ARTICLE

Genome-wide SNPs detect no evidence of genetic population structure for reef manta rays (*Mobula alfredi*) in southern Mozambique

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Abstract

Little is known about the extent of genetic connectivity along continuous coastlines in manta rays, or whether site visitation is influenced by relatedness. Such information is pertinent to defining population boundaries and understanding localized dispersal patterns and behaviour. Here, we use 3057 genome-wide single-nucleotide polymorphisms (SNPs) to evaluate population genetic structure and assess the levels of relatedness at aggregation sites of reef manta rays (*Mobula alfredi*) in southern Mozambique ($n = 114$). Contrary to indications of limited dispersal along the southern Mozambican coastline inferred from photo-identification and telemetry studies, our results show no evidence of population structure (non-significant $F_{ST} < 0.001$) for *M. alfredi* along this coast. We also found no evidence that individuals sampled at the same site were more related than expected by chance for males, females or across both sexes, suggesting that kinship may not influence visitation patterns at these sites. We estimated the effective population size (N_e) of this population to be 375 (95% CI = 369–380). Comparison to a distant eastern Indian Ocean site (Western Australia, $n = 15$) revealed strong genetic differentiation between Mozambique and Western Australia ($F_{ST} = 0.377$), identifying the Indian Ocean basin as a barrier to dispersal. Our findings show that genetic connectivity in *M. alfredi* extends for several hundred kilometres along continuous coastlines. We therefore recommend that the population in Mozambique be considered a discrete management unit, and future conservation plans should prioritize integrated strategies along the entire southern coastline.

Introduction

Knowledge of fine-scale population structure within a region is important for understanding localized dispersal patterns, determining the spatial extent of discrete populations, and assessing whether population declines may be mitigated

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through effective migration from adjacent areas (Seddon et al. 2014). Additional insights into dispersal patterns and behaviour can be gained by measuring relatedness between individuals, specifically whether relatedness influences spatiotemporal patterns of encounters and site fidelity (Lieber et al. 2020) or even social relationships (Lewis et al. 2013). Such information has proven to be particularly valuable for elasmobranch populations, where overexploitation, low reproductive rates and a lack of knowledge present conservation and management challenges (Dudgeon et al. 2012; Ovenden et al. 2018). These challenges may be magnified by low effective population sizes (N_e) (e.g. Andreotti et al. 2016; Dudgeon and Ovenden 2015; Reid-Anderson et al. 2019), which increase their vulnerability to loss of genetic diversity and allele-frequency fluctuations due to genetic drift.

One such threatened elasmobranch is the reef manta ray (*Mobula alfredi*), a wide-ranging zooplanktivorous filter feeder that inhabits pelagic environments in tropical and warm–temperate waters (Couturier et al. 2012). Manta rays experience considerable anthropogenic pressure from target

fisheries driven by the demand for their gill plates in Asian medicine markets (Croll et al. 2016), bycatch and indirect threats such as entanglement, boat strikes (McGregor et al. 2019), marine pollution (Germanov et al. 2019b) and the impacts of unregulated tourism (Murray et al. 2019; Venables et al. 2016). Moreover, their low reproductive rate hinders population recovery and exacerbates the risk of localized extinction in countries where fisheries operate (Dulvy et al. 2014). *M. alfredi* reliably aggregate at inshore-feeding sites and reef-cleaning stations throughout the Indian and Pacific Oceans, with residency and site affinity identified at multiple study locations (Couturier et al. 2011; Germanov et al. 2019a; Peel et al. 2019). The drivers behind such philopatry, particularly to cleaning stations, are not fully understood for *M. alfredi*. In addition to cleaning behaviour, visitation may be influenced by social or reproductive interactions with conspecifics (Perryman et al. 2019; Stevens et al. 2018), yet the role of kinship in group formation or repeated site visitation has not been explored for manta rays. Despite displays of residency and site preference, *M. alfredi* is highly mobile, capable of travelling >1100 km along continuous coastlines (Armstrong et al. 2019) and 450 km across island chains, connecting aggregation sites that were previously thought to be isolated (Germanov and Marshall 2014).

There is a paucity of published information on genetic structure and connectivity in *M. alfredi* populations and, until recently, population genetics studies were generally lacking. Hosegood (2020) found significant genetic differentiation between sites in the Indian (the Maldives, Seychelles and Chagos Archipelago) and Pacific (Hawaii, Fiji and eastern Australia) Oceans ($F_{ST} = 0.110–0.288$), yet did not detect evidence of genetic sub-structuring within populations in the Maldives or Hawaii. In addition, considerable variation in the frequency of melanistic individuals throughout other Indo-Pacific populations has been attributed primarily to genetic drift (Venables et al. 2019). While these findings are indicative of limited gene flow across ocean basins and large geographic distances, little is known about the levels of genetic connectivity outside of these locations at medium-to-fine spatial scales, particularly along continuous coastlines.

