could not be identified with our current sampling design: 1) a high gene flow that dilutes existing, recent population differentiation (Bailleul et al., 2018; Waples & Gaggiotti, 2006), 2) sex-biased dispersal where one sex obscures the philopatric signal (Fraser, Lippé, & Bernatchez, 2004), or 3) temporal structure caused by their biennial – triennial pupping behaviour (Waples, 1998).

Future work

The use of neonate and juvenile samples in this study is ideal to detect population structure in highly migratory species, but our sampling design and choice of markers did not allow us to fully investigate potential temporal- or sex-biased dispersal. Regional female philopatry has been suggested by Bester-van der Merwe et al. (2017) in South Africa; however, this has not yet been observed in Oceania (Francis, 2010; Hernández et al., 2015). Hernández et al. (2015) did not detect any sign of philopatry using mitochondrial markers, but using whole mitochondrial genome sequences instead of the control region might provide better insight (Feutry et al., 2014). Paternally inherited (Y-chromosome) markers

or the spatial distribution of siblings may also help detecting sex-biased dispersal (Feutry et al., 2017; Petit et al., 2002). Moreover, Pittwater, Tasmania, is currently the only known School Shark nursery area in Australia where pups can reliably be caught (others in Tasmania and Victoria currently yielding few or no pups). However, samples from other nurseries closer to the mainland of Australia and multi-year sampling could possibly reveal population structure between other regions of Australia and New Zealand. In any case, given the highly migratory nature of adult School Sharks, such fine-scale structure, if it existed, would only impact management practices if nursery areas were to be targeted by the fishing fleet, which is not the case. Thus, catches of the Tasmanian sharks in the fishery will not deplete the hypothetical Pittwater nursery population disproportionately compared to other nurseries.

2.1.5. CONCLUSION

In conclusion, this study has illustrated how kin bias can affect population structure inference if sampling is not randomly spread and we proposed several measures for identifying such biased sampling towards kin. The unbiased estimates of population connectivity could not reject the existence of a panmictic population between Tasmania and New Zealand School Sharks; yet possible caveats in the study have been pinpointed and the presence of small local populations may still be plausible. Overall, due to the migratory behavior of School Sharks we argue that potential population structure would only form a conservation issue if nursery areas were to be targeted by fisheries, which they currently are not.

2.2. CHAPTER 2 – Population structure in Bull Sharks from a global distribution

2.2.1. INTRODUCTION

Understanding population structure of a species and the barriers that disrupt dispersal are important questions to answer for the management of any species. This is especially true for species of commercial importance (Begg, Friedland, & Pearce, 1999) or conservation concern (Moritz, 1994). Threats, such as overfishing and habitat modifications, have impacted a wide range of marine species, but these threats have disproportionately impacted elasmobranchs (sharks and rays; Dulvy et al., 2014). Consequently, the need to accurately identify the appropriate spatial scale for management actions in elasmobranchs is high.

The power of genomic data has improved the population delineation of large, mobile elasmobranch species (Green et al., 2019; Ovenden et al., 2018). This is a critical task to assess the conservation status of any species and manage the risks of local extinctions where small, isolated populations are at greater risk of extinction than larger, well-connected ones (Frankham et al., 2017). Complexity-reduction genome-scan methods, such as ddRAD, GBS, or DArTseq (Jaccoud, Peng, Feinstein, & Kilian, 2001) and targeted DNA-capture approaches, for example rapture and DArTcap (Ali et al., 2016; Feutry et al., 2020), have shown increased power to assess reproductive connectivity in natural populations, compared to classical approaches such as microsatellites (Green et al., 2019; Layton et al., 2020). These methods have also been applied in DNA forensics or traceability studies to identify species, sex, provenance, and close-kin relationships (Arenas et al., 2017; Feutry et al., 2017; Nielsen et al., 2012; Stovall et al., 2018). Moreover, the sequencing of full mitochondrial genomes, as opposed to single genes, has been shown to increase the detectability of population structure along the matrilineal line (Feutry et al., 2014), especially in taxa with a low mitochondrial mutation rate, such as elasmobranchs (Martin, Naylor, & Palumbi, 1992).

The Bull Shark (*Carcharhinus leucas* Müller & Henle 1839) is a cosmopolitan species that occupies tropical to subtropical waters and has an important ecological role in freshwater, estuarine, and coastal environments (Matich, Heithaus, & Layman, 2011; Trystram, Rogers, Soria, & Jaquemet, 2017). This species experiences variable degrees of exploitation within its range and is currently assessed as Vulnerable on the IUCN Red List of Threatened Species (Rigby, Espinoza, Derrick, Pacoureau, & Dicken, 2021). Its main threats are similar to other elasmobranchs, such as small-scale and large-scale fisheries for meat and fins (Holmes, Steinke, & Ward, 2009; Tillett, Field, et al., 2012), and also shark control programs (Blaison et al., 2015; Dudley, 1997). Bull Shark harvesting has led this species to enter the global shark fin trade (Clarke, McAllister, et al., 2006; Fields et al., 2018). Under unsustainable levels of exploitation, these threats would result in population decline and negative ecosystem impacts

(Heithaus, Frid, Wirsing, & Worm, 2008; MacNeil et al., 2020). However, more detailed information about population connectivity is needed to assess potential negative impacts.

The Bull Shark has shown capacity for long-distance migrations (Brunnschweiler, Queiroz, & Sims, 2010; Daly, Smale, Cowley, & Froneman, 2014; Espinoza, Heupel, Tobin, & Simpfendorfer, 2016; Lea, Humphries, Clarke, & Sims, 2015) with genetic connectivity along continuous coastlines (Glaus et al., 2020; Pirog et al., 2019; Testerman, 2014); yet females exhibit philopatric behaviours to estuarine habitats at small spatial scales (~100 km; Karl, Castro, Lopez, Charvet, & Burgess, 2011; Sandoval Laurrabaquio‐Alvarado et al., 2021; Tillett, Meekan, Field, Thorburn, & Ovenden, 2012). The genetic connectivity of the Bull Shark has been extensively studied within ocean basin regions, such as the western Atlantic (Karl et al., 2011; Sandoval Laurrabaquio-A et al., 2019; Sandoval Laurrabaquio‐Alvarado et al., 2021) and the Indo-West Pacific regions (Deng, Chen, Song, Li, & Han, 2019; Glaus et al., 2020; Kitamura, Takemura, Watabe, Taniuchi, & Shimizu, 1996; Pirog et al., 2019; Tillett, Meekan, et al., 2012), yet no studies have investigated connectivity throughout its entire distribution using a full suite of next-generation sequencing (NGS) approaches.

This study aims to apply novel genomic techniques (full mitochondrial genomes, mitogenomes; and nuclear single nucleotide polymorphisms, SNPs) to infer population genetic structure at global and local scales. We hypothesise that gene flow is reduced by known biogeographical barriers and connectivity occurs along continuous coastlines. Therefore, we analysed a global set of samples with three different genomic sequencing approaches (DArTseq, DArTcap, and mitogenomes) which allow us to discuss 1) genetic diversity, and 2) population structure and connectivity. Additionally, we test the forensic power of the genomic data to assign provenance and establish a diagnostic SNP panel to aid monitoring of fisheries and trade.

2.2.2. MATERIAL AND METHODS

Sample collection and DNA extraction

A total of 922 putative Bull Shark samples were collected between 1980 and 2019 from 18 different countries or oceanographic areas in four ocean regions: the eastern Pacific (E-PAC), western Atlantic (W-ATL), eastern Atlantic (E-ATL), and the Indo-West Pacific (IWP; Fig. 2.2.1). Samples from Brazil, East Indian Ocean, Fiji, and numerous locations in Australia were sourced from previously published studies (Glaus et al., 2020; Karl et al., 2011; Pirog et al., 2019; Tillett, Meekan, et al., 2012). The sex-ratio at each location was roughly equal and the total length (TL) of sampled individuals ranged from 26.4 (*in utero*) to 410.0 cm (Appendix D1 section 2). DNA was extracted using the Qiagen Blood and Tissue kit following standard protocol (Qiagen Inc., Valencia, California, USA).

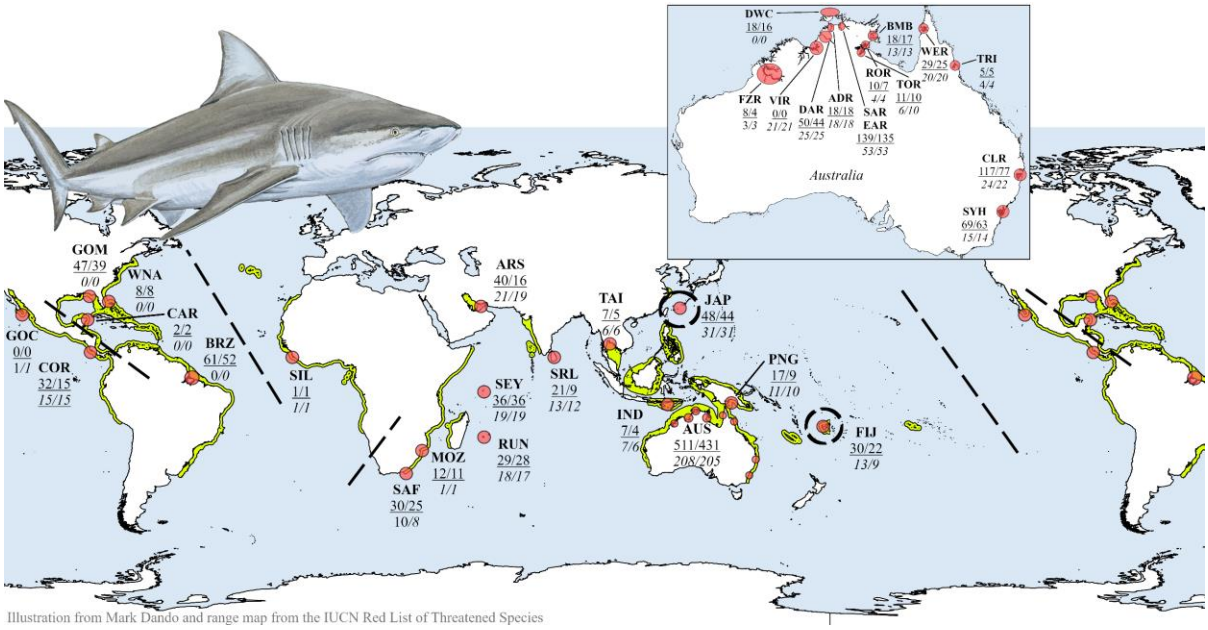

Figure 2.2.1: Map indicating the sampling sites with a red circle and the known species range distribution in yellow. The sample sizes for the SNP data are underlined with the number of samples before/after data filtering. The sample sizes for the mitogenome data are in *italic* with the number of samples before/after data filtering. Dashed lines indicate nuclear separation and known biogeographic barriers. GOC = Gulf of California; COR = Costa Rica; BRZ = Brazil; CAR = Caribbean Sea; GOM = Gulf of Mexico; WNA = Western North Atlantic; SIL = Sierra Leone; SAF = South Africa; MOZ = Mozambique; RUN = Reunion Island; SEY = Seychelles; ARS = Arabian Sea; SRL = Sri Lanka; TAI = Thailand; IND = Indonesia; PNG = Papua New Guinea; AUS = Australia; FZR = Fitzroy River; VIR = Victoria River; DAR = Daly River; ADR = Adelaide River; DWC = Darwin Coastal; SAR = South Alligator River; EAR = East Alligator River; BMB = Blue Mud Bay; ROR = Roper River; TOR = Towns River; WER = Wenlock River; TRI = Trinity Inlet; CLR = Clarence River; SYH = Sydney Harbour; and UNK = fisheries samples from unknown origin; JAP = Japan; and FIJ = Fiji.

SNP bait design and DArTcap genotyping

One hundred and eighty-eight samples, with a minimum of 10 samples per site, were first genotyped using the DArTseq approach (Feutry et al., 2017). *De novo* SNP calling was performed with proprietary software (DArTsoft14). The resulting markers were filtered using the 'filter_rad' function, as implemented in *radiator* v1.1.5 (supplementary material sections 4-6; Gosselin, Lamothe, Devloo-Delva, & Grewe, 2020). Of the resulting high-quality markers, we randomly selected 3,200 markers for DNA-capture bait development. Secondly, the unfiltered data was run through the 'sexy_markers' function from *radiator* (Chapter 3; Devloo-Delva et al., in prep) to identify sex-linked markers, which were also selected for bait development. The selected baits were checked to ensure they did not contain any short (<40 bp) or low-complexity sequences. One biotinylated RNA MYbait (Arbor Bioscience, USA) per sequence was synthesized. DArTcap hybridization and washing followed the MYbaits standard protocol. The DArTcap enriched libraries were sequenced on a HiSeq 2500 (Illumina) as described by Feutry et al. (2020).

DArTcap SNP and individual filtering

Overall, 1,014 samples, including 92 technical replicates, were genotyped using the DArTcap protocol. Additional filtering was performed to remove any unspecific enrichment or low-quality loci. This filtering was performed with the 'filter_rad' function as implemented in the *radiator* package (see Appendix D1). Briefly, the data were filtered for: 1) low DArT reproducibility based on the technical replicates, 2) monomorphic markers, 3) high missing data, high heterozygosity, and low sequencing coverage per individual, 4) low minor allele count, 5) low and high SNP sequencing coverage, 6) high missing data per SNP, 7) too many SNPs per sequence due to sub-optimal sequence clustering, 8) short-distance linkage by keeping one SNP per sequence, 9) individual DNA contamination based on heterozygosity, 10) duplicated samples, and 11) departure from Hardy-Weinberg equilibrium. Furthermore, the remaining sex-linked markers were removed from the data.

These filtering steps were applied with different thresholds to six different subsets of the data (see Appendix D1). A hierarchical approach was used for the clustering methods (Vähä, Erkinaro, Niemelä, & Primmer, 2007). The first DArTcap dataset was filtered under less stringent thresholds (e.g. allowing for more missing data and higher levels of heterozygosity) to permit for the identification of species that were not *C. leucas* (DATA1: 922 sharks). The second dataset only contained confirmed Bull Sharks (DATA2: 868 sharks). Since unequal sample size can introduce bias in clustering algorithms (Foster et al., 2018; Puechmaille, 2016), the third dataset included all sampling locations and a random subset of 60 samples from Australia to homogenize the sample sizes (DATA3: 430 sharks). The fourth and fifth datasets were comprised of sampling locations in the W-ATL and IWP regions, respectively (DATA4: 117 sharks; DATA5: 732 sharks). Lastly, for the sixth dataset, within the IWP, samples from Japan and Fiji were removed and 60 samples from Australia were again randomly selected to homogenize the sample sizes (DATA6: 221 sharks).

Mitogenome amplification and sequencing

Samples from the E-ATL and Indo-Pacific regions provided an opportunity to further investigate the matrilineal evolutionary history due to the good spatial sample coverage and the lack of previous studies in these areas. The full mitochondrial genome of 384 putative Bull Sharks (Fig. 2.2.1) was amplified with two primer pairs (A and B fragments; Appendix D1 section 13.1; Chapter 5; Devloo-Delva et al., in prep). Polymerase chain reactions (PCR) were performed in 30 µL reactions, following the standard proofreading Takara LA Taq protocol (Takara, Otsu, Shiga, Japan). PCR conditions were set to 1 min at 94 °C for initial denaturation; then 40 cycles of denaturation (94 °C, 30 s), annealing (55 °C, 30 s) and extension (68 °C, 10 min); concluding with a 10 min extension at 72 °C. PCR products were cleaned following the Agencourt AMPure XP magnetic bead protocol (Beckman Coulter Inc., Indianapolis, Indiana, USA). Amplicons were quantified with a NanoDrop 8000 Spectrophotometer

(Thermo Fisher Scientific, Waltham, Massachusetts, USA), and the purified A and B fragments were pooled at equimolar concentrations for each individual. Subsequently, these amplicons were simultaneously fragmented and barcoded with the Nextera XT DNA Sample Preparation kits and 96-sample Nextera Index kit (Illumina, San Diego, California, USA). The libraries were quantified with the Qubit dsDNA BR assay kit (Life Technologies, Carlsbad, California, USA) and normalized. Libraries were then pooled and sequenced on a Miseq desktop sequencer using the 2x250 bp paired-end reads MiSeq reagent kit v2 (Illumina, San Diego, California, USA).

Mitogenome assembly and alignment

Demultiplexed fastq files were imported into Geneious prime software v2020.1 (Biomatters Ltd., Auckland, New Zealand) and the reads were paired. The Nextera adapters were removed and the reads quality trimmed at a phred score < 20 for a Kmer of 20 using the BBDuk tool as implemented in Geneious. Reads shorter than 60 bp after trimming were discarded from subsequent analyses. Merged reads for each individual were then mapped onto a previously published reference sequence (Chen, Liu, Peng, & Shi, 2015) using the 'Map to Reference' tool in Geneious with the 'high sensitivity' parameters and 10 iterations. The majority rule consensus (>50 % of mapped reads for any single SNP, insertion, or deletion) for each shark was exported. All mitogenome sequences were aligned with the 'multiple align' tool and the MUSCLE algorithm (Edgar, 2004).

Species identification

The mitochondrial genomes were blasted (*megablast*) in Geneious against the GenBank nr/nt database. All sequences with an identity ≥98 % were identified to species level. Samples that were only genotyped with the DArTcap approach were identified using less stringent filtering thresholds (DATA1) and a principal component analysis (PCA) as implemented in *Adegenet* v2.1.1 (Appendix D1 section 7; Jombart & Ahmed, 2011). Here, the species identified with the mitogenome served as a baseline to identify the species clusters with the PCA. To provide certainty, individuals needed to cluster on multiple principal component (PC) axes with a mitochondrial-verified species. Where this was not possible, we marked the species as 'unknown'. All species other than *C. leucas* were excluded from further analyses.

Genetic diversity

Allelic richness (A_r), observed heterozygosity (H_O) and unbiased expected heterozygosity (uH_E) were calculated for each sampling location using the R package *diveRsity* v1.9.90 (Keenan et al., 2013). Inbreeding coefficients (F_{IS}) were calculated with *hierfstat* v0.04−22 (Goudet, 2005), using 1,000 bootstraps to obtain the 95 % confidence interval. Nuclear nucleotide diversity (π_{nu}) was computed with the 'pi' function in *radiator* (Nei & Li, 1979). Mitochondrial nucleotide diversity (π_{mt}), haplotype

diversity (h), and parsimony haplotype network were calculated with the *pegas* v0.14 package (Nei & Li, 1979; Nei & Tajima, 1981; Paradis, 2010; Templeton, Crandall, & Sing, 1992). All analyses were performed in R 3.5.1 (R Core Team, 2020) and provided in the Appendix D1.

Population structure analyses

Fixation indices (Φ_{ST}, F_{ST}) were calculated between sampling locations and between regions with the 'popStructTest' function in the *strataG* v2.4.905 package (Archer, Adams, & Schneiders, 2017; Excoffier, Smouse, & Quattro, 1992; Weir & Cockerham, 1984) and their significance assessed with 1,000 permutations.

Next, dimensionality-reduction clustering analyses were conducted with *Adegenet* (PCA and Discriminant Analysis of Principal Components, DAPC; Jombart & Ahmed, 2011; Jombart, Devillard, & Balloux, 2010). Initially, individuals were grouped according to the data, using the successive K-means algorithm implemented in the 'find.clusters' function. The goodness-of-fit, determined by the Bayesian information criterion (BIC), was employed to find the best number of clusters (K). To avoid overfitting, the optimal number of principal components was selected through cross-validation with a 10 % hold-out set and 1,000 replicates for all DAPC analyses. Only sample sites with a sample size larger than one were used for this training-hold-out method.

Provenance assignment

The provenance assignment success of the DArTcap markers was tested with *AsssignPOP* v. 1.2.2 (Chen, Marschall, et al., 2018) and *Rubias* v.0.3.2 (Anderson, Waples, & Kalinowski, 2008; Moran & Anderson, 2019). Assignment accuracy was tested with *AssignPOP*, using both the Monte-Carlo and K-fold cross-validation procedures to test the assignment of a hold-out dataset with 1,000 iterations. The power of the markers was tested by selecting a subset of high F_{ST} loci (proportion = 0.05, 0.1, 0.5 and 1) to train the assignment model. Similarly, the assignment accuracy of simulated individuals, based on a reference leave-one-out dataset, was evaluated with *Rubias*. Known simulated proportions for each reporting unit were compared with the numbers estimated by *Rubias*. Populations with a sample size of one were excluded from these analyses. We also examined the minimum number of informative markers needed to assign provenance by subsampling 5-500 markers according to highest loading contributions of each PC from the DAPC analysis and testing the assignment accuracy with *Rubias*.

Kinship assignment

Kinship has the power to inform about contemporary reproductive dispersal, given that sample sizes are large enough to allow accurate allele frequency estimation (Bravington et al., 2016; Feutry et al., 2017). Consequently, close-kin relationships were investigated in each geographic region with a large

sample size, namely W-ATL and IWP. Kinship was tested using a log-likelihood-ratio (LLR) approach developed by Bravington et al. (2016), and applied by Hillary et al. (2018) and Feutry et al. (2020). A statistical threshold was set to reduce the number of false positive detections due to a large number of pairwise comparisons. In addition, replicated or recaptured individuals were identified based on the number of loci with the same genotype for each pair.

2.2.3. RESULTS

SNP genotyping, baiting success, and data filtering

One sample from Papua New Guinea (PNG) failed DArTseq library construction, resulting in 187 samples for DArTseq sequencing and genotyping. An average of 2,182,162 reads per sample were obtained from the sequencing and a total of 250,945 SNPs, located on 168,810 unique loci, were called by the DArTsoft14 program. Data filtering discarded 240,546 SNPs and 45 sharks, leaving a total of 10,399 high quality SNPs. These were further filtered for cluster sizes smaller than five, sequences longer than 60 bp, and sequences with less than seven consecutive nucleotide repeats. Of those, we randomly selected 3,200 loci with at least one SNP (Appendix D1 sections 4-6). We also identified 469 sex-linked markers (three Y-linked and 466 X-linked markers; Appendix D1 section 4.2); two and 208 Y- and X-linked markers, respectively, showed sufficient polymorphisms to include for DArTcap bait design.

Five samples failed DArTcap library construction. On average, 583,809 reads per sample were obtained from the DArTcap sequencing. After sequence clustering and SNP calling, we obtained 37,537 SNPs found on 26,335 loci. Quality-filtering was applied to the dataset (DATA1: 1,014 sharks including replicates; 5,053 SNPs), the data with confirmed Bull Sharks (DATA2: 769 sharks; 3,409 SNPs), the Bull Sharks data with only 60 samples from Australia (DATA3: 382 sharks; 1,849 SNPs), the W-ATL data (DATA4: 91 sharks; 931 SNPs), the IWP data (DATA5: 635 sharks; 3,416 SNPs) and the IWP data with only 60 samples from Australia (DATA6: 189 sharks; 1,785 SNPs). Here, we presented the results of DATA2 (genetic diversity, F_{ST}, and provenance assignment) and DATA3 (clustering analyses), while the other results are presented in the Appendix D1. After applying the filtering steps for population genetic analyses, DATA2 and DATA3 contained 78 and 88 of the selected DArTcap baits, respectively (Appendix D1 sections 8.4 and 9.4).

Mitogenome sequencing and assembly

The mitogenome was sequenced for 384 samples with an average of 49,766 reads per sample. Six samples from South Africa (SAF), Sri Lanka (SRL), PNG, and Australia (Clarence River and Wenlock River) had low coverage (<5,000 mapped reads) and were subsequently removed from analysis. All reads that mapped to the reference genome were checked for ambiguous base calls at an 85 % threshold

(where a base needed to be present >85 % of mapped reads to be called unambiguous) to detect cross-contamination, barcode slippage, or heteroplasmy. Seven samples were removed due to signs of DNA contamination (originating from SAF, PNG, Seychelles, Reunion, and Australia). Overall, the mitogenome length was 16,707–16,708 bp (Appendix D1 section 13).

Species identification

The mitogenome identified 10 samples from the Arabian Sea, Reunion, Sri Lanka, PNG, and Fiji as different species: the Pigeye Shark (*Carcharhinus amboinensis*), the Spinner Shark (*Carcharhinus brevipinna*), the Graceful Shark (*Carcharhinus amblyrhynchoides*), the Grey Reef Shark (*C archarhinus amblyrhynchos*), the Smalltail Shark (*Carcharhinus porosus*), the Dusky Shark (*Carcharhinus obscurus*), and the Speartooth Shark (*Glyphis glyphis*). In addition, the PCA analysis identified an additional 54 samples that consisted of these species and four samples that could not be assigned to any of these seven species (Appendix D1 section 7.8). Consequently, these samples were omitted, resulting in a total of 769 confirmed Bull Sharks in the filtered DArTcap data (DATA2) and 361 Bull Sharks in the filtered mitogenome data.

Genetic diversity

More than half the markers were monomorphic at almost all locations (Table 2.2.1). We noted that the H_O of the DArTcap data was approximately three times lower compared to the DArTseq data. This may be an artefact of the bait design, where many fixed homozygous markers between locations were selected from the DArTseq data (Appendix D1 5.6 vs 6.2.3). Regardless, both datasets show that the H_O for the E-PAC (H_O = 0.036) and the W-ATL (H_O = 0.048−0.052) regions are lower than the E-ATL and IWP regions (H_O = 0.056−0.69); the nuclear π followed a similar pattern (Table 2.2.1). Three sampling sites with small sample sizes (Caribbean Sea, Thailand, and Indonesia) exhibited low inbreeding coefficients (F_{IS} = -0.018−-0.084), but none were significantly different from zero (Table 2.2.1). Other sites showed a slightly positive F_{IS}, with the highest values exhibited by Costa Rica, South Africa, Mozambique, and Fiji (F_{IS} = 0.041−0.068).

A total of 165 polymorphic sites were identified across all 361 mitogenome sequences, with an average nucleotide diversity of 0.001 and a haplotype diversity of 0.890 (Appendix D1 section 13.3.1). Mitochondrial nucleotide diversities were slightly higher compared to the π_{nu} (π_{nu} = 0.0002−0.0007 vs π_{mt} = 0.0003−0.0030). Of the 165 polymorphic sites, most were only present within the IWP group (Appendix D1 section 13.3.4). Haplotype diversities were high in almost all sampling locations (h = 0.80−0.99), but the lowest values were detected in the Seychelles, Trinity Inlet, and Japan (h = 0.50−0.70).

Population structure

The DAPC indicated that the optimal number of clusters for DATA3 was K = 3 (Appendix D1 section 9.9). These clusters corresponded to the differentiation between the E-PAC, W-ATL, and E-ATL/IWP/Japan/Fiji. In addition, further investigation within the latter group (i.e. DATA5; Appendix D1 section 11.9) revealed that the optimal number of clusters was K = 3 (IWP, Japan, and Fiji). Both PCA and DAPC clearly differentiated the groups (Fig.2.2.2). While the DAPC grouped the E-ATL (one sample from Sierra Leone) with the IWP cluster, the PCA showed this location as distinct from the IWP region (Fig.2.2.2 B). The fixation indices showed similar patterns with high F_{ST} values between ocean basins (F_{ST} = 0.14−0.71; Table 2.2.2). These values were lower within the IWP, ranging from 0.07 between Japan and Fiji to less than 0.04 among other IWP locations. Interestingly and concordant with the PCA and DAPC results, we saw high and significant differentiation between the Japanese locations (Okinawa and Urauchi River). Comparisons within the Indian Ocean yielded low F_{ST} values (F_{ST} < 0.02). Similarly, nuclear differentiation between sites in the W-ATL region was small (F_{ST} < 0.007); although the clustering analysis showed signs of differentiation between the southern (Brazil) and the northern (Gulf of Mexico and Western North Atlantic) sites (Appendix D1 section 10). Other, unbiased, differentiation estimators, such as D_{est} and F'_{ST}, followed the same patterns as F_{ST} (Appendix D1).

The mitogenome Φ_{ST} values showed extremely high differentiation at an ocean-basin level (Φ_{ST} = 0.67−0.97; Table 2.2.2). Even within the Indian Ocean, differentiation was strong between the western (W-IO) and eastern (E-IO) sites (Φ_{ST} = 0.70−0.97), while the northern Indian Ocean (N-IO) sites showed intermediate divergence (Φ_{ST} = 0.46−0.52). The haplotype network demonstrated four major clusters (Fig. 2.2.3): E-PAC, E-ATL, W-IO, and the eastern Indian Ocean and western Pacific combined (E-IO/W-PAC). Within the W-IO and E-IO/W-PAC groups signals of smaller haplotype clusters exist, where each sampling site hosts its own unique haplotypes (Fig. 2.2.3). Two haplotypes, sampled from the Fitzroy River (Australia) and Fiji, clustered with the W-IO group and one haplotype from South Africa grouped with the E-IO/W-PAC group. Further, N-IO samples (from Sri Lanka and Thailand) were split between the two groups. Within Australia, the mitochondrial Φ_{ST} showed structure between western (Fitzroy River), northern (Victoria River, Daly River, Adelaide River, Alligator Rivers — encompassing the South Alligator River and East Alligator River —, Blue Mud Bay, Roper River, Towns River, Wenlock River) and eastern Australian sites (Trinity Inlet, Clarence River, and Sydney Harbour; Table 2.2.2). The haplotype network indicated that most haplotypes were unique to a sampling location (Appendix D1 section 13.4.2.4). Three major haplotype clusters could be identified; however, no apparent geographic pattern was visible in each of these clusters.

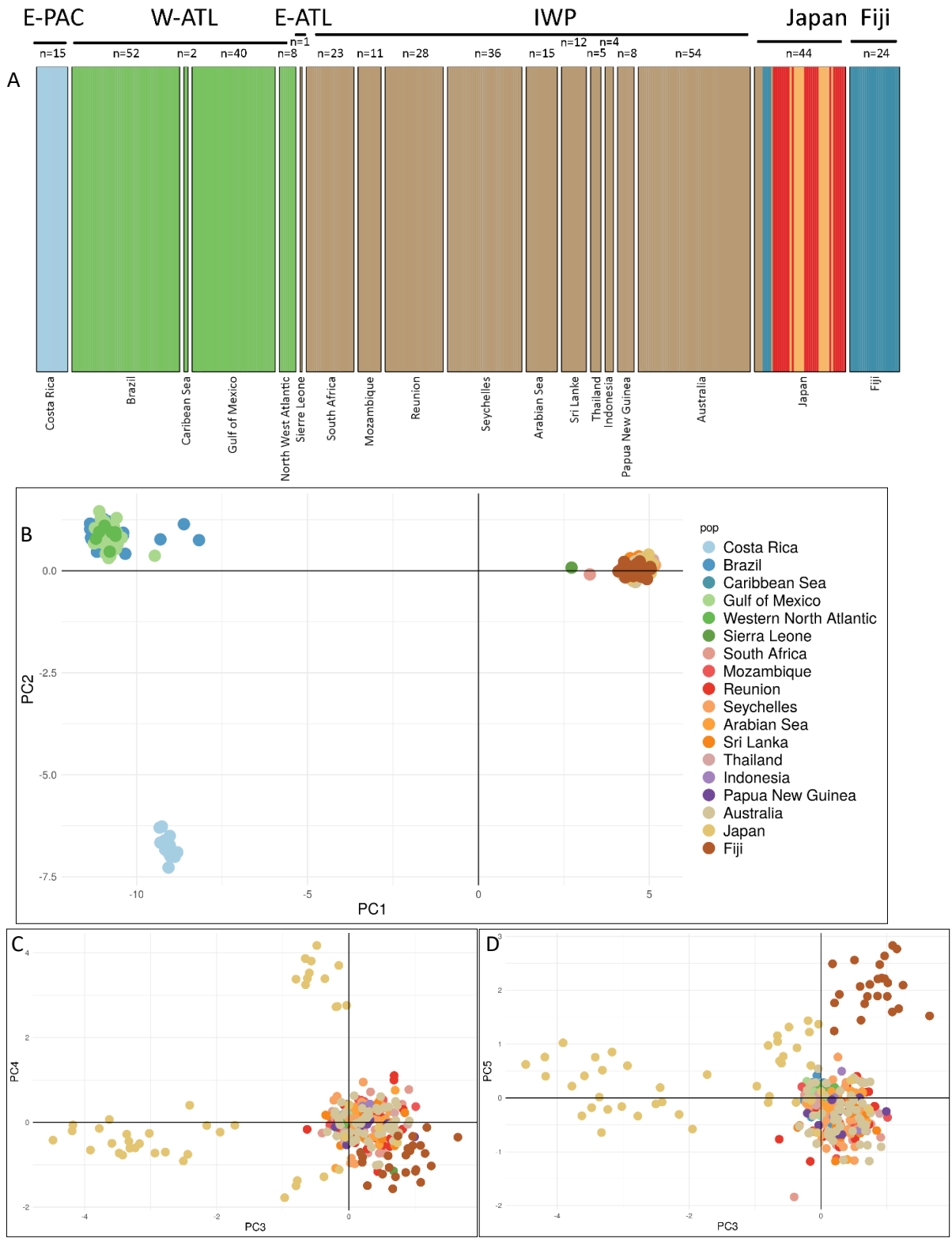

Figure 2.2.2: Population clustering analysis for the global dataset with an Australian subsample (DATA3: 382 sharks; 1,849 SNPs). Panel A shows the Discriminant analysis of principal components (DAPC) assignment barplot for K=6 and 51 principal components (PC). Each bar represents an individual and is coloured according to the posterior membership probabilities. Panel B-D represent the principal component analysis (PCA) scatterplot, where each dot represents an individual shark and colours represent the sampling country or oceanographic location. B) PCA scatterplot with PC1 on the x-axis and PC2 on the y-axis. C) PCA scatterplot with PC3 on the x-axis and PC4 on the y-axis. D) PCA scatterplot with PC3 on the x-axis and PC5 on the y-axis.

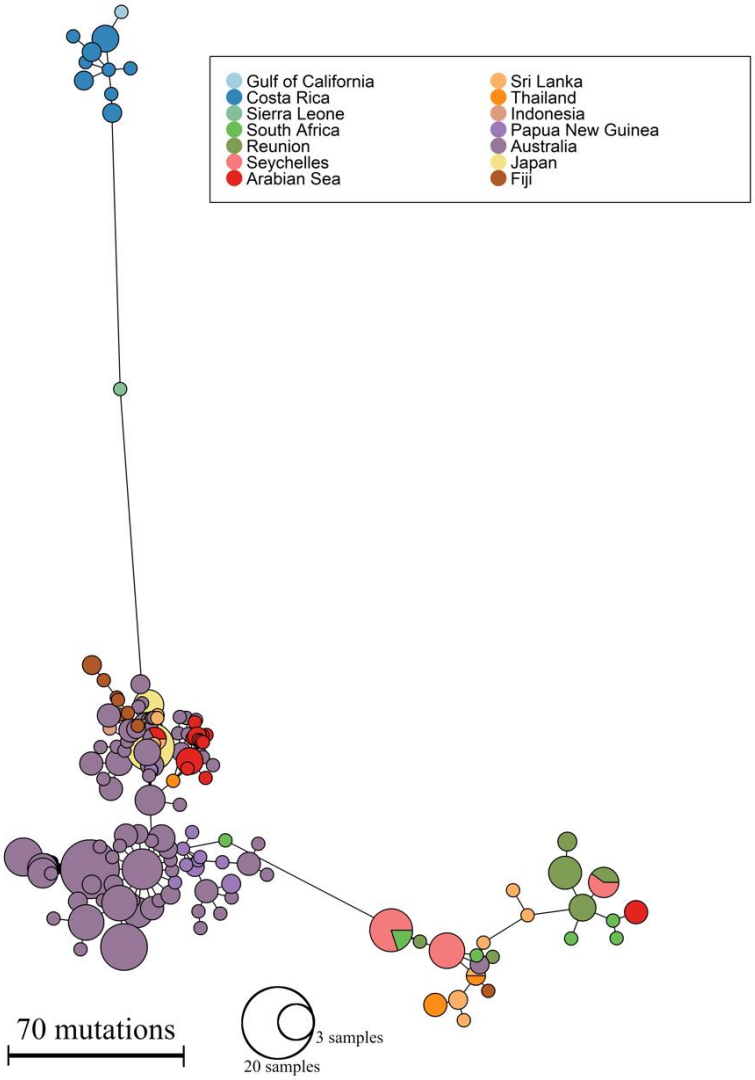

Figure 2.2.3: *Carcharhinus leucas* mtDNA haplotype network. The size of the circles is equivalent to the square root of the number of individuals that share this haplotype. The distance between haplotypes reflects the number of mutations between them. Each haplotype is coloured according to the sampling country or oceanographic location at which they were sampled.

Provenance assignment

After population structure was identified with the nuclear DArTcap markers, we tested the accuracy to assign provenance to a hold-out dataset (Fig. 2.2.4). Using the marker contributions of the DAPC analysis, we saw that a minimum of 50-100 high F_{ST} markers were needed to obtain a reliable assignment (>80 %) to each population (Appendix D1 section 8.9). Only a small number of markers (5-50) were required to differentiate the E-PAC, W-PAC, and E-ATL, but sites within the IWP required up to 100 markers. Samples from Japan and Fiji were unlikely to be assigned to the correct population with fewer than 500 markers.

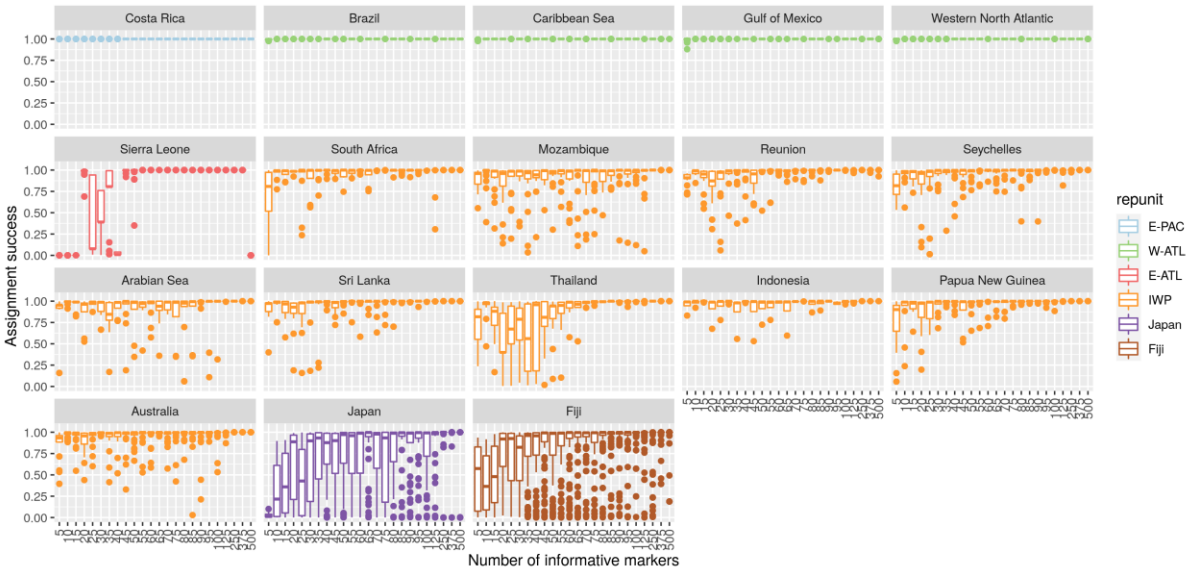

Figure 2.2.4: Reassignment success with respect to the number of informative markers to assign simulated individuals to their population of origin (E-PAC = eastern Pacific; W-ATL = western Atlantic; E-ATL = eastern Atlantic; IWP = Indo-West Pacific; Japan, and Fiji). The simulated individuals are based on a leave-one-out resampling technique in *rubias*. The assignment success was tested based on 5-500 informative markers that were selected according to their DAPC loadings contribution from the *adegenet* package.

