INSTITUTO POLITECNICO NACIONAL
CENTRO INTERDISCIPLINARIO DE CIENCIAS MARINAS

Indirect evidence on the spatio-temporal use of nurseries by *Carcharhinus limbatus* adult females in the Galapagos Marine Reserve

TESIS

QUE PARA OBTENER EL GRADO DE
MAESTRÍA EN CIENCIAS EN MANEJO DE RECURSOS MARINOS

PRESENTA

YASUNÍ TRINIDAD CHIRIBOGA PAREDES

LA PAZ, B.C.S., DICIEMBRE DE 2022

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REP 2017

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ACTA DE REGISTRO DE TEMA DE TESIS
Y DESIGNACIÓN DE DIRECTOR DE TESIS

La Paz, B.C.S., a 30 de Noviembre del 2022

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1.- Se designa al aspirante el tema de tesis titulado:

"INDIRECT EVIDENCE ON THE SPATIO-TEMPORAL USE OF NURSERIES BY *Carcharhinus limbatus* ADULT FEMALES IN THE GALAPAGOS MARINE RESERVE"

Objetivo general del trabajo de tesis:

To reconstruct the kinship in between neonates and juveniles by using single nucleotide polymorphisms (SNPs) and a maximum-likelihood based parental analysis to infer adult female

2.- Se designa como Directores de Tesis a los profesores:

Directora: DRA. DIANA ALEXANDRA PAZMIÑO JARAMILLO 2° Director: DR. ROGELIO GONZÁLEZ ARMAS

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"INDIRECT EVIDENCE ON THE SPATIO-TEMPORAL USE OF NURSERIES BY *Carcharhinus limbatus* ADULT FEMALES IN THE GALAPAGOS MARINE RESERVE"

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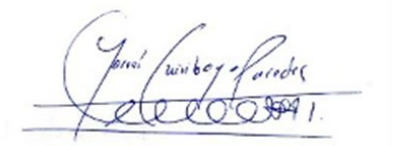

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List of Abbreviations

Abbreviation	*Definition*
AMOVA	Analysis of Molecular Variance
BIC	Bayesian Information Criterion
CB	Cerro Brujo
DAPC	Discriminant Analysis of Principal Components
DA	Discriminant Analysis
ETP	Eastern Tropical Pacific
FS	Full sibling
GMR	Galapagos Marine Reserve
GNPD	Galapagos National Park Directorate
HS	Half sibling
H_O	Observed Heterozygosity
H_E	Expected Heterozygosity
IUCN	International Union for Conservation of Nature and Natural Resources
LT	La Tortuga
LS	La Seca
MAATE	Ministerio del Ambiente, Agua y Transición Ecológica del Ecuador
MG	Manglecito
MAF	Minor Allele Frequency
PCA	Principal Component Analysis
PC	Principal Component
PG	Puerto Grande
RB1	Rosa Blanca 1
RB2	Rosa Blanca 2
SNP	Single Nucleotide Polymorphism
TL	Total length

Abstract

Philopatry refers to the behavior of an adult individual to return to the same place or region it was born to pup. This behavior can limit gene flow within the same population. Currently, studies on the shark's reproductive behavior in the Galapagos are scarce. This study uses single nucleotide polymorphisms (SNPs), and a maximum likelihood-based parentage analysis to reconstruct siblingship between *Carcharhinus limbatus* juvenile sharks to infer philopatry from adult females in San Cristóbal Island. Between 2016 and 2019, 185 individuals were captured in seven potential nursery areas: two to the east and five to the west of the island. For the analyses, 180 individuals and 4,300 SNPs were used as highly informative (call-rate > 95%, MAF > 10%). Sibship was found in 18.9% of individuals (10 siblings and 23 half-siblings). Most individuals were related in a biennial cycle (2016-2018 and 2017-2019), suggesting a female reproductive behavior every two years. Additionally, cross-bay full siblings were found between nearby bays (<10 km), evidencing genetic connectivity between close bays. This suggests that adult females have a tendency of straying between nearby bays when giving birth, potentially using larger areas. In addition, significant genetic differences ($p<0.05$) were found between eastern and western bays, suggesting a certain family structure because of a potential female philopatric behavior. However, a half-sib pair detected between an eastern and a western bay indicates the existence of genetic connectivity. This suggests that, while females are philopatric to a specific side of the island, males are potential vectors of gene dispersal around San Cristóbal Island. The results of this study reveal patterns of reproductive behavior for this species, and it is suggested that they can be taken into consideration when delineating strategies for conservation and management of nursery areas in the Galapagos Marine Reserve.

Key words: SNPs; nursery grounds; kinship analysis

Resumen

La filopatría se refiere al comportamiento de un individuo adulto de regresar al mismo lugar o región en que nació para tener a sus crías. Este comportamiento puede limitar el flujo de genes dentro de la misma población. Actualmente, los estudios sobre comportamientos reproductivos de tiburones en Galápagos son escasos. Este estudio utiliza polimorfismos de un solo nucleótido (SNPs) y un análisis de parentesco basado en máxima verosimilitud para reconstruir relaciones de hermandad entre tiburones juveniles punta negra, *Carcharhinus limbatus,* e inferir la filopatría de hembras adultas en la isla San Cristóbal. Entre 2016 y 2019, se capturaron 185 juveniles en siete áreas potenciales de crianza: dos al este y cinco al oeste de la isla. Para los análisis, se utilizaron 180 individuos y 4.300 SNPs por ser altamente informativos (call rate > 95%, MAF > 10%). Se encontraron relaciones de parentesco en el 18.9% de los individuos (10 hermanos y 23 medios hermanos). La mayoría de los individuos se relacionaron en un patrón bienal (2016-2018 y 2017-2019), lo que sugiere un comportamiento reproductivo de las hembras cada dos años. Además, se encontraron hermanos entre bahías próximas (< 10 km), lo que evidencia conectividad genética entre bahías cercanas. Esto sugiere que las hembras adultas presentan una cierta tendencia a extraviarse entre bahías cercanas al dar a luz, potencialmente utilizando áreas más extensas. Adicionalmente, se encontraron diferencias genéticas significativas ($p < 0.05$) entre las bahías del este y oeste de la isla, sugieriendo la existencia de una cierta estructura familiar como consecuencia de un potencial comportamiento filopátrico de las hembras. Aún así, un par de medios hermanos detectados entre una bahía del este y otra del oeste, indican la existencia de conectividad genética. Esto sugiere que, mientras las hembras son filopátricas a un lado específico de la isla, los machos son potenciales vectores de dispersión de genes alrededor de la isla San Cristóbal. Los resultados de este estudio revelan patrones de comportamiento reproductivo para esta especie y se sugiere puedan ser tomados en consideración al delinear estretegias de conservación y manejo de zonas de crianza en la Reserva Marina de Galápagos.

Palabras clave: SNPs; zonas de crianza; análisis de parentesco

Introduction

In a narrow sense, philopatry is defined as the preference of an individual to return to its natal region or site to reproduce (Feldheim *et al.* 2013). Such behavior can be common for both females and males that return to a site because mating and breeding take place in the same natal region of an individual, in which case we refer to it as regional philopatry (Chapman *et al.* 2015). When this behavior is common for both sexes, it contributes to the development of a closed population (Secor, 2002). On the other hand, natal philopatry occurs when an individual returns to its exact birth site (e.g. estuary, lagoon, island) to reproduce (Chapman *et al*. 2015). Since males do not necessarily return to the same natal site to mate, natal philopatry has been limited to describe an adult female behavior (Feldheim *et al*. 2002; Mourier & Planes, 2013; Marie *et al*. 2019) .

Geographical specificity is an essential property of philopatry. This quantifies how close an individual returns to its birth site or region, determining the scale in which a population may become closed (Secor, 2002). According to this, philopatric behavior could lead to population isolation at different geographical scales (Feldheim *et al*. 2013). Geographical specificity is crucial when assessing fishing stock units and creating conservation strategies because fishing pressures and habitat degradation could dramatically impact closed populations (Hueter *et al*. 2005).

Direct evidence for natal philopatry is not commonly described in the literature because efforts require the frequent monitoring of juvenile females from their birthplace to where they breed as adults in order to document the transgenerational use of a specific site for pupping (Chapman *et al*. 2015). Tagging approaches such as ultrasonic tagging and traditional capture-mark-recapture methods are useful for understanding short to mid-term behavioral ecology of individual movements. Furthermore, these methods are ineffective when understanding processes in a generational scale (Planes & Lemer, 2011; Klein *et al*. 2019). However, genetic markers can follow an individual's genotype across generations providing long-term data (Dudgeon *et al*. 2012). New approaches using individual-based genetic identification in the context of kinship analysis (Planes

& Lemer, 2011) provide additional information on the spatial distribution of close relatives, contributing to a novel understanding on the fine-scale movements of juveniles and adults (Feutry *et al*. 2017) widening the understanding of reproductive behaviors, such as philopatry.

Traditionally, studies assessing kinship often use microsatellite markers as a genetic tool (Feldheim *et al.* 2004, 2013; Hueter *et al.* 2005; Mourier & Planes, 2013; Bester-van der Merwe *et al.* 2019; Klein *et al.* 2019; Elizondo-Sancho *et al.* 2022). However, improvements in molecular techniques, as well as the decrease in DNA sequencing costs, opened the possibility of using single nucleotide polymorphisms (SNPs) (Attard *et al.* 2017). SNPs offer great power when inferring kinship because of the increased amount of molecular markers found across the entire genome of an individual (Devloo-Delva *et al.* 2019). SNPs can reveal kinship beyond parent-offspring (*e.g.* full and half-siblings) without the need of adult sampling (Feutry *et al.* 2017). To demonstrate this, Feutry *et al.* (2017) sampled only juveniles of the critically endangered speartooth shark, *Glyphis glyphis*, in a single time period at different river systems in Queensland, Australia. Kinship analysis from SNP data revealed the spatial distribution of kin dyads across and within rivers. Distribution of cross-cohort halfsiblings across the same river system and between rivers, demonstrated parental movement (most likely of males) to reproduce. Additionally, Marie *et al.* (2019) reconstructed the kin relationships in between neonate and juvenile scalloped hammerhead sharks, *Sphyrna lewini,* by using SNPs in order to determine the population mating system at Rewa Delta, Fiji. Results revealed additional insights in the reproductive behavior of this species, showing several cases of multiple paternity, and a series of matings from the same male with different females.