Previous studies at aggregation sites have largely focused on telemetry and photo-identification (hereafter, photo-ID) techniques to understand movements, demographics and population trends (see Stewart et al. 2018 for an overview). While ecological connectivity can be inferred from such methods, molecular approaches are required to assess the level of gene flow within and between study regions and confirm genetic connectivity (or isolation). In the Inhambane Province of southern Mozambique, long-term photo-ID and acoustic telemetry studies indicate regular interchange of *M. alfredi* between two regions (Praia do Tofo and Zàvora, Fig. 1) ~100 km apart, but limited movement
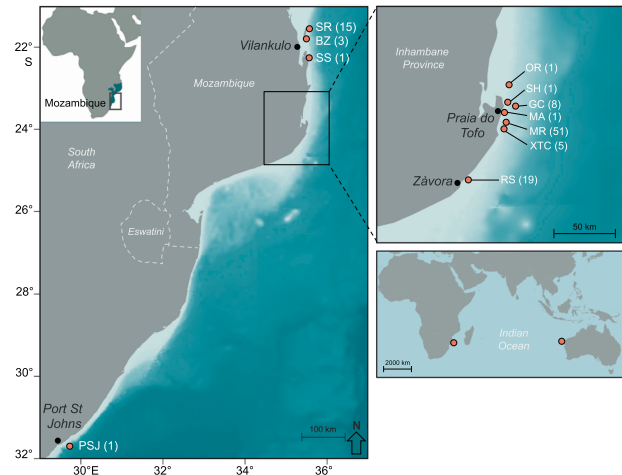

Fig. 1 Map showing sampling sites in Mozambique and South Africa. Orange circles represent sample sites and numbers in parentheses denote the number of samples collected. SR Spaghetti Reef, BZ Bazaruto Shallows, SS San Sebastian, OR Office Reef, SH Sherwood, GC Giant's Castle, MA Marble Arch, MR Manta Reef, XTC XTC reef, RS Red Sands, PSJ Port St Johns. The inset shows the collection location of Western Australian site relative to Mozambique. Note: 14 samples from Praia do Tofo lacked precise sampling location.

between these sites and the Bazaruto Archipelago, up to 300 km further north. Indeed, only 2% of the 1209 identified individuals have been sighted in both the Praia do Tofo/Zàvora and Bazaruto Archipelago regions over a 16-year period, despite these locations being situated along a continuous coastline with no apparent physical barriers (Marshall and Holmberg 2019; Venables et al. 2020).

Broadly, our study aims to examine the concordance of ecological connectivity inferred from photo-ID and acoustic telemetry with genetic population structure, and to investigate whether kinship plays a role in visitation patterns to aggregation sites. To achieve this, we use genome-wide single-nucleotide polymorphism (SNP) markers to (1) evaluate the genetic structure of *M. alfredi* along the coastline of southern Mozambique, (2) examine pairwise relatedness of sampled individuals to infer kinship and assess within-group relatedness, to determine whether individuals sampled at the same site were on average more related than expected by chance, (3) assess broad-scale population structure using a reference group of individuals from Western Australia and (4) provide the first estimate of the effective population size (N_e) for *M. alfredi* in southern Mozambique.

Materials and methods

Sample collection and DNA extraction

We collected 120 tissue samples from *M. alfredi* individuals using a custom-made stainless-steel biopsy probe mounted on

a modified Hawaiian sling. Samples were collected between 2006 and 2017, from ten sites in the Inhambane Province, southern Mozambique (22.913°S, 35.780°E) and one site off Port St John's, South Africa (31.643°S, 29.583°E, Fig. 1). Sample details are outlined in Table S1. Prior to biopsy sampling, a ventral identification photo was taken of each individual for photo-ID purposes. Where possible, we recorded sex (determined by the presence/absence of claspers), maturity (determined by clasper size and calcification for males and presence of mating scars and/or pregnancy for females) and the approximate disc width at the time of sampling (Table S1); see Marshall et al. (2011) for detailed photo-ID methodology. Tissue samples were stored in >90% ethanol at −20 °C prior to DNA extraction. We extracted genomic DNA using a DNeasy Blood & Tissue kit (Qiagen) and examined the quantity and quality of extracted DNA using fluorometric quantitation (NanoDrop DN-1000, Thermo Scientific & Qubit, Life Technologies) and a 0.8% agarose gel, to prevent degraded samples causing downstream issues.