Individual and kinship assignment

In total, 102 duplicated individuals were identified. Most represented the technical replicates that were intentionally included, yet we also detected six recaptured sharks in Sydney Harbour, Japan, and Fiji. These recaptures were always assigned to the same cohort where length data were available. Within the populations with larger samples sizes (W-ATL and IWP), we tested for close-kin relationships. Within the W-ATL, we performed 4,095 pairwise comparisons and found two full-sibling pairs (FSP) in Brazil. The IWP revealed more kin pairs due to the large number of pairwise comparisons (201,295). Overall, we found 17 FSPs, all within the same sampling location (Brazil, Reunion, Indonesia, Fiji, and within Australia: Daly, South Alligator, East Alligator, Towns, and Clarence Rivers), and 40 half-sibling pairs (HSPs) of which eight were across locations within northern Australia (Daly, Adelaide, Alligators, Towns and Wenlock Rivers) and 32 were found within the same river.

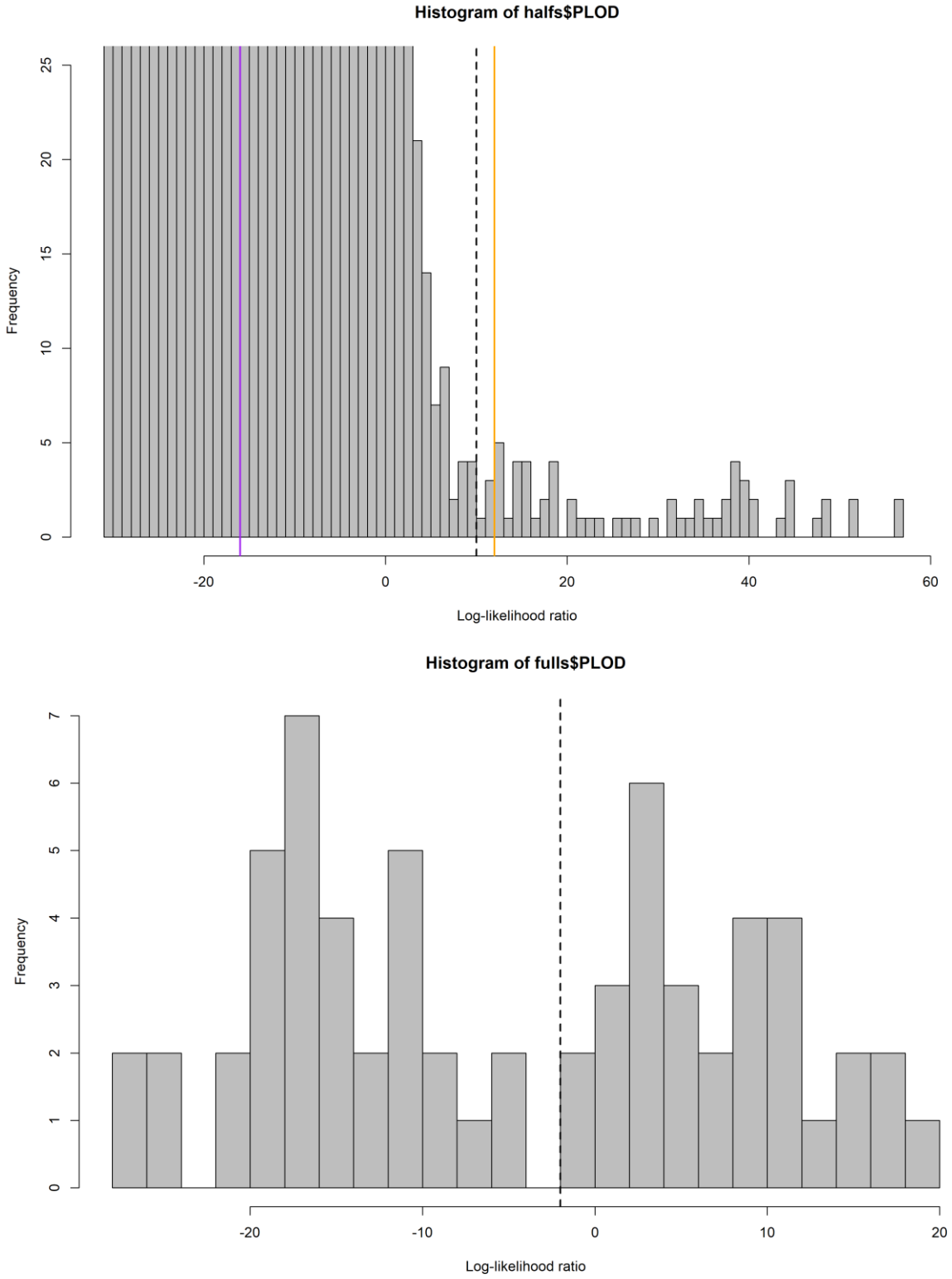

Figure 2.2.5: *Carcharhinus leucas* pairwise log-likelihood ratios (LLR) in the Indo-West Pacific region. Top: all pairwise comparisons, the histogram has been cropped at y = 25 for improved visualisation of kin pair frequencies. Purple line indicates the expected mean for unrelated pairs, orange line indicates the expected mean for half-sibling pairs, and dash line indicates false-positive cut-off (pairs retained as true kin are on its right side of LLR = 10). Bottom: comparisons between pairs retained as true kin. Half-sibling pairs have LLR values below -2, full sibling or parent-offspring pairs have LLR values above -2.

Table 2.2.1: Description of the genetic variation for the full data (DATA2: 769 sharks; 3,409 SNPs) and the full mitochondrial genomes (361 sharks; 16,708 bp). Diversity indices include the number of samples for the nuclear data (N_{nu}), the allelic richness (A_r), the observed heterozygosity (H_O), the expected heterozygosity corrected for low sample size (uH_E), the nuclear nucleotide diversity for the 70 bp reads (π_{nu}), the inbreed coefficient (F_{IS}) and its 2.5 % and 97.5 % confidence intervals ($F_{IS}2.5$, $F_{IS}97.5$), the number of monomorphic markers (M_O), the number of samples for the mitochondrial data (N_{mt}), the nucleotide diversity for the mitogenomes (π_{mt}), the haplotype richness (H) and the haplotype diversity (h). 'NA' values indicate that no or too few samples were available to estimate genetic variation. Diversity indices are divided by geographical regions in dark grey (E-PAC = eastern Pacific; W-ATL = western Atlantic; E-ATL = eastern Atlantic; IWP = Indo-West Pacific; JAP = Japan, and FIJ = Fiji). These regions are further separated by country or oceanographic location in light-grey(GOC = Gulf of California; COR = Costa Rica; BRZ = Brazil; CAR = Caribbean Sea; GOM = Gulf of Mexico; WNA = Western North Atlantic; SIL = Sierra Leone; SAF = South Africa; MOZ = Mozambique; RUN = Reunion Island; SEY = Seychelles; ARS = Arabian Sea; SRL = Sri Lanka; TAI = Thailand; IND = Indonesia; PNG = Papua New Guinea; and AUS = Australia). Finally, Australian sampling sites (in white) were: FZR = Fitzroy River; VIR = Victoria River; DAR = Daly River; ADR = Adelaide River; DWC = Darwin Coastal; SAR = South Alligator River; EAR = East Alligator River; BMB = Blue Mud Bay; ROR = Roper River; TOR = Towns River; WER = Wenlock River; TRI = Trinity Inlet; CLR = Clarence River; SYH = Sydney Harbour; and UNK = fisheries samples from unknown origin. Japan samples were sourced from: OKI = Okinawa Churaumi Aquarium; and URR = Urauchi River.

	E_PAC	GOC	COR	W-ATL	BRZ	CAR	GOM	WNA	E-ATL/SIL	IWP	SAF	MOZ	RUN	SEY	ARS	SRL	TAI	IND	PNG
N_{nu}	16	0	16	97	50	2	37	8	1	586	22	11	28	36	15	12	5	4	9
A_r	1.1540	NA	1.1540	1.2580	1.0950	1.0650	1.0870	1.0840	NA	1.3450	1.1120	1.0860	1.1080	1.1120	1.1140	1.1100	1.1090	1.0970	1.1050
H_O	0.0360	NA	0.0360	0.0500	0.0520	0.0490	0.0480	0.0500	NA	0.0610	0.0600	0.0560	0.0590	0.0600	0.0620	0.0610	0.0660	0.0610	0.0590
uH_E	0.0410	NA	0.0410	0.0550	0.0570	0.0510	0.0530	0.0540	NA	0.0640	0.0630	0.0640	0.0630	0.0630	0.0650	0.0640	0.0660	0.0610	0.0620
π_{nu}	0.0003	NA	0.0003	0.0004	0.0004	0.0003	0.0004	0.0004	0.0002	0.0005	0.0004	0.0004	0.0004	0.0004	0.0004	0.0004	0.0004	0.0004	0.0004
F_{IS}	0.0750	NA	0.0750	0.0536	0.0576	-0.0481	0.0638	0.0369	NA	0.0472	0.0465	0.0959	0.0572	0.0398	0.0370	0.0356	-0.0069	-0.0098	0.0285
$F_{IS}2.5$	0.0781	NA	0.0781	0.0748	0.0694	-0.0306	0.0724	0.0327	NA	0.0421	0.0467	0.0985	0.0528	0.0312	0.0273	0.0126	-0.0320	-0.0293	0.0192
$F_{IS}97.5$	0.1401	NA	0.1401	0.1082	0.1060	0.1257	0.1165	0.0984	NA	0.0579	0.0811	0.1568	0.0893	0.0606	0.0668	0.0614	0.0378	0.0551	0.0703
M_o	1370	NA	2562	3340	1771	2811	2075	2512	3340	301	2081	2443	2010	1914	2195	2353	2699	2835	2512
N_{mt}	16	1	15	0	0	0	0	0	1	302	7	0	16	18	19	12	6	6	15
π_{mt}	0.0003	NA	0.0003	NA	NA	NA	NA	NA	NA	0.0021	0.0019	NA	0.0003	0.0003	0.0018	0.0030	0.0029	0.0004	0.0006
H	10	1	9	NA	NA	NA	NA	NA	1	142	6	NA	6	3	13	11	4	6	13
h	0.9250	NA	0.9143	NA	NA	NA	NA	NA	NA	0.9873	0.9524	NA	0.8083	0.6601	0.9415	0.9848	0.8000	1.0000	0.9810

	AUS	FZR	VIR	DAR	ADR	DWC	SAR	EAR	BMB	ROR	TOR	WER	TRI	CLR	SYH	UNK	JAP	OKI	URR	FIJ
N_{nu}	444	3	0	49	18	15	88	47	18	9	10	24	5	83	65	10	44	9	35	25
A_r	1.1160	1.0830	NA	1.1140	1.1120	1.1160	1.1130	1.1110	1.1160	1.1000	1.1060	1.1120	1.1110	1.1200	1.1130	1.1080	1.2800	1.1020	1.1090	1.2820
H_O	0.0610	0.0600	NA	0.0600	0.0600	0.0640	0.0600	0.0590	0.0650	0.0590	0.0590	0.0600	0.0690	0.0650	0.0600	0.0610	0.0610	0.0590	0.0620	0.0570
uH_E	0.0640	0.0620	NA	0.0630	0.0630	0.0670	0.0630	0.0620	0.0660	0.0650	0.0630	0.0640	0.0680	0.0660	0.0630	0.0640	0.0610	0.0600	0.0600	0.0640
π_{nu}	0.0005	0.0004	NA	0.0004	0.0004	0.0005	0.0004	0.0004	0.0005	0.0004	0.0004	0.0004	0.0004	0.0005	0.0004	0.0004	0.0004	0.0004	0.0004	0.0004
F_{IS}	0.0440	-0.0006	NA	0.0470	0.0392	0.0275	0.0409	0.0383	0.0212	0.0648	0.0434	0.0456	-0.0145	0.0301	0.0448	0.0342	0.0155	-0.0026	0.0006	0.0809
$F_{IS}2.5$	0.0387	-0.0016	NA	0.0448	0.0338	0.0190	0.0370	0.0333	0.0034	0.0614	0.0449	0.0441	-0.0430	0.0062	0.0431	0.0270	-0.0151	-0.0273	-0.0440	0.0955
$F_{IS}97.5$	0.0544	0.0940	NA	0.0704	0.0695	0.0588	0.0596	0.0591	0.0410	0.1174	0.0948	0.0744	0.0249	0.0296	0.0676	0.0768	0.0170	0.0315	-0.0057	0.1358
M_o	395	2914	NA	1727	2178	2204	1360	1779	2120	2517	2480	2033	2681	1320	1550	2452	1969	2653	2133	2114
N_{mt}	203	5	21	25	18	0	38	17	13	4	5	19	4	21	13	0	31	10	21	9
π_{mt}	0.0006	0.0031	0.0004	0.0006	0.0007	NA	0.0002	0.0005	0.0001	0.0001	0.0001	0.0006	0.0000	0.0003	0.0006	NA	0.0004	0.0006	0.0001	0.0014
H	85	4	14	16	12	NA	18	12	7	3	3	14	2	12	11	NA	4	3	3	8
h	0.9777	0.9000	0.9333	0.9367	0.9216	NA	0.9346	0.9412	0.8718	0.8333	0.7000	0.9649	0.5000	0.9333	0.9744	NA	0.6903	0.7111	0.5286	0.9722

Table 2.2.2: Pairwise comparison of fixation indices. F_{ST} values are presented in the bottom diagonal and Φ_{ST} are in the top diagonal. 'NA' values indicate that no or too few samples were available to estimate genetic differentiation. Asterisks (*) indicate statistical significance of p<0.05. No pairwise comparisons were significant after Bonferroni correction. Fixation indices are divided by geographical regions in dark grey (E-PAC = eastern Pacific; W-ATL = western Atlantic; E-ATL = eastern Atlantic; and IWP = Indo-West Pacific). These regions are further separated by country or oceanographic location (GOC = Gulf of California; COR = Costa Rica; BRZ = Brazil; CAR = Caribbean Sea; GOM = Gulf of Mexico; WNA = Western North Atlantic; SIL = Sierra Leone; SAF = South Africa; MOZ = Mozambique; RUN = Reunion Island; SEY = Seychelles; ARS = Arabian Sea; SRL = Sri Lanka; TAI = Thailand; IND = Indonesia; PNG = Papua New Guinea; FZR = Fitzroy River; VIR = Victoria River; DAR = Daly River; ADR = Adelaide River; DWC = Darwin Coastal; SAR = South Alligator River; EAR = East Alligator River; BMB = Blue Mud Bay; ROR = Roper River; TOR = Towns River; WER = Wenlock River; TRI = Trinity Inlet; CLR = Clarence River; SYH = Sydney Harbour; and UNK = fisheries samples from unknown origin; OKI = Okinawa Churaumi Aquarium; URR = Urauchi River; and FIJ = Fiji. Fixation indices range between 0 and 1, where low values are indicated in green and high values in red.

	E-PAC		W-ATL				E-ATL	IWP																						Japan		Fiji
																	Australia																
	GOC	COR	BRZ	CAR	GOM	WNA	SIL	SAF	MOZ	RUN	SEY	ARS	SRL	TAI	IND	PNG	FZR	VIR	DAR	ADR	DWC	SAR	EAR	BMB	ROR	TOR	UNK	WER	TRI	CLR	SYH	OKI	URR	FIJ
GOC		0.124	NA	NA	NA	NA	NA	0.788	NA	0.968	*0.967	0.804	0.664	0.677	0.951	0.931	0.649	*0.951	*0.934	0.923	NA	*0.977	0.944	0.988	0.988	0.985	NA	*0.932	1.000	*0.966	0.936	0.937	0.989	0.848
COR	NA		NA	NA	NA	NA	0.958	*0.913	NA	*0.969	*0.968	*0.877	*0.832	*0.887	*0.965	*0.951	*0.891	*0.959	*0.947	*0.944	NA	*0.975	*0.956	*0.979	*0.973	*0.974	NA	*0.949	*0.975	*0.968	*0.954	*0.957	*0.981	*0.925
BRZ	NA	*0.348		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
CAR	NA	0.429	0.002		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
GOM	NA	*0.369	*0.005	0.000		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
WNA	NA	*0.396	0.004	0.000	0.000		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
SIL	NA	0.701	0.623	0.636	0.645	0.640		0.788	NA	0.968	0.967	*0.802	0.662	0.676	0.950	0.930	0.646	*0.950	*0.933	0.922	NA	*0.977	0.943	0.988	0.988	0.985	NA	0.931	1.000	0.966	0.935	0.937	*0.989	0.846
SAF	NA	*0.662	*0.652	0.640	*0.665	*0.649	0.149		NA	0.211	*0.159	*0.590	0.113	0.048	*0.747	*0.782	0.276	*0.828	*0.813	*0.784	NA	*0.897	*0.808	*0.846	*0.736	*0.757	NA	*0.800	*0.741	*0.849	*0.781	*0.777	*0.884	*0.643
MOZ	NA	*0.677	*0.653	*0.637	*0.668	*0.650	0.139	0.001		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
RUN	NA	*0.658	*0.652	*0.640	*0.663	*0.648	0.159	*0.003	*0.005		*0.606	*0.776	*0.456	*0.520	*0.941	*0.919	*0.713	*0.933	*0.915	*0.911	NA	*0.958	*0.929	*0.963	*0.953	*0.954	NA	*0.918	*0.956	*0.946	*0.925	*0.931	*0.967	*0.864
SEY	NA	*0.653	*0.650	*0.641	*0.661	*0.648	0.164	0.000	*0.005	*0.005		*0.776	*0.395	*0.399	*0.937	*0.916	*0.678	*0.929	*0.912	*0.907	NA	*0.955	*0.925	*0.959	*0.949	*0.950	NA	*0.915	*0.952	*0.943	*0.922	*0.928	*0.964	*0.862
ARS	NA	*0.670	*0.654	*0.638	*0.667	*0.650	0.158	0.002	*0.004	0.002	0.001		*0.292	*0.387	0.135	*0.286	*0.203	*0.322	*0.252	*0.224	NA	*0.483	*0.256	*0.392	0.256	*0.287	NA	*0.227	0.228	*0.362	*0.272	*0.256	*0.326	*0.219
SRL	NA	*0.677	*0.654	*0.641	*0.669	*0.653	0.155	0.001	0.003	0.003	0.000	0.000		0.000	*0.391	*0.504	0.000	*0.563	*0.552	*0.500	NA	*0.692	*0.524	*0.561	*0.404	*0.434	NA	*0.520	*0.393	*0.585	*0.490	*0.476	*0.610	*0.334
TAI	NA	*0.696	*0.656	0.640	*0.673	*0.658	0.155	0.000	0.000	0.003	0.005	0.000	0.000		*0.550	*0.648	0.030	*0.713	*0.691	*0.646	NA	*0.825	*0.677	*0.729	*0.544	*0.580	NA	*0.666	*0.539	*0.741	*0.638	*0.621	*0.780	*0.451
IND	NA	*0.707	*0.661	0.659	*0.679	*0.670	0.186	NA	0.011	0.013	*0.015	*0.012	0.011	0.019		*0.424	*0.345	*0.463	*0.320	*0.255	NA	*0.705	*0.389	*0.762	*0.651	*0.673	NA	*0.308	*0.629	*0.571	*0.409	*0.425	*0.660	*0.321
PNG	NA	*0.689	*0.658	*0.650	*0.674	*0.661	0.169	0.002	*0.011	*0.006	*0.004	*0.007	0.004	0.004	0.017		*0.405	*0.290	*0.245	*0.153	NA	*0.486	*0.277	*0.475	*0.343	*0.380	NA	*0.257	*0.429	*0.427	*0.116	*0.411	*0.639	*0.339
FZR	NA	*0.711	*0.662	0.654	NA	*0.670	0.155	0.000	0.000	0.000	NA	0.000	0.005	0.008	0.023	0.004		*0.439	*0.433	*0.375	NA	*0.611	*0.404	*0.469	0.197	*0.256	NA	0.405	0.288	*0.543	*0.399	*0.431	*0.666	*0.210
VIR	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA		*0.063	0.039	NA	*0.042	0.030	*0.133	0.009	0.073	NA	*0.069	*0.502	*0.440	*0.317	*0.501	*0.667	*0.410
DAR	NA	*0.647	*0.648	*0.639	*0.657	*0.645	0.158	0.000	*0.005	*0.004	*0.002	0.002	0.002	0.003	NA	0.001	0.002	NA		0.025	NA	*0.206	0.005	*0.268	0.145	*0.194	NA	*0.029	*0.377	*0.366	*0.259	*0.386	*0.514	*0.358
ADR	NA	*0.669	*0.654	*0.642	*0.667	*0.652	0.161	0.002	0.002	*0.005	*0.002	0.002	0.001	0.003	NA	0.004	0.000	NA	0.001		NA	*0.223	0.028	*0.234	0.110	*0.156	NA	*0.018	*0.335	*0.338	*0.184	*0.356	*0.514	*0.312
DWC	NA	*0.664	*0.650	*0.629	*0.663	*0.642	0.145	0.001	*0.005	*0.008	*0.003	*0.006	0.002	0.004	0.008	0.003	0.004	NA	*0.003	*0.004		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
SAR	NA	*0.641	*0.646	*0.641	*0.654	*0.646	0.164	0.001	*0.006	*0.007	*0.002	*0.003	0.001	0.002	NA	0.002	0.001	NA	*0.001	0.001	*0.002		*0.148	*0.147	0.062	*0.148	NA	*0.233	*0.720	*0.603	*0.527	*0.687	*0.802	*0.594
EAR	NA	*0.651	*0.650	*0.644	*0.660	*0.650	0.168	0.001	*0.005	*0.006	0.001	*0.003	0.000	0.004	*0.013	0.003	0.004	NA	0.000	0.001	*0.004	0.000		*0.245	0.103	0.163	NA	*0.044	*0.449	*0.411	*0.294	*0.435	*0.615	*0.363
BMB	NA	*0.662	*0.651	*0.632	*0.663	*0.644	0.158	*0.013	*0.012	*0.015	*0.012	*0.012	*0.008	0.011	NA	*0.011	0.012	NA	*0.011	*0.009	*0.013	*0.012	*0.012		*0.296	*0.331	NA	*0.236	*0.873	*0.646	*0.528	*0.691	*0.883	*0.512
ROR	NA	*0.684	*0.656	*0.639	*0.671	*0.654	0.144	0.002	0.003	*0.008	0.003	0.003	0.004	0.000	0.011	0.004	0.002	NA	0.003	0.004	0.002	*0.004	*0.004	*0.012		0.000	NA	*0.132	*0.911	*0.578	*0.394	*0.574	*0.880	*0.326
TOR	NA	*0.684	*0.657	*0.644	*0.672	*0.657	0.158	*0.008	*0.010	*0.014	*0.008	*0.009	*0.007	0.011	0.023	0.009	NA	NA	*0.009	*0.010	0.005	*0.010	*0.009	*0.019	*0.011		NA	*0.175	*0.885	*0.597	*0.428	*0.598	*0.879	*0.367
UNK	NA	*0.681	*0.656	*0.641	*0.671	*0.654	0.156	0.003	0.003	*0.006	0.003	0.001	0.001	0.003	0.012	*0.009	NA	NA	0.003	0.002	0.003	0.002	0.001	*0.010	0.002	*0.007		NA	NA	NA	NA	NA	NA	NA
WER	NA	*0.661	*0.653	*0.640	*0.664	*0.649	0.160	0.002	*0.009	*0.005	*0.003	0.001	0.002	0.004	*0.013	0.003	0.000	NA	*0.002	*0.003	0.001	*0.002	*0.002	*0.012	0.003	*0.009	0.003		*0.357	*0.348	*0.255	*0.378	*0.529	*0.328
TRI	NA	*0.696	*0.659	*0.638	*0.676	*0.659	0.162	*0.011	0.007	*0.016	*0.008	*0.011	*0.013	0.008	0.017	*0.018	0.005	NA	*0.007	*0.011	0.005	*0.010	*0.007	0.017	0.003	0.013	0.006	*0.007		0.000	0.186	*0.551	*0.882	0.000
CLR	NA	*0.631	*0.639	*0.629	*0.646	*0.635	0.155	*0.002	*0.005	*0.005	*0.003	0.002	0.002	0.001	NA	*0.004	0.000	NA	*0.001	0.001	0.002	*0.001	*0.001	*0.012	*0.005	*0.008	0.001	*0.002	*0.007		*0.188	*0.570	*0.750	*0.178
SYH	NA	*0.642	*0.646	*0.638	*0.654	*0.644	0.158	*0.001	*0.003	*0.006	*0.003	0.002	0.002	0.001	NA	0.003	0.000	NA	*0.001	0.000	*0.003	*0.002	0.000	*0.011	*0.003	*0.008	0.001	*0.004	*0.009	*0.001		*0.446	*0.666	*0.178
OKI	NA	*0.697	*0.663	*0.664	*0.680	*0.670	0.205	*0.024	*0.033	*0.030	*0.026	*0.029	*0.028	*0.035	*0.045	*0.034	0.034	NA	*0.026	*0.029	*0.028	*0.028	*0.027	*0.034	*0.032	*0.039	*0.029	*0.032	*0.040	*0.028	*0.026		*0.555	*0.389
URR	NA	*0.669	*0.662	*0.660	*0.673	*0.664	0.226	*0.045	*0.046	*0.047	*0.040	*0.042	*0.044	*0.050	NA	*0.048	NA	NA	*0.044	*0.043	*0.044	*0.040	*0.041	*0.053	*0.048	*0.053	*0.043	*0.040	*0.051	*0.040	*0.042	*0.057		*0.611
FIJ	NA	*0.659	*0.652	*0.639	*0.664	*0.648	0.161	*0.030	*0.033	*0.033	*0.032	*0.027	*0.032	*0.026	NA	*0.031	*0.018	NA	*0.032	*0.032	*0.032	*0.032	*0.030	*0.042	*0.028	*0.036	*0.029	*0.033	*0.038	*0.030	*0.032	*0.056	*0.073	

2.2.4. DISCUSSION

Our study is one of the few that applies both DArTseq, DArTcap and mitogenome data to evaluate the broad and fine-scale population delineation of a large mobile species. By using a combination of markers and analytical methods, we were able to identify genetically distinct populations at both inter and intra-ocean-basin scales. We also highlighted the application of SNP markers for species, sex, provenance, and kinship identification.

Genetic diversity

We found lower observed and expected heterozygosity in the E-PAC and W-ATL sites, compared to the sampling locations in the E-ATL and IWP. Similarly, these regions also had more monomorphic loci, suggesting lower genetic variability in these regions. However, we did not identify any significant indications of inbreeding. Compared to our DArTseq results, other large vagile species, such as the Galapagos Shark (*Carcharhinus galapagensis*), the Blacktip Reef Shark (*Carcharhinus melanopterus*), and the Grey Reef Shark, had slightly higher nuclear SNP diversity (H_O = 0.20−0.40; Maisano Delser et al., 2019; Momigliano et al., 2017; Pazmiño et al., 2018); whereas the Silvertip Shark (*Carcharhinus albimarginatus*) and the Scalloped Hammerhead (*Sphyrna lewini*) exhibited similar levels of heterozygosity (H_O = 0.11−0.16; Green, 2019; Green et al., 2019). The mitogenome genetic diversity was generally high and similar to the mitogenome observations in the W-ATL (Sandoval Laurrabaquio-Alvarado et al., 2021), and slightly higher than the single-gene estimates (π <0.002 and h<0.85; Deng et al., 2019; Karl et al., 2011; Pirog et al., 2019; Sandoval Laurrabaquio-A et al., 2019; Tillett, Meekan, et al., 2012). High nucleotide diversities in South Africa, Sri Lanka, Thailand, and the Fitzroy River (Australia) were due to the presence of haplotypes from different mitochondrial lineages in these regions and may represent evidence of secondary contact.

Observed heterozygosity and SNP nucleotide diversity was generally smaller in the DArTcap data (DATA1) compared to the DArTseq data. This reflects an underrepresentation of heterozygous alleles in the selected baits, although baits were selected randomly from the filtered DArTseq data. However, when the data were split by geographical region (DATA4 and DATA6), the H_O was similar to the DArTseq data (Appendix D1 sections 10.6 and 12.6), indicating that most homozygous alleles were fixed between regions. This may explain why the recently published genetic diversities, obtained through DArTseq, (Glaus et al., 2020) were higher than the observed values in our DArTcap data, but similar to our DArTseq data.

Population structure

Our DArTcap results show five distinct clusters (E-PAC, W-ATL, IWP, Japan, and Fiji), with indication that the E-ATL may also hold a separate Bull Shark population. Within Japan, we see that individuals

group into three separate clusters, where samples from the Okinawa aquarium group more closely with the IWP and samples from the Urauchi River separate into two groups. Opposite to Testerman (2014), we show that the E-PAC is strongly differentiated from the W-ATL. Minor separation was detected between the southern and northern W-ATL locations. This concurs with the studies that showed mitochondrial population structure is present in the W-ATL (Karl et al., 2011; Sandoval Laurrabaquio‐Alvarado et al., 2021) and that long-distance movement is rare (Carlson, Ribera, Conrath, Heupel, & Burgess, 2010). Similar within-region separation may be present in the IWP (as suggested by Pirog et al., 2019), but large population sizes and recent separation could be obscuring our ability to detect nuclear genetic differences (Waples, 1998).

The mitogenome results demonstrated maternal structure at an even finer scale, supporting the growing evidence of long-term female-philopatric behaviour of the Bull Shark (Karl et al., 2011; Sandoval Laurrabaquio-A et al., 2019; Tillett, Meekan, et al., 2012). We see similar structure as for the nuclear DNA, yet the mtDNA shows a clear separation between the W-IO and E-IO/W-PAC and a stronger signal of differentiation from the E-ATL sample. Interestingly, we see that haplotypes from the Arabian Sea are more similar to the E-IO/W-PAC, rather than geographically closer locations in the W-IO. This could reveal a signature of colonization from the E-IO/W-PAC group, or oppositely, that the Arabian Sea was the source population for the E-IO/W-PAC locations. Conversely, we see that certain haplotypes from Sri Lanka and Thailand are grouped across the W-IO and E-IO/W-PAC groups. This suggests secondary contact of historically-separated lineages at these sampling locations. Haplotypes from Japan and Fiji are similar to the E-IO/W-PAC group, confirming these are recently established populations. Remarkably, we see that each haplotype is unique to each sampling location (except for locations within Australia). This could indicate that each site has been separated sufficiently long for the mitogenome to mutate, without female reproductive connectivity.

Within Australia, where the sample size of juveniles was largest, we find no clear nuclear structure; yet the mitogenome differentiation shows three separated clusters (western, northern, and eastern Australia). This signal is not clear from the haplotype networks and may indicate multiple historical isolation, followed by connectivity, events. Historical barriers, such as the land-bridge between Australia and PNG, may have caused the mitogenome to differentiate between groups. Subsequently, the opening of this barrier during the early Holocene (following the Last Glacial Maximum) allowed individuals with distinct haplotypes to move between the northwestern and eastern Australian groups. Collectively, mitochondrial DNA suggests females within Australia may be philopatric to the northwestern and eastern sites. Nonetheless, on a demographic timescale based on the kinship distribution within Australia, we find that all FSPs and most HSPs are found within a sampling location

and would indicate contemporary population structure to a finer scale. This result needs to be verified with larger sample sizes.

Population structure in large coastal sharks tends to be driven by environmental barriers, movement ecology, and habitat preference (Dudgeon et al., 2012). Generally, our results show six populations which are likely separated by several known marine biogeographic barriers, i.e. the Isthmus of Panama, the Mid Atlantic Barrier, the Benguela Upwelling and the East Pacific Barrier (Dudgeon et al., 2012; Rocha et al., 2007). Previously, it was hypothesized that connectivity between the E-PAC and W-ATL may occur through the Panama Canal (Pirog et al., 2019; Testerman, 2014), but our results indicate these populations have been long-separated (e.g. since the closure of the Isthmus of Panama) and that connectivity through the Panama Canal is rare if existent at all. Further, the higher divergence between the W-ATL and E-ATL, compared to the E-ATL vs. IWP, would suggest that the Mid Atlantic Barrier forms a stronger and longer barrier than the Benguela Upwelling System. Next, Fiji and New Caledonia have previously been identified as differentiated island populations, although New Caledonia showed less differentiation than Fiji (Glaus et al., 2020; Pirog et al., 2019). This was attributed to the long open-ocean distances to the islands; yet contrary to direct observations that the Bull Shark can move across open-ocean expanses to pup (Lea et al., 2015). In our study, individuals from Japan differentiate significantly from the IWP cluster, supporting the notion that this is an insular population and that deep-water ocean distances may drive differentiation. Moreover, three distinct groups were found within Japan. The sharks from the Okinawa Aquarium were known to be related and most similar to the IWP group, yet no biological explanation (sex or temporal differentiation) can be found for the two groups within the Urauchi River. The sample size within this river was too small to accurately estimate kinship; thus, a family structure due to extremely small population size cannot be excluded (e.g. Devloo‐Delva et al., 2019; Feutry et al., 2017). The minor nuclear separation between the northern and southern W-ATL sites would suggest that a recent barrier to dispersal may be present in this region, such as oceanographic features (e.g. Caribbean Current) or reproductive asynchrony (Carrillo, Johns, Smith, Lamkin, & Largier, 2015; Carrillo et al., 2017; Castro, 2011; Jenson, 1976). Other than physical barriers, we see that females exhibit a behavioural tendency to return to the same nursery areas; a behaviour that is observed throughout the species' distribution. Philopatry has been described in a range of shark species and likely poses a strong barrier to female dispersal (Chapman et al., 2015).

We hypothesize that the barriers we find for the Bull Shark may affect elasmobranch species with a similar large dispersal capability and a global distribution as well. To date, the global population structure of only a few large-bodied shark species has been investigated (e.g. Galapagos Shark, *C. galapagensis*; Sandbar Shark, *Carcharhinus plumbeus*; and Scalloped Hammerhead, *S. lewini*). Known

biogeographic barriers have similarly affected these species. For example, the Galapagos Shark, which inhabits insular habitats, still shows limited connectivity across oceanic barriers, such as the East Pacific Barrier (Pazmiño et al., 2018). Similarly, both the Scalloped Hammerhead and Sandbar Shark exhibited increased connectivity within ocean basins with continuous coastlines, but showed significant divergence due to the East Pacific Barrier and closure of the Isthmus of Panama (Daly-Engel et al., 2012; Portnoy, McDowell, Heist, Musick, & Graves, 2010). Consequently, our results may provide a foundation to construct hypotheses to test in unstudied species with similar vagility, ecology and life-history (such as the Spinner Shark, the Graceful Shark, or the Dusky Shark).

Species, sex, provenance, and kinship identification

The results showed that our DArTcap panel was able to identify species, sex, provenance, and kinship. Species identification relied on the availability of samples with known species identification, and for this, the mitogenome was imperative. We were able to identify most of our samples to species level using the DArTseq and DArTcap data combined with mitochondrial information (i.e. Bull Shark, Pigeye Shark, Spinner Shark, Graceful Shark, Grey Reef Shark, Smalltail Shark, Dusky Shark, and Speartooth Shark). Given the importance of trade monitoring and the morphological similarity (at least at juvenile stage) between these species, a selection of these markers could be developed into a rapid tool for species identification (Johri et al., 2019; Liu et al., 2017). Previously, the species composition of the shark trade has been studied using mtDNA genes (Cardeñosa et al., 2017; Clarke, Magnussen, Abercrombie, McAllister, & Shivji, 2006; Fields et al., 2018), although some species, like the Galapagos and Dusky Shark, show little mitochondrial divergence (Corrigan et al., 2017; Naylor et al., 2012) and nuclear SNPs could help resolve such issues (e.g. Kyne & Feutry, 2017; Liu et al., 2017).

Similarly, the presence of sex-linked markers, and specifically Y-linked markers, allowed us to identify the genetic sex of our DArTseq samples when the visual sex information was missing. Unfortunately, the DArTcap baits did not capture these markers appropriately, yet future studies could determine the genetic sex from fisheries or trade samples by redesigning the Y-linked marker baits (e.g. Bilton et al., 2019).

The DArTcap panel also showed great promise and power to re-assign samples to their respective populations (ocean-basin scale), and we estimated that a minimum of 100 markers are needed to accomplish a power > 80 %. To date, few studies have investigated the power of markers to assign individuals to the population of origin, and most have employed mtDNA for genetic stock identification (e.g. Cardeñosa, Fields, Shea, Feldheim, & Chapman, 2021; Chapman et al., 2009; Fields et al., 2020). Given the philopatric behaviour of sharks, mtDNA generally has the advantage of assigning provenance to a finer scale. Nonetheless, mtDNA, and especially hypervariable sites such as the

control region, can be subjected to homoplasy (Hassanin, Lecointre, & Tillier, 1998; Levin, Zhidkov, Gurman, Hawlena, & Mishmar, 2013).

The DArTcap panel was able to identify close-kin relationships. These are important to estimate contemporary dispersal patterns (Feutry et al., 2017) or total adult abundance of a population (Bravington et al., 2016). Nonetheless, this application requires a large sample size (respective to the total population size) and sufficient biological information (such as age-at-length and age-at-maturity). Whilst this was limited in our current dataset, kin pairs still provided further insight into local connectivity in Australia.

Overall, these results suggest a diagnostic SNP panel with few markers (100-500) could be designed to identify species, sex, and provenance from tissue samples. New genotyping technologies, such as DNA microarrays, may provide a more cost-efficient method for monitoring of Bull Shark exploitation/harvest (Wenne, Drywa, Kent, Sundsaasen, & Lien, 2016).

Management considerations

Although the Bull Shark is currently not listed as threatened with extinction, it faces a wide range of threats throughout its global distribution, such as commercial, recreational, and artisanal fisheries, meat and fin trade, and nursery habitat modification (Rigby et al., 2021). This was exemplified when the Bull Shark was listed as Endangered within the Arabian Sea due to reported declines in numbers and ongoing fishing pressures (Jabado et al., 2017). Management actions should focus on preserving access to nursery habitat, as well as regulating sustainable exploitation of the Bull Shark. Our study shows the existence of genetically distinct island populations (Japan and Fiji) which possibly have smaller population sizes and currently face a number of threats, such as targeted or bycatch fisheries (Glaus, Adrian-Kalchhauser, Burkhardt-Holm, White, & Brunnschweiler, 2015). The lack of connectivity would indicate that local depletion in these areas would unlikely be stabilized by inflow from neighbouring populations. Further, weak or absent nuclear population structure signals within ocean basins (such as within IWP), may not necessarily mean they should be managed as a single stock. Population structure may exist but must be too weak or too recent to be detected. These areas may require improved genetic techniques (such as whole-genome haplotyping; Browning & Browning, 2011) and an integrated approach (including tagging, parasite fauna, or microchemistry analyses) to quantify the amount of fine-scale connectivity (Begg & Waldman, 1999). As such, threat assessments and management actions should be considered at the smallest spatial scales, in accordance with the mitogenome results, which would prevent a local depletion of philopatric females. To aid fisheries and trade monitoring of Bull Shark populations, the DArTcap panel has proven a promising tool to identify species, sex, and provenance of tissue samples. However, to be used as an enforcement tool this panel

needs more robust testing, which involves obtaining more samples from under-sampled locations (e.g. E-PAC and E-ATL) and testing the accuracy on newly acquired samples.