The blacktip shark, *Carcharhinus limbatus,* is a cosmopolitan coastal species that occurs in tropical and subtropical waters (Castro, 1996) and it is listed as globally vulnerable on the IUCN Red List of Threatened Species (IUCN, 2022). Its reproductive strategy is described as placental viviparity, with a biennial reproductive cycle, a gestation period that lasts 12 months, and litter size in between 4-11 pups (Castro, 1996). Despite its capacity for long–range movements (up to 2148km in the Atlantic US

Coast) (Kohler *et al.* 1998), this species presents natal philopatry to protected coastal nursery grounds for pupping (Castro,1996; Heupel & Hueter, 2002; Keeney *et al.* 2003, 2005; Bester-van der Merwe *et al.* 2019).

In the Galapagos Marine Reserve (GMR) research on this species has focused mainly on the movement patterns of adults. Studies using satellite and acoustic telemetry, demonstrated that blacktips are resident to the archipelago, displaying movements within the marine reserve boundaries: between the far north (Darwin and Wolf Islands) to the far south (San Cristóbal and Santa Cruz Islands) (Hearn *et al.* 2014; Peñaherrera-Palma, 2016). However, little is known about the reproductive behavior of this species in the GMR, mainly due to the inherent difficulty of sampling gravid females, and because fisheries catch-data is not available. To overcome these difficulties, studies have focused efforts on the juvenile stage.

It is well known that neonates and juveniles spend their first years of life at protected nursery bays in order to increase survival chances (Capapé *et al.* 2004; Heithaus, 2007). According to Heupel *et al.* (2007) an specific area is defined as a nursery when it fulfills three criteria: (i) juveniles are more abundant in that area than in others, (ii) juveniles remain or return to the same area for extended periods, (iii) the area is used across years. In Santa Cruz and San Cristóbal Islands, research revealed that adult females use coastal mangrove-fringed bays as potential nurseries (Llerena-Martillo *et al.* 2009, 2010, 2013; Jaenig, 2010; Hirschfeld, 2013; Chiriboga-Paredes, 2018; Goodman, 2020). However, in an attempt to define which bays satisfy Heupel *et al.* (2007) criteria as nurseries for blacktips in San Cristóbal, Goodman (2020) assessed the relative abundance of juvenile blacktip sharks comparing drone surveys and traditional gillnet methods. From 14 sampled sites around the island, four of them showed greater abundance and density of juveniles, fulfilling the first criteria (Heupel *et al.* 2007). Additionally, based on the monthly abundance peaks, this study suggests that the breading season for blacktips starts in late February. However, additional studies are needed to understand the reproductive behavior of adult females in nursery grounds to completely address Heupel *et al.* (2007) criteria and define specific nurseries for this species around the island.

Despite industrial fishing being banned in the GMR, and sharks being protected by law since 1989 (prohibiting the fishing, transport, and sale of sharks and all their products, MICIP, 1989), by-catch remains an important issue at potential nurseries around the archipelago (Hirschfeld, 2013; Hearn *et al.* 2014). According to a fish-assemblage study carried out using gillnets in Santa Cruz Island, species of greater commercial value (*e.g.* mullets, *Mugil galapangesis*), represent 10-38% of fishing capture's contribution, while juvenile blacktip unintentional capture vary between 15-45% (Llerena-Martillo *et al.* 2018). Furthermore, mangroves habitats, used as key areas during early life stages of this species, are highly used by the tourism industry in the Galapagos. Tanner *et al.* (2019) suggests a monthly mangrove-visit range of 400 – 14,000 tourists, and an annual income of approximately $62.5 million dollars for mangrove related-tourism activities. All these activities and their associated impacts threaten mangrove bays. Impacts include plastic pollution, sea floor disturbances (because of the recurrent anchoring of boats) and, in some cases, constant hull cleaning and repair inside mangrove bays, which might lead to potential water contamination by heavy metals and hydrocarbons.

Given the current threats to mangrove habitats, and the lack of studies related to the reproductive behavior of blacktips in the GMR, this study aims to reconstruct the kinship in between neonates and juveniles by using single nucleotide polymorphisms (SNPs) and a maximum-likelihood based parental analysis to 1) infer adult female philopatry 2) assess if adult females differentiate between bays or use the Island as a whole nursery unit. The results of this study will contribute to efforts in the attempt to define nursery grounds for this species around the Island.

Methods

Study area

The Galapagos Marine Reserve is located in the Eastern Tropical Pacific (ETP) approximately 1000 km east from continental Ecuador and encompasses a total area of 138,000 km^2 (Danulat & Edgar, 2002). Recently, the creation of Hermandad Marine

Reserve expanded the protected area by 60,000 km^2 protecting some migratory routes for a variety of marine species (Hearn et al, 2022).

The GMR is under the influence of three major oceanic currents. The Panama current brings waters with sea surface temperature between 25–30 ^oC (Banks, 2002) determining the warm-rainy season from December to April. The Humboldt current lowers sea surface temperature between 14-23 ^oC (Banks, 2002), shaping the cold-dry season, from June to October. May and November correspond to transition months in between warm and cold seasons. And the submerged Cromwell current that creates upwellings from cold and rich nutrient water from depth, causing high primary productivity areas along the western (Fernandina and Isabela Island) and the central area of the archipelago (Schaeffer *et al.* 2008).

The Galapagos coastline is characterized by exposed rocky shores, although mangrove habitats are associated with many islands. The existence of a west-east mangrove coverage pattern describes more mangrove coverage in younger islands compared to older ones (Moity *et al.* 2019). San Cristóbal is the oldest and easternmost island, with an estimated mangrove cover area between 10.5 and 118.10 hectares (Rivas-Torres *et al.* 2018; Moity *et al.* 2019). The western coast of San Cristóbal represents most of the mangrove present at the island (Moity *et al.* 2019) while the east coast is more rocky-exposed with less mangrove patches.

Diverse studies focused on assessing abundance and small-scale movements of juvenile blacktips mainly at San Cristóbal (Llerena-Martillo, 2009; Hirschfeld, 2013; Chiriboga-Paredes, 2018; Goodman, 2020). These studies suggested that potential nurseries are present, mainly at the west side of the island. Goodman (2020) assessed juvenile abundance at all reported west sites and added two less-explored bays at the east. Based on the consistent relative abundance and density obtained, the study concluded that three sites at the west: Puerto Grande, La Seca, and Manglecito, and one at the east: Rosa Blanca 1, are key areas as nursery grounds for blacktips. While three additional sites: Cerro Brujo, La Tortuga, and Rosa Blanca 2 need further research in order to address their importance as developing habitats for the species.

In this study, we sampled the seven fringed-mangrove bays reported by Goodman (2020). Five of them are at the west side of the island, which is characterized by calm waters: Manglecito (-0.830733333, -89.53925), La Seca (-0.8218167, -89.521867), Puerto Grande (-0.8047167, -89.47367), Cerro Brujo (-0.7726, -89.4613), and La Tortuga (-0.717383, -89.3884). Whereas two are situated at the east side of the island, characterized by exposed rocky shores and rough waters: Rosa Blanca 1 (- 0.8279667, -89.356283) and Rosa Blanca 2 (-0.8186917, -89.34694) (Figure 1). All these sampling sites are classified under the category of Extractive Use by the Zoning Scheme of the Galapagos Marine Reserve, allowing artisanal fishing inside the bays (Llerena *et al.* 2010).

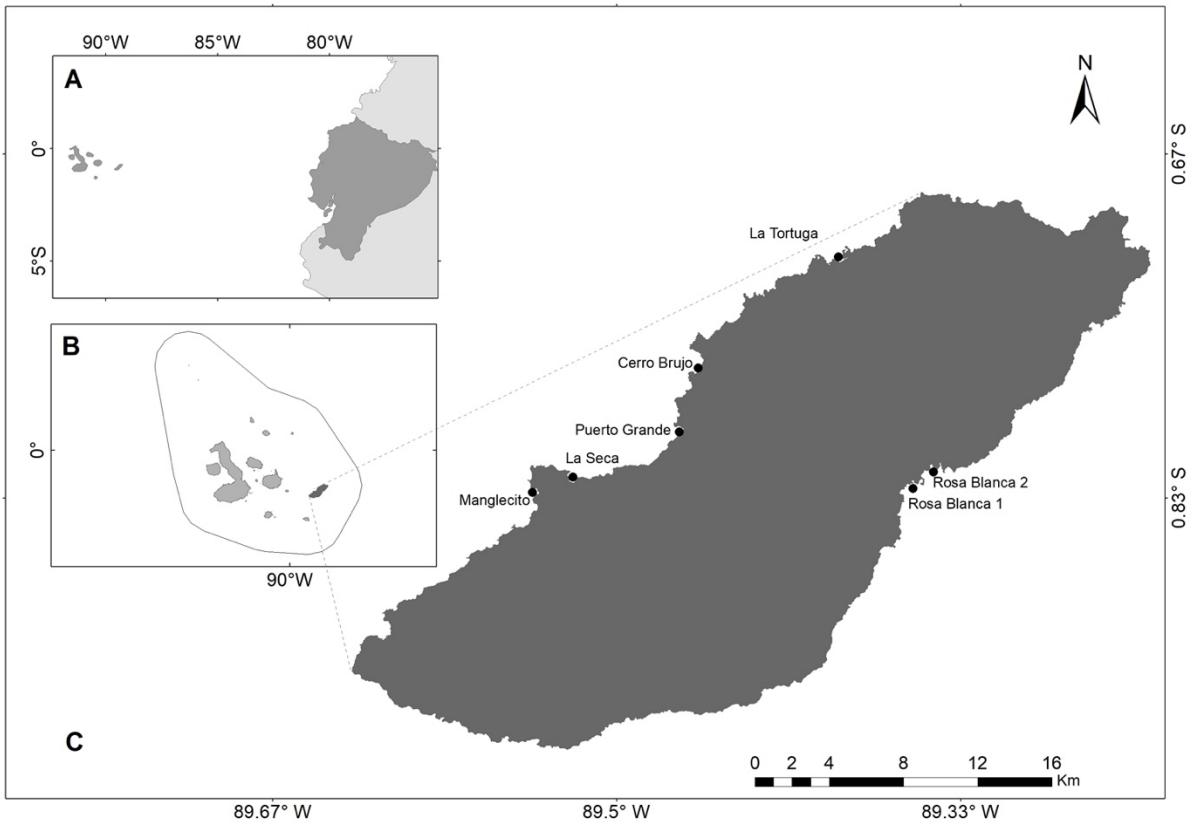

Figure 1. Study site. A) Continental Ecuador and the Galapagos Islands B) The Galapagos Marine Reserve is shown in a black solid polygon, and San Cristóbal Island is accentuated in grays. C) Inset of San Cristóbal Island. Sampled bays at west and east are represented by black dots.