SNP genotyping and filtering

SNPs were generated using the standard DArTseq^{TM} protocol by Diversity Arrays Technology Pty Ltd (DArT; Kilian et al. 2012). DArTseq^{TM} is a genotyping-by-sequencing approach based on enzymatic double-digest genome complexity reduction that combines DArT markers (Jaccoud et al. 2001) and next-generation sequencing on Illumina platforms to genotype thousands of SNPs homogeneously spaced across the genome. Sequencing was carried out as described by Georges et al. (2018), with the exception that PstI–HpaII restriction enzymes were used for the double digest of genomic DNA. SNPs were identified and called following the standard procedure in the proprietary DArT analytical pipeline DArTsoft14^{TM}. SNPs were called as part of a wider set of *M. alfredi* individuals sampled from Mozambique and Australia. As F_{ST} is a ratio (average within genetic diversity compared to total diversity), the inclusion of individuals from a broad spatial scale ensures that estimates of F_{ST} will be comparable with those from studies that use a similar experimental design. For the purpose of this study, our analysis focused primarily on data from Mozambique to assess population structure and estimate N_e. Further, to assess representative genetic diversity at a broader scale, we included an outgroup of 15 *M. alfredi* individuals sampled from Ningaloo Reef, Western Australia (WA, Fig. 1).

To reduce the effects of lower-quality SNPs, genotyping errors or large amounts of missing data on our analyses, we developed a data-filtering pipeline based on the methods described in Junge et al. (2019) and implemented in R

v3.6.1 (R Core Team 2019), with modifications outlined herein. Stepwise filtering is described in detail in Supplementary material (Table S2). Duplicate individuals were identified using the 'detect duplicate genome' function in the package 'radiator' (Gosselin 2019), removing the duplicate with the highest missing data. Individuals were also removed based on outlier statistics of individual-observed heterozygosity (<0.165 and >0.204) calculated using the 'detect mixed genomes' function in 'radiator'. Subsequent filtering steps were conducted within the 'dartR' package (Gruber et al. 2018) according to the following criteria: (1) >98% genotyping reproducibility, (2) no monomorphic loci, (3) <15% missing data per locus, (4) minor allele frequency >1%, (5) <15% missing data per individual, (6) average read depth between 8 and 50, (7) one SNP per fragment (randomly selected) to minimize linkage and (8) no loci out of Hardy–Weinberg equilibrium at >1 sampling site, with Bonferoni correction (α = 0.01). To ensure that loci unique to WA individuals were not included in the population structure assessment for Mozambique, we ran two separate datasets through the filtering pipeline. The first included individuals sampled in Mozambique only; the second included individuals from both Mozambique and WA. Summary statistics (H_E, H_O and F_{IS}) were calculated using the 'hierfstat' package (Goudet and Jombart 2015). We conducted locus outlier analysis using OutFLANK v0.2 (Whitlock and Lotterhos 2015), BayeScan v2.1 (Foll and Gaggiotti 2008) and LOSITAN (Antao et al. 2008) to meet the assumption of locus neutrality required by some analyses (e.g. STRUCTURE). Outlier detection was conducted on the filtered Mozambique dataset, due to high F_{ST} detected between Mozambique and Western Australia during initial data exploration. Loci were considered to be putatively under selection if they were identified by two or more approaches (see Table S3 for more detail).

Power analysis

To evaluate the theoretical statistical power of the Mozambique SNP dataset to detect various levels of genetic differentiation, we conducted a power analysis using POWSIM v4.1 (Ryman and Palm 2006). Settings of effective population size (N_e) and generations of drift (t) were selected to represent different F_{ST} values (0.0005–0.05) using the equation in Ryman and Palm (2006). We used the empirical number of loci and allele frequencies calculated using the R package 'PopGenReport' (Adamack and Gruber 2014), and empirical sample sizes across a scenario of three subpopulations. We ran POWSIM with 1000 dememorizations followed by 100 batches of 1000 iterations per run. Chi-square probabilities were used to test the significance ($p < 0.05$) of F_{ST} values for each replicate run, and the number of significant F_{ST} values in

200 replicate runs provided an estimate of the statistical power for a given level of genetic divergence.

Population structure

We assessed genetic differentiation using the R package '*StAMPP*', which generates pairwise fixation indices (F_{ST}), 95% confidence intervals (CIs), and significance values through bootstrapping over loci (Pembleton et al. 2013). Significance was assessed based on $\alpha = 0.05$. Population structure was first assessed at a site level, excluding sites with low sample sizes ($n < 5$). Based on the movement capacity of *M. alfredi* and photo-ID re-sightings of sampled individuals, we then pooled sites into broader geographic regions along the Mozambican coast: Bazaruto (SR, BZ and SS), Praia do Tofo (OR, SH, GC, MA, MR and XTC) and Zàvora (RS). See Fig. 1 for full site names. Cluster analyses were used to evaluate population structure in the form of (1) a principal coordinate analysis (PCoA), (2) a discriminant analysis of principal components (DAPC) and (3) Bayesian algorithms implemented in STRUCTURE v2.3.4 (Pritchard et al. 2000). We conducted the PCoA in R package '*dartR*' and the DAPC in R package '*Adegenet*', with the optimal number of clusters (K) selected based on the lowest Bayesian Information Criterion (BIC, Jombart and Ahmed 2011). A successive K-means algorithm was used to group individuals according to the optimal number of clusters. We ran an additional DAPC for each dataset, including a priori grouping based on sampling locations. We ran STRUCTURE using an admixture model with correlated allele frequencies and a burn-in and replicate run length of 15,000 and 100,000, respectively. We tested K values of 1–5 with ten iterations for each K, and determined the optimal number of clusters following the ΔK method of Evanno et al. (2005) using STRUCTURE HARVESTER (Earl and vonHoldt 2012), with visualization in CLUMPAK (Kopelman et al. 2015).