2.2.5. CONCLUSION

Our study is the first to combine various NGS techniques to demonstrate global and regional population structure in an apex predator. We demonstrated ocean-basin scale divergence for the Bull Shark, with potential reproductive connectivity along continuous coastlines. Full mitochondrial genomes revealed regional female philopatry, while close-kin relationships indicated contemporary philopatric behaviour in Australia at a river-scale. Given the similar characteristics of the Bull Shark to other carcharhinid species, we hypothesise that these may be affected by the same barriers to dispersal, such as open-ocean distance, strong temperature gradients, and historical land-bridges. In addition, the Bull Shark may be considered an indicator species regarding ecosystem health, considering its abundance and functional role in regulating trophic interactions. Current threats, such as fisheries and habitat modification, could potentially wipe out small isolated populations (e.g. Japan and Fiji), and in turn modify the trophic dynamics within those ecosystems. Therefore, threat assessment and management actions should be conducted at the smallest spatial scale to avoid local depletion. In addition, our diagnostic SNP panel may uncover the regional, sex-specific contribution of Bull Sharks in the meat and fin trade. Future studies should concentrate on quantifying the migration rates between the observed populations, and the historical and current range expansions of the Bull Shark to understand its ability to cope with a changing climate.

3. SECTION 3 – Sex-linked markers and sex-chromosome systems in elasmobranchs

Summary

- This section aimed to establish the importance of sex-chromosome systems (SCS) as a driver for the evolution of sex-biased dispersal (SBD) and to provide tools, in the form of sex-linked markers (SLM), to detect SBD. The identification of SLMs has important applications in the field of population genetics and dynamics due to their sex-specific inheritance patterns, and consequently the ability to identify sex in species or life stages that lack sexual dimorphism. Such markers will help infer the sex-chromosome system of a species. Brom et al. (2018) reintroduced the hypothesis that SBD can be a consequence of heteromorphic sex chromosomes (i.e. Y or W chromosomes); thus identifying such chromosomes could reveal potential drivers for SBD.
- In chapter 3, I developed a new bioinformatic tool (R function) to identify SLMs from large datasets produced from next-generation sequencing (DArTseq, DArTcap, or ddRAD). Specifically, this R function 'sexy_markers' investigates sex-specific patterns of SNPs based on i) presence/absence, ii) heterozygosity, and iii) read depth. The function was validated against a DArTseq dataset of 558 White Sharks, *Carcharodon carcharias* Linnaeus 1758, with 23,000 SNPs. Nine Y-linked and 406 X-linked markers were identified, of which 179 and 223 mapped to two White Shark reference genomes. In addition, the Y-linked markers allowed me to develop a quick a PCR assay (Y-amplicon = 650 bp) to identify the sex from tissue samples. I also found that 6.7 % of the samples had a phenotypic-genotypic sex mismatch, which may be explained by human error, but other sex-determining factors cannot be ruled out. Overall, the function proved useful to identify SLMs and sex chromosomes.
- In chapter 4, I evaluated the presence of SLMs and sex-chromosome systems in 21 elasmobranch species. By including several publicly available datasets in addition to my three study species, I investigated if these sex-chromosome systems have a common ancestral state or if they can switch between XY and ZW systems across the evolutionary tree. Results showed that 18 species exhibit signals of an XY-chromosome system and that this represents an ancestral state. These results provide insight into the genome evolution and speciation of elasmobranchs.

3.3. CHAPTER 3 – An R-based tool for identifying sex-linked markers from SNPs

Devloo-Delva, F.; Bradford, R.W.; Grewe, P. M.; Feutry, P. & Gosselin T. (In preparation) An R-based tool for identifying sex-linked markers from restriction site-associated DNA sequencing with applications to elasmobranch conservation.

Sex-linked markers (SLMs) are important in both theoretical and applied biological sciences, especially in conservation. For example, such markers can provide valuable insight into speciation and genome evolution (Charlesworth & Mank, 2010; Graves, 2008; Kitano & Peichel, 2012). Further, in the field of population genetics they allow the inference of sex-specific demographic events due to their sex-specific inheritance, for instance Y-linked markers are only paternally inherited (Greminger, Krutzen, Schelling, Pienkowska-Schelling, & Wandeler, 2010; Petit et al., 2002; Wilson Sayres, 2018). Lastly, sex ratio is a key component when evaluating population dynamics, particularly when females and males differ in life-history traits. Heterogametic markers (only present in one sex) also have the ability to identify sex from DNA samples, which is especially important in threatened species when sexual dimorphism is absent and destructive sampling is not an option (e.g. Stovall et al., 2018; Suda et al., 2019).

Despite the importance of SLMs, only a few methods and tools currently exist to identify them in non-model species. Most approaches are focused on presence-absence and heterozygosity patterns in next-generation sequencing (NGS) data to detect heterogametic (Y or W) and homogametic markers (X or Z), respectively (Fowler & Buonaccorsi, 2016; Gamble, 2016; Gamble & Zarkower, 2014a; Hill, Burridge, Ezaz, & Wapstra, 2018). Most of these workflows following this approach use demultiplexed RADseq reads and are based in STACKS or RADtools, which can be computationally intensive, or the faster C++ (Feron et al., 2021). Other studies showed that outlier detection methods (e.g. BayeScan; Foll & Gaggiotti, 2008), with the data partitioned by sex, can identify SLMs as well (Benestan et al., 2017).

Many elasmobranchs (sharks and rays) have been repeatedly shown to be threatened with extinction (Dulvy et al., 2014). Their low growth rates, late age-at-maturity, low fecundity, and low connectivity between populations, which is often male biased (Daly-Engel et al., 2012; Phillips et al., 2021), has instigated many conservation genetic and genomic studies in elasmobranchs (e.g. Devloo‐Delva et al., 2019; Feutry et al., 2020; Hillary et al., 2018). However, to date, no studies have investigated the utility of SLMs for sex identification and population genetics using these available genomic resources. In this study, we introduce a tool that can analyse the existing genomic data, such as Restriction-site associated DNA sequencing (RADseq), Diversity Arrays Technologies sequencing (DArTseq), or

genotyping by sequencing (GBS), to look for signals of sex chromosomes and identify SLMs on the sex chromosomes.

Specifically, we have designed a function, 'sexy_markers', as part of the '*radiator*' package (Gosselin et al., 2020) in the R environment (R Core Team, 2020) which tests three different hypotheses to find heterogametic and homogametic markers (see Appendix E1): (i) markers are only present in females or males (Fig. 3.3.1. top left), (ii) markers are homozygous in one sex while exhibiting an intermediate range of heterozygosity (0.1–0.5) in the other sex (Fig. 3.3.1. top right), (iii) markers have double the read depth in either females or males (Fig. 3.3.1. bottom). Here, the first hypothesis identifies heterogametic markers and the latter two detect homogametic markers. The function is based on a visual identification of SLMs from NGS data after minimal quality filtering. This function allows the re-assignment of genetic sex when heterogametic markers are identified. In addition, we demonstrated the work flow and accuracy of this function using a White Shark example (*Carcharodon carcharias*, listed as Vulnerable to extinction; Rigby et al., 2019), with a DArTseq dataset of 558 individuals and 23,393 single nucleotide polymorphisms (Hillary et al., 2018). We further compare our function to alternative approaches, such as outlier detection, that have been used in the literature (e.g. Benestan et al., 2017): OutFLANK (Whitlock & Lotterhos, 2015) and PCadapt (Luu, Bazin, & Blum, 2017). The X- and Y-linked markers were validated using polymerase chain reactions (PCR) with primers designed from SLMs that were blasted (*Megablast*) to the reference genomes from Marra et al. (2019) and the Vertebrate Genome Project (VPG; https://vgp.github.io/genomeark/). Beta-actin primers were also designed from the reference genome to act as a positive control between sexes.

Overall, we found nine Y-linked and 406 X-linked markers using the 'sexy_markers' function in less than 5 min computation time (Fig. 3.3.1 and Appendix E1). The nine heterogametic SLMs allowed us to assign sex to 43 individuals with unknown sex and showed a 6.7 % phenotypic – genotypic sex mismatch. The latter is most likely explained by human error, although hermaphroditic elasmobranchs have been identified (reviewed in Adolfi, Nakajima, Nóbrega, & Schartl, 2019). Further, the outlier methods identified seven SLMs, but none were in common with the 'sexy_markers' approach. We were able to confidently blast 179 SLMs (seven Y-linked and 172 X-linked markers) to 49 scaffolds from the Marra et al. (2019) genome, of which 47 SLMs mapped to five scaffolds (i.e. putative sex scaffolds). Eight Y-linked and 215 X-linked markers had confident BLAST hits (see supplement) to eight scaffolds from the VPG genome, with the majority (199 SLMs) mapping to three scaffolds. Overall, we conclude that 48 % of the 415 identified SLMs were located on putative sex scaffolds. These markers were considered as a reference to test the accuracy of the 'sexy_markers' function with sub-optimal data. By randomly sampling a range of individuals for a varying number of markers (Fig. 3.3.2), we showed that too few individuals (< 100) and too few markers (< 10,000 or 50 % of the total data) will yield false

positive results (Fig. 3.3.2 A-B). The discovery of false positive SLMs was even worse when the female:male sex ratio was skewed (2:1 or 1:2; Fig. 3.3.2 C-F). The X- and Y-linked markers were validated through multiplex PCR (Fig. 3.3.3). PCR results showed that only males amplified for the Y-chromosome fragments and females had a higher intensity for X-chromosome bands (compared to the autosomal beta-actin amplicons).

In general, these results confirm that the White Shark has morphologically distinct sex chromosomes (X and Y); an observation also obtained using karyotyping (Maddock & Schwartz, 1996), where the authors suggested the White Shark and other elasmobranchs possess X and Y sex chromosomes. Further, we showed the utility and robustness of the 'sexy_markers' function for species with morphologically distinct sex chromosomes. Finally, we developed a quick (~2 h) PCR assay to identify the sex of White Shark samples. This tool may prove useful to sex neonate sharks (which do not exhibit sexual dimorphism) or samples obtained through biopsies - without a chance to note the phenotypic sex. Chapter 4 of this thesis validates the R function on other species, and Chapter 6 utilises the sex-linked markers for population genetic studies.

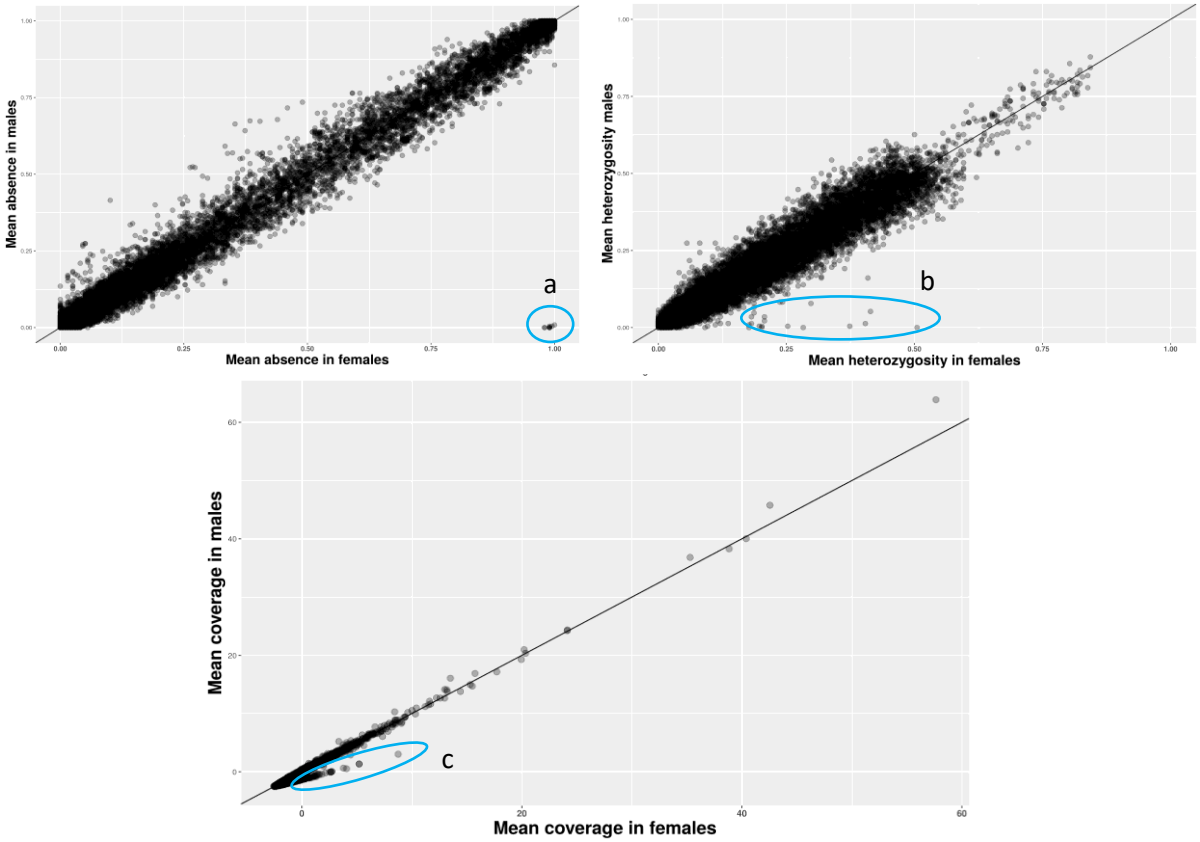

Figure 3.3.1: Identification of sex-linked markers in *Carcharodon carcharias*. Each data point represents a marker for the mean absence (top left panel), heterozygosity (top right panel) and coverage (bottom panel) between females (x-axis) and males (y-axis). Sex-linked markers that followed the expected patterns of presence/absence, heterozygosity, or coverage are highlighted with a blue circle: (a) markers that only present in one sex; (b) markers that are homozygous in one sex while exhibiting heterozygosity in the other sex, and (c) markers that have double the read depth in one sex.

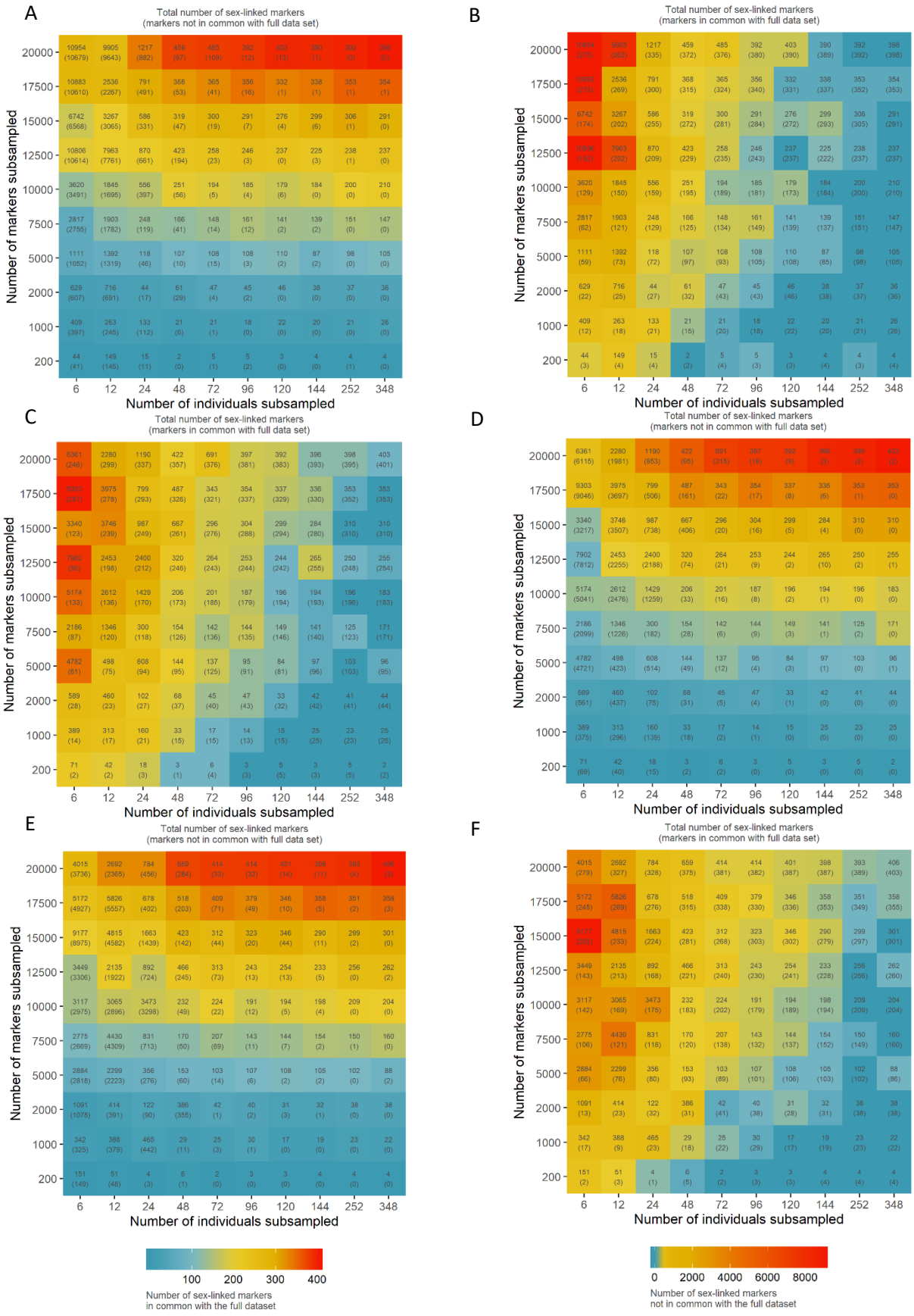

Figure 3.3.2: A heatmap of the robustness test for a female:male sex ratio of 1:1 (A, B), 1:2 (C, D) or 2:1 (E, F). Each square is coloured according to correct sex-linked markers (A, C, E) or false positives (B, D, F).

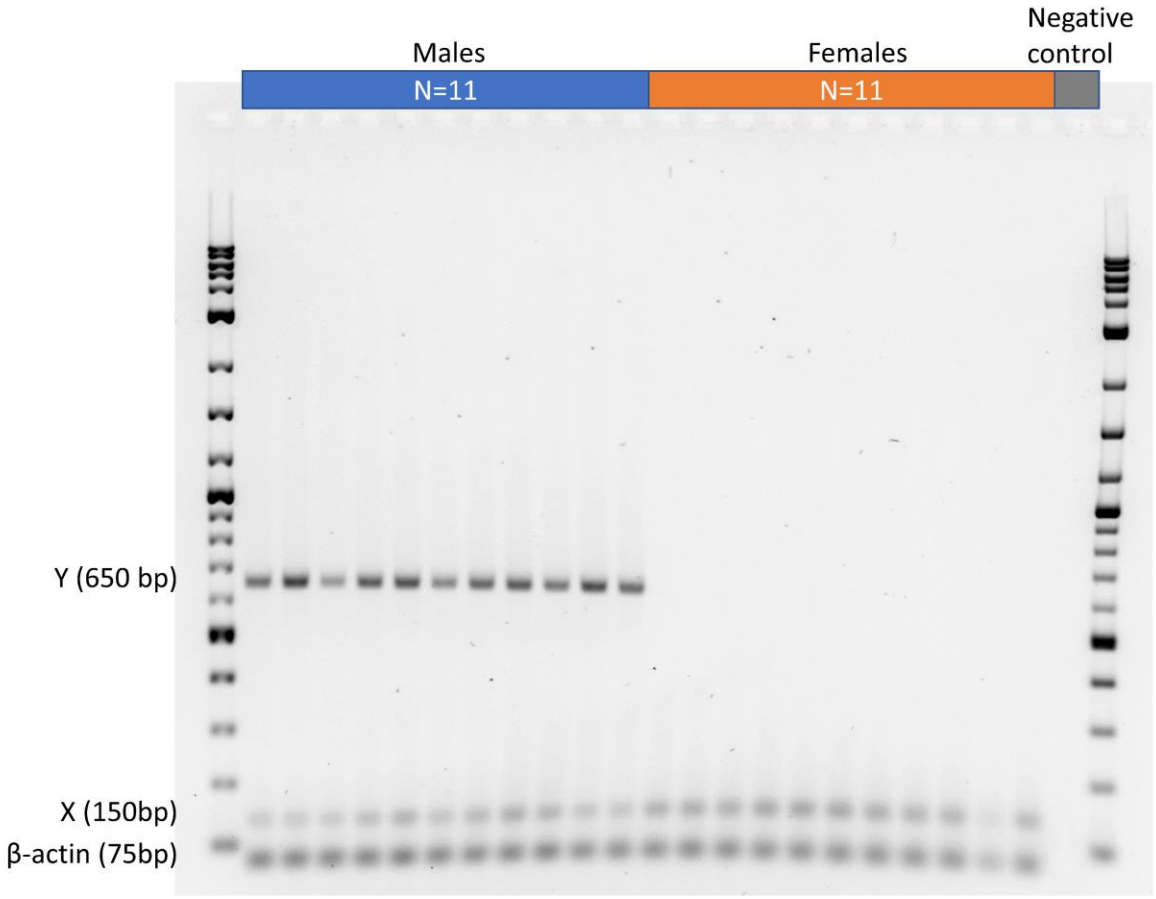

Figure 3.3.3 PCR results of Y- and X-chromosome amplicons between females and males showing 650 and 150 base pairs (bp) fragments, respectively. Beta-actin amplicons (75 bp) serve as a positive test and a reference of intensity.

3.4. CHAPTER 4 – The evolution of sex-chromosome systems in 21 elasmobranch species

3.4.1. INTRODUCTION

A wide variety of sex-determination mechanisms exist in the animal kingdom (see Angelopoulou, Lavranos, & Manolakou, 2012; Ashman et al., 2014; Bachtrog et al., 2014; Schartl & Herpin, 2018 for extensive reviews). These mechanisms range from environmental sex determination (ESD; e.g. crocodiles and turtles) to genetic sex determination (GSD; e.g. mammals and birds). Within GSD, the sex is often linked to the presence of heteromorphic sex chromosomes (i.e. sex-chromosomes system; SCS). Yet, in the presence of sex chromosomes, sex can still be environmentally determined (Capel, 2017; Charlesworth & Mank, 2010; Fernandino & Hattori, 2019; Hill et al., 2018; Holleley et al., 2015; Quinn, Sarre, Ezaz, Graves, & Georges, 2011; Schwanz, Georges, Holleley, & Sarre, 2020). In this regard, environmental or endocrine cues can sometime override the predetermined genetic sex during gonadogenesis (Capel, 2017; Fernandino & Hattori, 2019), which may have important implications in the light of climate change and for the persistence of populations and species (Boyle, Hone, Schwanz, & Georges, 2014).

Knowledge of GSD has applications in many different fields. First, understanding the mechanisms of GSD provides insights into sexual antagonistic selection, speciation, and genome evolution like recombination suppression (Charlesworth & Mank, 2010; Graves, 2008; Kitano & Peichel, 2012; Patten, 2019; Schartl & Herpin, 2018). For example, Kitano et al. (2009) demonstrated that an isolated group of Threespined Sticklebacks (*Gasterosteus aculaetus*) acquired an additional sex chromosome. Subsequently, increased selection on the genes within this chromosome led to increased phenotypic divergence and ultimately speciation. While nucleotide sequences from sex chromosomes have proven beneficial for addressing such questions, they often remain difficult to obtain, even for humans, due to many repetitive elements (Cioffi, Camacho, & Bertollo, 2011; Ezaz & Deakin, 2014; Tomaszkiewicz, Medvedev, & Makova, 2017). However, information on the SCS of an organism can help design sequencing techniques that target the sex chromosomes (Greminger et al., 2010; Tomaszkiewicz et al., 2017). Furthermore, sex chromosomes sequencing can lead to advances in population genetics, such as the quantification and evolutionary drivers of sex-biased gene flow (Brom et al., 2018; Petit et al., 2002; Wilson Sayres, 2018), or the identification of master sex determining genes — such as *sry* in mammals or *dmrt1* in other vertebrates (Graves, 2008; Schartl & Herpin, 2018). Knowledge on how sex is determined allows for a better understanding of the reproductive biology and sexual dimorphism within a species (Devlin & Nagahama, 2002). For economically important species, knowledge of the sex determination process enables the manipulation of sex and, consequently, productivity (Budd, Banh, Domingos, & Jerry, 2015; Fernandino & Hattori, 2019; Gui &

Zhu, 2012; Mei & Gui, 2015). Finally, the ability to determine sex using DNA assays (Bilton et al., 2019; Stovall et al., 2018) can benefit ecological and biological studies, and also species management, by enabling the addition of gender as a covariate (Gamble, 2016).

Sex chromosomes thought to have evolved from an autosomal pair, where one chromosome acquired a sex-determining gene and the chromosomes gradually differentiated through suppression of recombination (Ezaz, Srikulnath, & Graves, 2017; Graves, 2008). This has occurred independently on many occasions and from different autosomes (Bachtrog et al., 2014; Gamble et al., 2015). Nonetheless, the sex chromosomes within a pair usually retain a degree of homology and recombination at pseudo-autosomal regions (PARs), although this varies greatly among taxonomic groups (Graves, 2008). There are two main SCS identified: male heterogamety (XX female/XY male) or female heterogamety (ZW female/ZZ male), but more complex systems exist, such as $X_1X_1X_2X_2/X_1X_2Y$ or ZW_1W_2/ZZ, which are presumed to have evolved from male or female heterogamety through a series of translocations of sex determining genes (Kitano & Peichel, 2012). Mammals and birds have a relatively stable mechanism (XY- and ZW-dominated, respectively). Reptiles, amphibians, and bony fishes, on the other hand, exhibit multiple SCS that appear to have evolved repeatedly (Capel, 2017; Devlin & Nagahama, 2002; Ezaz, Stiglec, Veyrunes, & Marshall Graves, 2006; Gamble et al., 2015). For example, Gamble et al. (2015) inferred that XY and ZW SCS have evolved 17 to 25 times among 12 gecko species, and concluded that ESD was the ancestral sex determination mechanism from which heteromorphic sex chromosomes had evolved. It has also been suggested that these SCS can act as an 'evolutionary trap' through a lack of recombination and degeneration of the Y or W chromosomes, such that direct transitions between XY and ZW are rare (Bachtrog et al., 2014). Nonetheless, in some bony fishes ancestral sex chromosomes have reverted back to autosomes, and been replaced by new sex chromosomes (i.e. sex chromosome turnover; Kikuchi & Hamaguchi, 2013; Kitano & Peichel, 2012). This process may happen when a gene on another chromosome takes over the role of master sex determination gene, and could either maintain or shift the heterogametic sex (e.g. XY to XY or XY to ZW; Vicoso, 2019). Similarly, XY to ZW transitions have been shown to occur in ectotherms through an intermediate state with ESD-sex-reversed individuals (Ezaz et al., 2006; Quinn et al., 2011).

Cartilaginous fishes (Chondrichthyes) are the oldest living representatives of jawed vertebrates, comprising two extant subclasses, Elasmobranchii (sharks, skates and rays) and Holocephali (chimeras; Li, Matthes-Rosana, Garcia, & Naylor, 2012). Cartilaginous fishes represent one of the earliest divergences at the base of the vertebrate evolutionary tree, and can provide novel insights into the evolution of sex determination and SCS (Maddock & Schwartz, 1996). Nonetheless, their sex-determining mechanisms have seldom been studied (Heist, 2012) and it is unknown whether their SCS show similar diversity to other vertebrate lineages. The karyotypes of only 83 chondrichthyan species

(<6 % of the ~1260 species) have been investigated (Stingo & Rocco, 2001), and only those of 23 species allowed any karyological inference about their SCS (see Table 3.4.3 at the end of this chapter; and Uno et al., 2020), albeit hampered by low sample sizes. For example, Maddock and Schwartz (1996) could only confidently detect XY systems in two out of 13 species (*Rhinobatos productus* and *Platyrhinoidis triseriata*) and were unable to identify any heteromorphic chromosomes in two species (*Galeocerdo cuvier* and *Raja eglanteria*). In some instances sex chromosomes may be homomorphic or cryptic (Nygren & Jahnke, 1972; Ohno et al., 1969), and remain undetected by basic karyological surveys. To the best of our knowledge, SCS have only been inferred in 30 chondrichthyan species using a range of different methods (see Table 3.4.3). The presence of an XX/XY system was demonstrated in 12 species (Asahida, Ida, Terashima, & Chang, 1993; Chapman et al., 2007; Donahue, 1974; Maddock & Schwartz, 1996; Schwartz & Maddock, 1986; Uno et al., 2020), while the possibility of male heterogamety (XY) and female heterogamety (ZW) in 10 and one species, respectively, was merely implied due to low sample sizes per sex (Maddock & Schwartz, 1996; Nakamura, Wachtel, Lance, & Beçak, 1987). Alternative XY systems, such as $X_1X_2X_1X_2/X_1X_2Y$ or XX/XO, were suggested in six ray species (Asahida, Ida, & Hayashizaki, 1995; Cruz et al., 2021; Kikuno & Ojima, 1987; Paes da Cruz, Shimabukuro-Dias, Oliveira, & Foresti, 2011; Valentim, Porto, Bertollo, Gross, & Feldberg, 2013; Valentim, Porto, & Feldberg, 2019). Sixty-five species did not show any indication of heteromorphic chromosomes (Donahue, 1974; Maddock & Schwartz, 1996; Valentim et al., 2019).

In addition to the uncertainty surrounding SCS in chondrichthyans, intersex or hermaphroditic individuals (with both reproductive organs) have been recorded in a range of sharks and rays (Adolfi et al., 2019; Capapé, El Kamel-Moutalibi, Mnasri, Boumaïza, & Reynaud, 2012; Castro, 1996; Jones, White, & Potter, 2005; Santander-Neto & Lessa, 2013). The molecular or environmental triggers for sex change in chondrichthyans are poorly understood but could include temperature influences or environmental pollution (Katsu et al., 2010; Yano & Tanaka, 1989). Most chondrichthyan species are ectothermic, except for the Lamnidae (e.g. White Sharks, *Carcharodon carcharias*), and temperature is a common determinant of sex in ectotherms with ESD (Capel, 2017; Schartl & Herpin, 2018). Given the ecto- and endothermic nature of elasmobranchs, SCS-transitions could occur through intermediate ESD (Quinn et al., 2011). Nevertheless, gonadal plasticity is more commonly observed in teleost fishes compared to chondrichthyans (Adolfi et al., 2019), which would suggest that SCS-transition may occur less frequently in chondrichthyans.

While current knowledge of 30 species would suggest that an XX/XY SCS is predominant and likely the ancestral state in chondrichthyans, we aim to test this hypothesis using genomic datasets of 21 species (16 additional species to the 30 species with known SCS). These genomic data contain information about the presence/absence (PA) of reads (hereafter referred to as 'silico' data) and single nucleotide

polymorphisms (SNP) on those sequences. Specifically, if heteromorphic sex chromosomes exist, we expect to find (a) reads that are only present in one sex (i.e. sex-limited), (b) SNPs that are homozygous in one sex while exhibiting heterozygosity in the other sex, and (c) silico and SNP markers that have double the read depth in one sex. Collectively, homologous evolution of the SCS is investigated by identifying conserved sex-linked markers (SLMs) across species and ancestral state reconstruction based on the inferred SCS from the analysed datasets and information from previous Y-antigen, karyotyping, parthenogenesis, and next-generation sequencing (NGS) studies.

3.4.1. RESULTS

The 'sexy_markers' function in the *radiator* R package v1.1.5 (Gosselin et al., 2020) identified SLMs in 19 out of the 21 elasmobranch NGS datasets, ranging from 80 to 500 individuals and 3,500 to 1,500,000 markers (Table 3.4.1). In total, the presence/absence method revealed 78 male heterogametic markers (Y-linked; Fig. 3.4.1-a), whereas the heterozygosity and coverage methods identified 164 and 3,137 female homogametic markers (X-linked; Fig. 3.4.1-b,c), respectively. On average 0.12 % of the data consisted of SLMs, and 221 individuals (6.6 % of the total individuals) had a conflicting phenotypic and genotypic sex. These conflicts were most frequent in the School Shark (*G. galeus*), and Grey Nurse Shark (*C. taurus*) datasets, with 27 (14 %) and 102 (21 %) mismatches, respectively. We also identified several markers that did not fit the predicted pattern of a SLM (highlighted in Fig. 3.4.1-d,e,f). These include (d) markers that are absent in females, but not always present in males; (e) markers that are present in males, but not always absent in females, and (f) markers that are homozygous in females, but nearly always heterozygous (H_O>0.75) in males.

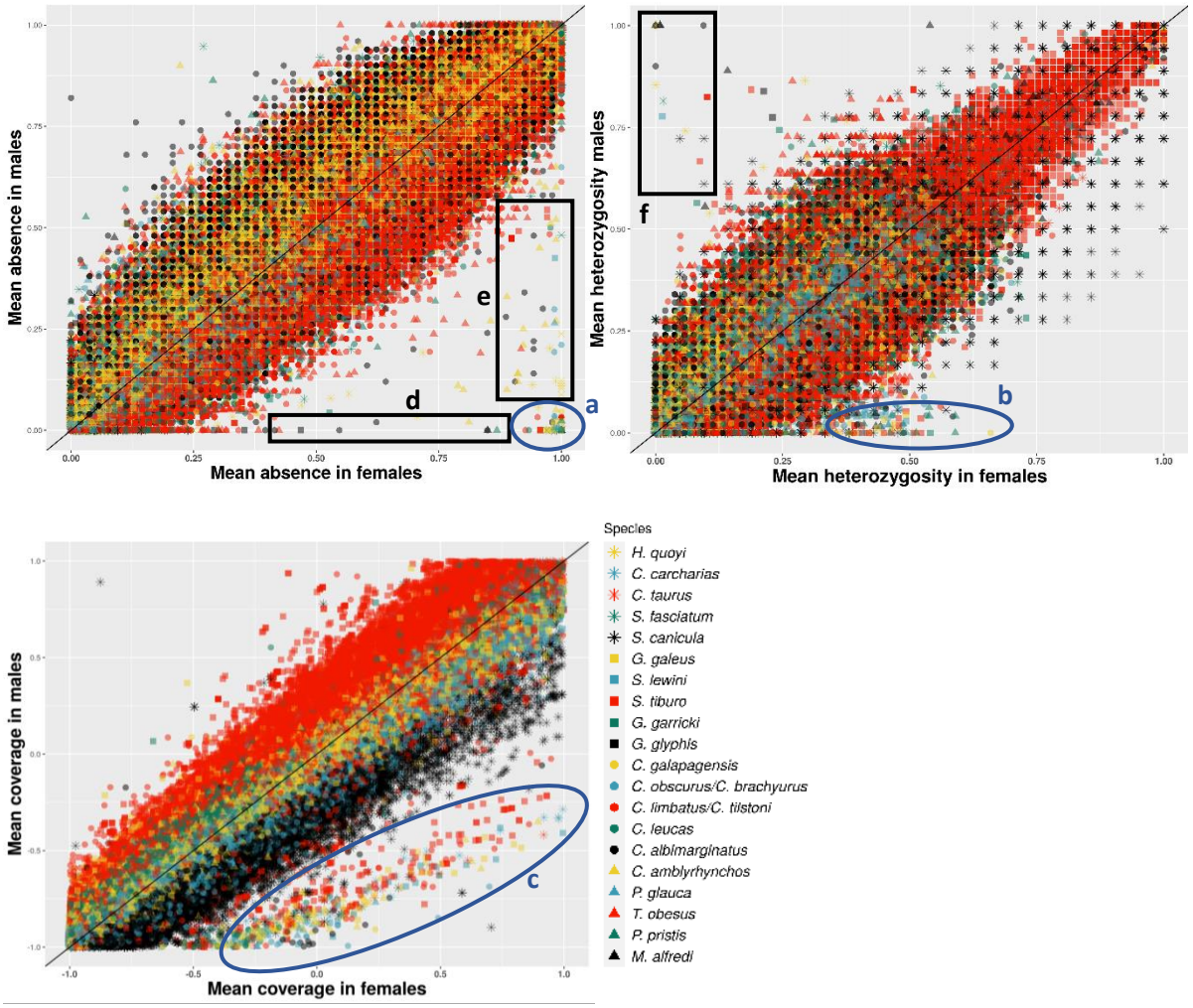

Figure 3.4.1: Combined results of sex-linked marker analysis for 21 elasmobranch species. Data points represent markers for the mean absence (top left panel), heterozygosity (top right panel) and coverage (bottom left panel) between females (x-axis) and males (y-axis). Sex-linked markers that followed the expected patterns of presence/absence, heterozygosity, or coverage are highlighted with a blue circle: (a) markers that are only present in one sex; (b) markers that are homozygous in one sex while exhibiting heterozygosity in the other sex, and (c) markers that have double the read depth in one sex. Sex-linked markers that did not follow the expected patterns of presence/absence or heterozygosity are highlighted with a black box: (d) markers that are absent in females, but not always present in males; (e) markers that are present in males, but not always absent in females, and (f) markers that are homozygous in females, but nearly always heterozygous (H_O>0.75) in males.

Evolutionary stability of SCS in Chondrichthyes

A male heterogametic SCS could be established in all but one of the 19 species for which we identified SLMs (see Table 3.4.3 and Table 3.41). Most species have SLMs that are only present in males (putative Y-linked marker), and only heterozygous in females and double the read coverage in females (putative X-linked markers); this indicates the presence of an XX/XY sex-chromosome system. For example, the School Shark data included 15 Y- and 112 X-linked markers. Only Y-linked markers were found in the Zebra Shark (*S. tigrinum*) and only X-linked markers were found in the Northern River Shark (*G. garricki*), suggesting a male heterogametic and female homogametic system, respectively. No SLMs were apparent in the Whitetip Reef Shark (*T. obesus*) and the Small-spotted Catshark (*S. canicula*).

Overall, this study provides the first SCS information for 12 species, while nine study species had been previously investigated karyologically, albeit with low sample sizes (less than two samples per sex). This brings the total number of chondrichthyans with heterogametic sex chromosomes to 46 species (Table 3.4.3). In total, 65 species have no apparent heteromorphic chromosomes based on previous karyotyping studies. Because 'evidence of absence' is difficult to verify, we only included 11 species, that were investigated for more than two individuals per sex, for further analysis (Table 3.4.3). The inferred states of male heterogamety, female heterogamety, or no apparent sex chromosomes, based on the 46 and 11 species, were added to the branch tips of their phylogenetic tree (Fig. 3.4.2). Ancestral state reconstruction was determined by a stochastic mapping approach with symmetrical transition rates and indicated that male heterogamety is the most likely ancestral state in Chondrichthyes (Fig. 3.4.2).