Field methods

All procedures for shark capturing, handling, and tissue sampling were approved by the Galapagos National Park Directorate (GNPD) and follow the protocol for the correct manipulation of juvenile sharks in the GMR (Llerena-Martillo, 2012). All field procedures are approved by the GNPD under the annual research permit numbers: PC-69-16, PC-34–17, PC-24–18, PC-13–19.

Capture and sampling

Juvenile blacktip sharks were caught between 2016 and 2019 during monthly monitoring surveys in the cool season (June-October), warm season (December-April), and transition months (May and November). Individuals were captured with a monofilament gill net approximately 100 m long, 3 m depth, and 3.5 inches mesh size. Each fishing set had a duration of approximately one hour at each of the seven bays for all sampling years.

During the one-hour net-soak, the gillnet was manually checked every 30 minutes to ensure that no sharks or other accompanying fishes were entangled. Upon juvenile shark capture, a small hose that pumped sea water was used to refresh the individuals to reduce stress levels during handling. Individuals were sexed and measured for total length (TL). A small tissue sample was taken from the first dorsal fin (approx. 1 cm^2) and placed in a plastic vial with 96% ethanol. The capture-release process took one and a half minutes in average, releasing all individuals in good conditions.

Laboratory methods

All laboratory procedures were carried out under the research permit number MAE-DNB-CM-2016-0041 approved by the Ministry of Environment and Water of Ecuador (MAATE).

DNA extraction

A subsample of 185 juveniles out of 693 captures was used for DNA extraction and genotyping. Methodology for subsampling prioritized choosing uniformly samples from each year rather than from each site. Priority was given to a temporal sampling in order to look for the biennial reproductive pattern of adult females and to be able to infer philopatry based on year after year data. However, this decision led to an uneven sampling per site (Table 1).

Total genomic DNA was extracted in the Galapagos Science Center Microbiology Lab by using the DNeasy Blood and Tissue Kits (QIAGEN) which provides a silica-based DNA extraction. Following their standardized protocols (www.corporate.quiagen.com), the tissue samples were lysed with proteinase K, then buffers (AL, AW1, AW2, AE) were used to provide optimal DNA-binding conditions to the silica-membrane. After precipitation and washing steps, samples were incubated for 1 min at 20-25°C and centrifuged at 8000 rev/min for 1 min to obtain genomic DNA.

Table 1. Summary of temporal and spatial subsampling for DNA extraction and genotyping. Number of subsampled individuals are shown.

Site	Location	2016	2017	2018	2019	Total
Rosa Blanca 1	East				12	12
Rosa Blanca 2	East				17	17
La Seca	West	18	18	18	23	77
Puerto Grande	West	18	16	17	20	71
La Tortuga	West				1	1
Manglecito	West				3	3
Cerro Brujo	West				4	4
Total		36	34	35	80	185

SNPs genotyping

Genomic DNA subsamples were exported to the Diversity Arrays Technology Facility Laboratory (https://www.diversityarrays.com/) in Canberra, Australia under exportation permits from the GNPD and MAATE.

All 185 subsamples were SNP genotyped using the DArTseq protocol, a new method of sequencing complexity reduction representations (Devloo-Delva *et al.* 2019). The DArTseq protocol used in this study is the same described in Grewe *et al.* (2015). In brief, DArTseq method aims to reduce the complexity of the genome using a combination of a double digest restriction enzyme (ddrRAD) that randomly selects DNA fragments cutting it into smaller pieces and then sequencing short (75 base-pairs) fragments containing one or more SNPs (Devloo-Delva, 2019 unpublished data). A raw database with a total of 30,000 SNPs was received from Diversity Arrays Technology Facility Laboratory.

Data Analysis

SNPs filtering process and outlier detection

Because of uneven subsampling across sites, we opted to split the raw database into two datasets: the first dataset included four sites with 176 individuals (sites with a more consistent sampling over the years: La Seca, Puerto Grande, Rosa Blanca 1, and Rosa Blanca 2), and a second dataset including seven sites with 185 individuals (including all sampled sites). From now on, the first dataset will be named as dataset A, and the second one as dataset B. This decision was made in order to work with a more consistent dataset (A), and to detect any interesting pattern when analyzing all sites together (B). Filtering process, outlier detection, and all analyses hereinafter were performed for datasets A and B.

Quality SNP filtering was performed in R software (R Core Team, 2016) using the dartR v1.9.9.1(Gruber *et al.* 2018) and Adegenet v2.1.4 packages (Jombart, 2008) following

the filtering protocol by Devloo-Delva (2019, unpublished data) in order to retain highly informative SNPs.

For dataset A and B, we filtered duplicated SNPs (*i.e.* multiple loci in the same sequence) to avoid linkage disequilibrium, and monomorphic SNPs which are uninformative in discriminating information (fixed over all individuals). Then, we filtered individuals and SNPs by call rate (below 98%) and heterozygosity (above 30%) because a low call rate might indicate bad DNA quality, and a high heterozygosity a contaminated DNA. SNPs with a genotyping reproducibility below 99%, and average read depth higher than 12 and lower than 42 sequences per locus were filtered out. Additionally, minor allele frequency (MAF) filtering was performed in both datasets. We varied MAF threshold from 0.02 to 0.50 to monitor the optimum threshold. Based on the results of SNPs filtering, we opted to conserve 0.02 and 0.10 MAF thresholds as informative enough. This way, SNPs from datasets A and B were sorted into two sub datasets based on chosen MAF thresholds for assessing kinship.

We also looked for candidate loci under selection (outliers) using OutFLANK v0.2 (Whitlock & Lotterhos, 2015) and PCAdapt v.4.3.3 (Luu *et al.* 2016; Privé *et al.* 2020) R packages. OutFLANK method separates the effect of different sources of variation among loci from the variation created by spatially heterogenous selection on specific loci (Whitlock & Lotterhos, 2015). This way the method can detect loci responsible for local adaptation by inferring the F_{ST} (genetic differentiation) distribution for all loci. It is expected that loci under selection strongly affects the tails of the F_{ST} distribution, showing higher-than-average F_{ST} values (Whitlock & Lotterhos, 2015).

PCAdapt is a method that calculates the Principal Component Analysis (PCA) of a scaled genotype matrix and tests how much each variant is associated with population structure, assuming that outliers are indicative of local adaptation (Privé *et al.* 2020). Through a series of regressions, it gets a matrix of Z-scores for each variant and for each principal component (PC). By computing the Mahalanobis distances for each Z-

score, it integrates all PCA dimensions in an approximate chi-squared distribution, obtaining a p-value for each genetic variant (Privé *et al.* 2020).

For OutFLANK as well as for PCAdapt, we used the False Discovery Rate correction (FDR) in order to prevent an excess of false positives when finding loci under selection (Whitlock & Lotterhos, 2015). This correction uses a measure of statistical significance called q-value based on the false discovery rate instead of the p-value which is based on the false positive rate (Storey & Tibshirani, 2003). For both packages, we used a q-value of 0.01 to identify significant loci under selection. For PCAdapt we used the q-value as a less conservative method, and additionally the Bonferroni Correction as a conservative comparative method. Resultant significant loci under selection were compared in between OutFLANK and PCAdapt. Outliers were removed to retain only neutral loci for posterior kinship analysis.

Population Structure Exploration

We ran a Discriminant Analysis of Principal Components (DAPC) as an initial assessment to explore broad-scale population structure before proceeding with kinship analyses. To run the analysis we used the Adegenet v2.1.4 package (Jombart *et al.* 2010; Jombart & Collins, 2021). DAPC is a multivariate method designed to identify and describe clusters of genetically related individuals (Jombart *et al.* 2010). This method combines the PCA approach and the Discriminant Analysis (DA). While PCA summarizes the overall variability among individuals and ensures that variables are not correlated, DA partitions variability into between-group and within-group, maximizing genetic differentiation between groups. To proceed with DA, prior groups need to be defined. When clusters are not previously defined, the method uses a K-means clustering of PC to create different models and an associated likelihood, and by using the Bayesian Information Criterion (BIC) the number of clusters (K) can be defined (Jombart *et al.* 2010).

We used datasets A and B (and their corresponding MAF thresholds subsets) and determined the optimal K using the lowest BIC value.

Kinship analysis

Sibship (*i.e.* assignment of individuals to sibling groups) was performed in Sequoia v2.3.5 package (Huisman, 2017) and COLONY v.2.0.6.8 software (Jones & Wang, 2010) as a method for results comparison. Sequoia reconstructs multigenerational pedigree using SNPs data and a likelihood ratio approach. The package uses a hill-climbing algorithm to assign relationships to putative pairs of relatives (Huisman, 2017). It can assign parents, half-siblings that share an unsampled parent (at least 40% of parent genotype is required to reconstruct HS), and assign grandparents to half-siblings (Huisman, 2017). It can deal with complex pedigree relationships such as overlapping generations and close inbreeding. The package accounts for genotyping errors and missing data.

In contrast, COLONY is a free software that uses a maximum likelihood method to infer parentage and sibship from multi-locus genotype data (Jones & Wang, 2010). Colony uses a simulated annealing algorithm (Wang, 2004) to search for the best configuration (Wang & Santure, 2009) and implements a pairwise and a full-pedigree likelihood approach. The last one considers the entire pedigree configuration to make comparisons in between all the individuals, rather than a dyad comparison, to infer clusters of parent-offspring, full-siblings (FS), half-siblings (HS) or unrelated. Colony works with dominant and codominant markers (SNPs) which are assumed to be in Hardy-Weinberg and linkage equilibrium, accounting for genotyping error (allelic dropout and others, such as mutations) at each locus (Jones & Wang, 2010).

Neutral A and B datasets (and their corresponding MAF subsets) were analyzed for sibship with Sequoia and Colony. For Sequoia, we used a genotyping error rate of 0.01 for each MAF threshold (Attard *et al.* 2017). Because Sequoia requires the individuals' birth year as a variable for analyses, we compiled age and growth literature (Smart *et al.* 2015) to create age categories based each on individual TL and sampling year in order to estimate individuals' birth year (Table 2).