Relatedness

To investigate relatedness and infer kinship of sampled individuals, we calculated pairwise relatedness estimates for *M. alfredi* sampled along the Mozambican coast. In the absence of a pedigree, kinship inferences for non-model species rely on measures of relatedness simulated from empirical data (Attard et al. 2018). Firstly, we used COANCESTRY v.1.0.1.9 (Wang 2011) to determine the most appropriate relatedness estimator for our data. We simulated 500 pairs of dyads based on empirical allele frequencies for each of four kinship categories—unrelated, half-sibling, full-sibling and parent–offspring. Pairwise relatedness was estimated for each simulated pair using the seven relatedness estimators available in COANCESTRY

(described in Wang 2011). We assessed the accuracy (proximity to the true value) of each estimator by calculating correlation to true relatedness using Pearson's correlation coefficient. We assessed precision (variation in estimated values) by calculating standard deviations around mean estimates and visualizing percentiles using box plots across the four kinship categories as per Attard et al. (2018). The maximum-likelihood estimators 'TrioML' and 'DyadML' were the most appropriate for our data with the highest correlation coefficients and lowest variance across kinship categories (Fig. S1). As they produced similar relatedness estimates, we report only the DyadML estimates (R_{dyadML}). We estimated pairwise relatedness across all possible dyads (i.e. pairs of individuals) using the '*related*' R package (Pew et al. 2015) and examined these estimates to infer kinship of related pairs.

To assess whether individuals sampled at the same location were more closely related than expected by chance, and whether this differed by sex, we used the '*grouprel*' function in '*related*' to compare the average observed within-group relatedness with the expected distribution. The function calculates the average pairwise relatedness within each predefined group (i.e. sampling site), as well as an overall within-group relatedness estimate. The expected distribution of average within-group relatedness is generated by randomly shuffling individuals between groups, while keeping each group size constant, using 1000 Monte Carlo simulations. This results in 1000 iterations of each group, where each iteration is one realisation of the expected relatedness value of the dataset. The observed mean relatedness is then compared to the simulated distribution to test the null hypothesis of groupings being random associations with respect to relatedness. We excluded sites with low sample sizes ($n < 5$) and then assessed within-site relatedness for each site separately and overall across sites, for all individuals ($n = 94$) and separately for females ($n = 62$) and males ($n = 28$).

Effective population size (N_e)

Individuals deemed to be from the same population (i.e. non-significant F_{ST}) were pooled for inclusion in genetic effective population size (N_e) estimation using the contemporary single-time-point Linkage Disequilibrium (LD) method as implemented in NeEstimator v. 2.1 (Do et al. 2014). We used the random mating model and report estimates for P_{crit} (the criterion for excluding rare alleles) = 0.01, with results for $P_{crit} = 0.02$ available in Supplementary material. To minimize the likelihood of linked loci and avoid downwardly biasing N_e estimates (Waples et al. 2016), we retained only one SNP per fragment during the data-filtering process. Genotyping was conducted de novo in the absence of a *M. alfredi* reference genome; therefore,

linkage could not be directly assessed. However, due to data filtering and the likely large genome size, we consider the potential impact of physically linked loci to be low, given the probability of physical linkage between loci decreases (assuming sparse sampling of nuclear loci) as haploid chromosome number increases. However, in the absence of detailed linkage information for *M. alfredi*, we have applied a physical linkage correction factor based on the likely haploid number of chromosomes, using the following formula adapted from Waples et al. (2016):

$$N_{e(adj)} = \frac{N_{e(raw)}}{0.098 + 0.219 \times \ln(chr)}.$$

As the number of chromosomes for *M. alfredi* is as yet unknown, we used the closest related species for which chromosome number was known (*M. japonica*; $2n = 66$), where (chr) was the haploid number of chromosomes $= 33$ (Chang et al. 1995; Gregory 2020). We report both the 'raw' and adjusted estimates of N_e, and the parametric and jackknifed 95% CIs (Jones et al. 2016).