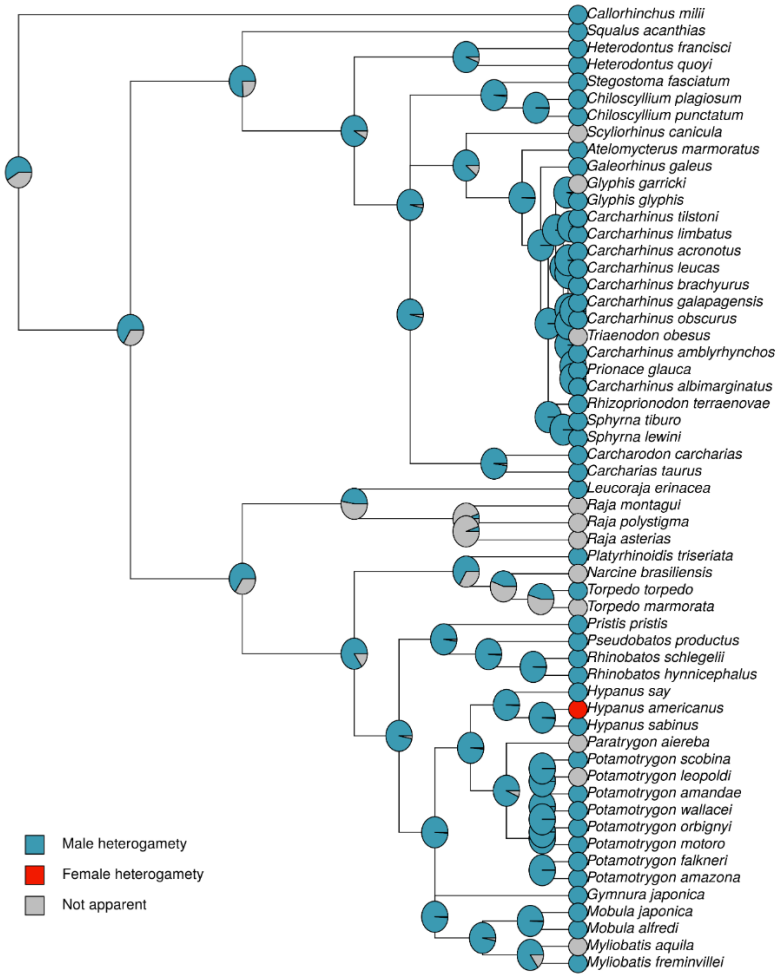

Figure 3.4.2: The phylogenetic relationship based on Stein et al. (2018), among the studied species and species with reliable karyotyping information (i.e. more than two samples per sex). Branch tips are labelled according to their inferred sex-chromosome system (male heterogamety, XY, or female heterogamety, ZW) and species without heteromorphic chromosomes are labelled 'not apparent'. The ancestral SCS states were reconstructed under symmetrical transition rates.

BLAST analysis

There were no noteworthy microbial hits (E value < $10e^{-13}$) for SLMs on NCBI GenBank, indicating that all SLMs are most likely from elasmobranch origin. Furthermore, after blasting the sequences against each other, we identified 758 unique sequences that were similar between SLMs from the same species, and 914 sequences (875 unique) that were conserved among the studied species, respectively (E value > $10e^{-20}$; Table 3.4.2). Ten heterogametic markers were conserved within the Carcharhinidae. Regarding the homogametic markers, 743 and 24 were conserved within the Carcharhiniformes and Lamniformes, respectively, while 19 were homologous between them. No conserved sequences were found between phylogenetically more distant groups.

In total 686 SLMs (389 unique) had reliable BLAST hits against at least one of 10 chondrichthyan reference genomes (Fig. 3.4.3): *Callorhinchus milii*, *Rhincodon typus*, *C. carcharias*, *S. torazame*, *Chiloscyllium punctatum*, *Chiloscyllium plagiosum*, *Pristis pectinata*, *Amblyraja radiata* and *Leucoraja erinacea*. Of those, all but 20 were X-linked. All reference genomes had scaffold that harboured putatively conserved sex-linked regions, but the *C. carcharias* genomes from Marra et al. (2019) and the Vertebrate Genome Project had the highest number of mapped SLMs (196 and 257, respectively), followed by *R. typus* (62 SLMs), *C. plagiosum* (45 SLMs), and *S. torazame* (45 SLMs).

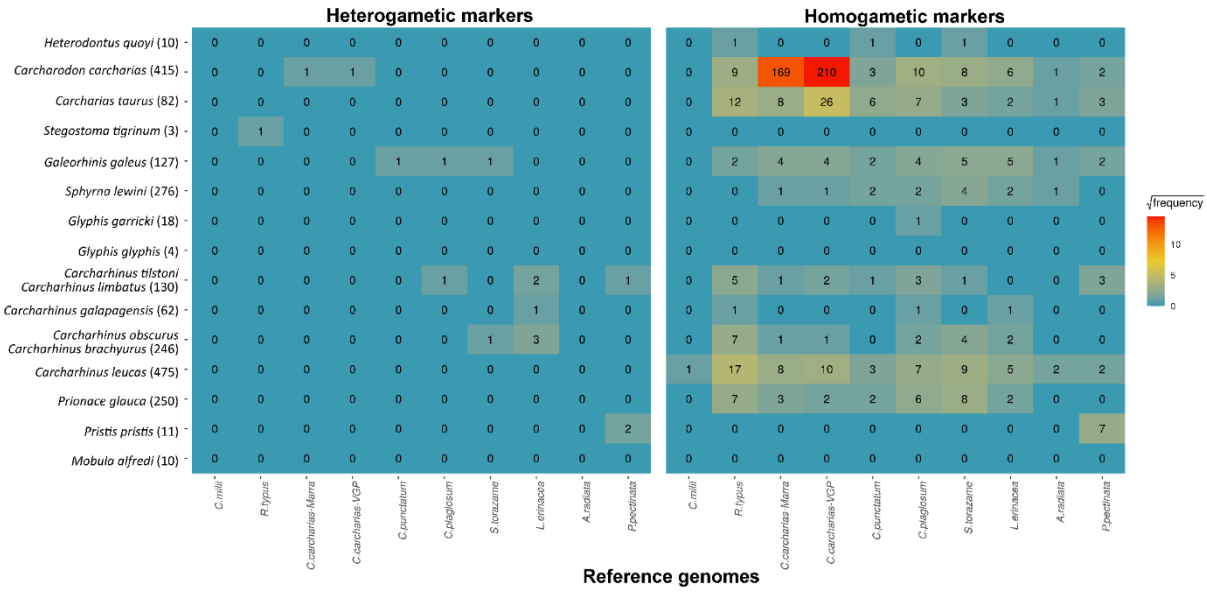

Figure 3.4.3: Heatmap of sex-linked markers with reliable BLAST hits against 10 chondrichthyan reference genomes. Studied species are presented on the y-axis, with the total number of SLMs per species in brackets, and the reference genome species are on the x-axis: *Callorhinchus milii*, *Rhincodon typus*, *Carcharodon carcharias* (includes the genomes from Marra et al. (2019) and the Vertebrate Genome Project), *Scyliorhinus torazame*, *Chiloscyllium punctatum*, *Chiloscyllium plagiosum*, *Amblyraja radiata*, *Leucoraja erinacea*, and *Pristis pectinata.* The colours represent the square root of the number of sex-linked sequences with high BLAST confidence (BLAST sequence length > 50 bp and E value < 10^{-20}).

Table 3.4.1: Summary of the next-generation sequencing data and results analysed in this study. The summary of the data includes the number of samples ($N_{samples}$) after quality-filtering (high missingness and heterozygosity), the number of samples with a conflicting phenotypic and genotypic sex ($N_{conflicting\ sex\text{-}ID}$), the total number of markers ($N_{markers}$) for SNP and presence/absence (silico) data after quality-filtering (monomorphic, short-distance linkage, and high missingness), and the number of sex-linked markers identified by each method and data type. Silico data refers to the presence/absence of reads (this data is specific to DArTseq and DArTcap) and SNP data refers to the single nucleotide polymorphisms on the sequences.

						Sex-linked markers						
Species	**Sequencing method**	**$N_{samples}$ after filtering**	**$N_{conflicting\ sex\text{-}ID}$ (sex ratio phenotypic/ genotypic sex)**	**$N_{SNP\text{-}markers}$**	**$N_{silico\text{-}markers}$**	**PA method (SNP)**	**PA method (silico)**	**heterozygosity method (SNP)**	**Coverage method (SNP)**	**Coverage method (silico)**	**Putative SCS**	**References of data sources**
Heterodontus quoyi	DArTseq	174	7 (0.93/0.96)	29,978	7,622	1	4	5	NA	NA	XX/XY	Hirschfeld *et al.* (in prep)
*Carcharodon carcharias**	DArTseq	428	29 (1.01/0.86)	18,709	116,206	1	8	17	81	308	XX/XY	Hillary et al. (2018)
Carcharias taurus	DArTseq	463	102 (1.51/1.05)	11,399	54,741	1	1	2	16	62	XX/XY	Bradford et al. (2018)
Stegostoma tigrinum	DArTseq	93	7 (2.88/2.44)	6,884	2,161	1	2	0	NA	NA	XX/XY	Dudgeon *et al.* (in prep)
Scyliorhinus canicula	2bRAD	77	NA (1.04/NA)	11,260	NA	NA	0	0	NA	NA	Not apparent	Manuzzi et al. (2018)
Galeorhinus galeus	DArTseq	163	27 (0.66/0.77)	24,094	30,090	2	13	11	68	33	XX/XY	Devloo-Delva et al. (2019)
Sphyrna tiburo	ddRAD	128	7 (0.96/1.06)	560,657	NA	3	NA	14	528	NA	XX/XY	Portnoy et al. (2015)
Sphyrna lewini	DArTseq	287	5 (1.05/1.06)	35,117	86,931	1	2	14	117	142	XX/XY	Green (2019)
Glyphis garricki	DArTseq	89	NA (0.89/NA)	3,180	NA	0	NA	1	14	NA	Female homogamety	Feutry et al. (2020)
Glyphis garricki	DArTcap	316	NA (0.95/NA)	2,215	3,749	0	0	1	2	0	Female homogamety	Feutry et al. (2020)
Glyphis glyphis	DArTseq	176	2 (1.84/1.79)	2,557	36,274	0	2	2	NA	NA	XX/XY	Feutry et al. (2017)
*Carcharhinus tilstoni***	DArTseq	54	2 (1.00/1.08)	22,964	40,992	0	9	0	73	48	XX/XY	Morgan *et al.* (in prep)
*Carcharhinus limbatus***	DArTseq	37	0 (2.08/2.08)	22,964	40,992	0	9	0	73	48	XX/XY	Morgan *et al.* (in prep)
Carcharhinus galapagensis	DArTseq	90	2 (1.03/1.09)	12,493	7,122	0	3	2	46	11	XX/XY	Pazmiño et al. (2018)
*Carcharhinus obscurus****	DArTseq	127	4 (1.28/1.23)	32,586	48,698	1	6	10	111	118	XX/XY	Junge et al. (2019)
*Carcharhinus brachyurus****	DArTseq	15	3 (1.00/1.50)	32,586	48,698	1	6	10	111	118	XX/XY	Junge et al. (2019)
Carcharhinus leucas	DArTseq	92	3 (1.13/0.96)	249,758	396,379	1	2	6	144	322	XX/XY	Devloo-Delva *et al.* (in prep)
Carcharhinus amblyrhynchos	ddRAD	190	16 (0.74/0.90)	566,971	NA	2	NA	13	402	NA	XX/XY	Green (2019)
Carcharhinus albimarginatus	ddRAD	103	3 (1.00/1.06)	343,123	NA	5	NA	2	277	NA	XX/XY	Green et al. (2019)
Prionace glauca	DArTseq	303	18 (0.93/0.91)	101,346	NA	NA	3	43	214	NA	XX/XY	Nikolic et al. (2020)
Triaenodon obesus	DArTseq	153	NA (2.12/NA)	16,478	7,647	0	0	0	0	0	Not apparent	Hirschfeld *et al.* (in prep)
Pristis pristis	DArTseq	87	4 (0.72/0.81)	7,169	5,948	0	2	9	NA	NA	XX/XY	Feutry *et al.* (in prep)
Mobula alfredi	DArTseq	176	7 (1.79/1.79)	10,476	NA	2	NA	8	NA	NA		Armstrong *et al.* (in prep)

*SLMs identified in Devloo-Delva, Bradford, Feutry, Grewe, and Gosselin (in prep)

**These species were sequenced and genotyped together

***These species were sequenced and genotyped together

Table 3.4.2: The number of sex-linked sequences that had high BLAST similarity (BLAST sequence length > 50 bp, E value < 10^{-20}) within and among species. The number of conserved homogametic (X-linked) sequences between species are on the lower diagonal and conserved heterogametic (Y-linked) sequences between species are on the upper diagonal. The total number of X-linked ($N_{X\text{-linked}}$) and Y-linked ($N_{Y\text{-linked}}$) markers for each species are presented adjacent to species names.

	$N_{Y\text{-linked}}$	*H. quoyi*	*C. carcharias*	*C. taurus*	*S. tigrinum*	*G. galeus*	*S. lewini*	*G. garricki*	*G. glyphis*	*C. limbatus/ C.tilstoni*	*C. galapagensis*	*C. obscurus/ C. brachyurus*	*C. leucas*	*P. glauca*	*P. pristis*	*M. alfredi*
	$N_{X\text{-linked}}$	**5**	**9**	**2**	**3**	**15**	**3**	**0**	**2**	**9**	**3**	**7**	**3**	**3**	**2**	**2**
Heterodontus quoyi	**5**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Carcharodon carcharias	**406**	0	106	0	0	0	0	0	0	0	0	0	0	0	0	0
Carcharias taurus	**80**	0	28	25	0	0	0	0	0	0	0	0	0	0	0	0
Stegostoma tigrinum	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Galeorhinus galeus	**112**	0	4	0	0	36	0	0	0	0	0	0	0	0	0	0
Sphyrna lewini	**273**	0	3	1	0	3	129	0	0	0	0	0	0	0	0	0
Glyphis garricki	**18**	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0
Glyphis glyphis	**2**	0	0	0	0	0	0	0	2	0	1	1	0	1	0	0
Carcharhinus limbatus/ C.tilstoni*	**121**	0	2	4	0	8	22	1	1	33	1	4	0	1	0	0
Carcharhinus galapagensis	**59**	0	0	1	0	3	19	0	0	16	10	2	0	1	0	0
Carcharhinus obscurus/ C.brachyurus*	**239**	0	4	1	0	18	54	1	0	72	69	122	0	1	0	0
Carcharhinus leucas	**472**	0	1	0	0	9	29	1	0	67	23	100	207	0	0	0
Prionace glauca	**257**	0	1	1	0	12	30	1	0	44	31	100	116	86	0	0
Pristis pristis	**9**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mobula alfredi	**8**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

*These species were sequenced and genotyped together

3.4.2. DISCUSSION

Based on previous knowledge, we hypothesised that male heterogamety (XY) may be the ancestral state in Chondrichthyes. This study has almost doubled the number of chondrichthyan species with known SCS by using a genomic data approach, allowing a more robust estimation of the evolutionary processes that shape SCS and sex determination.

Evolutionary stability of SCS in Chondrichthyes

Sex determination strategies in vertebrates range from temperature or hormone sensitivity to XY- or ZW-based SCS, with higher variability in ectotherms, such as reptiles, amphibians, and teleost fishes (Bachtrog et al., 2014; Devlin & Nagahama, 2002; Schartl & Herpin, 2018). All data combined, 46 chondrichthyan species exhibited an XY-based system and only 11 species displayed a lack of sex chromosomes. We identified 767 X- and Y-linked sequences that were conserved between species within the same order (Carcharhiniformes and Lamniformes). This provides evidence that their sex chromosomes likely evolved from the same autosome pair (i.e. ancestral state).

Gamble et al. (2015) demonstrated in geckos that once a SCS was acquired, all species within this group maintained the same SCS. On the other hand, Quinn et al. (2011) described the potential of transitions between XY and ZW systems in vertebrates if an intermediate step of viable and fertile sex-reversed individuals exists through ESD. The available evidence indicates that a male heterogametic system is dominant and likely ancestral in Chondrichthyes, consistent with the evolutionary trap model. Nonetheless, one species (*Hypanus americanus*) exhibited a female heterogametic SCS; which may have transitioned recently as its congener *Hypanus sabinus* exhibited an XY system. We acknowledge that current studies are biased toward investigating GSD, since breeding experiments under different temperatures on large sharks are impractical. In this respect, 11 chondrichthyan species did not display any heteromorphic chromosomes, suggesting that either i) sex chromosomes are cryptic or ii) sex can be determined by environmental, endocrine, or epigenetic mechanisms. Differences between small or cryptic heteromorphic sex chromosomes are difficult to observe with current karyotyping methods (Charlesworth & Mank, 2010; Devlin & Nagahama, 2002; Rocco, 2013b). Fluorescence in situ hybridisation (FISH) of sex-chromosome-specific sequences could solve this problem (Rocco, 2013b). Next, Katsu et al. (2010) showed that environmental estrogen-like hormones have the ability to disrupt reproductive activity and gonadal development in two shark species. In addition, we observed 221 mismatches between phenotypic and genotypic sex, mainly in the School Shark, White Shark and Grey Nurse shark datasets. These mismatches most likely reflect errors of morphological sex identification, but alternatively, environmental influences could be overriding the GSD. For example, White Sharks were phenotypically sexed as (sub)adults with a high confidence, yet the PCR results from Devloo-Delva et al. (in prep; Chapter 3) indicate that several phenotypic females

amplified for the 650 base pairs (bp) Y-linked amplicon and several males did not. This could indicate the existence of intersex or hermaphroditic individuals, potentially due to environmental effects (e.g. Yano & Tanaka, 1989). The existence of reproducing hermaphrodites has been recorded in several chondrichthyan species and could allow SCS to shift from XX/XY to ZW/ZZ through mating between sex reversed individuals (Quinn et al., 2011). To fully resolve this question, further investigation using full genome sequencing and experimental evaluation needs to be conducted on the evolutionary mechanism of SCS-transitions and the influences of environmental factors, such as temperature or hormones, on sex determination in elasmobranchs. Based on the limited sampling so far, the batoids, and more specifically Rajiformes and Myliobatiformes, appear to exhibit both i) higher turnover rates in their SCS, and ii) a lack of heteromorphic chromosomes (Table 3.4.3 and Fig. 3.4.2).

In mammals, the Y chromosome is slowly degenerating, due to loss of recombination and transfer of genes to other chromosomes, and the accumulation of repetitive sequences (Cioffi et al., 2011; Ezaz & Deakin, 2014). Hence, the differentiation between mammalian sex chromosomes have been considered proxies for their age (Graves, 2008). However, in other groups, sex chromosome pairs appear highly homologous regardless of their age (Kitano & Peichel, 2012).The size of the Y-chromosome and the amount of recombination in Chondrichthyes could be a proxy for the age of their SCS (Graves, 2015). Uno et al. (2020) did not identify any accumulation of repetitive sequences in the Y chromosomes of *C. plagiosum* and *C. punctatum*; which might indicate that the Y chromosomes originated from a recent sex chromosome differentiation. Our method does not allow an unbiased estimation of the chromosome size, but several of our species (*H. quoyi, S. tigrinum*, *S. lewini*, *S. tiburo*, *C. albimarginatus*, *C. amblyrhynchos, C. obscurus—C. brachyurus*, and *P. glauca*) show a presence/absence pattern that could be consistent with occasional recombination between the X and Y chromosomes (i.e. present in males, and occasional in females Fig. 3.4.1-e). This may be a sign of retreating pseudo-autosomal region (PAR) boundaries and degradation of the Y-chromosome (Graves, 2015). The absence of this pattern in other species may reflect a lack of PARs or a bias in sequencing. Overall, the large number of SLMs identified in this study show that the sex chromosomes are well-differentiated and may continue to lose PARs; which can be suggestive of an established SCS with low probability of sex chromosome turnover (see Kitano & Peichel, 2012).

Graves and Peichel (2010) stated that it is difficult to identify a vertebrate ancestral sex determination mechanism due to the lack of conserved sex-determination genes and a high variability of SCS. Nonetheless, mammalian XY systems may have evolved from an ancient reptilian ZW system, given an intermediate GSD – TSD state (Ezaz et al., 2017; Ezaz et al., 2006; Quinn et al., 2011). Our current results of highly conserved sex-linked sequences among taxonomically-distant species support the hypothesis that an XY system is ancestral in Chondrichthyes, which may have evolved from the same

autosomal chromosomes. However, the question of whether these represent the ancestral sex chromosomes within vertebrates remains to be answered through increased genome sequencing efforts.

Limitations of SLM detection and SCS inference

While the presence of hetero- and homogametic markers is evident in most species, some SLMs do not follow the hypothesised patterns (Fig. 3.4.1-d,e,f). For example, (d) the markers are always absent in the homogametic sex, but variably present in the heterogametic sex. This is likely explained by the existence of a mutation at the restriction-enzyme cutting site of the Y-linked marker. This was observed in the *H. quoyi, S. tiburo*, *G. glyphis*, *G. garricki*, *C. limbatus* - *C. tilstoni*, *C. leucas*, *C. albimarginatus*, *P. pristis*, and *M. alfredi* data. (e) The marker is always present in the heterogametic sex, but infrequently present in the homogametic sex. As discussed, this pattern can be caused by partial recombination between sex chromosomes, where the number of females where the sequence is present may provide an indication of the recombination rate between these sex chromosomes (e.g. ~25 % in *P. pristis*). (f) The heterogametic sex exhibits high heterozygosity (H_O>0.75), while the homogametic sex is homozygous. This pattern was observed in *H. quoyi*, *S. tigrinum*, *S. lewini*, *S. tiburo*, *G. glyphis*, *G. garricki*, *C. albimarginatus*, and *M. alfredi* and could be the result of X–Y homologous regions (not, or partially, subjected to recombination) with a mutation that is present in only one of the sex chromosomes. For all these unusual patterns, the likelihood of false positive results will be enhanced by issues with sampling design (e.g. low sample size or skewed sex ratios), genotyping method (e.g. choice of restriction enzymes or low sequencing coverage), SNP-calling parameters (e.g. clustering paralogous sequences) and data-filtering thresholds (e.g. individuals with excess missingness or heterozygosity).

This study could not confidently determine the SCS of three species. Two species (*S. canicula* and *T. obesus*) showed no evidence of SLMs, while *G. garricki* had only X-linked markers. First, the lack of SLMs may have resulted from a smaller number of SNPs in the dataset (N_{SNPs} = 10,000–20,000; Table 3.4.2) and does not confirm a lack of sex chromosomes. Second, the existence of XX/XO (or ZO/ZZ) systems would be impossible to distinguish from XX/XY (or ZW/ZZ), unless full genome coverage and random spread across the genome can be guaranteed. Failure to detect heterogametic markers does not always imply that heteromorphic chromosomes are absent. Moreover, an $X_1X_1X_2X_2/X_1X_2Y$ system is relatively common in fish, most likely arrived from the fusion between a sex chromosome and an autosomal chromosome (Kitano & Peichel, 2012). Such SCS would provide a similar pattern, in terms of heterozygosity and read depth, as an XX/XY system. Only by designing probes from the homogametic markers and FISH can the existence of multiple X chromosomes be proven (e.g. Rocco, 2013b; Uno et al., 2020).

We were able to use the 'sexy_markers' function to infer a male heterogametic system in 18 of 21 elasmobranch species, confirming previous findings for *C. limbatus*, *C. carcharias, S. tiburo* and *S. canicula*. However, the detection of SLMs and our SCS inference of five species (*C. obscurus, C. taurus*, *P. glauca*, *S. lewini* and *S. tigrinum*) contradicted previous karyotyping studies, albeit with low sample sizes. While both methods are subject to potential errors, the possibility exists that the sex chromosomes of these species have only recently differentiated and no morphological differences are visible (i.e. cryptic sex chromosomes). This may call for caution when using karyological information with insufficient replicates per sex to study the evolution of SCS.

Practical applications

Karyotyping is a time-consuming process to study chromosomal configurations and many difficulties exist (Uno et al., 2020). Our study takes advantage of available NGS data that was originally gathered for unrelated purposes to infer SCS in elasmobranchs; making this a very cost-effective approach which will, hopefully, accelerate the growing knowledge of SCS in Chondrichthyes. When SLMs are detected, the applications are numerous. For example, genotypic sex assignment of individuals is important for species management. Sex-specific PCR assays could be applied to monitor sex-specific catches or trade, where full carcasses are often unavailable (Cardeñosa & Chapman, 2018). Further, they could be used when phenotypic sex identification is difficult. The ability to assign sex to juveniles can help ecological and demographic studies to look at sex-specific movement or population growth (e.g. Pillans et al., 2021; Tsai, Sun, Punt, & Liu, 2014). The pipeline employed herein can also be used to remove SLMs prior to analyses that require autosomal loci (Benestan et al., 2017). SLMs can be used as anchoring points to develop new informative sex-chromosome markers to investigate historical, sex-specific demographic parameters, such as sex-biased dispersal and changes in effective population size (Petit et al., 2002; Wilson Sayres, 2018). The use of SLMs in elasmobranch population genetic studies is currently non-existent (Phillips et al., 2021) despite their proven value to infer sex-specific demography in other taxa (e.g. Hallast & Jobling, 2017; Sacks et al., 2013).

3.4.3. CONCLUSION

This study is the first to present insights in the existence and evolution of chondrichthyan SCS. A novel methodology allowed detection of X- and Y-linked markers in 19 of 21 studied elasmobranch species, which, in turn, provided understanding of their SCS. This extended the SCS knowledge in Chondrichthyes from 30 to 46 species, where 45 species exhibit male heterogamety. Strong sequence conservation was found between most chondrichthyan species, supporting the hypothesis that this group have ancestral sex chromosomes and transitions between SCS appear rare. This could indicate that male heterogamety is ancestral in vertebrates, but further research on the frequency of ESD in Chondrichthyes and more reference genomes are needed to confirm this hypothesis. Lastly, we

advocate and demonstrate the importance of SLMs to investigate fundamental and applied questions in biology.

3.4.4. MATERIALS AND METHODS

Techniques for inferring sex-chromosome systems

Previously, the inference of SCS relied on antigen and karyotyping techniques (e.g. see Maddock & Schwartz, 1996; Nakamura et al., 1987). However, both methods require fresh or well-preserved material, which makes it difficult to attain suitable samples with sufficient replication. Furthermore, the high salt and urea concentration in the blood of elasmobranchs causes problems with the preparation of samples for karyotyping (Maddock & Schwartz, 1996; but see Uno et al., 2020). On rare occasions, parthenogenesis studies have been useful for inferring the SCS (Chapman et al., 2007; Kinney, Wack, Grahn, & Lyons, 2013). For example, Chapman et al. (2007) proposed an XX/XY system for the Bonnethead shark (*Sphyrna tiburo*), through the occurrence of viable female pups from automixis (the fusion of eggs).

Advances in NGS and SNP genotyping have generated a large amount of data to investigate SCS in non-model organisms (Seeb et al., 2011). The discovery of NGS markers that are linked to sex has provided insights into SCS (Gamble & Zarkower, 2014b). SLMs have been identified from natural populations using restriction-site associated DNA (RAD) sequencing methods to genotype SNPs from large sample sizes of each gender (Gamble, 2016). For example, Fowler and Buonaccorsi (2016) identified 33 SLMs in Rockfishes from double-digestion (dd)RAD data by looking at the presence/absence patterns between genders. Hill et al. (2018) identified 206 SLMs based on PA pattern from DArTseq data, but also investigated differences in heterozygosity between sexes (one sex consistently homozygous). More recently, Devloo-Delva et al. (in prep; Chapter 3) identified nine Y-linked and 406 X-linked markers in the White Shark, *Carcharodon carcharias*, from DArTseq data using the 'sexy_markers' function in the *radiator* R package (Gosselin et al., 2020).

Data collection

SNP datasets were sourced from 20 published and unpublished studies comprising 21 elasmobranch species (see Table 1). Seventeen datasets were generated by the DArTseq and DArTcap protocols (Feutry et al., 2017; Feutry et al., 2020), three other datasets followed a ddRAD genotyping protocol (as described in Green et al., 2019; Portnoy et al., 2015) and one followed the 2bRAD protocol (Manuzzi et al., 2018). The DArT protocols were sequenced on an Illumina HiSeq platform and yielded reads that were 69 bp long. Unfortunately, reads from the ddRAD and 2bRAD datasets were not available. DArTseq and DArTcap genotyping also yielded dominant marker data, i.e. presence/absence

of sequences (silico). For both SNP and silico data a distinction was made between data with coverage information per marker and individual (i.e. count data) and without (i.e. genotype data).

SNP and shark genotyping, data filtering and sex-linked marker detection

All datasets were filtered for monomorphic markers, short-distance linkage, high individual missingness, and high individual heterozygosity using the 'sexy_markers' function in the *radiator* package using R v 3.5.1 (R Core Team, 2020). Filtering thresholds were selected after plotting the data (see Appendix F1). To identify SLMs several assumptions and prerequisites needed to be considered and fulfilled: heteromorphic sex chromosomes where recombination had, at least partly, stopped; sufficient and equal samples sizes for each sex; and random sequencing across the genome (see Devloo-Delva et al., in prep). Satisfying these conditions was necessary to allow us to discover SLMs and validate the function's utility. Settings and results per species and data type are presented in the Appendix F1. The SCS of each species were inferred if any reliable X(Z)- and Y(W)-linked markers were detected. When only Y-linked markers were identified the SCS was labelled male-heterogamety or vice versa.

Ancestral state reconstruction

A consensus phylogenetic tree was sourced from Stein et al. (2018; via http://vertlife.org/sharktree/ accessed on 23rd of April 2021). A subset of their phylogeny with 10,000 sampled trees was requested for 46 species with SCS information, and 11 species without heteromorphic chromosomes and more than two samples per sex (see Table 1). The consensus tree was plotted in R using the *ape* v5.3 and *PhyTools* v0.6-99 packages (Paradis, Claude, & Strimmer, 2004; Revell, 2012). Subsequently, we assigned the inferred SCS (i.e. female or male heterogamety) to the branch tips and used stochastic character mapping in *PhyTools* to infer the ancestral states for each node (see Appendix F1 for details). Equal rate (ER) or synonymous (SYN) transition rate models were tested and the model with the lowest log-likelihood was selected.

BLAST analysis

Once SLMs were identified and their respective sequences extracted, the sequences were blasted against the NCBI GenBank database to exclude the possibility of microbial contamination (E value < $10e^{-13}$). Subsequently, the sequences were blasted against each other to identify putative homology within and across species. A BLAST hit was considered matching if the E value < $1e^{-20}$ and the query matched over 60 of the 70 bp (Appendix F1).

All sex-linked sequences were blasted against 10 chondrichthyan genomes (Hara et al., 2018; Marra et al., 2019; Read et al., 2017; Venkatesh et al., 2014; Wang et al., 2012; and https://vgp.github.io/genomeark/). Therefore, results were grouped by the taxonomic family of the

queries. The results were filtered by query coverage, query length and E-value (Appendix F1). Thresholds for the E-value were family-specific. Repetitive section on the sex chromosomes could yield multiple BLAST hits per reference genome for a SLM query. To avoid a biased representation of the number of homologous SLMs, only the highest BLAST hit per query per reference genome was retained.

Table 3.4.3: Overview of all Chondrichthyes currently or previously studied for their chromosome composition and their possible sex-chromosome system. Confidence of the inferred sex-chromosome system are categorised 'low' if less than two samples per sex were investigated and 'high' when more than 10 samples per sex were examined. Intermediate samples sizes were labelled with 'medium' confidence. M = males, F = females. Asterisks (*) indicate species included in the phylogenetic tree for ancestral state reconstruction.

Sub/infra class	Order/family	Scientific name	Sex-chromosome system	Sample size (M/F)	Confidence	Method	References
Elasmobranchii Batoidea	Myliobatiformes Dasyatidae	*Dasyatis matsubarai*	Not apparent	1 (1/0)	low	karyotyping	Asahida and Ida (1990)
		Hemitrygon akajei	Not apparent	2 (1/1)	low	karyotyping	Asahida, Ida, and Inoue (1987)
		Hypanus americanus	Female heterogamety*	2 (0/2)	low	karyotyping	Maddock and Schwartz (1996)
		Hypanus sabina	XX/XY*	10 (7/3)	medium	karyotyping	Donahue (1974)
		Hypanus say	XX/XY*	4 (2/2)	medium	karyotyping	Donahue (1974)
		Pteroplatytrygon violacea	Not apparent	Unknown	low	karyotyping	Stingo and Capriglione (1986)
		Taeniura lymma	Not apparent	3 (1/2)	low	karyotyping	Rocco, Costagliola, Fiorillo, Tinti, and Stingo (2005) Rocco et al. (2007)
	Myliobatiformes Gymnuridae	*Gymnura micrura*	Not apparent	1 (0/1)	low	karyotyping	Schwartz and Maddock (2002)
	Myliobatiformes Myliobatidae	*Mobula alfredi*	XX/XY*	176 (63/113)	high	Sex-linked markers	This study
		Mobula japanica	XX/XY*	2 (1/1)	low	karyotyping	Asahida et al. (1993)
		Myliobatis aquila	Not apparent*	4 (2/2)	medium	karyotyping	Stingo (1979) Rocco et al. (2007)
		Myliobatis californica	Not apparent	1 (0/1) 3 (2/1)	low	karyotyping	Schwartz and Maddock (1986) Rocco et al. (2007)
		Myliobatis freminvillei	Male heterogamety*	1 (1/0)	low	karyotyping	Schwartz and Maddock (1986) Maddock and Schwartz (1996)
		Myliobatis tobijei	Not apparent	2 (1/1)	low	karyotyping	Asahida et al. (1987)
		Rhinoptera bonasus	Not apparent	1 (0/1)	low	karyotyping	Schwartz and Maddock (1986) Schwartz and Maddock (2002)
	Myliobatiforme Potamotrygonidae	*Paratrygon aiereba*	Not apparent*	4 (2/2)	medium	karyotyping	Valentim, Falcão, Porto, and Feldberg (2006)
		Plesiotrygon iwamae	Not apparent	4 (3/1)	low	karyotyping	Valentim et al. (2019)
		Potamotrygon amazona	Male heterogamety	1 (1/0)	low	karyotyping	Valentim et al. (2019)
		Potamotrygon constellata	Not apparent	2 (0/2)	low	karyotyping	Valentim et al. (2019)
		Potamotrygon falkneri	$X_1X_1X_2X_2/X_1X_2Y$*	34 (19/15)	high	karyotyping	Paes da Cruz et al. (2011)
		Potamotrygon leopoldi	Not apparent*	7 (3/4)	medium	karyotyping	Valentim et al. (2019)
		Potamotrygon motoro	Not apparent*	21 (7/14)	medium	karyotyping	Valentim et al. (2006)
		Potamotrygon motoro	$X_1X_1X_2X_2/X_1X_2Y$*	15 (7/8)	medium	karyotyping	Aichino et al. (2013)
		Potamotrygon aff. motoro	$X_1X_1X_2X_2/X_1X_2Y$*	30 (17/13)	high	karyotyping	Paes da Cruz et al. (2011)
		Potamotrygon orbignyi	Not apparent	7 (0/7)	low	karyotyping	Valentim et al. (2006)
		Potamotrygon orbignyi	XX/XY*	7 (0/7) 6 (5/1)	medium	karyotyping	Valentim et al. (2006) Valentim et al. (2019)
		Potamotrygon scobina	XX/XY*	3 (3/0)	medium	karyotyping	Valentim et al. (2019)

Sub/infra class	Order/family	Scientific name	Sex-chromosome system	Sample size (M/F)	Confidence	Method	References
		Potamotrygon wallacei	XX/XO*	45 (19/26)	high	karyotyping	Valentim et al. (2013)
		Potamotrygon aff. wallacei	Not apparent	1 (0/1)	low	karyotyping	Valentim et al. (2019)
	Myliobatiformes Urolophidae	*Urolophus aurantiacus*	Not apparent	2 (0/2)	low	karyotyping	Asahida et al. (1987)
		Urolophus halleri	Not apparent	1 (0/1)	low	karyotyping	Schwartz and Maddock (1986) Schwartz and Maddock (2002)
	Pristiformes Pristidae	*Pristis pristis*	XX/XY*	87 (48/39)	high	Sex-linked markers	This study
	Rajiformes Rajidae	*Amblyraja radiata*	Not apparent	1 (?/?)	low	karyotyping	Nygren and Jahnke (1972)
		Dipturus batis	Not apparent	1 (?/?)	low	karyotyping	Nygren, Nilsson, and JahnkeE (1971) Nygren and Jahnke (1972) Stingo (1979)
		Leucoraja erinacea	Male heterogamety*	4 (2/2)	medium	Antigen H-Y	Nakamura et al. (1987)
		Leucoraja erinacea	Not apparent	1 (0/1)	low	karyotyping	Schwartz and Maddock (2002)
		Leucoraja ocellata	Not apparent	1 (0/1)	low	karyotyping	Schwartz and Maddock (2002)
		Okamejei meerdervoortii	Not apparent	2 (2/0)	low	karyotyping	Makino (1937)
		Raja asterias	Not apparent*	5 (3/2)	medium	karyotyping	Schwartz and Maddock (2002) Rocco, Costagliola, and Stingo (2001) Rocco, Morescalchi, Costagliola, and Stingo (2002) Rocco et al. (2007)
		Raja clavata	Not apparent	1 (?/?)	low	karyotyping	Nygren et al. (1971)
		Raja eglanteria	Not apparent	2 (1/1)	low	karyotyping	Schwartz and Maddock (1986) Maddock and Schwartz (1996) Schwartz and Maddock (2002)
		Raja montagui	Not apparent*	7 (3/4)	medium	karyotyping	Stingo (1979) Rocco et al. (2005) Rocco (2007)
		Raja polystigma	Not apparent*	6 (3/3)	medium	karyotyping	Rocco et al. (2007)
	Rajiformes Rhinobatidae	*Platyrhinoidis triseriata*	XX/XY*	6 (3/3)	medium	karyotyping	Schwartz and Maddock (1986) Maddock and Schwartz (1996)
		Pseudobatos productus	XX/XY*	6 (3/3)	medium	karyotyping	Maddock and Schwartz (1996)
		Rhinobatos hynnicephalus	$X_1X_1X_2X_2/X_1X_2Y$ or XX/XO*	Unknown	low	karyotyping	Kikuno and Ojima (1987) Valentim et al. (2013)
		Rhinobatos schlegelii	$X_1X_1X_2X_2/X_1X_2Y$ or XX/XO*	Unknown	low	karyotyping	Asahida and Ida (1995) Valentim et al. (2013)
	Torpediniformes Narcinidae	*Narcine brasiliensis*	Not apparent*	10 (6/4)	medium	karyotyping	Donahue (1974)
	Torpediniformes Narkidae	*Narke japonica*	Not apparent	1 (0/1)	low	karyotyping	Stingo, Rocco, Odierna, and Bellitti (1995) Ida, Sato, and Miyawak (1985)
	Torpediniformes Torpedinidae	*Tetronarce californica*	Not apparent	2 (0/2)	low	karyotyping	Ida et al. (1985)