Table 2. Estimated age categories for juvenile blacktips to classify early stages of *C. limbatus* in the Galapagos based on Smart *et al.* 2015.

Total length (cm)	Category
≤ 62	Neonate
62.1 – 71.9	Between the first and third month of age
72 – 79.9	Between the fourth and eleventh months of age
≥ 80	Greater than one year of age

We estimated the birth year of the individuals by relating the total length with the provided age class category. Then by looking at the sampling year, we estimated when the individual was potentially born. For example, Individual ID 109, sampled on the 30th April 2017 in La Seca had a total length of 91 cm. According to the estimated age categories (Table 2), this individual is greater than one year of age. Consequently, the individual was not born in 2017 (sampling year), rather we estimated that it was born in the last months of 2015. This estimation was done for all measured individuals. In the case of those individuals that could not be measured, we provided Sequoia with the individual's sampling year.

For COLONY analysis, we run the program assuming polygamy for both parents, reproduction without inbreeding, dioecious species, and a full-likelihood analysis method with no sibship prior. We used an allelic dropout rate of 0.01 and a false allele rate of 0.01 because of high quality SNPs (Wang, 2004). As we did not count with parental genotype or known maternal/paternal siblings, we left those categories empty.

To interpret temporal sibling relationship results from Sequoia and COLONY, we used the birth year estimations for measured individuals, and sampling year for those that could not be measured at the field.

Population substructure

After kinship analysis we had a clearer panorama on how groups were clustered. Consequently, we explored a potential population substructure using Arlequin software v.3.1 (Excoffier *et al.* 2005). Arlequin integrates basic and advanced methods to analyze population genetics data. It computes intra and inter population analysis. The latter includes the assessment of population subdivision under the analysis of molecular variance (AMOVA) approach, with three levels: genes within individuals, individuals within demes, and demes within groups of demes (Excoffier *et al.* 2005). Arlequin also computes F-statistics values like the inbreeding coefficient (F_{IS}), the metapopulation fixation index (F_{IT}), the genetic differentiation index (F_{ST}), and others (Excoffier *et al.* 2005).

For the Arlequin substructure analysis, our null hypothesis proposed the existence of two groups around the island: west and east. Under this hypothesis, we grouped sites from dataset A as west group, including Puerto Grande (PG) and La Seca (LS), and east group: Rosa Blanca 1 (RB1), and Rosa Blanca 2 (RB2). For dataset B, the west group included Puerto Grande, La Seca, Manglecito (MG), Cerro Brujo (CB), and La Tortuga (LT). Whereas the east group corresponded to Rosa Blanca 1, and Rosa Blanca 2. We calculated the observed heterozygosity (H_o), expected heterozygosity (H_e), besides F_{IS}, F_{IT}, F_{CT}, and F_{ST}.

Results

Captured individuals

A total of 693 individuals were captured between 2016 and 2019 in all sampled sites. Here, we present results from the subsample of 185 individuals genotyped, from which 173 were measured and sexed. Sampled individuals registered a mean total length of 68.15cm (min = 52cm, max = 110cm) with a sex proportion of 1:1, M:F ($\chi^2 = 0.837$, $p > 0.05$) (Figure 2).

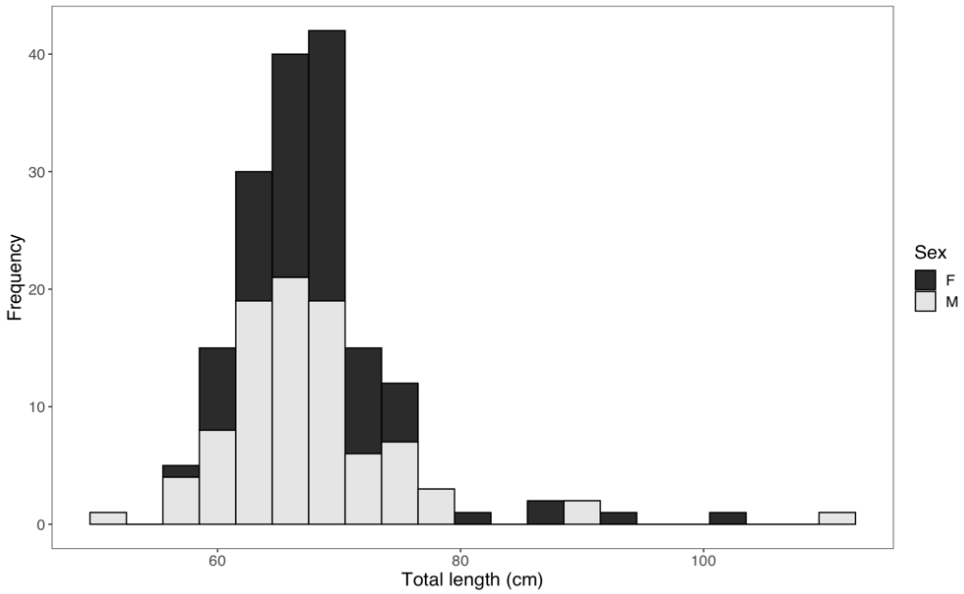

Figure 2. Histogram of total length frequency by sex of sampled juvenile blacktip sharks at seven sites around San Cristóbal Island.

Most individuals (n=116) belong to the 1-3 months age class (Table 3) according to estimated categories.

Table 3. Age categories for 185 subsampled neonates and juvenile sharks *C. limbatus* sampled during 2016 and 2019 around potential nurseries at San Cristóbal.

Number of individuals	Total length (cm)	Category
25	≤ 62	Neonates
116	62.1 – 71.9	Between the first and third month of age
24	72 – 79.9	Between the fourth and eleventh months of age
8	≥ 80	Greater than one year of age
12	NA	Not assessed

SNPs filtering process and outlier detection

A total of 185 individuals were genotyped for 36,159 SNPs by Diversity Arrays Technology Facilities. A total of five individuals were removed by quality filtering (one due to < 0.98 call rate, three due to > 0.3 heterozygosity, and one because of low heterozygosity). In dataset A, we reduced 176 individuals to 171, and from 185 to 180 individuals in dataset B. SNPs were also reduced in number depending on each applied MAF threshold (Table 4). Different studies conventionally use 0.02 MAF as an informative threshold (approx. 8,000 SNPs) (Feutry *et al.* 2017; Pazmiño *et al.* 2017). Then, we opted to conserve 0.02 (8,459 SNPs) as conventionally in studies, and 0.10 (4,915 SNPs) as a less conservative threshold to retain less but still informative SNPs for datasets A and B.

Table 4. Applied MAF thresholds and total retained SNPs for dataset A and B after quality filtering process.

MAF Threshold	Retained SNPs dataset A	Retained SNPs dataset B
0.02	8,459	6,512
0.05	6,739	5,512
0.10	4,915	4,374
0.15	3,958	3,579
0.20	3,230	2,952
0.30	1,974	1,803
0.40	921	855
0.50	14	10

Using a 0.02 MAF for dataset A, OutFLANK detected one outlier whereas PCAdapt detected 149 by q-value, and 27 by Bonferroni correction. Similarly, for dataset B,

OutFLANK identified two outliers and PCAdapt identified 150 (q-value) and 27 (Bonferroni correction). We checked for any overlap in between outliers from the two software tools for each dataset, and no coincidences were found. As OutFLANK is more stringent (Devloo-Delva *et al.* 2019), we chose to remove outliers based on OutFLANK only, to maintain neutral A and B datasets. On the other hand, no outlier SNPs were detected by OutFLANK neither PCAdapt for subset 0.10 MAF in datasets A and B.

Population Structure Exploration

The DAPC did not detect structured variance for MAF = 0.02 and 0.10 in datasets A and B. We retained 180 PCs, and the BIC suggested K = 1 as the best configuration (Figure 3). Consequently, the K-means DAPC suggested that one group is the best cluster configuration to explain the data. This was expected because we sampled related juveniles inside potential nurseries that were not too distant from each other (most nurseries < 10km apart) around a small island. All these variables contribute to a strong family signature, hiding the signal of a potential population structure.

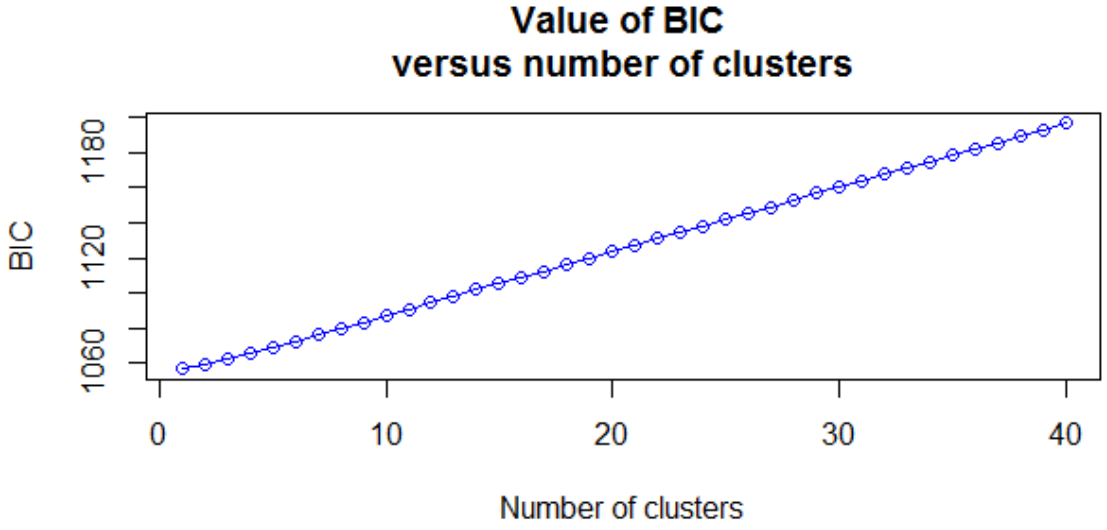

Figure 3. Bayesian Information Criterion (BIC) values for each number of clusters using the K-means based DAPC.

Kinship analysis

Sequoia and COLONY kinship analysis were performed with datasets A and B, and their corresponding subsets of 0.02 and 0.10 MAF thresholds. Neither Sequoia nor COLONY found FS or HS for Manglecito, Cerro Brujo, or La Tortuga (included in dataset B). We attribute this result to the small subsample size genotyped for these localities.