We corroborated our empirical estimates of N_e by comparing them to N_e estimated from a simulated *M. alfredi* population. We conducted simulations using NeoGen v1.3.0 (Blower et al. 2019), which simulates the demographic and genetic composition of a population using species-specific demographic, life history and genetic priors, and obtains N_e from the simulated genotypes using the LD N_e method (Waples and Do 2010). The population was simulated to be the approximate size of the overall population ($N = 1400$), based on the total number of identified individuals to date (1209, as of December 2019), which serves as a minimum estimate. The specified priors used in simulations are outlined in Table S4. NeoGen was also used to evaluate the statistical power of our empirical estimates by assessing the accuracy and precision of N_e estimates (obtained from the simulated population), based on user-defined sampling strategies, i.e. the number of individuals and number of loci (Blower et al. 2019). To evaluate the statistical power of our estimates, we tested a sampling strategy with samples sizes of 90, 100, 110 and 120 individuals, and loci quantities of 2100, 2600 and 3100 to encompass the actual number of loci (3057) and individuals (113) used for empirical estimates.

Results

Data filtering and outlier detection

The DArT analytical pipeline resulted in 14,764 polymorphic SNPs called from *M. alfredi* sampled from Mozambique and Australia. The final filtered dataset

Table 1 Measures of genetic diversity across sampling regions in Mozambique and Western Australia.

Location	n	H_o	H_e	F_{IS}
Bazaruto	18	0.284	0.277	−0.010
Praia do Tofo	78	0.285	0.276	0.001
Zàvora	17	0.286	0.275	−0.014
Western Australia	15	0.191	0.200	0.045

No F_{IS} values were significantly different from zero at $p < 0.05$.

n sample size, H_o observed heterozygosity, H_e expected heterozygosity, F_{IS} inbreeding coefficient.

comprised 3057 SNPs among 114 *M. alfredi* from Mozambique. No loci deviated from Hardy–Weinberg equilibrium (after prior filtering steps), and locus outlier analysis identified seven loci as putatively under selection, which were removed for all subsequent analyses. The remaining filtered SNPs were considered to conform to selective neutrality. With the inclusion of the WA outgroup, the filtered dataset comprised 3705 SNPs among 129 individuals; measures of genetic diversity are displayed in Table 1.

Power analysis

POWSIM simulations showed that given our sample sizes, number of loci and their allele frequencies, the Mozambique SNP dataset had sufficient statistical power to detect significant F_{ST} values as low as 0.01 in 100% of tests, $F_{ST} = 0.001$ in 93.5% of tests and $F_{ST} = 0.0005$ in 52% of tests (Table S5).

Population structure

We found no evidence of genetic differentiation between sampling sites along the Mozambican coast based on pairwise F_{ST} (Table S6). Similarly, no evidence of population structure was detected between geographic regions (nonsignificant $F_{ST} < 0.001$, Table 2). Cluster analyses aligned with pairwise F_{ST} results as the PCoA plot showed an overlap of individuals from all sampling regions and no evident clustering (Fig. 2a). Similarly, the *K*-means method identified a single cluster as optimal for the DAPC using only the Mozambique dataset based on BIC (Fig. 2c), and STRUCTURE plots showed no support for population substructuring in Mozambique (Fig. 2f). High genetic differentiation was evident between Mozambique and Western Australia when compared separately with each geographical region ($F_{ST} = 0.379–0.388$, $p < 0.001$, Table 2), and Mozambique overall ($F_{ST} = 0.377$, $p < 0.001$). This was supported by the two distinct and widely spaced clusters evident in the PCoA plot (Fig. 2b) and the DAPC, in which

two clusters were identified as the optimal clustering solution using the K-means method (Fig. 2d, e). The DAPC based on an a priori grouping showed the same overall clustering patterns as the K-means-based DAPC. STRUCTURE also revealed an optimal $K = 2$, with evidence of distinct structure between the two locations (Fig. 2g).

Relatedness

Average within-group relatedness over all individuals was highest at MR and RS ($R_{dyadML} = 0.025$). Similarly, the highest average within-group relatedness for females was at MR ($R_{dyadML} = 0.023$) and at SR for males ($R_{dyadML} =$

Table 2 Pairwise genetic differences (F_{ST}, below diagonal) and p values (above diagonal) calculated from single-nucleotide polymorphisms (SNPs) for *M. alfredi* among geographic regions in Mozambique and Western Australia.

	Bazaruto	Praia do Tofo	Zàvora	Western Australia
Bazaruto		0.726	0.756	0.000
Praia do Tofo	0.000		0.131	0.000
Zàvora	0.000	0.001		0.000
Western Australia	0.387	0.380	0.388	

Fig. 2 Population structure analyses results. Principal coordinate analysis (PCoA) plot for **a** Mozambique, and **b** Mozambique and Western Australia. Optimal number of cluster (K) selection, based on Bayesian Information Criterion (BIC) for **c** Mozambique and **d** Mozambique and Western Australia, **e** discriminant analysis of principal component (DAPC) plot for Mozambique and Western Australia (based on a single discriminant function as $K = 2$). STRUCTURE plots for **f** Mozambique and **g** Mozambique and Western Australia.