Sub/infra class	Order/family	Scientific name	Sex-chromosome system	Sample size (M/F)	Confidence	Method	References
		Tetronarce tokionis	Not apparent	1 (1/0)	low	karyotyping	Asahida and Ida (1990)
		Torpedo marmorata	Not apparent*	4 (2/2)	medium	karyotyping	Rocco et al. (2007)
		Torpedo nobiliana	Not apparent	Unknown	low	karyotyping	Rocco (2013a)
		Torpedo torpedo	XX/XY*	4 (2/2)	medium	FISH	Rocco, Bencivenga, Archimandritis, and Stingo (2009) Rocco (2013a)
Elasmobranchii Selachii	Carcharhiniformes Carcharhinidae	*Carcharhinus acronotus*	Male heterogamety*	1 (1/0)	low	karyotyping	Maddock and Schwartz (1996)
		Carcharhinus albimarginatus	XX/XY*	103 (50/53)	high	Sex-linked markers	This study
		Carcharhinus amblyrhynchos	XX/XY*	190 (100/90)	high	Sex-linked markers	This study
		Carcharhinus brachyurus	XX/XY*	15 (6/9)	medium	Sex-linked markers	This study
		Carcharhinus galapagensis	XX/XY*	90 (43/47)	high	Sex-linked markers	This study
		Carcharhinus leucas	XX/XY*	92 (47/45)	high	Sex-linked markers	This study
		Carcharhinus limbatus	Male heterogamety*	1 (1/0)	low	karyotyping	Maddock and Schwartz (1996)
		Carcharhinus limbatus	XX/XY*	37 (12/25)	high	Sex-linked markers	This study
		Carcharhinus tilstoni	XX/XY*	54 (26/28)	high	Sex-linked markers	This study
		Carcharhinus obscurus	Not apparent	1 (1/0)	low	karyotyping	Asahida et al. (1995)
		Carcharhinus obscurus	XX/XY*	127 (57/70)	high	Sex-linked markers	This study
		Carcharhinus plumbeus	Not apparent	2 (1/1)	low	karyotyping	Asahida et al. (1995)
		Galeocerdo cuvier	Not apparent	2 (1/1)	low	karyotyping	Schwartz and Maddock (1986) Maddock and Schwartz (1996) Schwartz and Maddock (2002)
		Glyphis garricki	Not apparent*	316(162/154)	high	Sex-linked markers	This study
		Glyphis glyphis	XX/XY*	176 (63/113)	high	Sex-linked markers	This study
		Prionace glauca	Not apparent	1 (1/0)	low	karyotyping	Yabu and Ishii (1984) Asahida et al. (1995)
		Prionace glauca	XX/XY*	303(159/144)	high	Sex-linked markers	This study
		Rhizoprionodon terraenovae	Male heterogamety*	1 (1/0)	low	karyotyping	Schwartz and Maddock (1986) Maddock and Schwartz (1996) Schwartz and Maddock (2002)
		Sphyrna lewini	Not apparent	1 (1/0)	low	karyotyping	Hinegardner (1976) Asahida et al. (1995)
		Sphyrna lewini	XX/XY*	287(139/148)	high	Sex-linked markers	This study
		Sphyrna tiburo	XX/XY*	4 (0/4)	low	Inference from parthenogenesis	Chapman et al. (2007)
		Sphyrna tiburo	XX/XY*	128 (59/69)	high	Sex-linked markers	This study
		Triaenodon obesus	Not apparent	153 (49/104	high	Sex-linked markers	This study
	Carcharhiniformes Pentanchidae	*Galeus eastmani*	Not apparent	1 (0/1)	low	karyotyping	Asahida and Ida (1989)
		Galeus nipponensis	Not apparent	1 (0/1)	low	karyotyping	Asahida and Ida (1989)
	Carcharhiniformes Scyliorhinidae	*Atelomycterus marmoratus*	XX/XY*	3 (2/1)	medium	FISH	Rocco (2013a)
		Cephaloscyllium umbratile	Not apparent	2 (2/0)	low	karyotyping	Asahida, Ida, and Inoue (1988)
		Cephaloscyllium ventriosum	Not apparent	1 (0/1)	low	karyotyping	Schwartz and Maddock (2002)

Sub/infra class	Order/family	Scientific name	Sex-chromosome system	Sample size (M/F)	Confidence	Method	References
		Scyliorhinus canicula	Not apparent*	1 (?/?)	low	karyotyping	Stingo (1979) Matthey (1937) Rocco et al. (2002)
		Scyliorhinus canicula	Not apparent*	77 (25/49)	high	Sex-linked markers	This study
		Scyliorhinus stellaris	Not apparent	1 (?/?)	low	karyotyping	Stingo (1979) Rocco et al. (2002)
		Scyliorhinus torazame	Not apparent	2 (1/1)	low	karyotyping	Asahida et al. (1988) Uno et al. (2020)
	Carcharhiniformes Triakidae	*Galeorhinus galeus*	XX/XY*	187	high	Sex-linked markers	This study
		Mustelus canis	Not apparent	1 (1/0)	low	karyotyping	Schwartz and Maddock (2002)
		Mustelus manazo	Not apparent	2 (0/2)	low	karyotyping	Asahida and Ida (1989)
		Triakis scyllium	Not apparent	2 (1/1)	low	karyotyping	Asahida and Ida (1989) Uno et al. (2020)
		Triakis semifasciata	Female or male heterogamety	1 (?/?)	low	karyotyping	Schwartz and Maddock (1986) Maddock and Schwartz (1996) Schwartz and Maddock (2002)
	Heterodontiformes Heterodontidae	*Heterodontus francisci*	Male heterogamety*	4 (2/2)	medium	Antigen H-Y	Nakamura et al. (1987)
		Heterodontus francisci	Not apparent	1 (?/?)	low	karyotyping	Schwartz and Maddock (1986) Schwartz and Maddock (2002)
		Heterodontus japonicus	Not apparent	Unknown	low	karyotyping	Ida, Asahida, Yano, and Tanaka (1986)
		Heterodontus quoyi	XX/XY*	174	high	Sex-linked markers	This study
	Lamniformes Lamnidae	*Carcharodon carcharias*	Male heterogamety*	1 (1/0)	low	karyotyping	Maddock and Schwartz (1996)
		Carcharodon carcharias	XX/XY*	428(230/198)	high	Sex-linked markers	Devloo-Delva et al. (in prep)
	Lamniformes Odontaspididae	*Carcharias taurus*	Not apparent	1 (?/?)	low	karyotyping	Schwartz and Maddock (2002)
		Carcharias taurus	XX/XY*	463(225/238)	high	Sex-linked markers	This study
	Orectolobiformes Hemiscylliidae	*Chiloscyllium plagiosum*	XX/XY*	6 (4/2)	medium	karyotyping	Uno et al. (2020)
		Chiloscyllium punctatum	XX/XY*	13 (9/4)	medium	karyotyping	Uno et al. (2020)
	Orectolobiformes Rhincodontidae	*Rhincodon typus*	Not apparent	2 (1/1)	low	karyotyping	Uno et al. (2020)
	Orectolobiformes Stegostomatidae	*Stegostoma tigrinum*	Male heterogamety*	93 (27/66)	high	Sex-linked markers	This study
		Stegostoma tigrinum	Not apparent	2 (1/1)	low	karyotyping	Uno et al. (2020)
	Hexanchiformes Hexanchidae	*Notorynchus cepedianus*	Not apparent	Unknown	low	karyotyping	Maddock and Schwartz (1996) Schwartz and Maddock (2002)
	Hexanchiformes Chlamydoselachidae	*Chlamydoselachus anguineus*	Not apparent	Unknown	low	karyotyping	Ida et al. (1986)
	Squaliformes Etmopteridae	*Etmopterus spinax*	Not apparent	Unknown	low	karyotyping	Nygren and Jahnke (1972)
	Squaliformes	*Oxynotus centrina*	Not apparent	Unknown	low	karyotyping	Stingo and Capriglione (1986)

Sub/infra class	Order/family	Scientific name	Sex-chromosome system	Sample size (M/F)	Confidence	Method	References
	Oxynotidae						
	Squaliformes	*Squalus acanthias*	Male heterogamety*	2 (1/1)	low	Antigen H-Y	Nakamura et al. (1987)
	Squalidae						
		Squalus acanthias	Not apparent	2 (1/1)	low	karyotyping	Schwartz and Maddock (1986) Maddock and Schwartz (1996) Rocco et al. (2007)
		Squalus suckleyi	Not apparent	2 (2/0)	low	karyotyping	Makino (1937)
	Squatiniformes	*Squatina californica*	Not apparent	3 (1/2)	low	karyotyping	Maddock and Schwartz (1996) Rocco et al. (2007)
	Squatinidae						
Holocephali	Chimaeriformes	*Callorhinchus millii*	Male heterogamety*	20 (10 /10)	high	Sex-linked markers	Kemper & Naylor (unpublished data)
	Chimaeridae						
		Chimaera monstrosa	Not apparent	2 (2/0)	low	karyotyping	Nygren and Jahnke (1972) Rocco et al. (2007)
		Hydrolagus colliei	Not apparent	1 (0/1)	low	karyotyping	Ohno et al. (1969)

4. SECTION 4 – Historical and contemporary sex-biased dispersal in sharks

Summary

- Sex-biased dispersal has been presumed in many elasmobranch species, yet convincing evidence is often missing (Phillips et al., 2021). With an integrated approach using both conventional and novel methods, these assumptions can be tested more rigorously. In this section I combined the results from Sections 2 and 3 to quantify sex-biased dispersal between populations in the Northern River Shark and the Bull Shark.
- The School Shark did not exhibit population structure and it has previously been shown that sex-biased dispersal is associated with strong spatial structure of the metapopulation (e.g. Brom et al., 2018). Due to the fact the School Sharks data also lacked large adult individuals, I could not use assignment testing to quantify the number of direct dispersers. Likewise, I did not identify any informative kinship relationships (parent-offspring or half sibling) in the data. Neither did I sequence any mtDNA regions, and the X- and Y-linked markers showed insufficient variation, thus I could not infer historical SBD using a mixed marker approach.
- The Northern River Shark showed strong regional population structuring with indication that the Van Diemen Gulf contains signals of divergence at smaller spatial scales. In Chapter 5, I sequenced the full mitochondrial data for 379 sharks in addition to DArTcap and the kinship data from Feutry et al. (2020), with allowed me investigate to sex-specific connectivity on both evolutionary and contemporary, or demographic, timescales. Almost all of the 11 rivers, creeks, embayments, or estuaries in five regions formed a distinct breeding unit, with locations in close geographical proximity sharing more kin. Both females and males were highly philopatric on historical and contemporary timescales; however, close-kin results suggested that males may disperse more often and further.
- The Bull Shark exhibited strong population divergence at an ocean-basin scale with signals of population structure within the Indo-West Pacific region. Chapter 6 further investigated sex-specific dispersal within this region by contrasting full mitochondrial genomes, autosomal SNPs, and X-linked SNPs. I looked for signals of direct migrants using a range of individual and population-level assignment approaches. The spatial distribution of close-kin was also investigated. Results indicated the presence of female philopatry with evidence from mtDNA, X-linked markers, and kinship. However, male-biased dispersal could only be established at small geographic scale, based on five cross-cohort, cross-river half siblings in Australia.

4.5. CHAPTER 5 – Sex-specific dispersal patterns of the Northern River Shark

Devloo-Delva, F.; Kyne, P. M.; Marthick, J. R.; Grant, M.; Gunasekera, R. M.; Johnson, G.; Morgan, D. L.; Pillans, R. D.; Saunders, T.; White, W. T.; Grewe, P. M. & Feutry, P. (under review) **Sex-specific dispersal patterns of the threatened Northern River Shark, *Glyphis garricki*.** Heredity.

4.5.1. INTRODUCTION

Information on population structure and genetic, or reproductive, connectivity can be crucial to assist the conservation and management of threatened species. Isolated populations may be susceptible to local extinction, whereas well-connected populations can maintain sufficient genetic variability and fitness (Caughley, 1994; Frankham et al., 2017; Ralls et al., 2020). Nonetheless, connected populations (i.e. the metapopulation) may still be prone to decline if sex ratios or dispersal are biased towards a specific sex (Caughley, 1994). The sex with the greatest dispersal is more likely to be subjected to a greater range of threats, while the least dispersing sex may be more vulnerable to localised threats for longer periods. Similarly, recently-established small populations may have lower arrivals from one sex and could be negatively impacted through elevated levels of inbreeding (Bonte et al., 2012).

Philopatry and sex-biased dispersal (SBD) are important traits that both describe the individual-level dispersal behaviour of a species within and among populations, resulting in population-scale dynamics (Dobson, 2013; Greenwood, 1980). We define philopatry as the return of reproducing individuals to their natal sites or region and SBD as the uneven reproductive movement of females or males between distinct populations which is estimated by gene flow (Chapman et al., 2015; Phillips et al., 2021). Both philopatry and SBD provide important information for understanding population dynamics that can be used to aid in species conservation and management (Dobson, 2013). Although these terms are commonly considered together, they are fundamentally distinct and not mutually exclusive in any given species. Dispersal is typically undertaken by both sexes, but dispersal rate and distance can differ between females and males (Pusey, 1987). For instance, Blundell, Ben-David, Groves, Bowyer, and Geffen (2002) illustrated that male River Otters (*Lontra canadensis*) dispersed more, but only to nearby areas whereas females dispersed less frequently but travelled greater distances.

Many methods have been used to infer philopatry and SBD (Goudet et al., 2002; Prugnolle & de Meeus, 2002). By following the matrilineal connectivity with mitochondrial DNA (mtDNA), studies have shown whether females historically revisited the same breeding or pupping grounds (i.e. female philopatry). The comparison of mtDNA against biparentally- or paternally-inherited nuclear DNA (nuDNA) can allow the 'indirect' inference of historical sex-biased gene flow when either nuDNA or mtDNA shows a different pattern of structuring between populations. On an intra-generational timescale, dispersal has been 'directly' measured by capture-mark-recapture (e.g. Hutchings & Gerber, 2002), telemetry

(e.g. Papastamatiou et al., 2013; Pillans et al., 2021), or assigning individuals to their population of origin (Goudet et al., 2002; Mossman & Waser, 1999). Nonetheless, these methods cannot inform if the dispersed individuals successfully breed in the new population, unless breeding and subsequent offspring are visually observed. A novel method to measure contemporary 'reproductive' dispersal from juveniles was proposed by Bravington et al. (2016). This reproductive dispersal can be measured 'directly' or 'indirectly' by analysing the spatial distribution of parent-offspring pairs (POP) or half-sibling pairs (HSPs), respectively. The latter was applied by Feutry et al. (2017) to study the Speartooth Shark (*Glyphis glyphis*); a euryhaline species known to occur in a limited number of macrotidal tropical rivers of northern Australia (Pillans et al., 2009), with limited juvenile movement between rivers. Feutry et al. (2017) identified 121 cross-cohort HSPs using 1,330 single nucleotide polymorphism (SNP) markers. Data from the full mitochondrial genome (mitogenome) indicated whether the HSPs were paternally or maternally related. In other words, if the HSPs had different mtDNA haplotypes, they were paternally related. Vice versa, if the HSPs shared a haplotype they were expected to be maternally related, although they may still be paternally related but identical-by-state rather than identical-by-descent due to insufficient mitogenome variation (e.g. incomplete lineage sorting; see Toews & Brelsford, 2012). Feutry et al. (2017) found that all 18 cross-cohort, cross-river HSPs were most likely paternally-related, proving that their father moved between populations (i.e. contemporary male-biased dispersal, MBD).

In elasmobranchs (sharks and rays), female philopatry and MBD have been recorded in several species. In total, 62 and 25 elasmobranch species have been shown to exhibit female-philopatric behaviour and MBD, respectively (Chapman et al., 2015; Flowers et al., 2016; Phillips et al., 2021). Many of these studies make this inference by comparing markers with different modes of inheritance (mtDNA vs nuDNA). Yet comparing haploid, non-recombining mtDNA against diploid, recombining autosomal DNA can lead to biased conclusions and such comparisons rely on the simplistic assumptions of a classical island model of populations that are never met in reality (see Lawson Handley & Perrin, 2007; Phillips et al., 2021; Toews & Brelsford, 2012). Approaches based on genetic profiling and kinship analyses such as those used by Feldheim et al. (2014) and Feutry et al. (2017) have made similar observations in sharks without these caveats.

The Northern River Shark (*Glyphis garricki*) is a threatened, euryhaline shark restricted to a small number of rivers and estuaries of northern Australia and southern Papua New Guinea (Feutry et al., 2020; Kyne, Heupel, et al., 2021). These rivers are known pupping and nursery areas, yet the location of mating aggregations are currently unknown (Pillans et al., 2009). Feutry et al. (2020) identified five distinct genetic populations across this distribution using 1,700 SNP markers: King Sound and Cambridge Gulf in Western Australia, Daly River and Van Diemen Gulf (VDG) in the Northern Territory,

and southern Papua New Guinea (PNG). This study showed that the King Sound population exhibited low genetic diversity. It also found both low historical and contemporary genetic connectivity among these populations and sampling locations (within populations) based on the number of migrants per generation (*Nm*) and kinship distribution. Whether there is a sex bias in dispersal among populations is currently unknown.

In this study, we investigate the maternal evolutionary history and assess the possibility of female philopatry and MBD in *G. garricki* with three different methods that capture population dynamics at different timescales. First, we evaluate the mitochondrial genetic variation among regions to detect historical female philopatry and demographic events, such as colonisation or genetic bottlenecks. Second, we compare full mitogenome data, which have a higher resolution than conventional regions (e.g. control region; Feutry et al., 2014), against the SNP data described by Feutry et al. (2020) to infer any historical demographic differences between females and males, driven by unequal gene flow or difference in effective population size (N_e). Third, we use mtDNA data to infer the paternal or maternal relationship between the HSPs to identify contemporary philopatry and SBD at a fine spatial scale. Through the combination of these approaches we explore how genetics can directly inform on population connectivity for the conservation and management of threatened species.

4.5.2. MATERIAL AND METHODS

Sample collection and DNA extraction

Between 2012 and 2016, a total of 379 *G. garricki* tissue samples were collected from 11 rivers, creeks, large marine embayments, or estuaries (hereafter referred to as sampling locations) in five different regions covering the entire known geographical range of the species (Fig. 4.5.1; Feutry et al., 2020; Kyne, Heupel, et al., 2021). Each shark was measured, sexed, and sampled for genetic material before it was released at the site of capture. The total length (TL) of all sharks ranged from 52 to 182 cm; most sharks were juveniles or sub-adults, with 20 males >141 cm TL (sexually mature i.e. possessing calcified claspers; Feutry et al., 2020). Sexual maturity in female sharks cannot always be assessed externally, but seven females >153 cm TL were assumed to be mature based on the established male size-at-maturity (Feutry et al., 2020; Pillans et al., 2009). Genomic DNA was extracted following the standard protocol of the DNeasy Blood and Tissue kit (Qiagen Inc., Valencia, California, USA).

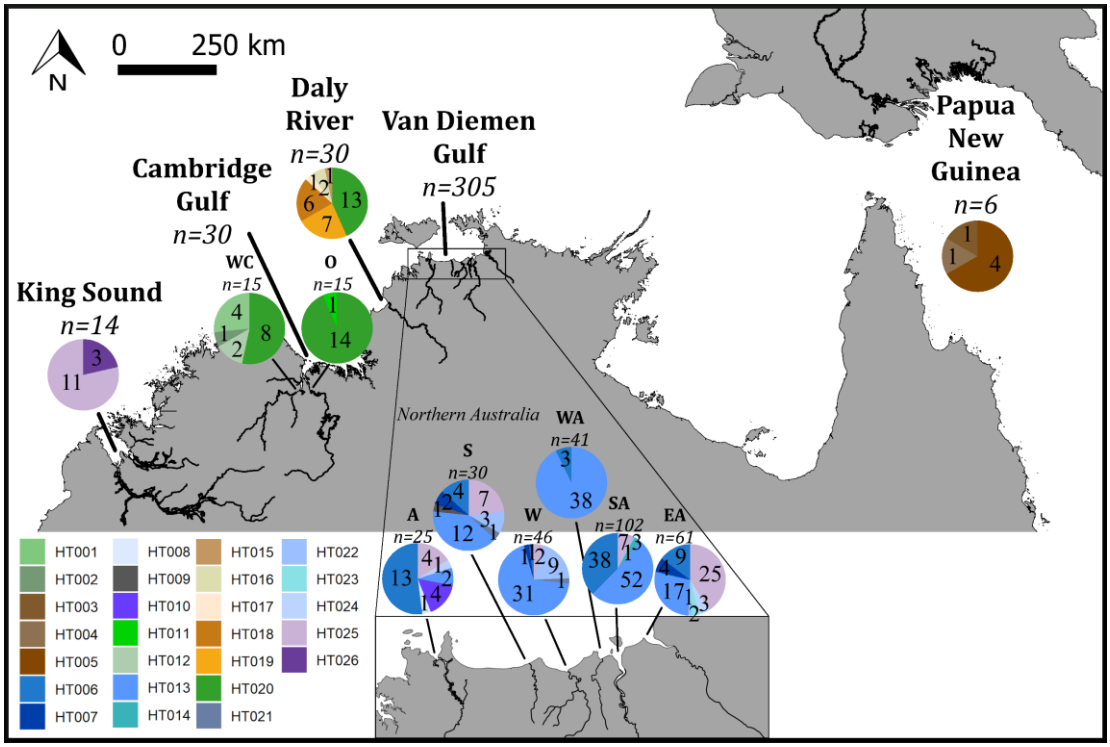

Figure 4.5.1: *Glyphis garricki* sampling regions and sampling locations, from west to east: King Sound, Cambridge Gulf (WC, West Cambridge Gulf; O, Ord River), Daly River, Van Diemen Gulf (A, Adelaide River; S, Sampan Creek; W, Wildman River; WA, West Alligator River; SA, South Alligator River; EA, East Alligator River), and Papua New Guinea. Each pie chart is coloured according to the haplotypes in the sampling location, with the haplotype frequency provided for each river.

Mitogenome amplification and sequencing

The full mitochondrial genome was amplified with two primer pairs (A and B fragments; Table 4.5.1), for all but eight samples that did not amplify sufficiently. For these samples, primers that target quarter fragments of the mitogenome were designed (A1, A2, B1, and B2 fragments; Appendix G1 section 1.2). Polymerase chain reactions (PCR) were performed in 30 µL reactions, following the standard proofreading Takara LA Taq protocol (Takara, Otsu, Shiga, Japan). PCR conditions were set to 1 min at 94°C for initial denaturation; then 40 cycles of denaturation (94°C, 30 s), annealing (55°C, 30 s), and extension (68°C, 10 min); concluding with a 10 min extension at 72°C. PCR products were cleaned following the Agencourt AMPure XP magnetic bead protocol (Beckman Coulter Inc., Indianapolis, Indiana, USA). Amplicons were quantified with a NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and the purified A and B fragments were pooled at equimolar concentration. Subsequently, these amplicons were simultaneously fragmented and barcoded with the Nextera XT DNA Sample Preparation kits and 96 sample Nextera Index kits (Illumina, San Diego, California, USA). The libraries were quantified with the Qubit dsDNA BR assay kit (Life Technologies, Carlsbad, California, USA) and normalized. Libraries were then pooled and sequenced on a Miseq desktop sequencer using the 2x250 bp paired-end reads MiSeq reagent kit v2 (Illumina, San Diego, California, USA).

Table 4.5.1: Primer pairs designed in this study to amplify fragments of the mitochondrial genome of *Glyphis garricki*.

Mitogenome fragment	Fragment size (bp)	Primer name	Primer sequence (5' – 3')
A	9 039 bp	BSh(1975L)_for	5'-AACACAAACTCCGCCTGTTTACCAAAAACATC
A	9 039 bp	BSh(10999H)_rev	5'-CAACCGGCAATTGGAGCTTCAACGTGGG
B	9 710 bp	BSh(9198L)_for	5'-GAGCCCATCATAGCTTAATAGAAGGTAAC
B	9 710 bp	16s(747-H)_rev	5'-GGTGTCTAAAGCTCCATAGGGTCTTCTCGTCT

Mitogenome assembly and alignment

Demultiplexed fastq files were imported into Geneious pro software v11.1.5 (Biomatters Ltd., Auckland, New Zealand), and the reads were paired. The Nextera adapters were trimmed and the reads were quality trimmed at a phred score <20 for a Kmer of 20 using the BBDuk tool as implemented in Geneious. Reads shorter than 50 bp after trimming were discarded from subsequent analyses. Reads for each individual were then mapped onto a previously published reference sequence (Feutry, Grewe, Kyne, & Chen, 2015) using the 'Map to Reference' tool in Geneious with the 'high sensitivity' parameters and 10 iterations. The majority rule consensus (>50 % of mapped reads for any single SNP, insertion, or deletion) for each shark was exported.

In addition to the 379 samples, we obtained another six *G. garricki* mitogenomes from NCBI Genbank (accession numbers: KF646786, KT698042, KT698044, KT698053, KT698059, NC_023361; Feutry, Grewe, et al., 2015; Li, Corrigan, et al., 2015). All mitogenome sequences were aligned with the 'multiple align' tool and the MUSCLE algorithm (Edgar, 2004).

Genetic variation and haplotype analysis

Mitogenome and SNP data were imported into R 3.5.1 (R Core Team, 2020) using the *apex* and *dartR* packages (Gruber et al., 2018; Jombart et al., 2017). Both mtDNA and SNP datasets were filtered so that they contained the same individuals, except the PNG mtDNA data that contained six different samples. Nucleotide diversity (π), haplotype diversity (h), and parsimony haplotype networks were calculated with the *pegas* package (Nei & Li, 1979; Nei & Tajima, 1981; Paradis, 2010; Templeton et al., 1992).

Genetic differentiation and historical demography

A global Analysis of Molecular Variance (AMOVA) was performed with *pegas* (10,000 permutations) to detect population differentiation among regions and sampling locations within regions (Excoffier et al., 1992). Fixation indices (Φ_{ST}, F_{ST}) were calculated with 10,000 permutations between sampling locations and between regions with the 'popStructTest' function in the *strataG* package (Archer et al., 2017; Excoffier et al., 1992; Weir & Cockerham, 1984). We analysed the Φ_{ST} and F_{ST} values from the

mitogenome and SNP datasets, and compared these against the evidence of contemporary connectivity based on kinship results.

The mismatch distribution and Watterson's estimator of theta ($\theta = 2N_{ef}\mu$) were calculated with the 'MMD' and 'theta.s' functions as implemented in *pegas* (Rogers & Harpending, 1992; Watterson, 1975). The mismatch distribution tests for population expansion were based on pairwise site differences between haplotypes, where mutation rate is assumed to be constant (Rogers & Harpending, 1992). θ measures historical female effective population size (N_{ef}) relative to mutation rate; a larger θ indicated a longer time to coalescence across population genealogies given the observed population growth. Mutation–drift equilibrium was tested with 'TajimasD' and 'Fusfs' functions from *strataG* and the 'R2.test' function from *pegas* (Fu, 1997; Ramos-Onsins & Rozas, 2002; Tajima, 1989). Tajima's D and R^2 compare θ values based on nucleotide site differences and Fu's F_S uses pairwise differences (Ramos-Onsins & Rozas, 2002). Significantly negative F_S or D values and R^2 values close to zero indicated an excess of low-frequency mutations. A departure from the null hypothesis in these tests can be problematic to attribute to differences in demographic history or selective pressures (Ballard & Whitlock, 2004). Fu's F_S, Tajima's D, and R^2 are arguably more robust than other tests, such as mismatch distributions (Ramos-Onsins & Rozas, 2002).

Kinship analyses

Using the SNP dataset, close-kin relationships were detected as described in Feutry et al. (2020). Briefly, each individual's genotype was compared against each other to calculate the likelihood that two sharks were related following Bravington et al. (2016). This likelihood was also used to control for false positive and false negative detections, which is essential when estimating connectivity based on kinship. Kinship was only investigated in the region with the largest number of samples (i.e. Van Diemen Gulf; VDG).

We analysed these kin pairs in light of their spatial distribution within and between six sampling locations (rivers and creeks; hereafter 'rivers') in the VDG and cohorts to infer sex-specific connectivity at a contemporary timescale. Reproductive dispersal was also directly observed from parent-offspring pairs that were distributed between sampling locations, where we assumed that the oldest individual (i.e. parent) dispersed. The occurrence of cross-river full-sibling pairs (FSP) was used to examine the incidence of juvenile dispersal. These are expected to be very low if dispersal only occurs in adults. We also assumed minimal juvenile dispersal based on the close association to nursery habitat, limited linear extent of river occupancy and the salinity preference of juvenile euryhaline sharks (Lyon, Dwyer, Pillans, Campbell, & Franklin, 2017; Pillans et al., 2020; Pillans et al., 2009).

Sex-specific dispersal was inferred with an indirect approach by comparing the haplotypes of HSPs. First, cross-cohort (i.e. born in different years), cross-river HSPs inform if parents moved between locations between breeding seasons. By comparing the haplotypes of each pair, we can infer which parent, the mother or the father, is more likely to have moved between sampling locations. Alternatively, cross-cohort, same-river HSPs reveal whether fathers or mothers are more likely to return to the same river between breeding seasons (i.e. philopatry). Specifically, the null hypothesis of 'no maternal philopatry' was statistically tested with an approximate likelihood ratio (Δ) test, developed by Feutry et al. (2017). Given that an abundant haplotype in the population has a higher probability to be shared between paternally-related HSPs, this test took the river-specific haplotype frequencies into account to calculate the likelihood that cross- or same-river HSPs are maternally- or paternally-related. Specifically, the likelihood to be paternally related is always the multiplication of the population-specific frequencies, while the likelihood to be maternally related is zero if the haplotypes are different. If haplotypes of a HSP were the same the most conservative probability to be maternally related is the lowest frequency. P-values were calculated by randomising the same and cross-river HSPs over 10,000 permutations. Additionally, since sample size influenced the number of kin pairs found, we corrected the number of HSPs by the number of pairwise comparisons performed (HSPcorr). The ratio of 'HSPcorr within' over the sum of 'HSPcorr between' sampling locations in the VDG ($HSPcorr_{within}/\sum HSPcorr_{between}$) gave an estimate of philopatric and dispersive behaviours. Specifically, a ratio larger than 1 indicated stronger philopatric behaviour and smaller than 1 implied higher connectivity.

Length-at-age function

The connectivity inference based on the spatial distribution of kin relied on the fact that we could assign each individual to the year it was born with a reasonable amount of certainty. No age and growth studies have been performed on *G. garricki*. There are limited opportunities to obtain such data as *G. garricki* is a protected species in Australia and ageing of elasmobranchs is commonly estimated by examining growth bands in large sample sizes of vertebrae. In order to assign the age cohorts, Bravington et al. (2019) fitted a von Bertalanffy growth function to 34 recaptured sharks with a maximum size of 155 cm TL and a maximum recapture interval of two years:

$$L_t = L_0 + (L_\infty - L_0)(1 - e^{-Kt})$$

Where L_t is the TL at capture (in mm), L_0 is the length at birth (500 mm), L_∞ is asymptotic length (1,547 mm), K is the growth coefficient (0.139 $year^{-1}$), and t is the age (in years). While limitations of the growth function are acknowledged (e.g. L_∞ is likely underestimated, leading to an overestimation of K), age cohort assignment for size classes closer to L_0 will be less prone to error as these age cohorts

will capture a wider length range. However, larger individuals approaching L_∞ will have an exponentially increasing likelihood of falling in their own age cohort. Because the majority of specimens used were small (juveniles or subadults <141 cm TL), sufficient assignment of individuals to age cohorts was achieved for the purpose of this study. Specimens of >154.7 cm TL (i.e. L_∞) could not be assigned to age cohorts and were excluded from this part of the analysis. The FSPs caught in the same period (two weeks) were used to assign a fixed standard deviation (SD) of length-at-age (43 mm) to the mean L_t of each age cohort (Appendix G1 section 10.2.2). An extra 0.5 year was added to the upper and lower ranges of t for each age cohort to account for instances where catch dates were not aligned with the austral summer pupping season (e.g. cohort 1 t = 0–0.5, cohort 2 t = 0.5–1.5 etc). This conservative approach aimed to capture the variable range of lengths that may occur in each age cohort resulting from varied individual growth rates and birth sizes.

4.5.3. RESULTS

Mitogenome assembly and alignment

The full mitogenomes of 379 samples were sequenced with an average of 98,360 reads sequenced per sample. Two samples from the South Alligator River (VDG) had low coverage (<1,000 mapped reads) and were omitted from further analyses. All reads that mapped to the reference mitogenome were checked for ambiguous base calls at an 85 % threshold (where a base needed to be present in >85 % of the mapped reads to be called unambiguous) to detect cross-contamination, barcode slippage or heteroplasmy. Overall, the mitogenome length was 16,702–16,703 bp and consisted of 38 polymorphic sites across the remaining 383 sharks. Twelve of these sites were only polymorphic due to ambiguous base calls and consequently imputed with the most common base call, yielding 26 polymorphic sites. These included two insertions in tRNA-Tyr (-/T, n = 3) and Control Region (-/A, n = 28), and one deletion in tRNA-Cyt (G/-, n = 1). One region between tRNA-Pro and the Control Region consistently had low coverage (<100 mapped sequences), most likely indicating the presence of secondary structure. This may explain why eight samples did not amplify well and had to be amplified in quarter sections (Appendix G1 section 1.2). These eight samples did not show any new mutations in their mitochondrial genome. All *G. garricki* sequences were uploaded to NCBI GenBank (accession numbers: MW652871 – MW653247).

Genetic variation and haplotype analysis

The average nucleotide diversity for the 383 aligned sequences was 0.000095 (variance < 0.000001). Most variable sites had a low diversity (π = 0.02–0.1), but three sites had a π = 0.25–0.5 (Appendix G1 section 4.1). The 383 sequences resulted in 26 haplotypes with a haplotype diversity (h) of 0.7670 (variance = 0.000233). The diversity indices per region and sampling location are summarised in Table

4.5.2 and Appendix G1 sections 4.2 and 4.3, respectively. Here, we see that Cambridge Gulf, specifically West Cambridge Gulf, had the highest π and King Sound had the lowest. The VDG had the highest haplotype diversity (h = 0.659). This pattern becomes most obvious when illustrated in a haplotype network (Fig. 4.5.2). Only five haplotypes were singletons, all but two haplotypes were private to a single geographical region (Appendix G1 section 4.3), and the haplotypes from PNG and two haplotypes from Cambridge Gulf were most distant (4–5 mutations). Other than these haplotypes, the network mainly shows a signal of expansion with few central haplotypes (HT25 and HT19) and the other haplotypes spreading out by one mutation at a time.

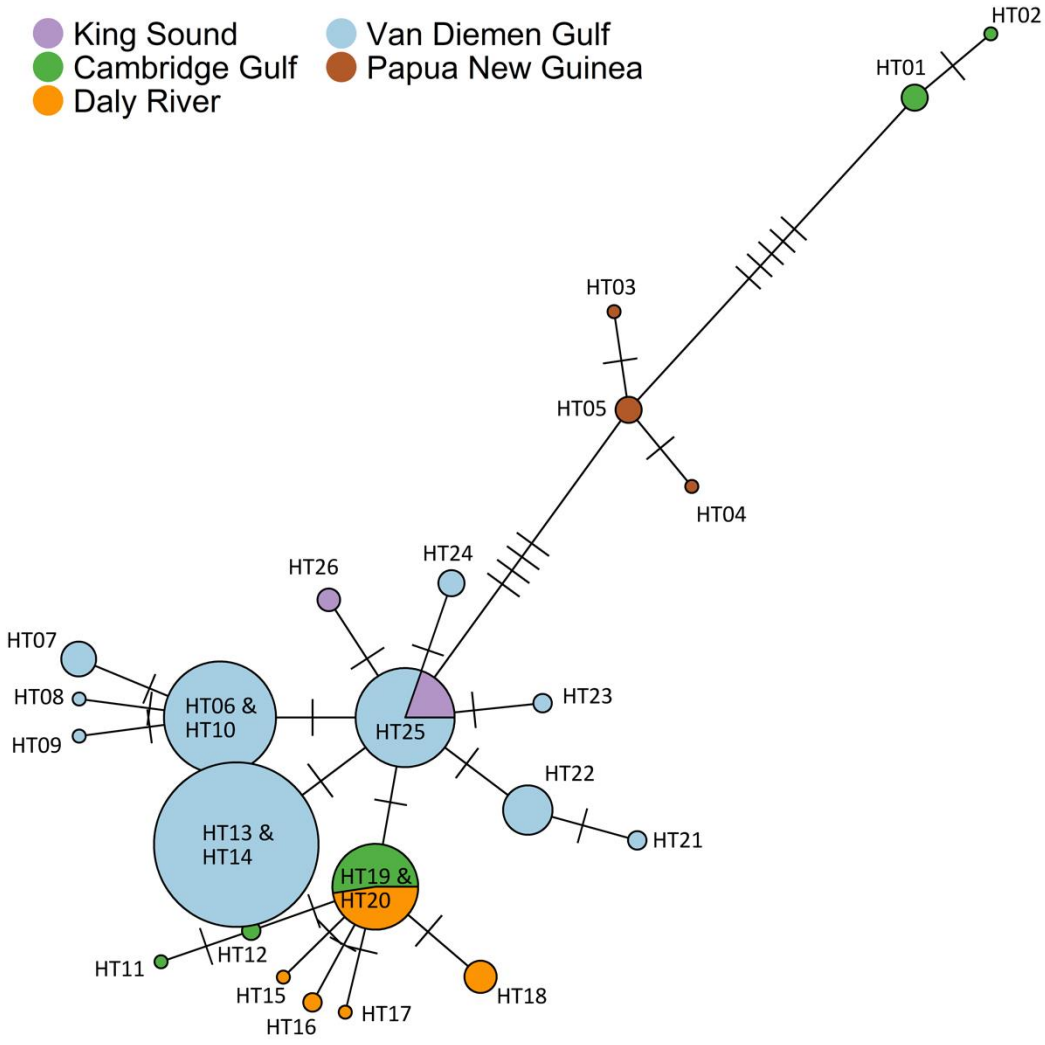

Figure 4.5.2: *Glyphis garricki* haplotype network. The size of the circles is equivalent to the square root of the number of individuals that share this haplotype and each hatch mark represents a mutation. Haplotypes due to indels (HT10, HT14 and HT20) are clustered with the most similar haplotypes. Each haplotype is coloured according to the region they were sampled from.

Genetic differentiation and historical demography

Overall, the AMOVA showed that the differentiation among regions and sampling locations within regions was highly significant (Φ_{ST} = 0.710; p = 0.000; Appendix G1 section 6.1). Specifically, the highest pairwise Φ_{ST} (0.77–0.94) was observed between PNG and all other sampling locations, except for West Cambridge Gulf (Table 4.5.3). The lowest Φ_{ST} was observed between close sampling locations, such as within the VDG (e.g. the Alligator Rivers). Interestingly, several sampling locations within the VDG (East Alligator River and Sampan Creek) appeared less differentiated from King Sound than geographically closer regions (i.e. the Daly River and Cambridge Gulf).

The mismatch distributions for Daly River, VDG, and PNG had unimodal distributions, similar to the expectation of a sudden population expansion. King Sound had a right-skewed mismatch distribution. Only Cambridge Gulf exhibited a bimodal mismatch distribution (Appendix G1 section 8.1). The θ value of Cambridge Gulf was highest (θ = 2.524), followed by VDG, Daly River, and PNG, while King Sound had the lowest θ value (θ = 0.315; Table 4.5.2). The tests based on nucleotide site differences (Tajima's D and R^2) were statically significant for Daly River and the VDG; while the test based on pairwise differences (Fu's Fs) was most negative for Cambridge Gulf and the VDG.