Full siblings

The number of FS pairs detected by Sequoia and COLONY did not vary when analyzing different datasets and their corresponding 0.02 and 0.10 MAF subsets. Sequoia identified a total of eight pairs of full siblings (4.7% from 171 individuals) distributed in eight different family groups (Table 5) for datasets A and B. Cross-bay FS were identified in between Puerto Grande and La Seca (west), and between Rosa Blanca 1 and Rosa Blanca 2 (east). No cross-bay FS were detected in between east and west. Given that Rosa Blanca 1 and Rosa Blanca 2 were subsampled just for 2019, all FS identified belong to the same 2019 cohort. On the other hand, for Puerto Grande and La Seca, where 2016-2019 years were sampled, we found FS related every two years (Figure 4).

COLONY identified 10 FS pairs (5.9% total sibship from 171 individuals) for datasets A and B. Two additional ones to those already detected by Sequoia (Table 6). COLONY was able to identify two new cross-bay FS pairs in between Puerto Grande and La Seca. The new full sibling pair ID 158 – 164, and ID 158 – 170 were related in a biennial cycle (2016 - 2018) (Figure 4). As we did not measure individual ID 158 we could not estimate its birth year, rather we used the sampling year to interpret temporal sibship with its sibling pairs.

Table 5. Full-siblings assignment results reconstructed by Sequoia for dataset A and B. For each family member the ID, sampling site, and total length are shown.

Full Sib Family #	Member 1 ID	Member 2 ID	Site member 1	Site member 2	TL (cm) member 1	TL (cm) member 2
1	CL164	CL170	Puerto Grande	Puerto Grande	62.5	70
2	CL061	CL058	Puerto Grande	La Seca	63	72
3	CL106	CL109	La Seca	La Seca	65	91
4	CL002	CL004	Rosa Blanca 1	Rosa Blanca 1	58.3	59.7
5	CL003	CL044	Rosa Blanca 1	Rosa Blanca 2	60.2	62
6	CL007	CL011	Rosa Blanca 1	Rosa Blanca 1	64	63.4
7	CL008	CL056	Rosa Blanca 1	Rosa Blanca 2	64.3	57.1
8	CL041	CL047	Rosa Blanca 2	Rosa Blanca 2	64	62.3

Table 6. Colony results for full siblings assessed in 2016-2019 around San Cristóbal Island. For each member the ID, sampling site, and total length are provided. (*) shows new FS pairs that were not recognized by Sequoia.

Full Sib Family #	Member 1 ID	Member 2 ID	Site member 1	Site member 2	TL (cm) member 1	TL (cm) member 2
1	CL164	CL170	Puerto Grande	Puerto Grande	62,5	70
	CL164*	CL158*	Puerto Grande	La Seca	62,5	NA
	CL170*	CL158*	Puerto Grande	La Seca	70	NA
2	CL061	CL058	Puerto Grande	La Seca	63	72
3	CL106	CL109	La Seca	La Seca	65	91
4	CL002	CL004	Rosa Blanca 1	Rosa Blanca 1	58,3	59,7
5	CL003	CL044	Rosa Blanca 1	Rosa Blanca 2	60,2	62
6	CL007	CL011	Rosa Blanca 1	Rosa Blanca 1	64	63,4
7	CL008	CL056	Rosa Blanca 1	Rosa Blanca 2	64,3	57,1
8	CL041	CL047	Rosa Blanca 1	Rosa Blanca 2	64	62,3

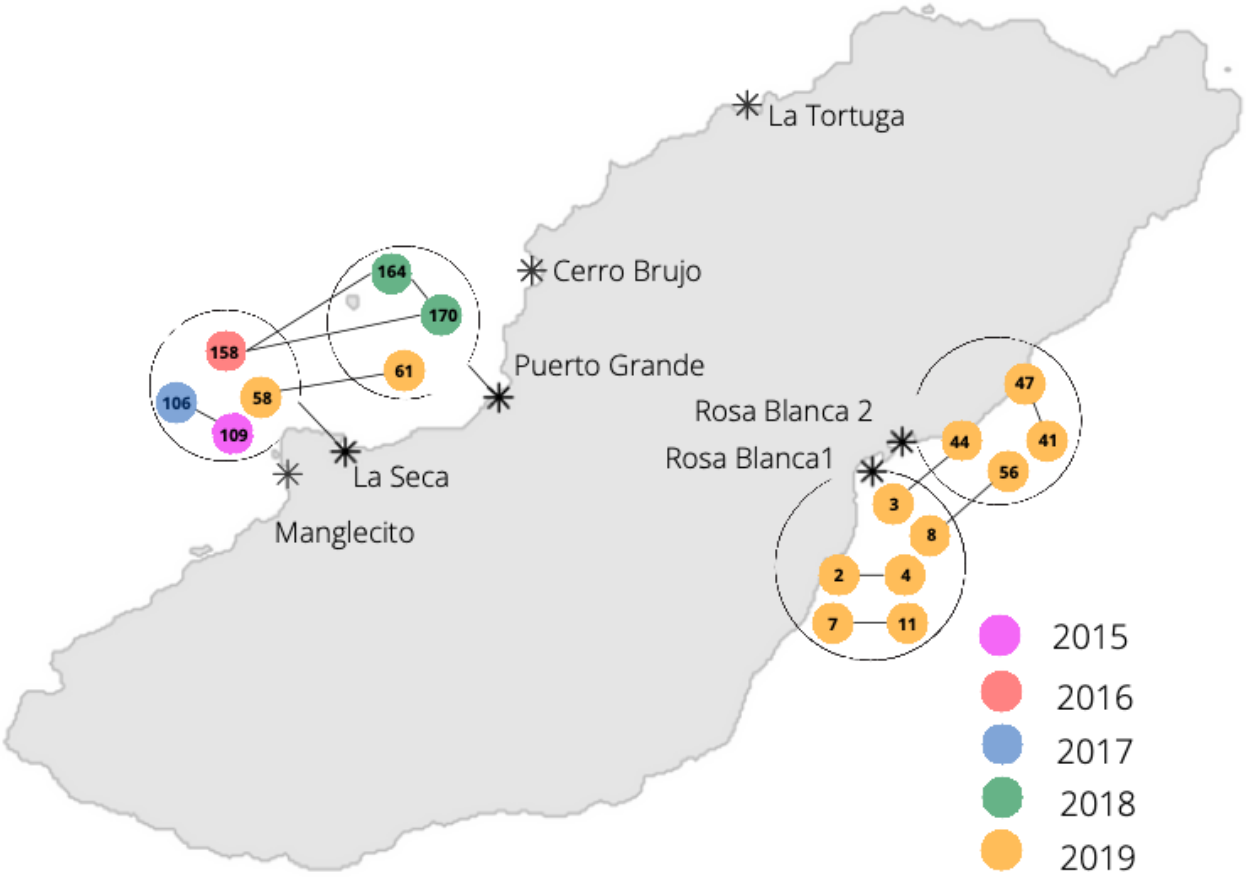

Figure 4. Full-sibship identified (solid black lines) by Sequoia and COLONY around San Cristóbal. Each individual ID is represented with a number, and its birth year with a different color (except for ID CL158 for which we used sampling year). Sampling *locations* appear as an asterisk on the map. Neither COLONY nor Sequoia identified FS for Manglecito, Cerro Brujo, or La Tortuga.

Half siblings

HS pairs detected by COLONY differed in between datasets A and B, and for each applied MAF threshold (Table 7). Then, we decided to use dataset B and 0.10 MAF threshold as the most informative to represent HS results. COLONY was able to identify 24 HS pairs spread out into 18 families (Table 8). Cross-bay and cross-year HS relationships were identified (Figure 5). Cross-bay HS pairs were registered in between Puerto Grande and La Seca, and in between Rosa Blanca 1 and Rosa Blanca 2.

Surprisingly, one half-sibling pair was identified in between Puerto Grande (west) and Rosa Blanca 1 (east). Individuals from both sites were estimated to be born in 2019, and because of their size (TL), they were catalogued as 1-3 months old.

Table 7. MAF threshold and HS pairs assigned by COLONY. Differences registered in the number of HS pairs assigned by COLONY for each dataset and MAF threshold.

Dataset	MAF Threshold	# HS pairs
A	0.02	21
	0.10	23
B	0.02	24
	0.10	24

Table 8. Half-sibling families identified by COLONY for 2016-2019 period. Here we present each member ID, sampling site, and total length.

Half Sib Family #	Member 1 ID	Member 2 ID	Site member 1	Site member 2	TL (cm) member 1	TL (cm) member 2
1	CL123	CL135	Puerto Grande	Puerto Grande	66	68
2	CL135	CL018	Puerto Grande	La Seca	68	67.4
	CL128	CL164	Puerto Grande	Puerto Grande	74.3	62.5
	CL128	CL170	Puerto Grande	Puerto Grande	74.3	70
	CL128	CL158	Puerto Grande	La Seca	74.3	NA
3	CL128	CL064	Puerto Grande	Puerto Grande	74.3	68.8
	CL164	CL064	Puerto Grande	Puerto Grande	62.5	68.8
	CL170	CL064	Puerto Grande	Puerto Grande	70	68.8
	CL064	CL158	Puerto Grande	La Seca	68.8	NA
4	CL131	CL019	Puerto Grande	La Seca	74.1	61
5	CL019	CL027	La Seca	La Seca	61	61.1
6	CL137	CL020	Puerto Grande	La Seca	68.5	70
7	CL140	CL142	Puerto Grande	Puerto Grande	63.5	88
8	CL143	CL078	Puerto Grande	Puerto Grande	64	65
9	CL185	CL071	Puerto Grande	Puerto Grande	66.1	72
10	CL185	CL154	Puerto Grande	La Seca	66.1	74.6
11	CL186	CL061	Puerto Grande	Puerto Grande	60	63
12	CL186	CL058	Puerto Grande	La Seca	60	72
13	CL175	CL161	Puerto Grande	La Seca	69	NA
14	CL072	CL013	Puerto Grande	Rosa Blanca 1	68	67.5
15	CL150	CL080	La Seca	La Seca	65	70
16	CL016	CL021	La Seca	La Seca	69.1	71.6
17	CL010	CL041	Rosa Blanca 2	Rosa Blanca 2	62.5	64
18	CL010	CL047	Rosa Blanca 2	Rosa Blanca 2	62.5	62.3