0.015). A comparison of the average within-group relatedness to the expected distribution for each site found no evidence that individuals sampled at the same site were more related than expected by chance for any of the major sampling locations, regardless of analysis across all individuals or separately by sex ($p = 0.23$–0.94; Fig. S2). Although most pairs were unrelated, we identified two pairs of likely first-degree relatives (parent–offspring or full-sibling; $R_{\mathrm{dyadML}} = 0.481$ and 0.469), with both pairs sampled at different sites (RS and MR) in different years. We also identified 15 pairs of likely second-degree relatives (half-sibling, avuncular or grandparent–grandchild; $R_{\mathrm{dyadML}} = 0.203$–$0.316$; Fig. S3). Nine of these pairs were sampled at different sites in different years; four were sampled at the same site (MR) in different years, one pair at different sites in the same year and the final likely second-degree pair was sampled at MR on the same day.

Effective population size (N_e) estimates

$N_{e(\mathrm{raw})}$ was estimated to be 325 (95% $CI_{\mathrm{parametric}} = 319$–$328$; $CI_{\mathrm{jackknife}} = 274$–$392$) using the contemporary single-time-point LD method ($P_{\mathrm{crit}} = 0.01$), based on 3057 loci and 113 (harmonic mean = 109) individuals. $N_{e(\mathrm{adj})}$ adjusted for bias from linkage was 375 (95% $CI_{\mathrm{parametric}} = 369$–$380$; $CI_{\mathrm{jackknife}} = 318$–$453$; Table S7). The 'true' LD N_e estimate from the simulated population ($N = 1400$) was 400 ($P_{\mathrm{crit}} = 0.01$), which is comparable to our empirical estimates (Figs. S4; S5). The output from simulations with alternative values of N ($N = 1300$, $N = 1500$) is included in Fig. S6. The simulations also confirm the statistical power in our N_e estimates, given the number of individuals and loci used (S110_L2600; Fig. S4). The CIs we report indicate high precision in our estimates, yet we recognize that precision is likely exaggerated due to the high numbers of non-independent pairwise comparisons between thousands of loci. This is evident in the reduction in the size of CIs surrounding N_e estimates as the number of loci used to generate estimates increases (Fig. S7). We report the ratio of N_e/N_c (annual adult abundance) in Supplementary material (Table S8).

Discussion

Using genome-wide SNP markers, we found no evidence of genetic structure for *M. alfredi* along the southern Mozambican coastline, demonstrating high genetic connectivity among aggregation sites. Acoustic telemetry and long-term photographic monitoring detected minimal movement between northern and southern aggregation sites (Venables et al. 2020), with only 2% of identified individuals recorded in both regions over a 16-year study period

(Venables 2020). Yet, our findings show that the apparent ecological segregation inferred from photo-ID and telemetry data does not reflect genetic structuring in this population. In addition to the lack of population structure, we found no evidence that individuals sampled at the same site were more related than expected by chance, indicating that kinship may not influence site visitation patterns in southern Mozambique. At a broader spatial scale, we detected high genetic differentiation between Mozambique and Western Australia, which identifies the Indian Ocean basin as a barrier to dispersal between these locations. Furthermore, we estimated the N_e of the *M. alfredi* population in southern Mozambique to be 375 (95% CI = 318–453).

We found a high level of genetic connectivity among *M. alfredi* sampled throughout their geographic range in Mozambique. Low F_{ST} among locations was supported by cluster analyses, which consistently showed no evidence of sub-structure. Classic population genetic analyses provide indirect estimates of connectivity only, and lack of genetic structure does not necessarily reflect an absence of barriers to gene flow. Indeed, it is important to acknowledge that if population structure was present, it was too low to be detected with the samples and markers used (e.g. see Bailleul et al. 2018). Nevertheless, our power analysis shows that the high number of markers used in our study provided high statistical power, with an ability to detect a significant F_{ST} as low as 0.001 in >90% of tests.

Low genetic differentiation along continuous coastlines has been demonstrated for other elasmobranchs, including grey nurse sharks (*Carcharias taurus*) (Reid-Anderson et al. 2019), grey reef sharks (*Carcharhinus amblyrhynchos*) (Momigliano et al. 2017) and tiger sharks (*Galeocerdo cuvier*) (Holmes et al. 2017). Similarly, no evidence of genetic sub-structuring was detected for *M. alfredi* among sites in the Maldives, spanning ~350 km across the archipelago (Hosegood 2020), an area comparable to the stretch of coastline examined here. This indicates a high degree of connectivity along continuous coastlines or interconnected island chains spanning within the dispersal capacity (>1000 km) of the species. Such information may be useful in other locations when defining management/conservation units or designing spatial management approaches in the absence of focused molecular studies.