Table 4.5.2: Genetic variation in the mitogenome of *Glyphis garricki* per geographic region. Abbreviations: n, number of samples per region; π, nucleotide diversity; h, haplotype diversity; θ, theta (where $\theta = 2N_{ef}\mu$) and σ^2_θ, variance of theta. Significant values are underlined.

	n	π	h	θ	σ^2_θ	Tajima's D	D pval	R^2	R^2 pval	Fu's Fs
King Sound	14	0.000022	0.363	0.315	0.099	0.217	0.409	0.163	0.413	-0.904
Cambridge Gulf	30	0.000122	0.453	2.524	1.171	-1.173	0.125	0.072	0.059	-4.678
Daly River	30	0.000036	0.526	1.262	0.437	-1.731	0.032	0.056	0.007	-1.245
Van Diemen Gulf	305	0.000066	0.659	1.749	0.390	-1.579	0.043	0.031	0.038	-5.698
Papua New Guinea	6	0.000040	0.600	0.876	0.468	-0.351	0.423	0.266	0.892	1.420

Table 4.5.3: Pairwise fixation indices per sampling location for *Glyphis garricki*. Mitochondrial DNA Φ_{ST} below diagonal and nuclear DNA F_{ST} above diagonal. Non-significant results after Bonferroni correction (p > 0.0009) are underlined. Sampling locations were King Sound (KS), Cambridge Gulf (WC, West Cambridge Gulf; O, Ord River), Daly River, Van Diemen Gulf (A, Adelaide River; S, Sampan Creek; W, Wildman River; WA, West Alligator River; SA, South Alligator River; EA, East Alligator River), and Papua New Guinea (PNG).

Φ_{ST}\ F_{ST}	KS	WC	O	D	A	S	W	WA	SA	EA	PNG
King Sound		0.297***	0.302***	0.290***	0.287***	0.274***	0.270***	0.271***	0.259***	0.268***	0.395**
West Cambridge Gulf	0.337***		0.008*	0.093***	0.122***	0.122***	0.120***	0.121***	0.122***	0.123***	0.156**
Ord River	0.768***	0.243*		0.096***	0.128***	0.128***	0.126***	0.126***	0.129***	0.128***	0.154**
Daly River	0.671***	0.313***	0.060*		0.091***	0.089***	0.088***	0.089***	0.089***	0.090***	0.180***
Adelaide River	0.473***	0.469***	0.728***	0.702***		0.013***	0.015***	0.015***	0.015***	0.014***	0.174**
Sampan Creek	0.178**	0.370***	0.543***	0.561***	0.233**		0.006**	0.004***	0.002**	0.001*	0.170***
Wildman River	0.392***	0.494***	0.653***	0.655***	0.491***	0.081*		0.007***	0.008***	0.006***	0.171***
West Alligator River	0.750***	0.638***	0.871***	0.822***	0.715***	0.294***	0.127**		0.005***	0.004***	0.173***
South Alligator River	0.326**	0.526***	0.609***	0.618***	0.249**	0.036	0.140**	0.221***		0.002***	0.172***
East Alligator River	0.126**	0.425***	0.545***	0.559***	0.222***	0.005	0.180***	0.365***	0.082**		0.172***
Papua New Guinea	0.901***	0.574***	0.931***	0.893***	0.862***	0.767***	0.828***	0.938***	0.818***	0.798***	

Kinship analyses

The SNP data identified 4 POPs, 34 FSPs, and 130 HSPs out of 108,811 pairwise comparisons (Feutry et al., 2020). These pairs could be merged into 73 family groups; 43 groups consisted of only single pairs (POP, FSP or HSP), but 30 contained a combination of POPs, FSPs, and HSPs per group (Appendix G1 section 10.1). In order to reduce the number of false positive detections a strict log-likelihood ratio (LLR) threshold was set for separating full and half siblings from unrelated individuals (LLR = 30; see Feutry et al., 2020); around 30 % of the true HSPs did not pass the cut-off. This became apparent when only one shark of a FSP was indicated as a half sibling with another individual, but its full sibling was not. Due to their obvious relationship and their value to our study, these pairs were reinstated in further analyses yielding a total of 139 HSPs.

The mitogenome of eight samples could not be amplified or had a low sequencing coverage, which resulted in 12 HSPs with missing haplotype information. All POPs had different haplotypes, since all were father-offspring relationships, and all FSPs had the same haplotype. Twenty-six and 101 HSPs had non-matching and matching haplotypes respectively, but due to the high frequency of HT13 in the VDG rivers, 29 HSPs had high probabilities of having the same haplotypes (0.475 – 0.859) even if they were paternally-related (Fig. 4.5.2; Appendix G1 sections 10.2 and 10.3.2).

All POPs were assigned to different cohorts and all but one FSPs were assigned to the same cohort, thus indicating that the growth function can assign individuals to an approximate cohort. One FSP from the Wildman River did not have overlapping cohorts (Appendix G1 section 10.3.1). This was the FSP with the largest body sizes (124.0–138.5 cm TL). Overall, 54 same-cohort and 77 cross-cohort HSPs were identified. Eight same-river HSPs and two cross-river HSPs could not be assigned to a cohort due to missing length data or a size that was too large (i.e. >154.7 cm TL) for the growth function.

One POP was distributed between the Wildman and South Alligator Rivers; the other three POPs were found between the East Alligator and South Alligator Rivers (Fig. 4.5.3). All FSPs were juveniles or sub-adults (<141 cm TL) and all, except one, were found within the same river. The cross-river FSP was caught between Sampan Creek and the South Alligator River, almost three years apart, with the largest shark a 108.5 cm TL female (Appendix G1 section 10.3.1). Since six other FSPs with a larger body size (108.5–138.5 cm TL) were found in the same river and three of those were caught more than two years apart, we assume juvenile dispersal between sampling locations is relatively rare.

Eight same-cohort, cross-river HSPs were identified; four had a different haplotype and four shared a haplotype (Table 4.5.4). Sixteen cross-cohort, cross-river HSPs were also identified, of which 10 had different haplotypes and six had matching haplotypes. However, the latter consisted of very common haplotypes (HT06, HT13 and HT25; frequencies = 24–92 %). Four cross-cohort HSPs were distributed

between the Adelaide River and the other rivers in VDG. Three out of the four pairs exhibited different haplotypes and were most likely paternally-related (Fig. 4.5.3). In addition, two cross-river HSPs with matching haplotypes included a sexually mature male and presumed mature female (>141 cm TL), which may have been capable of dispersal.

Sixty-one cross-cohort HSPs occurred in the same river; 50 shared a haplotype, six had a different haplotype, and five had missing haplotype data. A formal likelihood ratio test showed that HSPs with the same haplotype are more likely to be found in the same river than across river ($\Delta = 14.195$, $p = 0.000$), suggesting the presence of contemporary female philopatric behaviour.

When correcting the number of HSPs for the number of pairwise comparisons performed (Appendix G1 section 10.4, Fig 4.5.4), we found that two sampling locations in VDG (Adelaide and Wildman Rivers) showed a stronger philopatric signal (>1). In contrast, we saw that Sampan Creek and the East Alligator River shared more kin between sampling locations, than retained within (>1). The West Alligator and South Alligator Rivers had an approximately equal ratio of same-river and cross-river kin (~1).

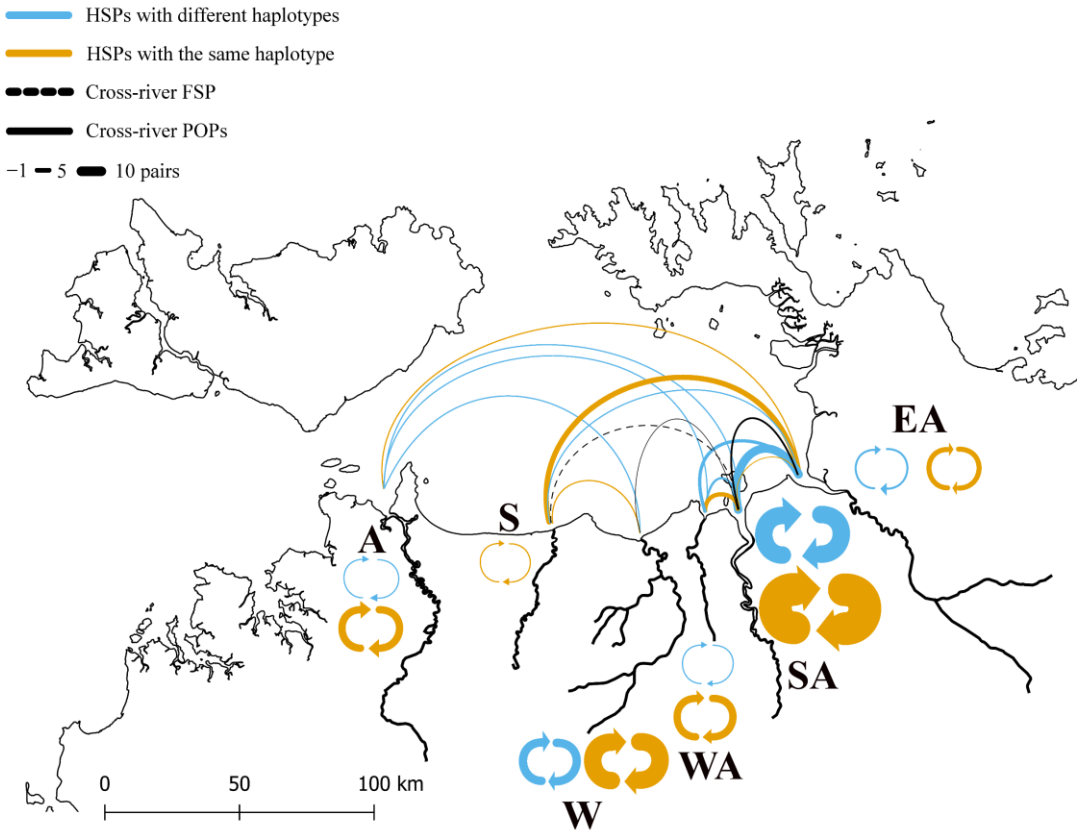

Figure 4.5.3: The distribution of *Glyphis garricki* kin pairs, as an indicator of contemporary connectivity, in Van Diemen Gulf, Northern Territory (A, Adelaide River; S, Sampan Creek; W, Wildman River; WA, West Alligator River; SA, South Alligator River; EA, East Alligator River). The line width represents the number of kin pairs distributed within and between sampling locations. HSP, half-sibling pair; FSP, full-sibling pair; POP, parent-offspring-pair.

Table 4.5.4: *Glyphis garricki* kinship distribution in Van Diemen Gulf, Northern Territory: POP + FSP + HSP with different haplotype\HSP with same haplotype*HSP with missing haplotype*. Below diagonal: cross-cohort, cross-river; above diagonal: same-cohort, cross-river; diagonal (bold) is a mix of cross-cohort and same-cohort. HSP, half-sibling pairs; FSF, full-sibling pairs; POP, parent-offspring pairs. Two cross-river HSPs were not included, because they were too large (total length > 154.7 cm) to estimate their cohort accurately.

Same-cohort / **Cross-cohort**	**A** **N= 25**	**S** **N= 30**	**W** **N= 45**	**WA** **N= 41**	**SA** **N= 101**	**EA** **N= 61**
Adelaide River (A)	**0+1+1\1*6***	0	0	0	0	0
Sampan Creek (S)	0	**0+1+0\1*0***	0	0	0+1+0\0*0*	0+0+0\3*0*
Wildman River (W)	0+0+1\0*0*	0+0+0\1*0*	**0+8+1\24*4***	0	0	0
West Alligator River (WA)	0+0+1\0*0*	0	0	**0+2+1\7*0***	0+0+1\0*0*	0+0+1\0*0*
South Alligator River (SA)	0+0+1\0*0*	0	1+0+0\0*0*	0+0+1\3*0*	**0+19+9\49*2***	0+0+2\1*0*
East Alligator River (EA)	0+0+0\1*0*	0+0+1\1*0*	0	0+0+2\0*0*	3+0+3\0*0*	**0+2+1\6*0***

Comparing mtDNA, nuDNA and kinship

When comparing fixation indices (Table 4.5.3; Appendix G1 section 11), both mitochondrial and nuclear markers had high and significant fixation values. Nonetheless, the Φ_{ST} values of the mtDNA were usually one order of magnitude higher than the F_{ST} value of the nuclear SNPs, even between close sampling locations (e.g. East Alligator vs. South Alligator Rivers; Fig. 4.5.4). In general, high fixation indices corresponded to few kin shared between locations, as was the case for the Adelaide and Wildman Rivers (Fig. 4.5.4). Vice versa, both Φ_{ST} and F_{ST} values for West Cambridge Gulf vs. Ord River and Sampan Creek vs. East Alligator River were non-significant, suggesting high connectivity. This was corroborated by the six kin pairs between Sampan Creek and East Alligator River, although no kin were found in the Cambridge Gulf. Generally, we found that Sampan Creek and East Alligator River share many kin with other VDG rivers, relative to number of the kin pairs found within these rivers (Table 4.5.4; Fig. 4.5.3; Appendix G1 section 10.4). Interestingly, we also observed some discordance between marker types. A non-significant Φ_{ST}, but significant F_{ST}, was observed between King Sound vs. East Alligator River, Ord vs. Daly Rivers, and Sampan Creek vs. Wildman River. Nineteen of the 177 kin pairs were not caught independently (i.e. within two weeks of each other and likely born in the same cohort) and could potentially generate a false signal of genetic structure due to biased sampling toward family members (see Devloo-Delva et al., 2019; Feutry et al., 2017). Most of these individuals were from the Wildman and South Alligator Rivers, where the sample sizes were large. Removing one individual per kin pair did not affect the fixation indices (Appendix G1 section 11.2). A disconnect was also observed between fixation indices and the number of shared kin pairs. For example, we found three, six, and nine kin pairs shared between the West Alligator, South Alligator and East Alligator Rivers (Fig. 4.5.3), but both Φ_{ST} and F_{ST} values were high and significant (Table 4.5.3).

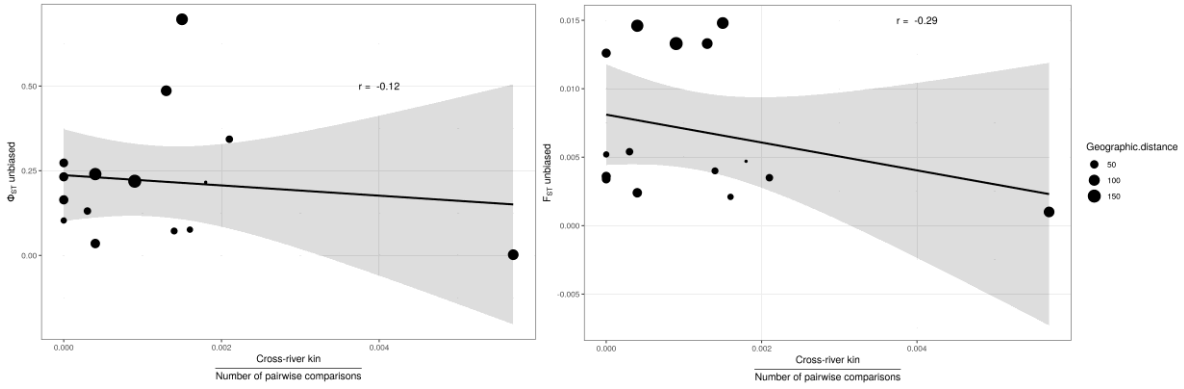

Figure 4.5.4: Correlation analysis between the pairwise fixation indices and the number kin shared between sampling locations (corrected over the number of pairwise comparisons). Left, the correlation for the unbiased Φ_{ST} values. Right, the correlation for the unbiased F_{ST} values. Each point represents a pairwise comparison between sampling location in the Van Diemen Gulf and their size corresponds to the linear geographical distance between the sampling locations.

4.5.4. DISCUSSION

This study provides new information on historical demographic events, as well as contemporary sex-specific dispersal of *G. garricki*, using nuclear SNPs to define a close-kin framework supplemented with additional information obtained from whole mitogenome sequencing data. Specifically, the mitogenome results provide evidence of historical colonisation events and range expansion, as well as secondary contact between two separated lineages. Based on the 177 kin pairs, we found that at least 63 % (10 out of 16) of the cross-cohort, cross-river HSPs have a high likelihood of being paternally-related and at most 89 % (50 out of 56) of the same-river, cross-cohort HSPs are likely maternally-related. Results also show that nearly each sampling location has significant differentiation for both the SNP and mitogenome data, suggesting historical population structure at a very fine spatial scale. Yet within the VDG, kinship data reveals greater connectivity from Sampan Creek and the East Alligator River, and stronger philopatric behaviour in the Adelaide and Wildman Rivers. Overall, we see that the three genetic approaches show similar findings, yet some discordance is also observed.

Maternal history, colonisation, and range expansion

The clear mitochondrial differentiation between all five sampling regions detected in this study is consistent with the lack of connectivity of both females and males reported by Feutry et al. (2020). However, low Φ_{ST} between the Ord and Daly Rivers is most likely explained by ongoing gene flow or the retention of ancestral haplotypes in recently diverged populations (incomplete lineage sorting; Toews & Brelsford, 2012). This concurs with results from Feutry et al. (2020) that *G. garricki* started its range expansion recently in the Gulf of Carpentaria and subsequently expanded both westwards and north-eastwards. In addition, the mitogenome analyses revealed that PNG was most different from all sampling locations. This would suggest that PNG and northern Australian sharks may have

been separated for a long period of time with limited female gene flow. Results also show two haplotypes in the Cambridge Gulf that are more similar to PNG; indicating secondary contact between sharks from Cambridge Gulf and PNG (or an unsampled, or extinct population). This is substantiated by the observation from Feutry et al. (2020) that the PNG samples tend to cluster with Cambridge Gulf using the SNP data. Finally, the mitogenome differentiation from the King Sound to other regions is less pronounced than in the nuclear data from Feutry et al. (2020), with the lowest differentiation between King Sound and the VDG (e.g. East Alligator River). Such 'mitonuclear discordance' is likely driven by range expansion with incomplete lineage sorting (Toews & Brelsford, 2012). This is again supported by the low genetic diversity in King Sound as suggested by Thorburn and Morgan (2004). Conversely, the high nuclear differentiation of the King Sound may be explained by an accumulation of mutations at the edge of a range expansion due to the effect of drift on a small and recently founded population (Peischl, Dupanloup, Kirkpatrick, & Excoffier, 2013).

The mismatch distributions show that Daly River, VDG, and PNG follow the expected model under sudden expansion, while King Sound showed signals of a recent expansion (e.g. due to colonisation) or bottleneck. As implied previously, the Cambridge Gulf deviates from this pattern due to hosting two distinctly-related haplogroups (with up to 13 mutations difference). Assuming that the mutation rates are similar between geographic regions, the θ values indicate that the historical N_{ef} are similar between the Daly River and VDG, but lower in King Sound. Similarly, the Tajima's D, R^2, and Fu's Fs tests suggest that the Cambridge Gulf, Daly River, and VDG are undergoing demographic expansion, whereas King Sound may be undergoing a sudden contraction or the mitogenome is experiencing balancing selection. However, we advise caution in the interpretation of results from regions with low sample sizes (i.e. King Sound and PNG) or signals of secondary contact (i.e. Cambridge Gulf).

Female philopatry and male-biased dispersal

On an evolutionary timescale, some disjuncture between mitochondrial and nuclear data is apparent. As noted before, this incoherence most likely reflects the colonisation history and range expansion from north-eastern Australia towards western Australia (Feutry et al., 2020). Such demographic events will affect the non-recombining haploid mtDNA, and recombining diploid nuDNA, differently (Lawson Handley & Perrin, 2007; Phillips et al., 2021) and this mitonuclear discordance may not be driven by SDB (Toews & Brelsford, 2012). Similarly, stronger purifying selection and/or lower mutation rates in the mitogenome may enhance the observed mitonuclear discordance (Ballard & Whitlock, 2004). Mitochondrial genes are expected to evolve under purifying selection given their importance for energy production and metabolism, and their four-fold lower effective population size augments the effect of the selective pressures. Nonetheless, the low mitochondrial diversity towards the edge of the range expansion (King Sound) indicates a low influx of genetically diverse females and/or a

disproportional amount of male colonisation. Overall, the high and significant mitochondrial and nuclear fixation indices would suggest that both females and males show historical philopatric behaviours.

On a contemporary timescale based on kinship, we see evidence that females are more likely to return to the same river in the VDG and we observe a slight bias towards male dispersal. The latter can be directly observed by the four father-offspring pairs across rivers. However, this may be biased since mature females are rarely caught and mother-offspring pairs are therefore less likely to be identified. Indirectly, we can infer trans-generational female philopatry from the result that 89 % of the same-river, cross-cohort HSP are more likely to be maternally-related than paternally-related. A small female population size with low haplotype diversity could confound this result, given that many paternally-related HSPs would have the same haplotype. Furthermore, based on the haplotypes of the cross-river, cross-cohort HSPs, we see that minimum 63 % of the dispersal was paternal and 37 % was putatively maternal (assuming negligible juvenile dispersal). Nonetheless, due to the high frequency of certain haplotypes in VDG, we cannot always call HSPs 'maternally-related' with a high likelihood. Similarly, potential PCR or sequencing errors or single-generation mutations may assign HSPs as paternally-related when they are not, although we consider this unlikely due to strict data filtering and quality control. Further, four same-cohort, cross-river HSPs are paternally-related, indicating the fathers reproduced with multiple females within one breeding season. Since little is known about the breeding grounds of *G. garricki,* these paternally-related HSPs could suggest that the fathers moved between sampling locations within a season or that mothers from different sampling locations aggregated at the same breeding ground. The four same-cohort, cross-river HSPs with the same haplotype prove more difficult to explain, as it seems implausible that the mother would have moved during pupping. The simplest explanation is that they are actually paternally-related, given their high haplotype frequencies, or they are cross-cohort HSPs. Otherwise, one individual (juvenile) of the HSP must have moved, which we presume rare. The possible misassignment of HSP cohorts is supported by the cross-cohort FSP within the Wildman River. This could be explained by differences between individuals in growth rate, but is most likely due to the limitations of assigning large individuals to age cohorts, stemming from inaccuracies in growth parameter estimates in the growth function used (see material and methods). Conversely, the small population size of the Wildman River (see Bravington et al., 2019) could increase the probability that the same female and male mated in different years. Sperm storage is another possible explanation for this observation. Unfortunately, the low number of intraspecific mutations across the *G. garricki* mitogenome obscure the maternal or paternal relationship between HSPs. As carcharhinids (which include *Glyphis* spp.) possess X and Y sex

chromosomes (Maddock & Schwartz, 1996; F. Devloo-Delva unpublished data), the inclusion of X- and Y-specific markers could help resolve kin relationships between females and between males.

Previously, Feutry et al. (2017) demonstrated that male *G. glyphis* are more likely to disperse between the Adelaide River and Alligator rivers. In the current study we showed a similar contemporary MBD pattern for *G. garricki* between the Adelaide River and the other VDG rivers (75 %). Yet, between more closely located VDG rivers, sex-biased dispersal was less pronounced. In addition, we could demonstrate contemporary female philopatry to each sampling locations, although we could not dismiss the occurrence of female dispersal. Close-kin results showed at the most 37 % of female reproductive movement. Based on the Φ_{ST} results, historical female migration rates could be estimated if the female population size is known; which can be determined by extending the close-kin mark-recapture model from Bravington et al. (2019) to assess sex-specific abundance from the maternal and paternal HSPs. Overall, this information on female philopatry and MBD adds to the growing theory of elasmobranch dispersal (Chapman et al., 2015; Flowers et al., 2016; Phillips et al., 2021). Twenty-five elasmobranch species have shown evidence of MBD (Phillips et al., 2021). Most of these species were evaluated for historical SBD, while only nine species showed contemporary MBD by assigning individuals to their population of origin (e.g. Gubili et al., 2014; Sandoval-Castillo & Beheregaray, 2015). Female philopatric behaviour was suggested in 62 elasmobranch species, but only one study (Feldheim et al., 2014) directly demonstrated natal philopatry and site fidelity from 20 years of genetic profiling.

Fine-scale population structure

Our study applied three different methods to assess fine-scale population structure at different timescales. We show that each geographic region forms its own genetically and demographically distinct population. Within VDG, we consider the Adelaide River and the Wildman River to host demographically independent entities, demonstrated by the higher fixation indices and the many kin pairs retained within these sampling locations. For the Adelaide River this can be explained by its geographic distance from suitable habitats, although it is not certain why sharks from the Wildman River are less likely to disperse. Demographic independence in an island model is often assumed when the migration rate between two populations is less than 0.1 (Hastings, 1993) or when less than one migrant per generation moves between populations (Spieth, 1974). Similarly, Bentzen (1998) argued that significant fixation indices provide strong evidence of demographic independence. In this study, we also found reproductive connectivity between spatially close rivers in VDG (i.e. low fixation indices and cross-river HSPs). For example, the increased connectivity of Sampan Creek and the East Alligator River is not explained solely by geographic proximity. Low mating opportunities, food availability, or environmental fluctuations may be responsible for the relatively few same-river kin pairs in these

sampling locations (Comins et al., 1980; Greenwood, 1980; Hamilton & May, 1977). Overall, when comparing the 26 cross-river HSPs to recent effective and adult population size estimates from VDG: N_e = 168 [16–1,411] (Feutry et al., 2020), N_{adults} = [582–1,116] (Bravington et al., 2019), we would expect these locations to be demographically dependent. Nonetheless, we see low, but significant fixation indices between most of these rivers. The small population sizes and powerful genomic markers (Waples & Gaggiotti, 2006), or the spatiotemporal stochasticity of demographic parameters (such as population growth rate or reproductive success; Lowe & Allendorf, 2010), may explain why significant differentiation is identified while these sampling locations are still demographically dependent. For example, Petit et al. (2001) showed that population structure can still be found in a bat, the Common Noctule (*Nyctalus noctula*), even if the number of migrants per generation is high (>50). Regarding our study, we consider each sampling region (King Sound, Cambridge Gulf, Daly River, VDG, and PNG) as separate populations in both the genetic and demographic sense, but we believe VDG forms a metapopulation where the sampling locations are variably connected.

In general, we see that the three different approaches enforce each other. When pairwise fixation indices are high, few kin are found between sampling locations and vice versa. On a few occasions we observed a discordance between marker types. Non-significant Φ_{ST}, but significant F_{ST}, were found between three pairwise comparisons. As mentioned before, this likely reflects the retention of ancestral polymorphisms in the mitochondrial genome, yet the statistical power to detect population structure of thousands of SNPs is also expected to be higher than a single mtDNA marker (Morin et al., 2009). Another conflict between methods was demonstrated by the many cross-river kin between the Alligator rivers as opposed to high and significant pairwise fixation indices. This could reflect that these sampling locations have only recently been connected, or that they are, in fact, still demographically independent while non-reproductive juvenile movement occurs between these adjacent sampling locations. Overall, we show that the close-kin method is a valuable tool for defining fine-scale population structure, provided that sampling is spatially and temporally extensive with sufficient covariate data (Bravington et al., 2016).

Conservation implications

Glyphis garricki is susceptible to population decline due to exposure to both riverine and marine pressures (Grant et al., 2019). Commercial and recreational fisheries likely pose the largest threat to *G. garricki* (Field et al., 2013; Kyne & Feutry, 2017), however this species is also vulnerable to habitat modification and degradation of riverine and coastal environments (Grant et al., 2019). Similar to Feutry et al. (2020), we see that the King Sound population has the lowest diversity and the lowest θ. This supports previous statements that *G. garricki* is susceptible to any environmental or anthropogenic changes in this area (Morgan et al., 2011; Thorburn & Morgan, 2004). Further, we

confirmed that each sampling location within VDG forms a unique genetic unit, with high female philopatry and MBD. In the Wildman River, for example, the similarity of allele frequencies to nearby locations indicate a recently established population, yet connectivity to these locations appears low. For these isolated populations, the immigration of males cannot compensate for the removal for local females and if a river were to be impacted by increased levels of mortality, natural recovery would not be guaranteed. As such, protection at the smallest spatial scale is essential to ensure local viability of populations, while allowing male dispersal to maintain genetic connectivity. Collectively, given the small population size estimates of *G. garricki* (Bravington et al., 2019), threats and their associated mortality rates need to monitored closely, as they may have disproportionate impacts on each sex.

4.5.5. CONCLUSION

This study provides the most comprehensive overview available on the spatial population structure of the threatened shark *G. garricki*. We investigated connectivity on both evolutionary and contemporary, or demographic, timescales. It is one of the first studies to provide novel insights into contemporary sex-specific dispersal using a close-kin framework, and shows that the full mitogenome can add a new dimension to the kinship analyses, as well as resolve important historical events, such as the connectivity between Cambridge Gulf and PNG or an unsampled population. In addition, it can inform us about potential sex-biased dispersal and fine-scale population structure. We found that females exhibit a strong philopatric behaviour and that both females and males disperse within the Van Diemen Gulf, yet males appear to disperse more often and further. We also found an unprecedented amount of fine-scale genetic population structure in an elasmobranch and evidence of contemporary demographic connectivity. This study indicates that each sampling location should be managed as a separate unit, since gene flow is not uniform and females tend to return to the same river. This study highlights the importance of a multi-method approach to provide crucial information for conservation and management.

4.6. CHAPTER 6 – Sex-specific dispersal patterns of the Bull Shark

4.6.1. INTRODUCTION

Philopatry and sex-biased dispersal are commonly described in elasmobranchs (sharks and rays), yet rarely validated using a range of genomic markers. Philopatry describes the return migration of individuals to a well-defined location, and is usually female-biased in elasmobranchs (reviewed in Chapman et al., 2015; Flowers et al., 2016). In contrast, the few studies that investigated sex-biased dispersal (SBD) have found that dispersal is male-biased (reviewed in Phillips et al., 2021).

The Bull Shark (*Carcharhinus leucas*) is no exception to these observed patterns of female philopatry and male-biased dispersal (MBD; e.g. Karl et al., 2011; Tillett, Meekan, et al., 2012). Generally, female philopatric behaviour is driven by the high maternal investment and patchy distribution of suitable nursery habitats (Chapman et al., 2015), while males may disperse to increase mating opportunities (Phillips et al., 2021). River systems have been identified as the primary nursery habitat for the Bull Shark. For example, most tagging and telemetry studies focusing on juveniles found that Bull Sharks younger than two to three years old (<150 cm total length, TL) remained close to their riverine natal area (Heupel, Yeiser, Collins, Ortega, & Simpfendorfer, 2010; Matich & Heithaus, 2015; Simpfendorfer & Milward, 1993). Physiological limitations, food availability, and predator avoidance have been proposed as drivers of the site residency of juveniles (Pillans et al., 2020; Pillans & Franklin, 2004). Satellite tagging and acoustic telemetry of adult sharks revealed medium to high site fidelity of large Bull Sharks (Brunnschweiler et al., 2010; Daly et al., 2014; Espinoza et al., 2016), even though the Bull Shark can travel up to 20.1 km per day (Kohler & Turner, 2001, 2019) and up to 2,000 km in six months (Lea et al., 2015). Using a maternally-inherited mitochondrial DNA (mtDNA) marker, this site fidelity or return migration of adult females was confirmed to be driven by female philopatric behaviour to these nursery habitats (Karl et al., 2011; Pirog et al., 2019; Sandoval Laurrabaquio-A et al., 2019; Tillett, Meekan, et al., 2012). In addition, most of these studies also included autosomal DNA (auDNA) markers, in the form of microsatellites. These auDNA markers did not identify population divergence at the same fine spatial scale as the mtDNA marker, and therefore these studies concluded that the signal of genetic connectivity from the auDNA must be driven by MBD. The comparison of mtDNA against auDNA is termed a 'mixed-marker' approach and can provide insights in the evolutionary demography of populations.

Other methods exist to infer and quantify SBD among geographically-distant locations, but these have not yet been applied to the Bull Shark. Alternative genetic markers with different inheritance patterns can be compared using the mixed-marker approach. The comparison between mtDNA and paternally inherited Y-chromosome DNA (yDNA) can reveal female- and male-specific gene flow. However, their

inheritance mechanism — haploid and uniparentally inherited — means that they each represent a single linked marker and that haplotypes are more easily lost due to genetic drift and natural selection (Webster & Sayres, 2016; Wilson Sayres, 2018). In this regard, contrasting auDNA against X-chromosome DNA (xDNA) — both diploid and biparentally inherited in females — is considered a more powerful approach since more unlinked markers can be used (Schaffner, 2004; Wilkins & Marlowe, 2006). In an ideal population (Wright, 1931), the genetic diversity of auDNA, xDNA, yDNA, and mtDNA is expected to follow a 4:3:1:1 ratio (Charlesworth, 2001). These methods have never been applied to elasmobranch research despite their proven utility in other vertebrates (Chen, Zhang, et al., 2018; Sacks et al., 2013; Yannic et al., 2012).

Another analytical approach, using multi-locus auDNA markers, detects evidence of contemporary SBD by assigning individuals back to their population of origin (i.e. individual-level assignment index, AI; Favre et al., 1997; Paetkau, Calvert, Stirling, & Strobeck, 1995). This method was later corrected for the levels of genetic diversity between populations (AIc; Goudet et al., 2002) and has only been applied to a handful of elasmobranch studies (Day et al., 2019; Sandoval-Castillo & Beheregaray, 2015). Similarly, fixation indices (F_{ST}), inbreeding coefficients (F_{IS}), and relatedness (R) can be compared between females and males using auDNA (Goudet et al., 2002). All these population-level metrics provide an estimate of SBD within the current generation since sex-specific signals are lost due to recombination between autosomal chromosomes. Goudet et al. (2002) concluded that the F_{ST} statistic is most powerful when dispersal is high, while the variance of AIc (vAIc) is best for low dispersal scenarios. Alternatively, the spatial correlation (r_{sp}) between genetic and geographic distances has proven a powerful tool to detect fine-scale SBD (Banks & Peakall, 2012). Lastly, the spatial distribution of genetic recaptures or related individuals can provide evidence for contemporary parental return-migration, site fidelity, philopatry or reproductive SBD (Feldheim et al., 2014; Feutry et al., 2017; Mourier & Planes, 2013). Formally, these dispersal behaviours can be tested by comparing the spatial distribution of parent-offspring pairs (POP), full-sibling pairs (FSP), and half-sibling pairs (HSP; e.g. Feutry et al., 2017; Feutry et al., 2020).

In this study, we aim to test previous findings of female philopatry and MBD using more powerful genomic markers (single nucleotide polymorphisms, SNPs; and full mitochondrial genomes, mitogenomes). Specifically, we estimate the amount of historical dispersal between populations using a mixed-marker approach (mtDNA vs auDNA and xDNA vs auDNA). Direct dispersal is quantified by identifying immigrant sharks, comparing population-level metrics (F_{ST}, F_{IS}, R, and r_{sp}) between sexes, and assessing the spatial distribution of cross-cohort HSPs.

4.6.2. MATERIAL AND METHODS

SNP and mitogenome data

Sequenced, genotyped, and filtered DArTcap SNPs and mitogenomes were sourced from Chapter 2 (Devloo-Delva et al., in prep) and X-chromosome SNPs were identified in Chapter 4 (Devloo-Delva et al., in prep). This included 3,443 autosomal and 148 X-linked SNPs, genotyped in 769 Bull Sharks across the globe (DATA2; Appendix D1 section 8) and mitogenome data (16,708 base pairs, bp) sequenced for 361 sharks from a global distribution, except for the western Atlantic Ocean. The highest sample coverage was in the Indo-West Pacific (IWP) region (N_{SNP} = 430, $N_{mitogenome}$ = 293).

Mixed-marker approach

The genetic variation between the mtDNA, xDNA and auDNA markers were compared in terms of their nucleotide diversities (π_{mt}, π_x, and π_{au}; Nei & Li, 1979). Analyses for the xDNA were separated by females and males since males were homozygous for the X-chromosome markers. Nucleotide diversities for the mtDNA were calculated with the 'nuc.div' function in the *pegas* package v0.14 (Paradis, 2010), while π_x and π_{au} were evaluated with the 'pi' in *radiator* v1.1.9 (Gosselin et al., 2020). Additionally, H_O and F_{IS} for the xDNA and auDNA markers were computed with the 'basicStats' function in the R package *diveRsity* v1.9.90 (Keenan et al., 2013), using a bootstrap of 1,000 to estimate the 95 % confidence intervals (Appendix D1 section 8.10.1.1). Next, pairwise F_{ST} values were calculated for the mitogenome, nuclear SNP and X-linked SNPs using the 'PopStrucTest' in the *strataG* package v2.4.905 (Archer et al., 2017; Weir & Cockerham, 1984). Collectively, the comparison of these values allowed inferences to be made of the sex-specific demographic history. Specifically, the maternal inheritance patterns of the mtDNA and xDNA alludes to the matrilineal evolutionary demography, whereas the auDNA bears signals of both paternal and maternal demographic events.

Individual and population level assignment tests

To identify direct evidence of contemporary SBD, the data was parsed by females and males, while only sharks capable of dispersal (i.e. >150 cm TL) were kept (Matich & Heithaus, 2012; Simpfendorfer & Milward, 1993). The individual AIc was calculated as the probability that each genotype of an individual will fit the observed allele frequency of that locus within the sampled location. Specifically, this analysis was performed with the 'aic' function in the *hierfstat* package v0.5-7 (Goudet, 2005). Direct migrants were individuals with an AIc lower than $q_{0.25}$−1.5 IQR of their sampled location, where $q_{0.25}$ is the first quartile and IQR is the inter quartile range of the AIc values.

The large adults and subadults capable of dispersal were grouped by sex. For each group, the differences in F_{ST}, F_{IS}, and the mean of AIc (mAIc) or differences in the ratio of vAIc were tested using the 'sexbias.test' in *hierfstat*. A statistical significance was obtained by randomising the sex (keeping

the sex ratio constant) and recalculating the test statistics for 100 iterations. The more dispersive sex is expected to exhibit lower F_{ST} and mAIc values, since allele frequencies from the dispersive sex are sampled from both resident and immigrant individuals and immigrants are expected to have reduced assignment success. Alternatively, a higher F_{IS} is anticipated in the dispersive sex because of the Wahlund effect (i.e. mixture of populations which results in a heterozygosity deficit), while a higher vAIc is caused by a mixture of both resident and immigrant individuals (Goudet et al., 2002). Fixation indices for each sex at each sampling site were also estimated with the 'PopStrucTest' function to further investigate specific site associations of a certain sex (Weir & Cockerham, 1984). Similarly, the correlation between sex-specific Euclidean genetic distance and shortest over-water distance was investigated for each site. A positive spatial correlation based on a Mantel test between genetic and geographic distances indicated a stronger site association between individuals of a specific sex, while a negative r_{sp} signified higher dissimilarity between individuals (Mantel, 1967). Lastly, relatedness according to Ritland (1996) was estimated between each individual pair using the 'coancestry' function in the *related* package v1.0 (Pew, Muir, Wang, & Frasier, 2015). The Ritland estimator was found to have the highest accuracy and robustness for estimating relatedness with SNP data (Attard, Beheregaray, & Moller, 2018). Pairwise relatedness was averaged over the within- or among-sex comparisons (female-female, FF; male-male, MM; or female-male, FM) for each sampling site. A lower relatedness was expected for the dispersing sex.

Kinship inference

Pairwise kinship relations were inferred using a pseudo-likelihood approach (Bravington et al., 2016) and a log-likelihood ratio (LLR) threshold was set to avoid false positive detection. This method relied on accurate estimation of allele frequencies. Therefore, kinship was only estimated in the IWP, within which populations had sufficient sample sizes and spatial coverage. Specifically, this study investigated the spatial distribution of cross-cohort HSPs, using the length-at-age function from Tillett, Meekan, Field, Hua, and Bradshaw (2011) to assign juvenile sharks to their age cohort. For example, if two pre-dispersed juveniles (<150 cm TL) are sampled in different locations and also identified as cross-cohort half siblings, this would imply one of the parents reproduced, moved and reproduced again between locations and breeding seasons. Additionally, mtDNA haplotype information allowed the inference to be made whether HSPs were paternally or maternally related. In other words, if the HSPs have different mtDNA haplotypes, they are paternally related, and the father most likely moved between locations. Vice versa, if the HSPs share a haplotype they are expected to be maternally related, although they may still be paternally related but identical-by-state rather than identical-by-descent. Finally, if juvenile FSPs are identified, these can be used to verify the assumption that small individuals do not stray from their natal area.