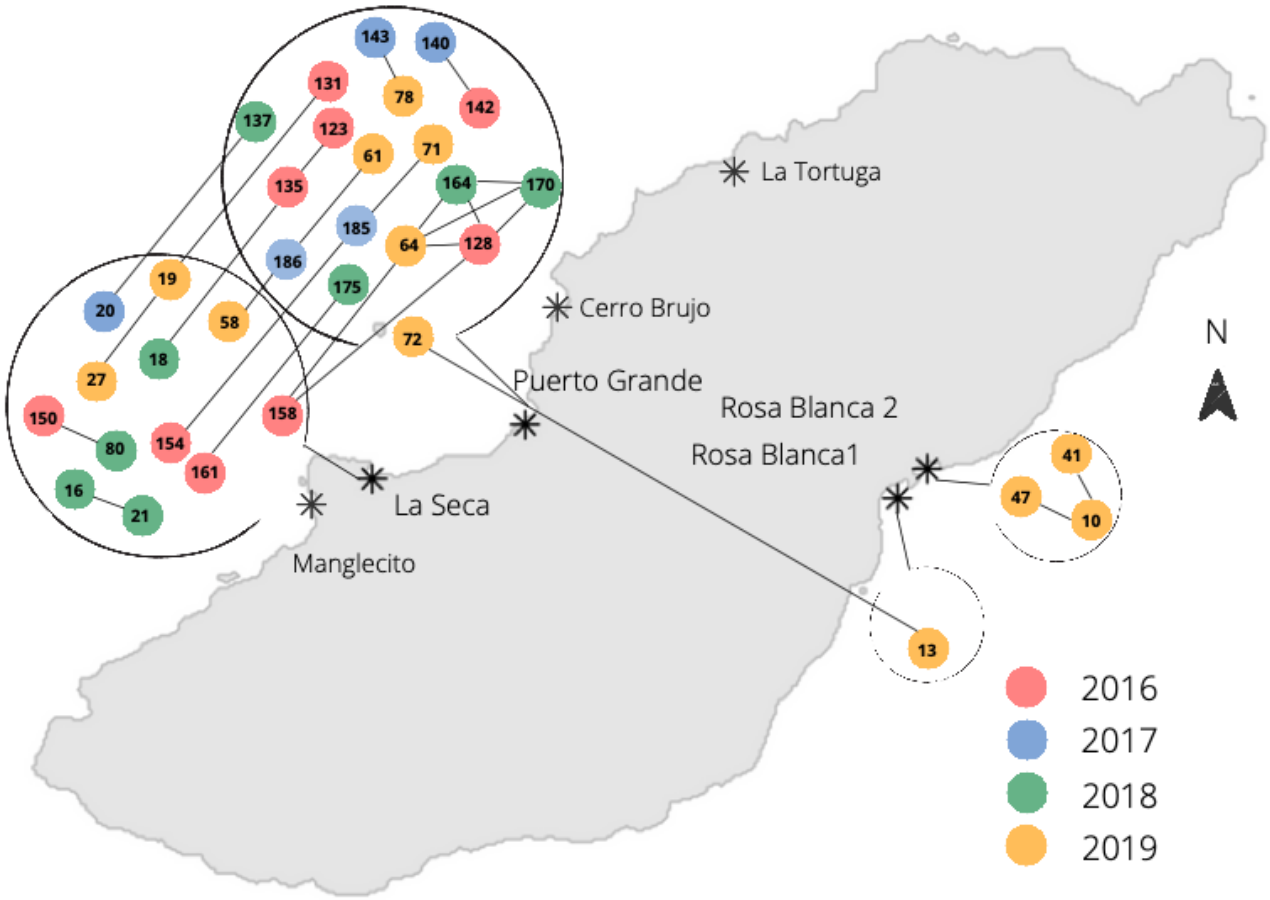

Figure 5. Half-siblings identified by COLONY software. Relationships are shown by solid black lines at San Cristóbal Island. Individuals are represented by its ID number, and a color that shows its estimated birth year (except for ID 158 and ID 161 whose sample year is shown). No HS were identified by COLONY in Manglecito, Cerro Brujo, or La Tortuga.

COLONY identified that at least 10 HS pairs were related every two years (biennial relationships) while five HS pairs were related on a consecutive year pattern (Figure 5). Additionally, COLONY identified a complex cross-year sibship in between HS family #3 (Table 8) and FS family #1 (Table 6). Individual ID128 was identified as HS for individual ID158, 164, and 170 (individuals related as FS). Similarly, individual ID 064 was identified as HS for ID128 and for the FS threesome ID158, 164, and 170 as well.

Because SNP markers are not sex linked, we were not able to determine if these HS relationships come from a maternal or a paternal line. Consequently, we illustrated the maternal and paternal possible scenarios for these half sibling relationships (Figure 6).

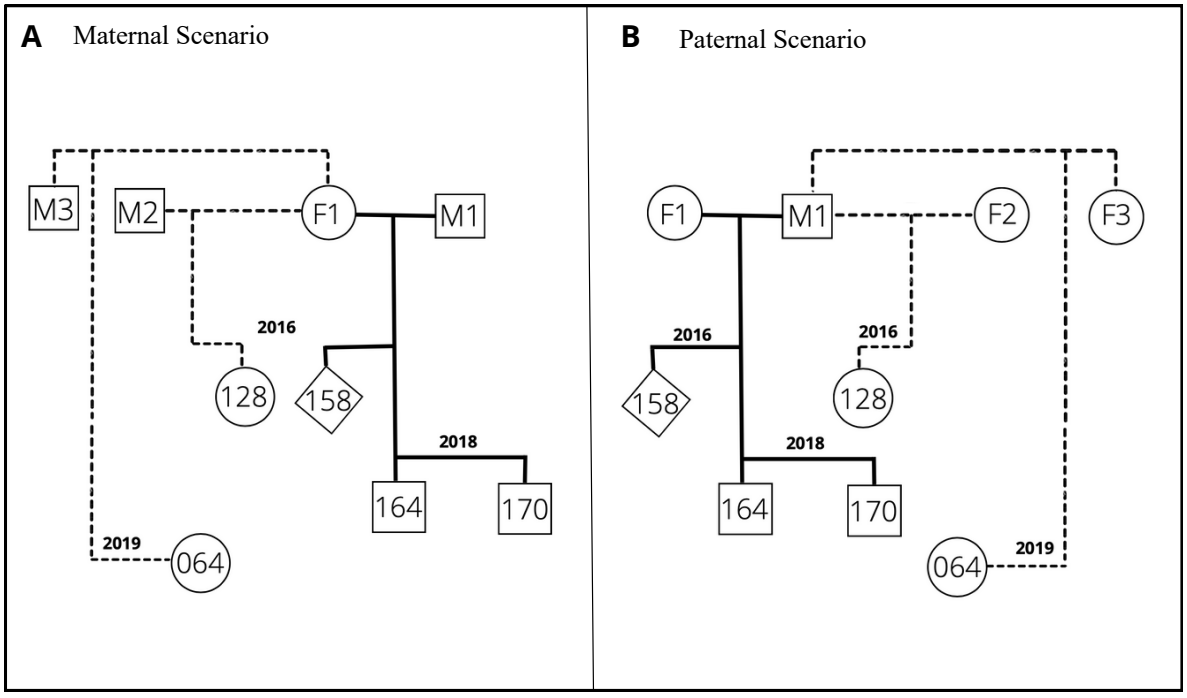

Figure 6. Cross-year half sibship complex. Here we describe a maternal and paternal scenario for half-siblings ID 128 and ID 064 with the full sibling threesome ID 158, 164 and 170. Putative parents are represented only for illustration purposes as F = Female, M = Male. Offspring is represented with its ID number, and circles for females, squares for males, and diamond for NA. 2016, 2018 and 2019 show offspring's birth years. Solid black lines represent family #1 full sibship, while dashed black lines represent potential half sibling relationships under two scenarios. A) Maternal scenario in which female F1 potentially reproduced with two additional males: M2 (offspring ID 128) and M3 (offspring ID 064). Consequently, ID 128 and ID 064 are related as maternal HS as well as with the full sibling threesome: ID 158, ID 164, and ID 170. B) Paternal scenario where male M1 reproduced with two additional females: F2 (offspring ID 128) and F3 (offspring ID 064). Then, ID 128 and ID 064 are paternal half siblings between them, and between the FS threesome.

Population substructure

The AMOVA showed significant differences among individuals within sites for datasets A (F_{IT} = 0.03269, $p < 0.05$), and B (F_{IT} = 0.02868, $p < 0.05$) (Table 9). Additionally, significant differences were found within individuals: F_{IS} = 0.02991, $p < 0.05$, and F_{IS} = 0.2593, $p < 0.05$ for datasets A and B, respectively (Table 10).

Table 9. Analysis of Molecular Variance (AMOVA) for dataset A. Assessment of potential population substructure in juveniles of *C. limbatus*. It shows results for dataset A (four sites), under the null hypothesis of genetic differences between west and east bays. PG = Puerto Grande, LS= La Seca (west), RB1 = Rosa Blanca 1, RB2 = Rosa Blanca 2 (east). Note that here the term "group" is used to define east and west clusters.

H_o: PG – LS, RB1 – RB2	% of variation	F-statistic	p-value
Among groups (west – east)	0.26	F_{CT} =0.00259	0.32574
Among sites within groups	0.03	F_{SC} =0.00028	0.35059
Among individuals within sites	2.98	F_{IS} =0.02991	0.02287
Within individuals	96.73	F_{IT} =0.03269	0.01653

Table 10. AMOVA results for dataset B (seven sites) to assess potential population substructure. Null hypothesis (H_o) supports genetic differences in between the east (RB1, RB2) and west (PG, LS, MG, CB, LT) sampled sites. PG = Puerto Grande, LS = La Seca, MG = Manglecito, CB = Cerro Brujo, LT = La Tortuga, RB1 = Rosa Blanca 1, RB2 = Rosa Blanca 2.

H_o: PG, LS, MG, CB, LT - RB1, RB2	% of variation	F-statistic	p-value
Among groups	0.24	F_{CT} = 0.00242	0.15445
Among sites within groups	0.04	F_{SC} = 0.00041	0.26686
Among individuals within sites	2.59	F_{IS} = 0.02593	0.03617
Within individuals	97.13	F_{IT} = 0.02868	0.03128

Observed and expected heterozygosity were calculated for both datasets. Dataset A maintained similar heterozygosity values for all four sites (Table 11) whereas for dataset B, heterozygosity was more variable when analyzing MG, CB, and LT (Table 12).