The single *M. alfredi* sampled off Port St Johns, South Africa, was encountered up to 1300 km from Mozambican sampling sites, yet cluster analyses grouped this individual within the Mozambican population. Infrequent sightings have been reported from Ponta do Ouro, Sodwana Bay and as far south as Aliwal Shoal along South Africa's east coast (A. Marshall, unpublished data). Similar patterns of extension into southerly latitudes along continuous coastlines have been recorded in Australia (Armstrong et al. 2020; Couturier et al. 2011); nonetheless, this finding is the first

indication of a population to span an international border. While inferences based on a single individual should be treated with caution, Mozambique is at the southernmost end of the east African distribution of *M. alfredi* (Marshall et al. 2019), and it is unlikely that individuals sighted in South Africa originate from other locations. We recommend the collection of additional samples from South Africa to better evaluate cross-border connectivity, which may have implications for joint management and protection strategies for *M. alfredi* in southeastern Africa.

We identified 17 pairs of likely first- and second-degree relatives, which would be expected given the small size of the study population. Individuals sampled at the same site were no more related than expected by chance, which suggests that visitation to aggregation sites along this coast was not influenced by kinship. Philopatry is common for *M. alfredi* (e.g. Couturier et al. 2011; Dewar et al. 2008; Germanov et al. 2019a), including in Mozambique, where tagged manta rays showed repeated visitation to cleaning station sites (Venables et al. 2020). In Raja Ampat, Indonesia, individuals showed preference to certain sites, despite close proximity (<2 km) of alternative sites (Perryman et al. 2019), with evidence of social relationships detected in site-associated groups. The drivers behind preferential site visitation are not fully understood, and ours is the first study to investigate relatedness at aggregation sites for *M. alfredi*. Close association of kinship groups at aggregation sites was detected in basking sharks (*Cetorhinus maximus*), suggesting that relatedness may be a consequence of, or a potential mechanism maintaining, site-specific re-encounters (Lieber et al. 2020). While our findings suggest that kinship does not play a role for *M. alfredi*, the Mozambican population is relatively transient (Venables 2020), with lower re-sighting rates compared to other study sites (Germanov et al. 2019a). Thus, we recommend additional assessments focusing on more resident populations, with higher re-sighting rates, to further investigate spatial association of related individuals.

Analyses of relatedness also have the potential to provide direct estimates of contemporary connectivity (Feutry et al. 2017) or estimate recent population sizes through close-kin mark-recapture (CKMR) techniques (Bravington et al. 2016; Hillary et al. 2018). While these techniques have proved valuable for some shark species, a higher number of close-kin pairs than identified here would be required to make robust conclusions. CKMR also relies on the collection of samples from juvenile individuals, and obtaining a sufficient juvenile sample size may prove challenging for *M. alfredi*, particularly in Mozambique where sightings of immature individuals are especially rare.

The absence of population structure observed here is consistent with the dispersal capacity of *M. alfredi* (Armstrong et al. 2019), yet contrasts ecological estimates of dispersal inferred from telemetry and photo-ID, which indicated minimal interchange between northern and southern regions in Mozambique (Venables et al. 2020). This highlights the difference between physical tagging (here photo-ID and acoustic tagging) that follows an animal during its lifetime only, sometimes for limited time periods, and genetic assessments, which provide essential additional information in terms of the reproductive patterns of previous generations. Here, we demonstrate the value of combining ecological and molecular methods, which suggest that although individuals may segregate to visit preferred inshore cleaning and feeding sites, movements between regions are frequent enough to maintain gene flow.

The substantial genetic differentiation between Mozambique and Western Australia is indicative of limited gene flow between these locations, likely due to geographic distance (~7800 km) and the Indian Ocean basin inhibiting dispersal. Ocean basins have been identified as barriers to dispersal in other elasmobranchs (Green et al. 2019; Momigliano et al. 2017; Vignaud et al. 2014), as reef-associated species rarely traverse extensive pelagic habitats (see Heupel et al. 2019). *M. alfredi* appears to be no exception. Strong association with inshore reef systems in coastal waters and island chains (Marshall et al. 2009) has resulted in a fragmented distribution, with demographically inferred populations typically separated by expanses of pelagic habitat (Lawson et al. 2017; Marshall et al. 2019). Significant genetic structure between sites in the Indian and Pacific Oceans (Hosegood 2020), further supports the notion that large areas of an open ocean inhibit the dispersal, and thus gene flow, of *M. alfredi*. An opportunistic sighting of a pregnant female *M. alfredi* in the eastern Pacific, some 6000 km from the nearest-known aggregation site, indicates that some individuals do traverse ocean basins (Arauz et al. 2019), yet the frequency of such movements and their role in the maintenance of gene flow over large geographic distances requires further research attention.