4.6.1. RESULTS

Mixed-marker approach

Nucleotide diversity was generally higher for the mtDNA compared to the nuclear DNA, whilst the π_{au} was higher than the $\pi_{x♀}$. Costa Rica and sites on both sides of the Atlantic Ocean exhibited the lowest $\pi_{x♀}$ and $\pi_{x♂}$ (Table 4.6.1). Under neutral expectations, the genetic diversity ratios should be $\pi_{x♀}/\pi_{au}$ = 0.75, and π_{mt}/π_{au} = 0.25. Our results showed that sites within the IWP followed the expectation for the $\pi_{x♀}/\pi_{au}$ ratios, while this was lower in Costa Rica and the Atlantic Ocean ($\pi_{x♀}/\pi_{au}$ = 0.30–0.50). On the other hand, π_{mt}/π_{au} ratios deviated from the expectation at all sites. The π_{mt}/π_{au} ratios were highest in the IWP, especially in South Africa, the Arabian Sea, Sri Lanka, and Thailand (π_{mt}/π_{au} = 4.40–6.97).

The pairwise F_{ST} values between the auDNA and the xDNA♀ displayed a similar pattern (Table 4.6.2), whereas the western Pacific, the eastern Atlantic, the western Atlantic and the IWP showed the strongest divergence. Nonetheless, the xDNA♀ was generally higher than the auDNA. Within the IWP, three pairwise comparisons had F_{ST} values for the X-linked markers that were nearly an order of magnitude higher than the autosomal F_{ST} values: Papua New Guinea vs Mozambique ($F_{ST\text{-}x♀}$ = 0.129 vs $F_{ST\text{-}au}$ = 0.011), Fiji vs Mozambique ($F_{ST\text{-}x♀}$ = 0.161 vs $F_{ST\text{-}au}$ = 0.033), and Papua New Guinea vs Indonesia ($F_{ST\text{-}x♀}$ = 0.162 vs $F_{ST\text{-}au}$ = 0.017).

Table 4.6.1: Description of the genetic variation and relatedness for the full data (769 Bull Sharks; 3,409 autosomal SNPs and 148 X-linked SNPs) and the full mitochondrial genomes (361 Bull Sharks; 16,708 bp). Diversity indices include the number of samples for the autosomal data (N_{au}), the autosomal nucleotide diversity for the 70 bp reads in percentage (π_{au}), the number of samples for the X-chromosome data of females ($N_{x♀}$), the X-chromosome nucleotide diversity for the 70 bp reads of females in percentage ($\pi_{x♀}$), the number of samples for the X-chromosome data of females ($\pi_{x♂}$), the X-chromosome nucleotide diversity for the 70 bp reads of males in percentage ($\pi_{x♂}$), the number of samples for the mitochondrial data (N_{mt}), the mitochondrial nucleotide diversity in percentage (π_{mt}), the ratio of female X-chromosomes nucleotide diversity over autosomal nucleotide diversity ($\pi_{x♀}/\pi_{au}$), the ratio of male X-chromosomes nucleotide diversity over autosomal nucleotide diversity ($\pi_{x♂}/\pi_{au}$), the ratio of mitochondrial nucleotide diversity over autosomal nucleotide diversity (π_{mt}/π_{au}), autosomal relatedness among females (R_{FF}), autosomal relatedness between females and males (R_{FM}), and autosomal relatedness among males (R_{MM}). 'NA' values indicate that no or too few samples were available to estimate genetic variation. Diversity indices are divided by geographical regions in grey (E-PAC = eastern Pacific; W-ATL = western Atlantic; E-ATL = eastern Atlantic; IWP = Indo-West Pacific; JAP = Japan, and FIJ = Fiji). These regions are further separated by country or oceanographic location (COR = Costa Rica; BRZ = Brazil; CAR = Caribbean Sea; GOM = Gulf of Mexico; WNA = Western North Atlantic; SIL = Sierra Leone; SAF = South Africa; MOZ = Mozambique; RUN = Reunion Island; SEY = Seychelles; ARS = Arabian Sea; SRL = Sri Lanka; TAI = Thailand; IND = Indonesia; PNG = Papua New Guinea; and AUS = Australia).

	E-PAC	W-ATL				E-ATL	IWP										JAP	FIJ
	COR	**BRZ**	**CAR**	**GOM**	**WNA**	**SIL**	**SAF**	**MOZ**	**RUN**	**SEY**	**ARS**	**SRL**	**TAI**	**IND**	**PNG**	**AUS**	**JAP**	**FIJ**
N_{au}	16	50	2	37	8	1	22	11	28	36	15	12	5	4	9	444	44	25
π_{au} (%)	0.028	0.040	0.027	0.038	0.036	0.024	0.044	0.043	0.044	0.044	0.045	0.044	0.042	0.038	0.042	0.046	0.043	0.045
$N_{x♀}$	9	0	0	18	6	1	9	5	13	28	9	7	3	0	4	193	25	22
$\pi_{x♀}$ (%)	0.008	NA	NA	0.018	0.014	0.007	0.030	0.028	0.029	0.031	0.031	0.031	0.032	NA	0.023	0.032	0.028	0.028
$N_{x♂}$	7	0	2	19	2	0	12	6	15	8	6	5	2	3	4	225	19	3
$\pi_{x♂}$ (%)	0.006	NA	0.009	0.013	0.025	NA	0.026	0.025	0.023	0.030	0.028	0.019	0.011	NA	0.019	0.028	0.018	0.017
N_{mt}	16	0	0	0	0	1	7	0	16	18	19	12	6	6	15	203	31	9
π_{mt} (%)	0.027	NA	NA	NA	NA	NA	0.195	NA	0.029	0.030	0.179	0.304	0.293	0.045	0.063	0.063	0.039	0.137
$\pi_{x♀}/\pi_{au}$	0.281	NA	NA	0.471	0.390	0.294	0.677	0.650	0.653	0.696	0.689	0.703	0.755	NA	0.553	0.689	0.649	0.620
$\pi_{x♂}/\pi_{au}$	0.230	NA	0.326	0.344	0.689	NA	0.591	0.588	0.526	0.664	0.629	0.439	0.256	NA	0.448	0.621	0.420	0.378
π_{mt}/π_{au}	0.956	NA	NA	NA	NA	NA	4.400	NA	0.654	0.674	4.012	6.973	6.884	1.165	1.517	1.378	0.899	3.072
R_{FF}	NA	NA	NA	-0.135	-0.148	NA	NA	-0.107	-0.154	-0.054	-0.103	-0.112	NA	NA	NA	-0.011	NA	-0.043
R_{FM}	NA	NA	NA	-0.140	-0.127	NA	NA	-0.103	-0.141	-0.061	-0.097	-0.105	NA	NA	NA	-0.011	NA	-0.041
R_{MM}	NA	NA	NA	-0.174	-0.135	NA	NA	-0.096	-0.138	-0.051	-0.084	-0.111	NA	NA	NA	-0.011	NA	-0.020

Table 4.6.2: Pairwise comparison of fixation indices. X chromosome F_{ST} values for females are presented in the bottom diagonal and autosomal F_{ST} values are in the top diagonal. 'NA' values indicate that no or too few samples were available to estimate genetic differentiation. Asterisks (*) indicate statistical significance of $p<0.05$. No pairwise comparisons were significant after Bonferroni correction. Fixation indices are divided by geographical regions in grey (E-PAC = eastern Pacific; W-ATL = western Atlantic; E-ATL = eastern Atlantic; IWP = Indo-West Pacific; JAP = Japan, and FIJ = Fiji). These regions are further separated by country or oceanographic location (GOC = Gulf of California; COR = Costa Rica; BRZ = Brazil; CAR = Caribbean Sea; GOM = Gulf of Mexico; WNA = Western North Atlantic; SIL = Sierra Leone; SAF = South Africa; MOZ = Mozambique; RUN = Reunion Island; SEY = Seychelles; ARS = Arabian Sea; SRL = Sri Lanka; TAI = Thailand; IND = Indonesia; PNG = Papua New Guinea; AUS = Australia; JAP = Japan; and FIJ = Fiji) Fixation indices range between 0 and 1, where low values are indicated in green and high values in red.

$F_{ST\text{-}au}$ / $F_{ST\text{-}x♀}$	E-PAC	W-ATL				E-ATL	IWP										JAP	FIJ
	COR	**BRZ**	**CAR**	**GOM**	**WNA**	**SIL**	**SAF**	**MOZ**	**RUN**	**SEY**	**ARS**	**SRL**	**TAI**	**IND**	**PNG**	**AUS**	**JAP**	**FIJ**
Costa Rica		*0.348	0.429	*0.369	*0.396	0.701	*0.662	*0.677	*0.658	*0.653	*0.670	*0.677	*0.696	*0.707	*0.689	*0.626	*0.661	*0.659
Brazil	NA		0.002	*0.005	0.004	0.623	*0.652	*0.653	*0.652	*0.650	*0.654	*0.654	*0.656	*0.661	*0.658	*0.636	*0.658	*0.652
Caribbean Sea	NA	NA		0.000	0.000	0.636	*0.640	*0.637	*0.640	*0.641	*0.638	*0.641	0.640	0.659	*0.650	*0.635	*0.654	*0.639
Gulf of Mexico	*0.520	NA	NA		0.000	0.645	*0.665	*0.668	*0.663	*0.661	*0.667	*0.669	*0.673	*0.679	*0.674	*0.642	*0.668	*0.664
Western North Atlantic	*0.583	NA	NA	0.000		0.640	*0.649	*0.650	*0.648	*0.648	*0.650	*0.653	*0.658	*0.670	*0.661	*0.639	*0.658	*0.648
Sierra Leone	0.893	NA	NA	0.806	0.815		0.149	0.139	0.159	0.164	0.158	0.155	0.155	0.186	0.169	0.157	0.207	0.161
South Africa	*0.777	NA	NA	*0.754	*0.724	0.170		0.001	*0.003	0.000	0.002	0.001	0.000	NA	0.002	0.001	*0.032	*0.030
Mozambique	*0.814	NA	NA	*0.775	*0.750	0.309	0.010		*0.005	*0.005	*0.004	0.003	0.000	0.011	*0.011	*0.004	*0.034	*0.033
Reunion	NA	NA	NA	*0.742	*0.715	0.267	0.000	0.004		*0.005	0.002	0.003	0.003	0.013	*0.006	*0.005	*0.035	*0.033
Seychelles	*0.727	NA	NA	*0.720	*0.698	0.188	0.000	0.023	0.018		0.001	0.000	0.005	*0.015	*0.004	*0.002	*0.029	*0.032
Arabian Sea	*0.761	NA	NA	*0.743	*0.707	0.175	0.000	0.013	0.000	0.003		0.000	0.000	*0.012	*0.007	*0.002	*0.031	*0.027
Sri Lanka	*0.782	NA	NA	*0.756	*0.724	0.140	0.000	0.027	0.004	0.001	0.000		0.000	0.011	0.004	0.001	*0.032	*0.032
Thailand	*0.810	NA	NA	*0.768	*0.736	0.150	0.000	0.038	0.005	0.014	0.000	0.000		0.019	0.004	0.001	*0.037	*0.026
Indonesia	*0.902	NA	NA	0.829	0.834	NA	NA	0.000	0.039	NA	0.000	0.000	0.000		0.017	NA	NA	NA
Papua New Guinea	*0.840	NA	NA	*0.793	*0.778	0.215	0.015	*0.129	*0.069	0.043	0.028	0.000	0.050	0.162		0.002	*0.035	*0.031
Australia	*0.693	NA	NA	*0.681	*0.681	*0.187	0.000	0.029	*0.014	0.001	0.000	0.000	0.000	NA	0.030		*0.029	*0.031
Japan	*0.755	NA	NA	*0.745	*0.726	*0.148	*0.030	*0.077	*0.087	*0.026	*0.054	0.020	0.073	NA	0.038	*0.030		*0.062
Fiji	*0.762	NA	NA	*0.750	*0.732	0.312	*0.055	*0.161	*0.095	*0.072	*0.059	*0.065	*0.066	0.095	*0.085	*0.060	*0.115	

Individual and population level assignment tests

Our data contained 209 sharks that had the capacity to disperse (>150 cm TL), including 106 females and 103 males. The AIc did not show any significant differences between females and males (Fig. 4.6.1). The average AIc for males was lower than for females ($AIc_{♂}$ = 5.03 and $AIc_{♀}$ = 12.01). This pattern was most evident in Australia ($AIc_{♂}$ = -46.78 and $AIc_{♀}$ = -35.27). Interestingly, most individuals showing a lower AIc compared to their sampled population were female (12 females and one male); nine of which were sampled in Fiji.

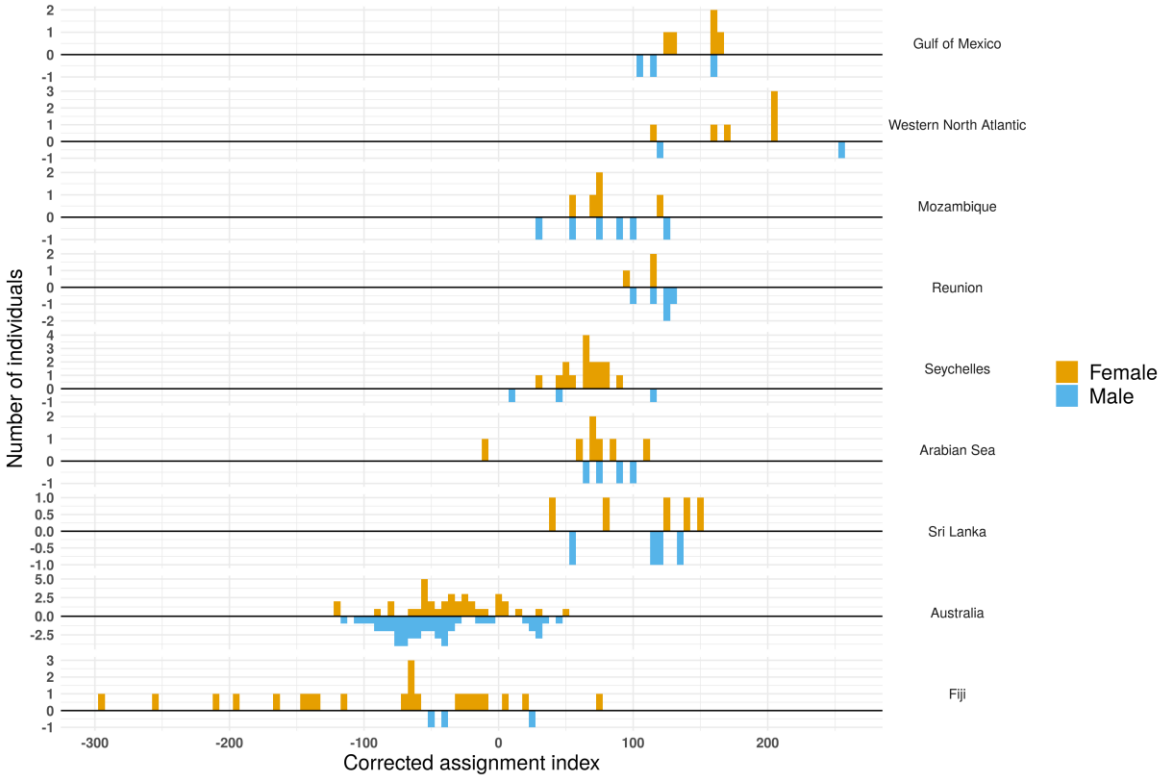

Figure 4.6.1: The corrected assignment index of all Bull Sharks larger than 150 cm total length. The number of female individuals (in orange) are indicated on the y-axis above the zero line, whereas the number of males (in blue) are indicated below the zero line. A lower assignment index indicated a lower probability of having been born in the sampled location.

At a population level, we found no statistically significant differences for F_{ST}, F_{IS}, mAIc, and vAIc between the sexes (Appendix D1 section 8.10.2). When comparing the F_{ST} per site for females and males separately, we did not detect any significantly lower values for males. Unexpectedly, slightly lower F_{ST} values were seen for females in the IWP (e.g. Fiji vs South Africa: $F_{ST♀}$ = 0.024, $F_{ST♂}$= 0.031; or Australia vs Seychelles: $F_{ST♀}$ = 0.000, $F_{ST♂}$= 0.008; Appendix D1 section 8.10.2), although these differences were not statistically significant. Similarly, we found that both females and males exhibited a high similarity between closely located sites and negative correlation between more distant sites (Fig. 4.6.2; top). Most r_{sp} values were not significantly different from zero, except for the closest (~2,500 km; $r_{sp} > 0$) and more distant (~17,000 km; $r_{sp} < 0$) locations. Males showed a significantly

higher correlation at a distance class of 10,000 km, while r_{sp} was not different from zero for females. Overall, we saw a significant correlation between Euclidean genetic distance and over-water distance (Fig. 4.6.2; bottom). Finally, relatedness among-females was similar to the among-males relatedness, except in the Gulf of Mexico where relatedness was lower among males and in Fiji where males exhibited higher relatedness (Table 4.6.1). The total relatedness was highest in Australia with no differences between sexes.

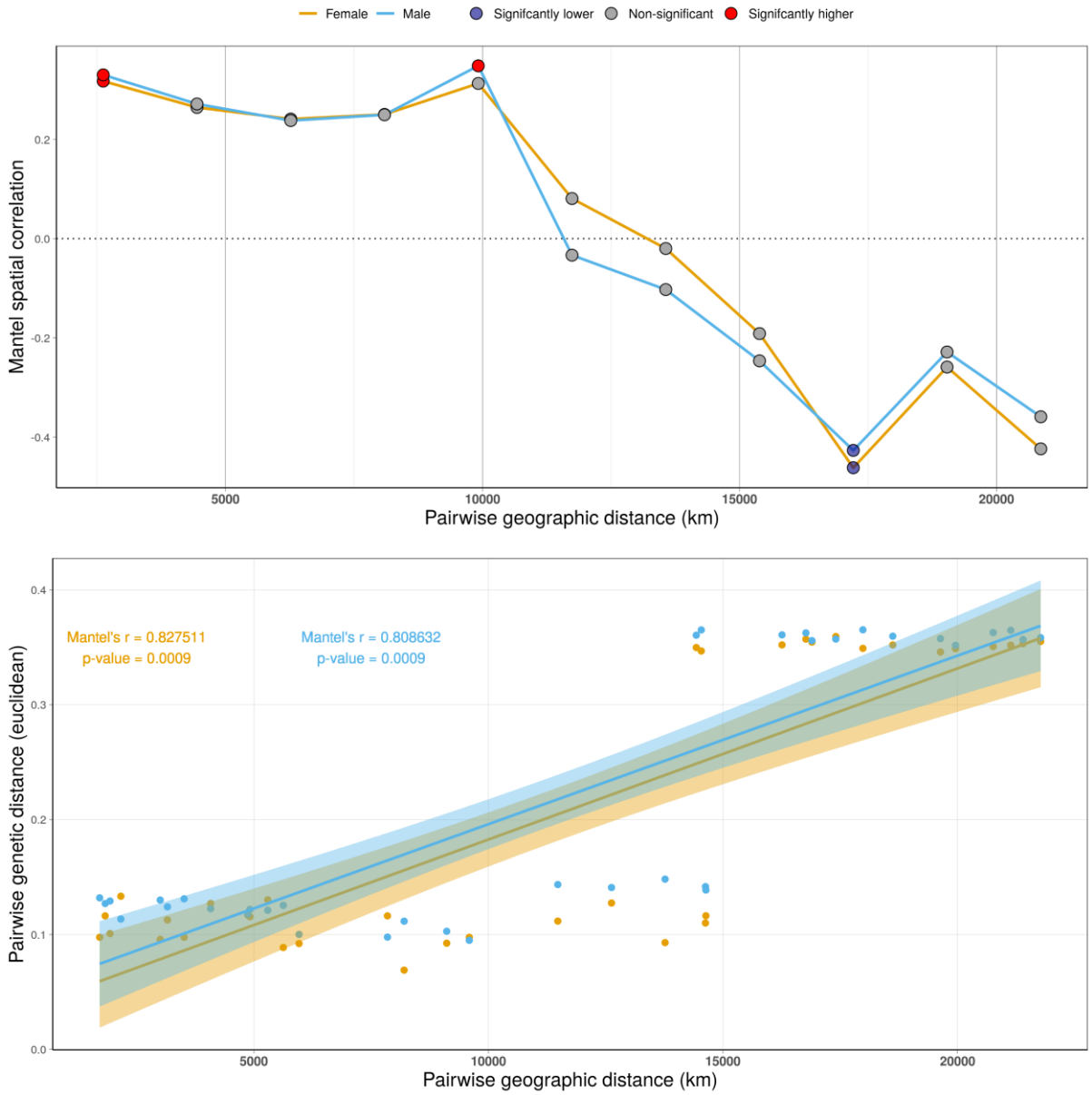

Figure 4.6.2: Top: Mantel spatial correlation between Euclidean genetic distance and the shortest over-water distance between sampling locations. A positive Mantel statistic (r_{sp}) indicates a stronger site association and a negative spatial correlation coefficient signifies a large genetic dissimilarity between geographically distant locations. Red and blue circles show a significant difference from the null hypothesis of no spatial structure (r = 0). Bottom: the correlation between the Euclidean genetic distance and the shortest over-water distance between sampling locations. The overall Mantel correlation statistic and associated p-values are presented for each sex separately.

Kinship

Overall, we found 18 FSPs and 40 HSPs. All FSPs were juvenile sharks (<127 cm TL) collected from the same location and all shared the same haplotype. All but eight HSPs were sampled from the same location and 11 of those 32 HSPs were born in a different year. Nine same-river, same-cohort HSPs had the same haplotype and two had different haplotypes (nine pairs had no haplotype information). Of the 14 same-river, cross-cohort HSPs, six and one had the same or differing haplotypes, respectively (seven had missing haplotype information). Lastly, we identified eight HSPs that were distributed across locations within northern Australia (Fig. 4.6.3; Daly, Adelaide, South Alligator, East Alligator, Towns and Wenlock rivers). Seven of those eight HSPs were juveniles (<125 cm TL) from different age-cohorts, of which five pairs had different haplotypes.

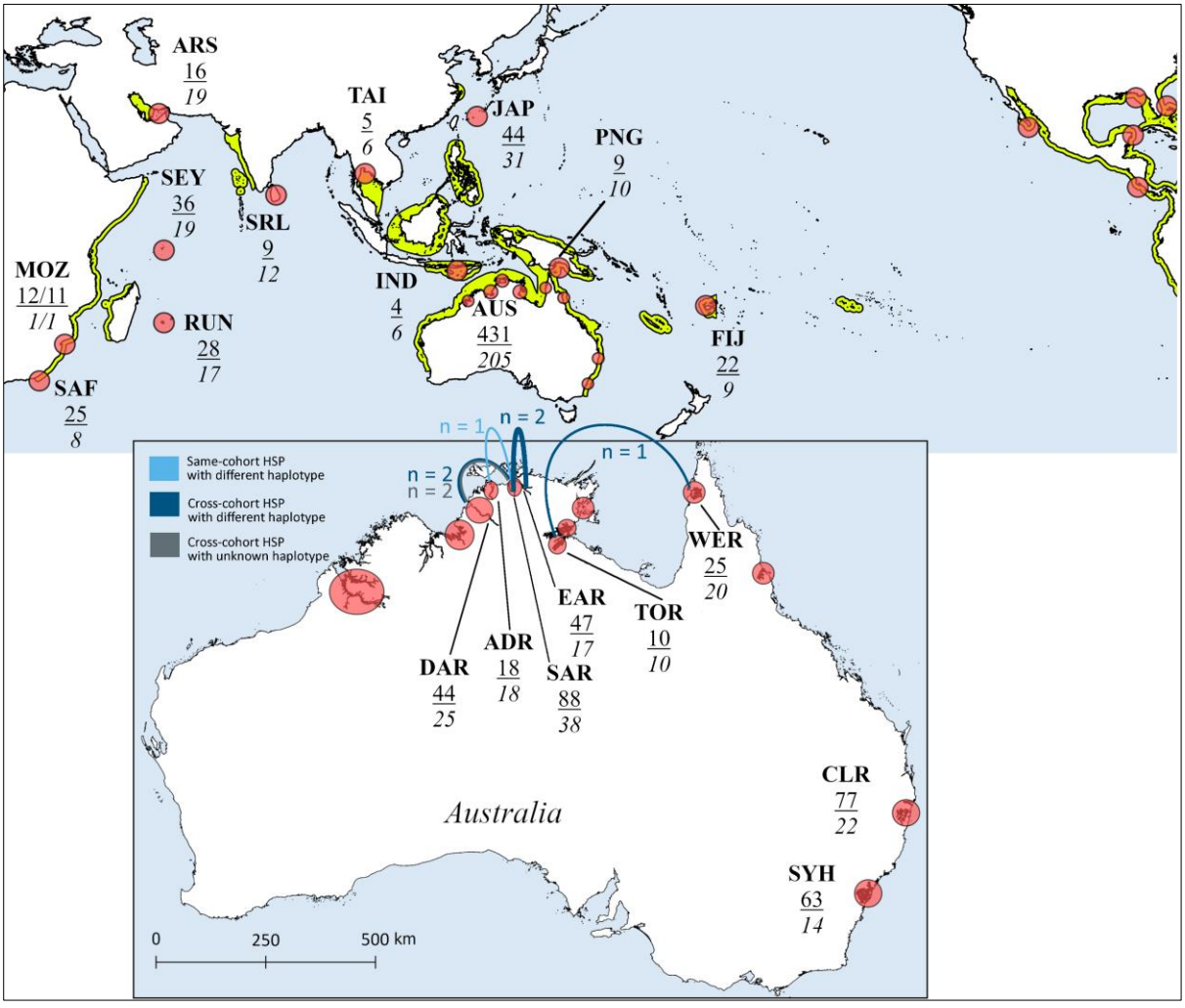

Figure 4.6.3: Map indicating the sampling sites in the Indo-West Pacific region with a red circle and the known species range distribution in yellow. The sample sizes for the SNP data are underlined with the number of samples after data filtering. The sample sizes for the mitogenome data are in *italics* with the number of samples after data filtering. Countries or oceanographic locations are coded as: are SAF = South Africa; MOZ = Mozambique; RUN = Reunion Island; SEY = Seychelles; ARS = Arabian Sea; SRL = Sri Lanka; TAI = Thailand; IND = Indonesia; PNG = Papua New Guinea; AUS = Australia; JAP = Japan; and FIJ = Fiji. Within Australia, sampling locations that contained close-kin relationships are specified: DAR = Daly River; ADR = Adelaide River; SAR =

South Alligator River; EAR = East Alligator River; TOR = Towns River; WER = Wenlock River; CLR = Clarence River; SYH = Sydney Harbour. Only half-sibling pairs (HSP) across sampling locations are indicated with an arch. Same-cohort HSPs with a different mitochondrial haplotype are indicated in light-blue, cross-cohort HSPs with a different mitochondrial haplotype are coloured dark-blue, and cross-cohort HSPs without mitochondrial haplotype information are coloured grey.

4.6.2. DISCUSSION

Mixed-marker

The 148 X-chromosomes markers indicated high and significant differentiation between ocean basins, corroborating previous results showing low ocean-basin connectivity (Chapter 2; Devloo-Delva et al., in prep; Pirog et al., 2019; Testerman, 2014). In addition, $F_{ST\text{-}x♀}$ values were generally higher than the autosomal F_{ST}. Between several pairwise comparisons $F_{ST\text{-}x♀}$ was significant, whereas $F_{ST\text{-}au}$ was not, suggesting a stronger matrilineal divergence. Within the IWP, our results indicated extremely high π_{mt}/π_{au} ratios, while the $\pi_{x♀}/\pi_{au}$ ratios were similar or lower than the expectation across different sampling locations. Genetic variation between mtDNA, xDNA, yDNA, and auDNA can deviate from the expected 4:3:1:1 ratio due to i) SBD patterns, ii) unequal sex ratios, iii) a reproductive skew between sexes (e.g. due to differences in mating systems), iv) sex-specific differences in generation time, v) population size changes over time (e.g. due to bottlenecks), vi) effects of purifying, positive, or balancing selection, and vii) difference in the mutation rate between markers (Webster & Sayres, 2016; Wilson Sayres, 2018). Previously, we considered the high mtDNA nucleotide diversities in South Africa, Sri Lanka, and Thailand to be caused by the process of secondary contact (Chapter 2, Devloo-Delva et al., in prep). Most likely these regions were historically isolated and have experienced more recent female connectivity. Regardless, none of the π_{mt}/π_{au} ratios are close to the 1/4-expectation. Potential reasons have been carefully considered by Karl et al. (2011) in the western Atlantic Ocean, where they concluded that female philopatry and MBD drove the observed mtDNA and auDNA discordance.

Concerning our data, differences in sex ratios, variance in reproductive success, changes in population size, or natural selection are likely not driving the high mtDNA nucleotide diversity as both positive and purifying selection are expected to decrease diversity while balancing selection maintains diversity (Wilson Sayres, 2018). Sex ratios have been reported as approximately equal (e.g. Blaison et al., 2015; Cliff & Dudley, 1991). Further, multiple paternity — where multiple sires are detected in a litter — has been observed in the Bull Shark (Pirog, Jaquemet, Soria, & Magalon, 2017). In theory, this leads to a higher male reproductive success and subsequently to higher nuclear effective population size (N_e) and nucleotide diversities (Karl, 2008), thus contradicting our observation of a higher than expected mtDNA diversity. Also, natural selection on the nuclear genome can reduce genetic diversity, but this process is not expected to have an equal effect across all sampling sites and is unlikely to explain our results. Nonetheless, due to differences in recombination between xDNA and auDNA,

natural selection could be driving the lower $\pi_{x♀}$ and higher $F_{ST\text{-}x♀}$ in certain regions. The most likely explanations for these elevated π_{mt} ratios are high female site philopatry, different generation times, a high mtDNA mutation rate, or possibly combinations of these factors. Overall, we see a clear bias in sex-specific demography, which could be driven by female philopatry. On the other hand, male-biased gene flow could not be verified due to the lack of informative Y-linked markers. Future studies should aim to include this marker to advance our understanding of the male-specific demographic history.

Individual and population level assignment

Both individual- and population-level tests, based on AIc, F_{ST}, F_{IS}, r_{SP}, and R did not identify any significant sex-bias in contemporary movement. The individual assignment test found a bias towards female migrants in Fiji, yet on average the male AIc was lower. Collectively, these results may be an indication that females move over larger distances and show a stronger discordance to the allele frequencies of the newly arrived location. Oppositely, the average lower AIc observed in males could be explained by more frequent movements, but over smaller distances. This study considered direct movement in 209 sharks capable of dispersal, which is a small sample size relative to the large N_e estimates in the IWP (e.g. ~1,000–50,000; Pirog et al., 2019; Tillett, Meekan, et al., 2012). Future assignment testing studies would benefit from larger sample sizes and finer spatial coverage. Overall, our results concur with previous studies showing that both females and males move over large distances (Brunnschweiler et al., 2010; Lea et al., 2015). However, these signals of contemporary movement do not verify that breeding occurs at these sites or if sharks sampled return to their natal sites to breed.

Kinship

The spatial distribution of close-kin, and the cross-cohort HSPs in particular, has proven valuable to identify contemporary dispersal followed by reproduction at the new site of arrival (e.g. Feutry et al., 2017). This study investigated the close-kin distribution of within the IWP and found that juvenile FSPs were always sampled from the same location, verifying the assumption that juvenile Bull Sharks stay in or near their nursery areas (Heupel et al., 2010; Matich & Heithaus, 2015; Simpfendorfer & Milward, 1993; Wiley & Simpfendorfer, 2007). We identified seven cross-cohort HSPs across Australian rivers (10–500 km apart); five with differing haplotypes. This implies that their fathers moved between breeding grounds, indicating a bias towards male reproductive dispersal. Nonetheless, this result is only based on five cross-cohort HSPs between six rivers and needs to be verified with larger sample sizes and wider geographic scope.

4.6.1. CONCLUSION

This study is a first investigating contemporary and historical sex-biased dispersal patterns using a suite of genomic markers, including autosomal SNPs, X-linked SNPs, and full mitochondrial genomes, as well as a range of analytical approaches (mixed-marker, assignment tests, and kinship). Female philopatric behaviour has been confirmed within the Indo-West Pacific region, with evidence from mtDNA, xDNA and kinship. However, MBD could only be established at fine geographic scale, based on five cross-cohort half siblings. Future research should strive to include Y-chromosome markers to measure male-specific demography. In addition, with 148 X-linked SNPs, we were able to demonstrate a higher and significant differentiation between sampling locations. Future studies should include more unlinked markers on the X chromosome to increase statistical power. Increased full-genome sequencing efforts should provide important resources to improve future SBD studies. In general, philopatry and SBD were found at small spatial scales using the kinship approach (i.e. river or estuary-level; ~10–100 km), confirming the value of this approach to provide national management advice regarding contemporary connectivity of populations.

5. SECTION 5 – General discussion

In this thesis, I aimed to investigate sex-biased dispersal (SBD) in elasmobranchs to understand the patterns and drivers of their movement. The research in this thesis has provided new theoretical knowledge of elasmobranch dispersal, as well as direct applicable management advice for the studied species.

Below, I describe the main patterns of dispersal (rate, distance, and sex-bias) that emerged from the three species studied here: Northern River Shark, School Shark, and Bull Shark. I further discuss the potential ecological and evolutionary forces that may have driven the observed patterns of population structure and SBD. In addition, I provide evidence for how imperfect experimental design can confound inferences about dispersal. I assess the strengths and weaknesses of genetic tools and analytical approaches used in this thesis and propose key considerations for future studies on dispersal. To conclude, I summarise how this thesis has advanced the field with both methodological improvements and specific management directions. Finally, I provide suggestions for future research.

5.1. Patterns and drivers of dispersal in three shark species

This thesis started with the hypothesis that dispersal, and specifically male-biased dispersal (MBD), is common in sharks. This is based on both empirical observations (reviewed in Heist, 2012; Phillips et al., 2021) and theoretical expectations. Regarding the latter, dispersal is expected under high competition, inbreeding, or environmental stochasticity and reduced dispersal costs (Ronce, 2007). The intensity and the spatial scale at which these selective pressures operate, as well as the biological dispersal capacity (e.g. body size) will determine the rate and distance of dispersal. Further, MBD is assumed in species with i) a polygynous mating system, ii) large female investment in reproduction with patchy nursery habitats, iii) small population sizes, and vi) heterogametic sex chromosomes (See General Introduction). If this generalisation is true for my study species, I would expect a high level of dispersal in all species, where males have a higher rate of dispersal and move larger distances compared to females.

5.1.1 OBSERVED PATTERNS

My datasets on three shark species — covering different spatial scales and investigated using different genomic tools — provided several interesting insights into the rate, distance, and sex-bias of dispersal. All species can be considered relatively large-bodied (reaching a maximum size of 160–400 cm TL), yet they occupy different habitats and ranges. For example, both the Northern River Shark and the Bull Shark are coastal/euryhaline species, however the Bull Shark is circum-globally distributed, while the

Northern River Shark has a narrow range in Australia and southern PNG. The School Shark, on the other hand, is a fully marine and demersal species with a global temperate distribution.

The rate and distance of dispersal of my three studied species was mostly inferred from population genetic structure. First, high dispersal rates were identified for the School Shark and along continuous coastlines for the Bull Shark. For example, no significant population structure was observed between Tasmanian and New Zealand School Sharks (~3,000 km; Chapter 1). Other studies on this species also demonstrated the lack of genetic structure between these regions (Hernández et al., 2015; Ward & Gardner, 1997), suggesting this species has a strong dispersive behaviour. The Bull Shark showed similar high connectivity within ocean basins (~1,000–10,000 km), but distinct population genetic structure and lower dispersal rates on a trans-oceanic scale (>10,000 km; Chapter 2). In general, acoustic telemetry studies established partial site fidelity for adult Bull Sharks throughout their range (Brunnschweiler et al., 2010; Espinoza et al., 2016). Although, their ability to move large distances has also been demonstrated using satellite tagging data (Lea et al., 2015). While such tagging or telemetry studies do not provide associated information about reproduction, my results from the nuclear SNPs indicate that reproductive dispersal occurs — although in lesser amounts for females. Using a close-kin framework, I established that most Bull Shark kin pairs remained in the same river (32 out 40 kin pairs in Australia), which implies that the signal of contemporary dispersal was more localised than what could be inferred from the genetic differentiation based on the SNP and mitogenome data which operate at an evolutionary timescale. This was demonstrated in Chapter 2 and Chapter 6 of this thesis. Despite sharing the euryhaline lifestyle of the Bull Shark, the Northern River Shark exhibited finer nuclear and mitochondrial population structure (<100 km; PNG and within northern Australia). For this species, the number of migrants per generation (Nm) were directly measured. Historical migration was low between regions (Nm = 0.37–2.50), compared to estimates in other carcharhinid species (e.g. *Carcharhinus melanopterus*, Nm = 8.7–48.1 over ~1,000–10,000 km; Maisano Delser et al., 2019). Similarly, the kinship results found that only 27 out of 177 kin pairs were distributed across sampling locations within the Van Diemen Gulf (VDG; 10–100 km). Overall, the strong genetic differentiation would suggest that Northern River Sharks disperse less frequent and over smaller distances compared to the School Shark and the Bull Shark.

Male-biased dispersal was detected for the Northern River Shark and the Bull Shark, generally within the population boundaries that were inferred from the nuclear data (Section 4). The School Shark dataset did not indicate any nuclear population structure, but no mitochondrial DNA was investigated to infer female genetic structure at finer scales and philopatric signals could be overlooked. Previous studies have suggested female philopatry and MBD in this species based on a mixed-marker approach (microsatellites vs mtDNA; Bester-van der Merwe et al., 2017; Chabot, 2015). The Northern River

Shark demonstrated some discordance between the nuclear and mitochondrial data (lower mtDNA divergence, Chapter 5), but a recent colonisation history, with insufficient time for haplotypes to diverge, most likely explains this pattern. Nevertheless, at a smaller spatial scale (10-100 km) and using a CKMR framework, the Northern River Shark showed a convincing male-bias in dispersal rates and distances. Bull Shark also showed a discrepancy between their nuclear and mitochondrial DNA within the IWP region (Chapter 2). While the nuDNA clustered the Indian Ocean and western Pacific samples together (~10,000 km), the mtDNA categorised each sampling country or oceanographic location as distinct (~1,000km). This mitonuclear discordance is similar to other genetic observations in the Bull Shark (Karl et al., 2011; Tillett, Meekan, et al., 2012). Conventionally, this difference is associated with MBD, but other explanations, such as demographic or spatial expansions or unequal sex ratios among populations, are possible (see General Introduction, Section 1.2.; Lawson Handley & Perrin, 2007; Lowe & Allendorf, 2010; Toews & Brelsford, 2012). The Bull Shark data also contained 148 X-linked markers, which allowed me to further test the hypothesis of female-driven population structure at smaller spatial scales. The X chromosome is expected to occupy more time (three times longer) in the female population, and because of recombination these multi-locus markers are less affected by selection and provide higher statistical power (Schaffner, 2004; Wilkins & Marlowe, 2006). The X-linked markers in both females and males showed a similar pattern to the auDNA, but F_{ST} values for the xDNA were generally higher, suggesting strong female philopatry (Chapter 6). However, the direct methods to measure contemporary dispersal (e.g AIc, F_{ST}, F_{IS}, R, or r_{sp}) did not identify any sex-specific movement patterns of adult Bull Sharks. The kinship results, however, found that parental dispersal of half siblings was mainly male-mediated. Overall, I found convincing evidence of female natal philopatry and MBD in the Northern River Shark and the Bull Shark, but could not substantiate SBD in the School Shark. These patterns could indicate that different factors are driving the propensity of dispersal in these species.