Table 11. Observed and expected heterozygosity in dataset A (four sampled sites).

	Ho	He
Puerto Grande	0.3608	0.3718
La Seca	0.3601	0.3720
Rosa Blanca 1	0.3619	0.3747
Rosa Blanca 2	0.3611	0.3722

Table 12. Observed and expected heterozygosity in dataset B (seven sampled sites).

	Ho	He
Puerto Grande	0.3635	0.3725
La Seca	0.3626	0.3731
Rosa Blanca 1	0.3649	0.3754
Rosa Blanca 2	0.3638	0.3733
Cerro Brujo	0.4131	0.4313
Manglecito	0.4522	0.4645
La Tortuga	1.0000	1.0000

Pairwise F_{ST} comparisons between east and west groups were assessed using datasets A and B. After 1023 permutations, F_{ST} for dataset A showed significant differences ($p < 0.05$) between sites located at the west: PG and LS, and those from

the east: RB1 and RB2 (Table 13). Additionally, F_{ST} values for dataset B showed the same east-west pattern as for dataset A. However, no significant differences were found in between the additional western sites (Cerro Brujo, Manglecito, and La Tortuga) and the east sites (Table 14).

Table 13. Group pairwise F_{ST} comparisons for dataset A (four sites) around San Cristóbal Island. F_{ST} values are shown below the diagonal, whereas p-values are presented above. Significant p-values appear as (*) under significance level of 0.05.

	Puerto Grande	La Seca	Rosa Blanca1	Rosa Blanca 2
Puerto Grande	-	0.46582	0*	0.03418*
La Seca	0.00022	-	0*	0.03711*
Rosa Blanca 1	0.00669	0.00620	-	0.35742
Rosa Blanca 2	0.00194	0.00174	0.00275	-

Table 14. F_{ST} paired comparisons for dataset B (seven potential nurseries) around the island. F_{ST} values are shown below the diagonal, whereas p-values are presented above. Significant p-values appear as (*) under significance level of 0.05.

Group	Puerto Grande	La Seca	Cerro Brujo	Manglecito	La Tortuga	Rosa Blanca 1	Rosa Blanca 2
Puerto Grande	-	0.34234	0.75676	0.00901*	0.99099	0*	0.05405*
La Seca	0.00022	-	0.88288	0.14414	0.99099	0*	0.03604*
Cerro Brujo	0.00020	0.00015	-	0.83784	0.99099	0.29730	0.67568
Manglecito	0.00561	0.00496	0.00081	-	0.99099	0.09910	0.32432
La Tortuga	0.01384	0.01023	0.00798	0.01634	-	0.35135	0.37838
Rosa Blanca 1	0.00634	0.00618	0.00739	0.01072	0.01274	-	0.28829
Rosa Blanca 2	0.00184	0.00171	0.00062	0.00495	0.01374	0.00262	-

Discussion

This study is the to first to apply genotyping-by-sequencing methods to assess the use of potential nursery grounds by adult *Carcharhinus limbatus* females at multiple spatial and temporal scales in the Galapagos Marine Reserve. Our results suggest a potential tendency of adult females to stray between proximate bays when pupping, potentially using extended areas rather than specific bays. Additionally, potential female philopatry is suggested for east and west bays around San Cristóbal Island. Finally, the spatial distribution of juveniles and its connectivity allows for indirect estimation of males' reproductive movements, suggesting a male-mediated gene dispersion.

Indirect evidence for female philopatric behavior

Mitochondrial and microsatellite markers using parental and/or offspring information have been largely used to understand philopatric behavior for some shark species (Hueter *et al.* 2005; Tillett *et al.* 2012; Feldheim *et al.* 2013). However, this is one of the few studies using genomic SNPs markers from juvenile sharks only in wild populations to analyze kinship (Feutry *et al.* 2017; Marie *et al.* 2019).

In total, we identified 10 FS and 24 HS pairs (18.9%) with more than 98% of certainty given the large number of genotyped SNPs. This low percentage of identified relatives could indicate a large population, in which the number of unrelated pairs exceed the number of true kin-pairs (Bravington *et al.* 2016). However, as this research does not have a population study focus, this aspect needs further exploration.

Because SNPs are not sex-linked markers, they provide biparental information. Thus, for half-siblings we could not identified the parent (whether it is mother or father) that contributes to the individuals' genomic information. Consequently, we interpreted the FS information only in order to infer philopatry, reducing the bias that HS might produce.

We identified biennial full sibships for two pairs between Puerto Grande - La Seca, and for one pair within La Seca. This temporal pattern suggests that adult females return to nurseries in a two-year cycle for parturition. This findings coincide with the two-year reproductive cycle reported by Castro (1996) for this species in South Carolina and Florida. This is explained as a large reproductive cycle in which after parturition females take one year to rest and to store sufficient energy before the next parturition (Castro, 1996).

We identified three cross-bay full sibling pairs in between PG – LS, and two in between RB1 – RB2. These results show genetic connectivity among proximate bays. Similarly, Keeney (2003) evidenced genetic connectivity in between proximate (aprox.120km apart) blacktip shark nurseries in the Florida Gulf coast. The author suggests that a great straying degree of gravid females among proximate bays could result in homogenized genotype frequencies. In our case, straight distance in between Puerto Grande and La Seca is 6.20km, while in between Rosa Blanca 1 and Rosa Blanca 2 it is 1.45km. These short distances in between bays, added to non-significant F_{ST} genetic differences in between PG - LS and RB1 - RB2, suggests more likely that gravid females present a high degree of straying from their natal nurseries, potentially using proximate bays as extended areas rather than specific sites. However, we recommend that further studies analyze the correlation in between geographic and genetic distance in order to assess if straying events are frequently enough for increasing genetic similarities in between proximate bays (Tillett *et al.* 2012).

Furthermore, we do not discard that juveniles' spatial distribution is a result of their own small-scale movements in between bays. Evidence from Hirschfeld (2013) and Chiriboga - Paredes (2018) showed cross-bay movement in between Puerto Grande and La Seca for individuals in between 65 - 74cm. Nevertheless, neither study registered movements outside the bay from neonates. The reported small-scale movements have been described as exploratory, demonstrating that after exploring, juveniles return to their home nursery for extended periods, showing high residency rates and a high preference for limited areas. These results are congruent with the

reported ontogenetic dietary shifts for early stages of this species at the Galapagos (Páez-Rosas *et al.* 2021). The study showed that changes in ^{13}C values for larger age classes are associated with exploratory feeding movements outside nurseries. Meanwhile, neonates presented enriched values of $^{13}C/^{15}N$ from mother-yolk reserves, used as energy source until the development of foraging skills, suggesting limited foraging activity for neonates. Additionally, Heupel *et al.* (2004) demonstrated by passive acoustic telemetry that juvenile blacktips in Florida expand their home range when they are three to four months old due to changes in foraging behavior.

In our study, we found that most cross-bay full sibships corresponded to individuals from one to three months old, and neonates from the same cohort. Then, short-age cross-bay siblings could be a result of a straying of adult females in between close nurseries, rather than juvenile movement per se. However, further studies focusing on just neonate and female adults sampling are needed in order to adequately address these genetic connectivity patterns in between proximate bays.

Pairwise F_{ST} showed significant genetic differences in between western (PG and LS) and eastern (RB1 and RB2) bays. According to Keeney (2003, 2005) genetic differentiation in between nurseries could be explained as a strong tendency of adult females to return to specific breeding areas generation after generation. Consequently, philopatric behavior entails a decrease in the genetic variability among individuals and populations, increasing the inbreeding intensity and speeding the chance for alleles' fixation (Shields, 1982). This coincides with our significant inbreeding coefficients ($F_{IS} = 0.02991$, $p < 0.05$) that suggest a decrease in individual heterozygosity given a potential female philopatric behavior. Then, genetic differences in between western and eastern bays could be explained as alleles differentially fixed in between the two sides of the Island by philopatric females.

Nevertheless, we cannot only attribute these genetic differences to philopatry solely, geophysical and environmental barriers to dispersal need further investigation. However, Hearn *et al.* (2014) reported that adult blacktip sharks are resident to the Galapagos archipelago, displaying movements all around the marine reserve: from the far north to the south east. According to these, blacktip sharks are highly mobile in

between different bioregions inside the archipelago. Then, it would be unlikely that genetic differences in between east and west bays are a result of the incapability of females or males to move all around San Cristóbal. Additionally, Peñaherrera - Palma (2016) evidenced that ultrasonically tagged blacktip adult females in the Galapagos display a strong use of areas < 500m deep at the north of Santa Cruz Island where potential nurseries have been reported (Jaenig 2010; Llerena-Martillo *et al.* 2013) suggesting that females remain close to nurseries given potential philopatry.

Indirect evidence on the reproductive system and adult movements

In our study, we identified 24 HS pairs in total, from which nine were biennial HS, two were related in a triennial cycle, and five pairs related in consecutive years. While most evidence report a biennial reproductive cycle for females (Castro, 1996), Dudley & Cliff (1993) showed that in South Africa this cycle could also be triennial. However, based on the biparental nature of SNPs markers, half-sibling's temporal patterns must consider a male-female perspective in order to be interpreted.

South African population of *C. limbatus* has been described as polygynandrous, meaning that both, males and females, are able to mate with more than one partner and male mating success is more variable than that of females (Klug, 2011; Bester-van der Merwe *et al.* 2019). Then, assuming a biennial reproductive cycle for females (Castro, 1996), as demonstrated by the full siblings in our study, it would be more likely that consecutive year HS share the same sire rather than the same mother. This hypothesis fits with the multiple paternity behavior demonstrated by Bester-van der Merwe *et al.* (2019) for this species in South Africa, registering a multiple paternity frequency in between 50-71% for 14 genotyped litters using microsatellites. However, multiple paternity frequency can differ in between populations. Then, complementing this study using maternal inherited markers (*e.g.* mitochondrial DNA, mitogenome) will provide direct evidence to clarify multiple paternity and comprehend the mating system of *C. limbatus* in the Galapagos.

Inferring adult movements based on the spatial distribution of juvenile HS pairs has been demonstrated as a useful tool for connectivity studies (Feutry *et al.* 2017) where difficulties for sampling or finding adults make adult movement information scarce.