At a regional scale, genetic connectivity between Mozambique and neighbouring locations (i.e. Mayotte, the Seychelles and the Red Sea) is yet to be investigated for *M. alfredi*, but photo-ID comparisons imply demographic isolation (Marshall and Holmberg 2019). Mayotte represents the closest aggregation site, situated ~1600 km across the Mozambique Channel. Given that deep water and large distances evidently restrict dispersal, genetic interchange between Mozambique and Mayotte is likely to be limited. A comparison of other western Indian Ocean sites (the Maldives, Seychelles and Chagos Archipelago) found significant differentiation between Seychelles and the other sites (F_{ST} = 0.05–0.06), but not between the Maldives and Chagos (F_{ST} = 0.012, non-significant) (Hosegood 2020). In the context of Mozambique, an assessment of genetic connectivity to nearby western Indian Ocean sites is an important next step to

determine whether genetic diversity could be maintained through gene flow, or whether this population is effectively isolated, with clear implications for conservation management.

The contemporary N_e for the Mozambican *M. alfredi* population was estimated to be 375. Given our findings of panmixia, the occurrence of this population at the most southerly extent of its range on the east African coast and the likely genetic isolation from adjacent western Indian Ocean populations, our estimates likely apply to a population, sensu Fisher-Wright (Wright 1931). Estimates of N_e can be subject to bias from various factors; the most applicable to our study is the effect of overlapping generations as *M. alfredi* is iteroparous (Waples et al. 2014). Therefore, it would be more pragmatic to consider the magnitude of our estimate (i.e. several hundred), particularly when assessing a species of conservation concern. According to a revision of the '50/500' guideline based on IUCN criteria (Frankham et al. 2014), the breeding population in Mozambique is large enough to prevent inbreeding depression, yet may have limited long-term evolutionary potential. For long-lived species with extended generation spans, there is an inherent time lag for N_e estimates as N_e is calculated for the generations preceding the sampled generation (Nunziata and Weisrock 2018). Our estimate of N_e represents the average breeding population size for generations preceding the sampled generation; thus, any decline that has occurred in the current generation (see Rohner et al. 2013; Venables 2020) would not be reflected in our estimates.

The N_e we report here is comparable to those estimated for populations of other elasmobranch species (e.g. Andreotti et al. 2016; Dudgeon and Ovenden 2015; Lieber et al. 2020; Reid-Anderson et al. 2019), which ranged between 333 and 400. The only available estimate of N_e for *M. alfredi* at the time of writing was for Japan's Yaeyama Islands ($N_e = 89$, 95% CI = 45–378) (Kashiwagi 2014). Despite the small breeding population, photo-ID monitoring over 23 years indicated demographic stability (Kashiwagi 2014). While this estimate is substantially lower than that reported here, the Yaeyama population occupies a small, isolated island habitat, which contrasts the expansive coastline of Mozambique. A more appropriate comparison for our findings would be to productive continental coastlines (Australia) or large, interconnected archipelagos (Indonesia, the Maldives) where interregional movement has been detected and the potential for genetic connectivity is higher.

The preservation of genetic diversity is a key objective in conservation management. Based on our findings, we recommend that the *M. alfredi* population in southern Mozambique be considered a single, stand-alone, management unit until a better understanding of population structure in the western Indian Ocean is obtained. Management measures should therefore be integrated along the entire southern coastline, as opposed to adopting local, site-specific approaches. Manta rays have one of the lowest known population growth rates for elasmobranchs, and populations have a diminished ability to recover from depletion (Dulvy et al. 2014). Declining census estimates at major aggregation areas (Venables 2020), combined with limited long-term evolutionary potential indicated by N_e and potential genetic isolation, call for immediate and effective management actions to address anthropogenic threats. Current regional protection for marine species, including manta rays, is limited to the Bazaruto Archipelago National Park and the Vilanculos Wildlife Sanctuary in the north of the Inhambane province. The rest of the coastline, including the high-usage areas of Praia do Tofo and Zàvora, is left open to unsustainable fishing practices. If protection is not extended to additional areas of critical habitat along this coast, *M. alfredi* could be reduced to a small, fragmented sub-population in the Bazaruto Archipelago. Similar to the vast population decline and range reduction observed for dugong (*Dugong dugon*) in Mozambique (Findlay et al. 2011). Spatial management planning should consider the development of a protected area network, as well as restrictions on the use of indiscriminate fishing gears, such as gill nets (see Venables et al. 2020 for more detail), in order to maintain the genetic diversity of this population and prevent localized extinction.

Data availability

Data are available in the DRYAD digital repository: https://doi.org/10.5061/dryad.m37pvmd0j.

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