5.1.2 PLAUSIBLE DRIVERS

The drivers of dispersal can be classified into ecological and evolutionary factors. Ecological drivers include environmental and behavioural influences, and often interact to shape the observed dispersal patterns. Evolutionary drivers, such as competition or inbreeding avoidance, determine how dispersal has evolved.

A first important consideration is the presence of biogeographic barriers to dispersal. These barriers can be categorised into ‘soft’ and ‘hard’ barriers. Hard barriers, such as land bridges, physically isolate two populations, while soft barriers include oceanographic properties, such as linear geographic distance, ocean currents, suboptimal thermal regimes, and other hydrological conditions, that reduce the movement of individuals (Cowman & Bellwood, 2013; Hirschfeld, Dudgeon, Sheaves, & Barnett,

2021; Luiz et al., 2012; Pyron & Burbrink, 2010). The dispersal potential, which can be approximated by maximum depth of occurrence, maximum body size and habitat, determines how strongly each barrier will affect connectivity between populations (Hirschfeld et al., 2021). In theory, smaller demersal species, such as the School Shark, will be affected by both soft and hard barriers, while larger coastal species, such as the Bull Shark, are more likely to overcome soft barriers (Fig. 5.1.1). For example, this is illustrated by the lower divergence between Bull Sharks from the E-ATL and IWP, despite the presence of the Benguela Upwelling Barrier (i.e., thermal barrier). Curiously, School Shark dispersal does not seem obstructed by soft barriers, such as the distance across the Tasman Sea, which would be expected if movement scales positively with body size. However, this could be attributed to the maximum depth of occurrence (-826 m; Walker et al., 2020). Conversely, the Northern River Shark — which grows up to 250 cm — showed strong population divergence across seemingly homogeneous environments, suggesting that factors other than biogeography are involved.

Behavioural and physiological preferences may also decrease the inclination to disperse. For example, the Northern River Shark has a strong euryhaline habitat-association — at least as juveniles (Pillans et al., 2009). In general, species with niche habitat requirements, such as riverine elasmobranchs (river sharks *Glyphis* spp. or sawfishes *Pristis* spp.), demonstrate stronger population structure, due to discontinuous habitat. Similarly, gravid females require a habitat with few predators and ample food resources, acting as a nursery for their pups (Heupel, Carlson, & Simpfendorfer, 2007; Heupel, Kanno, Martins, & Simpfendorfer, 2019). These habitats generally include river systems, mangroves, estuaries, or embayments, which are typically fragmented in distribution. Where these habitats are stable over time, females may prefer to continuously return to these areas for parturition (i.e. female philopatry), leading to higher survival rates of the pups and increased fitness of the mother. In general, males do not make the same migrations to the nursery areas — with the exception of aggregations at nursery sites for breeding purposes (e.g. Fisher, Call, & Grubbs, 2013).

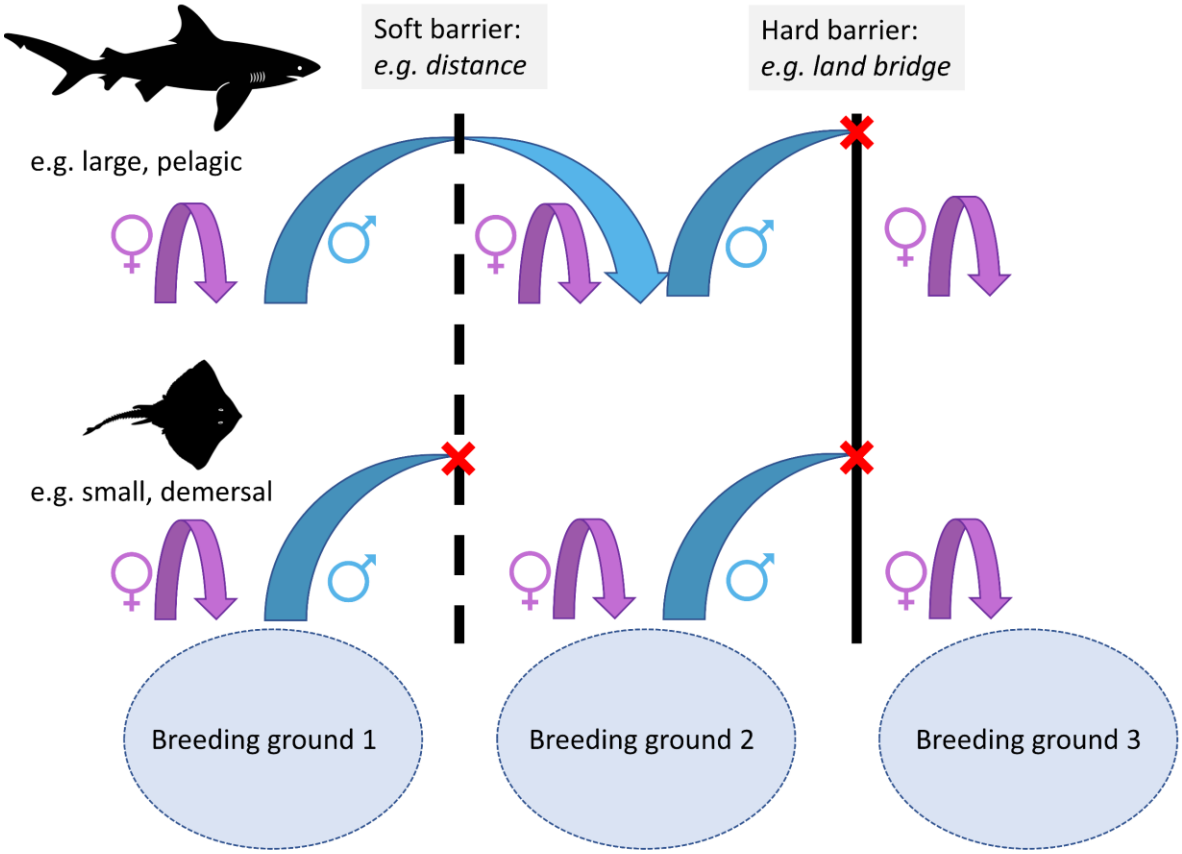

Figure 5.1.1: Dispersal patterns from i) large, vagile sharks (e.g. Bull Sharks) and ii) smaller or demersal species (e.g. Northern River Shark) in respect to environmental and behavioural barriers. Soft barriers represent permeable oceanographic properties (e.g. temperature gradient), easily overcome by large vagile species, allowing males to mate with (philopatric) females in new breeding grounds. Hard barriers represent more permanent changes, such as historical land bridges, that are impermeable to all species.

Theory would predict dispersal, and MBD, evolved under a scenario involving competition for mates in a polygynous system, small populations with associated higher inbreeding, competition between kin, and strong environmental fluctuations (e.g. heatwaves) between patches of suitable habitat (Greenwood, 1980; Hamilton & May, 1977; Perrin & Mazalov, 2000). On the other hand, dispersal may be selected against when the costs of dispersal are high, population densities are low combined with large population sizes (i.e. low competition and inbreeding), and individuals have direct benefits from philopatry (e.g. kin cooperation, habitat familiarity and food availability; Bonte et al., 2012; Lawson Handley & Perrin, 2007). The potential drivers which steered the evolution of dispersal remain difficult to disentangle, since little information is available from the past populations (e.g. structure, demography, or ecology) and because evolutionary drivers often interact. Here, I will assume that historical populations somewhat reflected the patterns we see today.

One notable strategy to increase fitness in sharks is to produce more offspring with multiple mates. Dispersal may increase chances of finding multiple mates, especially when local competition is high (Dobson, 2013; Greenwood, 1980). Previous studies have identified polyandry (i.e. litter sired by multiple males, also termed multiple paternity) in Bull Sharks and School Sharks (Hernández, Duffy, Francis, & Ritchie, 2014; Pirog et al., 2017). Such studies have not been conducted in the Northern River Shark, but the close-kin results from Chapter 5 identified 59 same-cohort HSPs; in which 43 of these pairs had the same mitochondrial haplotype and 16 had a different haplotype. These could indicate both polyandry and polygyny (i.e. mating with multiple females in one breeding season) in the Northern River Shark. In general, polyandry is a common observation in elasmobranchs (Lamarca, Carvalho, Vilasboa, Netto-Ferreira, & Vianna, 2020) and supports the hypothesis that males will disperse more frequently to avoid mating competition. Another form of competition avoidance — kin competition — may also drive the evolution of dispersal in sharks by increasing the available resources and subsequently the survival of offspring; yet this would require kin-recognition (Brom et al., 2016; Hamilton & May, 1977). Chapters 5 and 6 showed that the Northern River Shark and the Bull Shark exhibit a high number of full and half siblings within the same sampling location at juvenile life stage. Under the kin competition avoidance theory, the high number of juvenile kin could explain why adult males move away from their natal areas. If these signals were also true in historical populations and under the assumption that kin recognition exists, kin competition avoidance could be a substantial driver for dispersal in the Northern River Shark and the Bull Shark. The influence of kin competition avoidance as a driver for dispersal remains inconclusive for the School Shark due to the lack of evidence on natal philopatry and kin abundance.

Local inbreeding usually results in the accumulation of deleterious mutations which will decrease fitness and may drive individuals to disperse away from close relatives. This is especially true in species exhibiting natal philopatry and small population sizes (Perrin & Mazalov, 1999, 2000). I established that the Northern River Shark and the Bull Shark are philopatric to a specific river, while the School Shark did not display philopatry to their nursery areas. This would suggest higher levels of inbreeding in both the Northern River Shark and the Bull Shark. Both the Bull Shark and the School Shark exhibit large effective population sizes ($N_e \approx$ 10,000–100,000; Chabot & Allen, 2009; Karl et al., 2011; Pirog et al., 2019; Tillett, Meekan, et al., 2012), while N_e of the Northern River Shark in the VDG is approximately 10 to 100-fold lower (Feutry et al., 2020). If mating is assumed random, this preliminary evidence would suggest that higher inbreeding levels are expected in the Northern River Shark populations, due to both natal philopatry and smaller population sizes. Oppositely, the School Shark species seems least subjected to inbreeding due to the lack of apparent natal philopatry and large population sizes. Jointly, these observations would predict that inbreeding avoidance acts a driver for

dispersal and MBD in the Northern River Shark and the Bull Shark, but most likely not in the School Shark.

The effects of environmental or demographic stochasticity to promote dispersal have been demonstrated in several theoretical and modelling studies (Cadet, Ferrière, Metz, & Van Baalen, 2003; Comins et al., 1980; Hamilton & May, 1977; Henry et al., 2016). However, this remains more challenging to prove empirically due to the lack of data on these stochastic events. For example, the Northern River Shark tends to show a pattern of stronger dispersal in the Sampan Creek and East Alligator River (Chapter 5). Although, inferring historical habitat fluctuation as causation for dispersal is difficult. Temporal and spatial stochastic habitat perturbations have also been observed in the Bull Shark's distribution (e.g. extreme weather events; Matich & Heithaus, 2012; Matich, Strickland, & Heithaus, 2020), but again it remains difficult to ascribe those environmental events as a cause of dispersal as they may be obscured by other drivers (e.g. competition for mating). Periodic monitoring of population structure and dispersal may help clarify the role of stochastic events on dispersal.

Another driver that is rarely empirically investigated, is the presence of dispersal-related genes on the sex chromosomes. Under the assumption of equal sex-ratios, new Y-linked mutations that influence dispersal are expected to be more abundant throughout the populations than X-linked mutations, given the higher mutation rate on yDNA, uniparental inheritance, and influence of genetic drift (Brom et al., 2018; Schaffner, 2004). Theory predicts that these Y-linked genes would drive MBD as an effect of enhanced kin competition between siblings from the heterogametic sex, due to higher relatedness at the Y chromosome (Brom et al., 2018). While my results from Chapter 4 indicated that an XX/XY sex-chromosome system is likely ancestral and still dominant in Chondrichthyes, no dispersal-related genes (e.g. body size; Tan et al., 2021) have been detected on the sex chromosomes. Nonetheless, this theory cannot be dismissed without further research, such as additional genome and transcriptome sequencing, or genome-wide association studies.

To summarise, the effects of physical and behavioural barriers explain the patterns of dispersal quite well. Similarly, factors such as female habitat requirements, male mating competition, and the presence of sex chromosomes seem to be plausible drivers for the evolution of female philopatry and MBD in Northern River Shark and the Bull Shark, and perhaps also in other shark species with similar ecology and life history to my study species. Additional rigorous research remains essential to determine the level of importance of each driver. For example, to understand the role of resource competition avoidance, further study is needed on foraging energetics, habitat familiarity, and the fitness consequences of malnutrition. Also, additional investigation into the genes on sex chromosomes and their function will reveal the role of an XX/XY system in the evolution of SBD.

5.1. Confounding variables and methodological improvements in dispersal research

5.1.1 CONFOUNDING VARIABLES

There are several confounding factors that may obscure the observed patterns and hence the inferred drivers of dispersal. These confounding factors can be categorised as i) dispersal characteristics ii) analysis methods, and iii) experimental design (Fig. 5.1.2).

Dispersal characteristics

Like speciation, the formation of a new population is not a discrete process. When two groups of individuals become isolated from each other it may take many generations before differences in allele frequencies can be genetically detected (Bailleul et al., 2018; Waples & Gaggiotti, 2006). The rate of genetic drift is strongly related to the population size and the migration rate between both groups (Gagnaire et al., 2015). These demographic features will affect the detection of dispersal and could possibly explain why strong genetic isolation was detected for the Northern River Shark but not for the School Shark and the Bull Shark.

Analysis methods

The results of dispersal studies are also strongly impacted by the methods used; more specifically, the choice of genetic markers and analytical methods. The statistical power required to detect fine-scale population structure varies according to the dispersal rate and population size (Waples, 1998; Waples & Gaggiotti, 2006). In elasmobranch studies, often small mtDNA regions or microsatellites with few alleles are used (Green et al., in press; Heist, 2012; Phillips et al., 2021), which often failed to detect population structure (e.g. Feutry et al., 2014; Green et al., 2019) In this thesis I found that both SNP and mitogenome markers constitute powerful tools to measure connectivity between populations and offer a wide range of applications.

In terms of analytical methods, I have used a range of approaches to investigate (sex-biased) dispersal; namely, individual assignment, spatial distribution of close relatives and mixed-marker approaches. The individual assignment tests — such as population assignment, or within-sex relatedness — were only applied in the Bull Shark data, which contained subadult and adult individuals that are capable of dispersal and consequently represent potential immigrants. Despite a large sample size and good spatial coverage in the IWP, long-distance movement was rarely detected in the Bull Shark. These individual assignment methods rely on exhaustive sampling and powerful genetic markers. While assignment tests have proven very useful in other studies of natural populations (e.g. Blundell et al., 2002), they may be less powerful when applied to elasmobranchs given the limitations to acquiring ample adult samples from all putative populations. Further, results from the Northern River Shark and the Bull Shark studies provide evidence that CKMR can identify population structure and dispersal at

smaller spatiotemporal scales than expected based on previous population genetic studies. For example, I found high genetic connectivity (small and non-significant F_{ST}) values for the Bull Shark between adjacent sampling locations in Australia. However, the observation that 11 out of 19 cross-cohort HSPs are retained within the same rivers indicates philopatric behaviour to a much smaller geographic scale. Close-kin distribution is arguably a better indicator of demographic independence than a small but significant F_{ST} value, which may be difficult to interpret due to the interacting effects of population size and gene flow on population divergence. My thesis also shows the added advantage to integrating the kinship data with mitogenome sequencing to infer putative maternal and paternal relationship, and subsequently SBD.

Experimental design

Sampling must aim to capture a true representation of the population (Shringarpure & Xing, 2014). My thesis has highlighted the importance of experimental design and the risks associated with sampling inconsistencies. The high power of genomic data makes it more sensitive for skewed sampling towards age, sex, family members, or sampling year or season, and can inflate the population structure signal. For example, the School Shark exhibits aggregating behaviour by size and sex (Olsen, 1954), and closely-related neonates have a high probability to be caught together (e.g. within two weeks from each other; Chapter 1). Additionally, the School Shark exhibits a 2–3 year reproductive cycle (Olsen, 1984), which could cause temporal cohort-specific structure in this species if the timing of sampling scheme follows the same periodicity. Some species, like the Sharptooth Lemon Shark (*Negaprion acutidens*; Pillans et al., 2021), also exhibit strong seasonal movement and detection of dispersal or population structure may fail if sampling occurs in only one season (Latch & Rhodes, 2006). Also, population structure can be obscured when analysing a dataset that includes a mix of individuals that could not yet disperse (i.e. juveniles) and individuals that could have been migrants (i.e. adults; see Klein et al., 2019). In Chapter 2, I was able to identify fine-scale population structure in Australia, where most Bull Shark samples were young-of-the-year or juveniles sampled from their nursery areas. Lastly, unequal sample sizes between sampling locations has been proven to interfere with the ability to identify population divergence based on the clustering of individual genotypes (Chapter 2; Feutry et al., 2020; Foster et al., 2018; Puechmaille, 2016). Consequently, all the parameters mentioned above should be considered when studying SBD, as it may bias the findings.

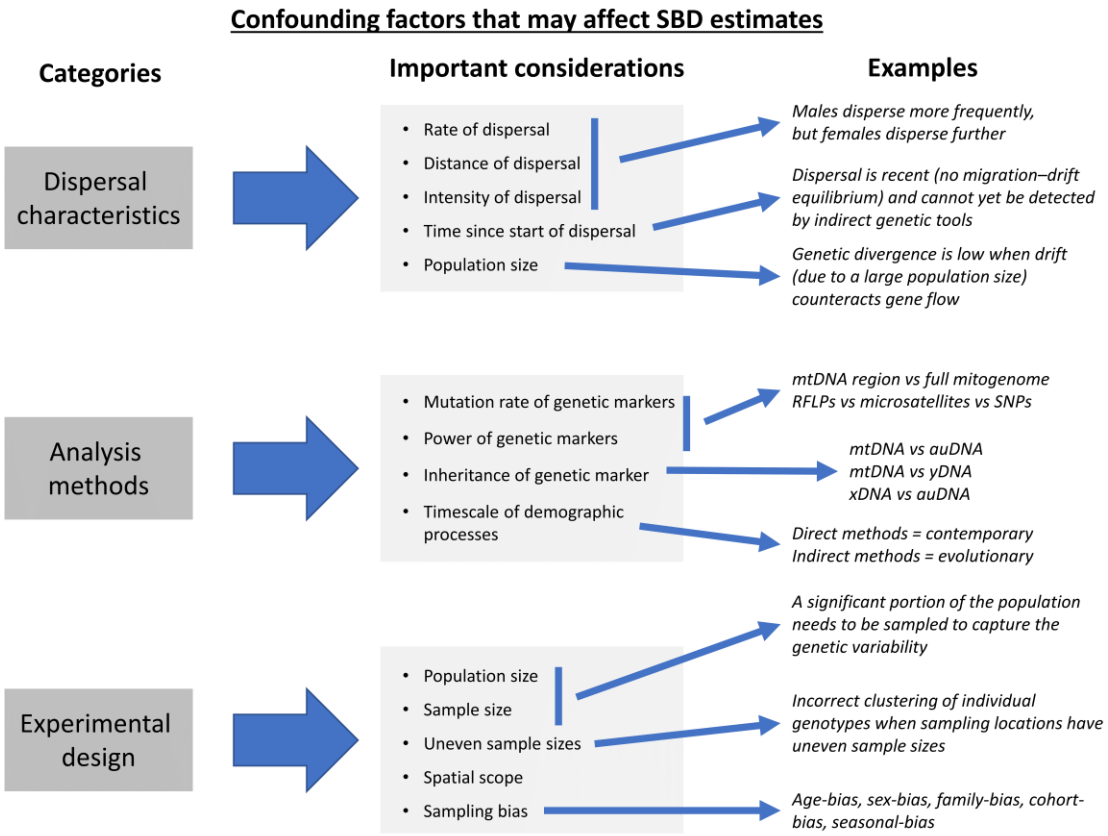

Figure 5.2.1: Conceptual figure that summarises the confounding factors influencing sex-biased dispersal analyses: i) dispersal characteristics, ii) analysis methods, and iii) experimental design. For each category several key factors and an example are listed that should be considered when starting a study on sex-biased dispersal.

5.1.1 METHODOLOGICAL IMPROVEMENTS

Future improvements to measure dispersal include, starting with having several well-defined and testable hypotheses, an appropriate sampling scheme to examine these hypotheses, using high resolution molecular markers and the appropriate analytical tools. Below, I describe some important considerations when developing an experimental design and choosing the appropriate analytical methods to study SBD.

Hypothesis testing

The genetic signature of population divergence in a species is often the result of a complex evolutionary history, such as sex-specific demography, range expansions or contractions, and natural selection. On top of that, confounding influences from sample collection or analysis method often impede the interpretation of results even further. Consequently, a well-defined hypothesis considering the impact of these obscuring variables is paramount. Often a closely-related species or a species with similar life-history or ecological characteristics may provide a starting point to construct such a hypothesis. Specifically, knowledge of contemporary and historical barriers, and a species' vagility and range may provide an indication of the spatial coverage required to measure dispersal. For example, Chin et al. (2017) proposed several hypothetical connectivity routes for hammerhead

sharks between Australia, Indonesia and PNG, based on an Integrated Assessment Framework which combined data from fisheries, genetics, tagging, telemetry, and biogeography. These pre-defined hypotheses provided a foundation for empirical testing by Green (2019) and Heupel et al. (2020) with genetic, parasite fauna, and telemetry tools. With the appropriate spatial scales and sample sizes the authors concluded that the connectivity between these regions likely occurs along the continental shelves with limited movement across deep water.

Sample collection

Sample collection is also constrained by several prerequisites. The locations where tissues are obtained should reflect the spatial scale of posed hypothesis. Additionally, enough samples to capture the variability of a population should be retrieved. Previous estimates of the (effective) population size can indicate the number of samples required from each location for an accurate representation of the true allele frequencies of a population. The Northern River Shark and the Bull Shark were studied across their known range, with large sample sizes in Australia (~5–50 % of the estimated N_e), which provided detailed knowledge on the rate, distance and sex-biased patterns of dispersal. The School Shark, on the other hand, included a sample size that covered roughly ~0.1 % of the estimated N_e, and only a small part of the species' distribution. The small sample size could explain why population structure and SBD were not detected in the School Shark, which highlights the importance of appropriate sampling effort respective to the population size and intensity of dispersal.

The location of sample collection (i.e. breeding, nursery, or foraging ground) will rely on both the posed hypothesis and the analytical approach used. For example, the CKMR approach based on HSPs, requires sampling of pre-dispersal individuals — in shark, these are often juveniles from their natal sites. This guarantees that HSPs across sampling locations did not disperse directly, but rather that one of their parents did. Conversely, the individual assignment approach depends on sampling adults, preferably from mating grounds. This may provide direct evidence of F_0 dispersal for reproductive purposes. While information is available about nursery and foraging areas in various elasmobranch species, the location where breeding occurs remains enigmatic in most species, limiting detailed investigation of their breeding behaviour.

Skewed sampling towards sex, age, family members, or the timing of sampling has proven to confound inference of population structure (e.g. Chapter 1; Benestan et al., 2017; Feutry et al., 2017; Fraser et al., 2004; Klein et al., 2019; Latch & Rhodes, 2006). Ideally, sample sizes should be similar between locations and without any bias. Nevertheless, sampling in elasmobranch research is often opportunistic given the sparse distribution of individuals. Luckily, if sampling effort was approximately random, ad-hoc sample selection can help reduce the bias from age, sex, and sample season and year.

Genomic markers

The higher statistical power of genomic markers compared to genetic markers has previously been established (e.g. Waples, 1998), and verified in sharks (see Feutry et al., 2014 for mtDNA vs mitogenomes; Green et al., 2019 for microsatelites vs SNPs). Similarly, Attard et al. (2018) demonstrated for the Pygmy Blue Whale (*Balaenoptera brevicauda*) that relatedness estimates from SNPs were more precise than the microsatellite estimates. The use of new DNA-capture approaches (e.g. Rapture; Ali et al., 2016) has decreased sequencing cost and allows larger sample sizes; which is essential when quantifying dispersal. For example, I used the DArTcap method for SNP genotyping (Chapter 2, 5, and 6), reducing the cost per sample from AU$50 (DArTseq) to AU$15 (DArTcap) and allowing me to sequence up to 1,000 individuals, thus maximizing my ability to capture the variation in populations.

The increased power of genomic markers, combined with a large number of samples, could result in additional bias when the data contains family members or sex-linked markers. For example, due to schooling behaviour in sharks, the sampling of related juveniles might be expected and one individual per pair should be removed from the dataset (see Chapter 1). However, if the sampling of kin pairs is independent and caused by the underlying effect of a small population size, these siblings should not be purged from the dataset (Waples & Anderson, 2017). Furthermore, the presence of SLMs has been shown to cause a false signal of population structure, if the sampling is biased towards a certain sex (Benestan et al., 2017). With genomic SNP data, closely related individuals and SLMs can be detected and removed from the dataset, if necessary.

Mixed-marker approach

Indirect methods framed as a 'mixed-marker' approach comparing mtDNA against auDNA has been the most common way to investigate sex-biased gene flow in elasmobranch due to the lower sampling requirements (Phillips et al., 2021). However, differences in genetic architecture between these markers (haploid vs diploid and uniparentally vs biparentally inherited) means that these markers are differentially affected by processes other than SBD, including genetic drift, selection, and population size changes (See General Introduction, Section 1; Ballard & Whitlock, 2004). Markers on the Y chromosome have been proposed to reveal male-specific gene flow patterns (Petit et al., 2002). Yet, similarly to mtDNA, the yDNA is haploid and uniparentally inherited, making these single linked markers. Alternatively, contrasting X-linked versus autosomal markers has shown to be 20 % more powerful when multiple polymorphic and unlinked markers can be targeted (Wilkins & Marlowe, 2006). In Chapter 4, I demonstrated that an XX/XY sex-chromosome system is predominant among Chondrichthyes. Further, the random sequencing of the nuclear genome allowed me to identify X-linked markers in the Bull Shark, which I used to examine the female population structure with less

bias from historical demographic events, such as range expansion or a population bottleneck (Chapter 6). Nonetheless, genetic variation of these marker types (mtDNA, yDNA, xDNA, and auDNA) will ultimately be shaped by multiple and interacting processes which makes it difficult to disentangle the presence of SBD. In such cases, direct methods — which investigate movement at a contemporary timescale — can provide additional certainty.

Spatial analysis of close-kin

Direct methods can be employed when quantitative dispersal information is needed to aid population monitoring (e.g. stock or threat assessments; Ovenden, Berry, Welch, Buckworth, & Dichmont, 2015). Conventionally, this is done with physical markers, such as tagging or parasite fauna composition (Begg & Waldman, 1999). More recently, the spatial distribution of close-kin has been used (Feutry et al., 2017). Overall, the CKMR approach has proven powerful, given that the probability of sampling HSPs is higher than the probability to sample full siblings or recaptured individuals in a random mating scenario (Bravington et al., 2016; Bruce et al., 2018). Although close-kin can quantify fine-scale contemporary reproductive movement between populations, this method has several limitations. Indeed, it requires i) a large sample size with sex and age data, ii) sufficient biological information (age-at-maturity, and growth data), and iii) high resolution genomic data (Bravington et al., 2016; Conn et al., 2020). Future improvements include the analysis of more distant kin, such as cousin or thiatic (aunts and uncles) pairs, to increase the sample size of kin and subsequently statistical power. Although such complex scenarios can easily become too ambiguous to be informative.

Overall, the choice of analytical approach relies on the aims of the study, but in general, a multi-method approach, using both direct and indirect methods, could provide a comprehensive representation of dispersal and SBD. For example, direct non-genetic methods (such as CMR and telemetric approached) can be combined with a close kin analysis to identify the percentage of successful reproduction after dispersal, which in turn can provide new information on the fitness benefits of dispersal (e.g. Aguillon et al., 2017).

5.2. Significance of findings

5.2.1 METHODOLOGICAL SIGNIFICANCE

This thesis used a range of methodological tools that are rarely applied in elasmobranch genetic research. A first important finding of my thesis is the high resolution and statistical power of genomics (i.e. full mitogenome and nuclear SNP data). While past population studies employed single mtDNA region (e.g. Control Region), studies such as Martin et al. (1992) and Feutry et al. (2014) have shown that the mtDNA may be evolving more slowly in elasmobranchs and short mtDNA fragments exhibit few mutations, which subsequently lowers the power to accurately detect population structure. For

example, Feutry et al. (2014) found that the Speartooth Shark contained 19 mutations across the whole mitogenome, where the traditionally-preferred Control Region only showed two mutations. Similarly, my Chapters 2 and 6 showed that fine-scale population structure in the Bull Shark would have been overlooked if only a single mtDNA gene was used, since many sampling sites in the IWP had only one or two segregating nucleotide mutations. Less powerful markers, in turn, may lead to the false conclusion of high connectivity between populations, resulting in management actions at inappropriate spatial scales. Further, at the start of my thesis, nuclear genome-wide data, such as SNPs, had rarely been applied in elasmobranch research (e.g. Feutry et al., 2017; Maisano Delser et al., 2016; Momigliano et al., 2017; Portnoy et al., 2015). Currently, more studies are becoming available (to date ~25 studies that I am aware of) that apply these methods for studying population structure, hybridization, and taxonomic studies of elasmobranchs. Nuclear genome-wide markers allowed me to confidently detect population structure in Chapters 2 and 6. However, the combination of methods also allowed greater confidence when no structure was identified (e.g. School Shark, Chapter 1). In the era of genomics, the study of large genomic data improves our understanding of dispersal, however, large datasets can prove a particular bioinformatic challenge. For this reason and to endorse reproducibility, I included an exhaustive R markdown for each chapter (Appendix B-G) that describes the code and results for each type of analysis I performed. Another important advancement of genomics is the identification of close-kin. The spatial distribution of parent-offspring pairs and cross-cohort half siblings allowed me to detect population structure at very small spatial scales, but also to quantify reproductive SBD within one generation (Feutry et al., 2017).

The application of genetic markers for trade and fisheries monitoring (i.e. species, provenance, and sex identification) is a significant outcome of my thesis. While species identification is widely applied using mtDNA, the recent separation of two sister species (e.g. *Carcharhinus obscurus* and *Carcharhinus galapagensis*) occasionally prevents accurate mtDNA species identification (Corrigan et al., 2017; Naylor et al., 2012). In such cases, SNPs can be a useful tool (Kyne & Feutry, 2017; Liu et al., 2017). In my thesis, this was demonstrated for the Bull Shark, where seven different species were identified in the dataset, supposedly composed of Bull Shark tissue samples only. Similarly, I demonstrated that Bull Shark provenance can be detected with the DArTcap SNPs. Traditionally, mtDNA has been used for such purposes (Cardeñosa et al., 2021; Chapman et al., 2009; Fields et al., 2020). One advantage of the mtDNA is that it reflects female philopatric behaviour and allows provenance to be found at very fine spatial scales. On the other hand, the drawbacks include that mtDNA requires population structure to be long established (e.g. >1,000 generations) and is more susceptible to homoplasy (Hassanin et al., 1998; Levin et al., 2013). Nuclear DNA contains more unlinked SNPs, potentially allowing us to overcome these drawbacks (Cardeñosa & Chapman, 2018). Lastly, I showed in my thesis

that elasmobranchs predominantly have an XY sex-chromosome system — which may have evolved from the same autosomal chromosome pair. The presence of male-specific Y-chromosome fragments in White Sharks and available reference genomes allowed me to develop a PCR-based test to identify the sex from tissue samples (Chapter 3). Results from Chapter 4 suggest that such male-specific markers could be designed to cover all species from the Carcharhinidae (whaler shark) family. Jointly, these nuclear SNP markers can be combined in a diagnostic panel that allows fast and cost-effective assessment of species, provenance, and sex to enforce wildlife trafficking regulations and monitor fisheries catches (Cardeñosa & Chapman, 2018).

5.2.2 IMPLICATIONS FOR MANAGEMENT

Although this thesis is not focused on management policies, the results provide valuable information which could be used to determine future strategies. Dispersal of large vagile elasmobranchs forms energetic links between ecosystems, improves the resilience of those systems, and regulates community biodiversity (Bauer & Hoye, 2014). The functional role of elasmobranchs, as well as their inherit vulnerability to threats, highlights why management actions are particularly pressing for this group. With recent genetic advances, genetic structure and demographic inference have gained more traction in informing management strategies. For instance, indirect methods can provide evidence of the evolutionary distinctiveness and genetic variability of a population (i.e. evolutionary significant unit, or ESU; Moritz, 1994). These ESUs can be important to evaluate the risk of localised extinction and will answer questions related to how a population will be able to cope with changes to its environment. Nevertheless, it still needs to be stressed that management actions should also reflect contemporary demographic events. In this regard, my thesis demonstrated some discrepancies between population structure measured with genetic markers and that measured with kinship distribution (i.e. evolutionary vs demographic timescales). For example, the Bull Shark showed population structure ranging from small (river-level, CKMR) to medium (country-level, mtDNA) to large (ocean-basin, nuDNA) spatial scales. This most likely reflects the demographic history (e.g. colonisation or changes in population size) of the species. Arguably, managers and stakeholders are more interested in identifying contemporary fine-scale population structure; but the large sample size required may not always be feasible for species with large populations or species without commercial interest. In those cases, indirect genetic methods based on mtDNA or auDNA genomic markers, combined with telemetry, parasite fauna composition, or microchemistry studies may prove a useful substitution (Begg & Waldman, 1999).

Results from my thesis support the existence of female philopatry and MBD in the Northern River Shark and the Bull Shark. This conclusion may warrant the need for sex-specific assessment of threats, since the philopatric sex is likely impacted by local threats for extended periods while the dispersing

sex has increased exposure and spatial overlap to a wider variety of threats across their dispersal range (Chapman et al., 2015; Speed, Field, Meekan, & Bradshaw, 2010). Currently, SBD is rarely included in any management or conservation planning, due to the complexity of the information and the high cost of analysis. The philopatric behaviour of females to specific nursery sites represents the isolation of half the reproducing individuals. Consequently, local depletion of females in these areas or loss of habitat would be catastrophic for recruitment and re-establishment by straying females could take a long time (Hueter et al., 2005). Further, males often move more frequently and over longer distances across different jurisdictional boundaries, which makes them more exposed to certain threats, such as fisheries, and will be more complex to manage efficiently (e.g. Queiroz et al., 2016). Given the importance of SBD to gene flow and possibly to the persistence of small local populations (Clobert et al., 2012), protection of the dispersing sex may be necessary. For example, catches of sharks are rarely reported with sex information. Results from Section 2 in this thesis could allow ad-hoc sex identification of catches to aid sex-specific stock assessment. In summary, threats need to be assessed in respect to their sex-specific impact and consequently could require sex-specific management actions.

5.3. Future directions

My thesis identified the presence of MBD in two out of my three study species and discussed the available evidence of MBD in 26 of the ~1260 chondrichthyan species, albeit with data largely skewed towards Carcharhiniformes (ground sharks, n=16). Based on results from a suite of genomic tools, I considered potential ecological and evolutionary drivers, such as sex-chromosome systems, of male-bias in dispersal. However, most empirical evidence on these drivers is also skewed towards Carcharhiniformes (e.g. 15 out the 46 species with known sex chromosomes). Future research should focus on collecting information from a wider taxonomic coverage. For instance, the Holocephali (chimeras) are poorly represented in chondrichthyan research (e.g. Shiffman et al., 2020), but this is especially noticeable in population genetic and sex chromosome studies. Moreover, several technological advances, such as sex-chromosome and DNA-methylation sequencing, will allow us to discover the underlying molecular mechanisms that regulate dispersal in the Chondrichthyes (Brom et al., 2018; Greminger et al., 2010; Saastamoinen et al., 2018). As mentioned, the sequencing of Y-chromosome DNA would improve the discovery of historical MBD in sharks. Here, the detection of Y-linked markers as anchoring points for genome-walking techniques could prove a promising direction. Lastly, chondrichthyan species are particularly threatened with extinction and increasingly more species demonstrate a sex-bias in their dispersal. Consequently, future management action plans should include sex-specific assessment of threats.

Appendices

All appendices are available at: https://cloudstor.aarnet.edu.au/plus/s/mBEZ8rG5M1cUXxT

Appendix A – Paper 1

1. A1: Manuscript from Phillips et al. (2021)
    a. https://link.springer.com/article/10.1007/s11160-021-09673-9
2. A2: Supplementary material: Literature review

Appendix B – Paper 2

1. B1: Published paper from Feutry et al. (2020)
    a. https://onlinelibrary.wiley.com/doi/10.1111/1755-0998.13204
2. B2: Supplementary material S1 from Feutry et al. (2020):
    a. Rmarkdown
3. B3: Supplementary material S2 from Feutry et al. (2020)
    a. S2.1: Quality control prior to population analyses
    b. S2.2: Nucleotide diversity
    c. S2.3: Model posterior probabilities
    d. S2.4: Cross-validation test to check for the validity of our model selection procedure
    e. S2.5: Distinction between full sibling pairs and half-sibling pairs
    f. S2.6: Origin of the range expansion
    g. S2.7: Estimate of the variation of the Ne through time with singletons.
    h. S2.8: Estimate of the variation of the Ne through time without singletons.
    i. S2.9: Posterior distributions of the demographic parameters
    j. S2.10: Posterior predictive distribution of the global F_{ST}.

Appendix C – Chapter 1

1. C1: Published paper from Devloo-Delva et al. (2019)
    a. https://onlinelibrary.wiley.com/doi/full/10.1002/ece3.5012
2. C2: Supplementary material S1 Devloo-Delva et al. (2019)
    a. Rmarkdown
3. C3: Supplementary material S2-5 Devloo-Delva et al. (2019)
    a. S1. Full analysis in Rmarkdown
    b. S2. COLONY2 settings for School Sharks analyzed in the present study and in Bester-van der Merwe et al. (2017)
    c. S3. STRUCTURE results for the dataset with full siblings included
    d. S4. STRUCTURE results for the dataset with full siblings excluded
    e. S5. Kinship results of COLONY2 for nine Tasmanian and 20 New Zealand School Sharks from Bester-van der Merwe et al. (2017)
    f. Raw and filtered SNPs with associated metadata. Data for this study are available at: https://doi.org/10.5061/dryad.pd8612j

Appendix D – Chapter 2 & Chapter 6

1. D1: Supplementary material from Devloo-Delva et al. (in prep)
    a. Rmarkdown
    b. Chapter 6: See section 8.10

Appendix E – Chapter 3

1. E1: Supplementary material from Devloo-Delva et al. (in prep)
    a. Rmarkdown

Appendix F – Chapter 4

1. F1: Supplementary material from Devloo-Delva et al. (in prep)
    a. Rmarkdown

Appendix G – Chapter 5

1. G1: Supplementary material from Devloo-Delva et al. (in prep)
    a. Rmarkdown

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