In our study, we identified one HS pair in between west (Puerto Grande) and east (Rosa Blanca 1) bays. The spatial distribution of this HS shows that despite the existence of genetic differences in between east and west, there is still some sort of genetic connectivity around the island. According to Keeney *et al.* (2005) males of *C. limbatus* may present lower levels of fidelity to nurseries, moving wider distances and dispersing genes as a homogenizer mechanism. Then, we suggest that genetic connectivity around the island could be explained as a male-mediated gene dispersal. However, caution is needed when interpreting these results given the small subsample size for eastern bays. There is the need that further studies incorporate and genotype more individuals to the subsample (n = 20 individuals per year) from RB1 and RB2. Additionally, it would be of interest to sample adult individuals from the island, as well as juveniles, females, and males from other islands of the archipelago to have a clear understanding about a potential male-mediated gene dispersal around the island and the whole archipelago.

Implications for conservation and management

The use of nursery grounds by elasmobranchs is likely an evolutionary strategy to increase juvenile development and survival (Heupel *et al.* 2007; 2018). Large, slow-growing, and few offspring sharks, as *Carcharhinus limbatus,* make great use of coastal nurseries in order to increase juvenile survival (Heupel & Hueter, 2002). However, human coastal degradation has advanced at an alarming rate, generating mangrove habitat loss in at least 1% per year in a global rate, and between 18-32% at local rates (Dulvy *et al.* 2003). Although interest in coastal management and conservation has been raising, funding is scarce (Beck *et al.* 2001). Consequently, knowledge on key habitats and sites is urgently needed to prioritize its conservation (Beck *et al.* 2001).

In San Cristóbal Island, a series of studies identified potential nurseries for a series of elasmobranch species, including batoids (Llerena-Martillo *et al.* 2009, 2013; Hirschfeld, 2013; Chiriboga-Paredes, 2018; Goodman, 2020; Pazmiño *et al.* unpublished data). However, the current coastal marine reserve zoning scheme allows for artisanal fishing in most of the island's coast, including potential nurseries identified for elasmobranchs. Research efforts in San Cristóbal had the objective to test Heupel *et al.* (2007) criteria and identify specific habitats, and sites within them, to suggest authorities' suitable management. Goodman (2020) tested the first abundance criteria for 14 sites around the island. The study showed that only four mangrove bays: Puerto Grande, La Seca, Manglecito, and Rosa Blanca 1, qualify for the first criteria holding the greater juvenile abundance compared to the other sampled sites. Additionally, Hirschfeld (2013) and Chiriboga-Paredes (2018) reported evidence for juvenile residency and site-fidelity at Puerto Grande and La Seca, concluding that these two bays fulfill the second Heupel *et al.* (2007) criteria. Finally, our study provide evidence that Puerto Grande, La Seca, Rosa Blanca 1, and Rosa Blanca 2 are sites repeatedly used over the years by juveniles and adult females, satisfying the third criteria. According to these results, Puerto Grande and La Seca bays fulfill the three criteria to be defined as nursery grounds for *Carcharhinus limbatus* at San Cristóbal. However, our study suggests a shift in the current spatial understanding of nurseries as isolated sites. We propose the hypothesis that adult females are potentially straying among proximate sites. Then, La Seca and Puerto Grande should be managed as one nursery unit. Additionally, we suggest treating Rosa Blanca 1 and Rosa Blanca 2 as just one big bay because of its proximity. However, further studies regarding the environmental variables that determine nursery habitat selection are needed.

In terms of management and conservation, our study states the need of full protection for Puerto Grande and La Seca. However, as Puerto Grande has been historically used as a recreational and fishing site by the local community, we suggest seasonal regulations for these activities. We suggest authorities to stop tourism, recreational, and fishing activities inside the bay during the pupping season from February to March

(Goodman, 2020) when natural mortality of neonates is higher and has the potential to increase with human activities' interaction.

Study limitations

Inconsistent subsampling

Because of financial constraints, we only genotyped a subsample of 185 individuals from 693, prioritizing a temporal sampling and choosing uniformly samples from each year rather than from each site. This decision led to an uneven sampling per site (La Tortuga n = 1, Manglecito n = 3, and Cerro Brujo n = 4). Consequently, we had an underrepresentation of La Tortuga (LT), Manglecito (MG), and Cerro Brujo (CB) when identifying full and half-siblings. In our study, we opted to separately analyze the SNPs information obtained from all seven sites (including LT, MG, CB), and from four sites (excluding LT, MG, CB) to explore how different temporal and spatial subsampling might affect the final siblingship results. However, when analyzing all seven sites and four sites only, we did not find any half or full sibling at LT, MG or CB. These results did not allow to determine neither genetic connectivity with other bays, nor the temporal and spatial use of female adults at these bays.

Nevertheless, we hypothesize that given the proximity among Manglecito and La Seca, and among Cerro Brujo and Puerto Grande, these four bays will potentially present genetic connectivity. However, further studies are needed. On the other hand, La Tortuga is further away; approximately 15 km from Puerto Grande, and 19km from La Seca in a straight distance. Considering that Chiriboga-Paredes (2018) did not registered movements from juveniles in between La Tortuga and the other sampled western bays, it would be of interest to assess the relatedness of juveniles among this site and the others around the island.

Despite the great resolution that SNPs markers provide to reveal kinship between juveniles, a consistent temporal and spatial sampling is key to obtain quality results.

Considering that Goodman (2020) reported that Manglecito should be considered as a nursery bay for blacktip sharks, and that La Tortuga and Cerro Brujo need further studies to assess their role as nursery habitats, there is the need to add and genotype a consistent number of individuals from LT, MG, ad CB from 2016 - 2019 in future studies.

Sampling juveniles

Traditionally, kinship analysis rely on adult and juvenile sampling (Bravington *et al.* 2016). However, sampling adults in the wild from highly mobile species as sharks could become a difficult task when fisheries catch-data is not available. A recent study by Feutry *et al.* (2017) developed an approach using SNPs and mitogenomes to reveal the contemporary patterns of gene flow using only samples from juveniles of the *Glyphis glyphis* sharks at northern Australia. This approach tackles the need of adult sampling and outstands as a novel genomic approach to be used in the management of threatened species.

This study motivated our work; however, our juvenile sampling presents several limitations. First, we were able to only use SNPs' markers, obtaining only biparental genomic information. Consequently, in the case of HS, we were not able to reach solid conclusions about sex-specific adult movements around the island. However, information on the life-history of the species played a major role in order to make inferences regarding adult movements (Feutry *et al.* 2017). Nevertheless, we recommend that further studies sampling only juvenile sharks consider using comparative genomic markers as SNPs and other sex-linked (*e.g.* mitogenomes) in order to obtain a complete sibship spectrum and resolve some complex kinship categories (HS, grand-parent, grand-child) that could provide valuable information (Bravington *et al.* 2016).

The second limitation presented was the sampling of all early life stages: from neonates to juveniles of the year. Because studies (Heupel *et al.* 2003; Hirschfeld, 2013; Chiriboga-Paredes, 2018) have shown that juveniles of this species make exploratory

movements outside its bay, we got a blurry panorama when inferring the reasons of genetic connectivity in between proximate bays. Is it a result of a potential straying of adult females among proximate bays? Or is it a reflection of juvenile exploratory movements? We recommend that further studies focus on sampling neonates only (≤ 62 cm) to avoid misinterpretations regarding genetic connectivity. Additionally, to the extent possible, we suggest sampling adult females to accurately understand the reproductive female behavior around the Island.

Temporal clustering of individuals

Age data is of major importance for kinship analysis (Bravington *et al.* 2016). However, studies on age-and-growth for *C. limbatus* are still missing in the Galapagos and in the Eastern Tropical Pacific, making it difficult to accurately estimate the age of individuals in our study. Nevertheless, Páez-Rosas *et al.* (2021) proposed an age classification for this species in the Galapagos based on a worldwide compilation of age-and-growth studies for this species. However, the study suggests similarities with individuals from the Gulf of Mexico and the US South Atlantic coast. In order to assess the use of this classification in our study, we compared the total lengths from age-and-growth literature reported for this species (Killiam and Parsons, 1989; Carlson *et al.* 2006; Smart *et al.* 2015) and the lengths obtained during the monitoring program of neonates and juveniles in San Cristóbal (Chiriboga-Paredes *et al.* 2020, unpublished data) and from adult blacktip sharks ultrasonically tagged in the Galápagos (Peñaherrera-Palma, 2016). Lengths from the Galápagos individuals look way more similar to those reported for Indonesia and South Africa (Smart *et al.* 2015) rather than those reported for the Gulf of Mexico or the US Atlantic coast. Additionally, we found evidence for significant genetic population differentiation between blacktips from an Indo-Pacific origin (Australia, South-Africa, and the Pacific) and those from the western Atlantic (Keeney & Heist, 2006; Bester-van der Merwe *et al.* 2019).

Based on this information, we created an age category classification similar to the one from African and Indonesian populations in order to estimate the birth year and age of our individuals in the Galápagos and interpret temporal sibships.

Nevertheless, we address the need of further studies to focus on age-and-growth parameters for *C. limbatus* in the Galapagos or in the Eastern Tropical Pacific to avoid bias in this kind of studies. New perspectives and technology, demonstrated that DNA methylation is a valid alternative to infer broad range of ages in sharks (Beal *et al.* 2022)

Conclusions

In this study we demonstrated how genomic information from only juvenile sharks can be used to reconstruct kinship in a sibling level to infer the spatial and temporal patterns that adult females present at nursery grounds in San Cristóbal Island, Galápagos. This represents a significant advance in the knowledge of the reproductive biology of the species in the Galápagos Marine Reserve, as well as regionally. In addition, the less invasive methodology applied in this study encourages the use of genomic tools, specially inside Marine Protected Areas, where advances in sampling techniques should contemplate less intrusive procedures. For *Carcharhinus limbatus* in San Cristóbal, we found that 1) there is a biennial reproductive cycle for females; 2) it is likely that females present a straying behavior betwixt proximate bays when pupping every two years; 3) we infer that genetic differences in between east and west bays are a result of a potential female philopatric behavior; 4) we infer that reproducing males likely move all around the island, dispersing the genes (although direct evidence is lacking); 5) The study recognized that only Puerto Grande and La Seca satisfy all Heupel *et al.* (2007) criteria in order to be identified as nursery grounds. We suggest authorities from the Galapagos National Park to take this study into consideration when delineating conservation and management strategies to mitigate the current tourism and fishing impacts that threatens nursery habitats in the Galapagos Marine Reserve.

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