Comparative population genomics of manta rays has global implications for management

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Abstract

Understanding population connectivity and genetic diversity is of fundamental importance to conservation. However, in globally threatened marine megafauna, challenges remain due to their elusive nature and wide-ranging distributions. As overexploitation continues to threaten biodiversity across the globe, such knowledge gaps compromise both the suitability and effectiveness of management actions. Here, we use a comparative framework to investigate genetic differentiation and diversity of manta rays, one of the most iconic yet vulnerable groups of elasmobranchs on the planet. Despite their recent divergence, we show how oceanic manta rays *(Mobula birostris)* display significantly higher genetic diversity than reef manta rays *(Mobula alfredi)* and that *M. birostris* populations display higher connectivity worldwide. Through reconstructing modes of colonisation, we reveal how both contemporary and historical forces have likely influenced these patterns, with important implications for population management. Our findings highlight the potential for fisheries to disrupt population dynamics at both local and global scales and therefore have direct relevance for international marine conservation.

Teaser

Population genomics of manta rays reveals striking differences in differentiation and diversity between two recently diverged species.

Main text

Introduction

Understanding the extent to which populations are connected is key to exploring population dynamics, predicting extinction risk and informing conservation management (*1*–*3*). In species with isolated populations characterised by limited dispersal, the risk of extirpation from local depletion is high (*4*). In such cases, local and regional scale management will be most appropriate for preventing and reversing population declines (*5*). In contrast, species with high rates of gene flow are potentially demographically and genetically more resilient to extrinsic factors (*3*, *6*). However, in order to maintain connectivity and mitigate genetic diversity loss in these taxa, management measures must be coordinated and encompass migratory corridors. As overexploitation and habitat destruction threaten to disrupt population dynamics at a global scale, characterising genetic variation and connectivity has become more important than ever before (*7*–*9*).

In widely distributed marine species with high dispersal potential, genetic differentiation is often found to be subtle or non-existent (*5*, *10*, *11*). Such patterns can arise from a range of mechanisms – from high contemporary gene flow through to recent divergence of historically large populations (*5*, *12*, *13*) – and can therefore be difficult to interpret. The latter scenario reflects a disconnect between demographic and genetic connectivity and has important implications for species resilience (*3*, *11*, *14*). This is because populations that appear genetically connected may not operate as single demographic units, making them more vulnerable to overexploitation. High-resolution SNP datasets go some way to addressing this problem by providing greater power to detect subtle differences at both neutral and adaptive loci (*15*, *16*). However, since population genetic differentiation can be affected by past, as well as contemporary patterns, parallel inference of historical relationships and genetic diversity can allow the relative contribution of historical processes to be explicitly evaluated (*17*–*19*). Furthermore, when carried out within a comparative framework, such an approach can provide powerful insights into the drivers of population divergence and therefore improve recommendations for conservation management (*20*).

Manta rays are large, mobile elasmobranchs inhabiting tropical and sub-tropical oceans (*21*) (Figure 1A, C) and provide an excellent opportunity to evaluate the genomic consequences of historical and contemporary population processes within a comparative framework. They comprise two described species estimated to have diverged less than 0.5 Mya as a result of

distinct habitat preferences (*22*). The reef manta ray (*Mobula alfredi)* frequents near-shore tropical reef environments, such as coral atolls and barrier reefs (Kashiwagi *et al.* 2011), with a high degree of residency (Deakos *et al.* 2011; Jaine *et al.* 2014; Braun *et al.* 2015; Setyawan *et al.* 2018; Peel *et al.* 2019; Knochel *et al.* 2022b; Germanov *et al.* 2022). In contrast, the oceanic manta ray (*Mobula birostris)* can also be found ranging into sub-tropical habitats along continental coastlines and at oceanic islands, often adjacent to productive deep-water upwellings areas (Kashiwagi *et al.* 2011; Andrzejaczek *et al.* 2021). As a result of these differences in habitat use, *M. alfredi* and *M. birostris* have long been considered to display marked differences in their migratory abilities and levels of gene flow. Yet, only a handful of long-distance movements have ever been recorded in *M. birostris* (*23*, *24*) alongside observations of site-fidelity (*25–27*), raising questions about the extent to which population structure and genetic diversity may differ across species. To date, assessments of genetic differentiation in *M. alfredi* have focussed on local and regional patterns (*28–30*) and we have little understanding of how genetic diversity is distributed across the species' range. In, *M. birostris*, the situation is even less clear, with studies reporting both widespread connectivity and population differentiation (*31–33*). Critically, these differences and uncertainties exist against a background of ongoing global exploitation and uncertain implications for management.

Targeted and incidental fisheries, driven in part by increasing demand for mobulid gill plates (*21*, *34*), have led to widespread population declines in manta rays (*35–39*). Currently, both species are managed through a patchwork of local, regional and international measures with varying levels of implementation and enforcement (*40–42*). To determine the appropriateness of management measures and assess population vulnerability, a global assessment of management units is urgently required (*40*, *43*). Here, we undertake a comparative genomic analysis of manta ray populations from across their global distribution to investigate connectivity, genetic diversity, and historical relationships with an aim to guide effective fisheries management.

Results

We generated double digest restriction-site associated DNA (ddRAD) sequence data for 173 individuals from 12 locations to represent the global distribution of each species (Figure 1A, C). For *M. alfredi* (*n* = 91), these comprised the Chagos Archipelago (n = 5), the Maldives (n = 36), Seychelles (n = 22), Australia Pacific (n = 3), Fiji (n = 8) and Hawaii (n = 17). For *M. birostris* (*n* = 82), these comprised Sri Lanka (n = 37), the Philippines (n = 22), South Africa (n

= 3), Mexico Caribbean (n = 4), Mexico Pacific (n = 9) and Peru (n = 7). Quality controlled sequencing reads were *de novo* assembled using the STACKS v2.64 pipeline and a total of 15,312 high-quality SNPs were called across both species. See Materials and Methods and Supplementary Information for details.

Contrasting patterns of population structure at a global scale

To investigate population differentiation within each species we used four complementary approaches: discriminant analysis of principal components (DAPC), admixture, pairwise F_{ST} and isolation by distance analysis. In *M. alfredi*, all methods supported the presence of strong population structure at both global and regional scales. Populations inhabiting different ocean basins displayed the highest degree of differentiation in the DAPC, with Pacific and Indian Ocean populations forming distinct clusters along PC1 (Figure 1B). Regional differentiation was also detected, with Seychelles, Chagos and the Maldives clustering apart along PC2, and Hawaii separating from Australia Pacific and Fiji along PC3 (Figure 1B and Figure S1A). These patterns were reinforced in the admixture analysis which highlighted two major ancestral source populations, inferred an optimal value of $K = 4$ and resolved hierarchical structure up to $K = 7$ (Figure S2 and Figure S3A). Pairwise F_{ST} estimates between ocean basins were on average over two times higher than those within (mean pairwise F_{ST} between ocean basins = 0.30, mean pairwise F_{ST} within ocean basins = 0.13, Figure 2A) yet all population comparisons were found to be significant (Figure S4A, mean = 0.23, min = 0.08, max = 0.43). Finally, we detected a significant relationship between pairwise F_{ST} and geographic distance (Mantel's $r = 0.84$, $P = 0.02$) indicating a strong effect of isolation by distance (Figure 2B).

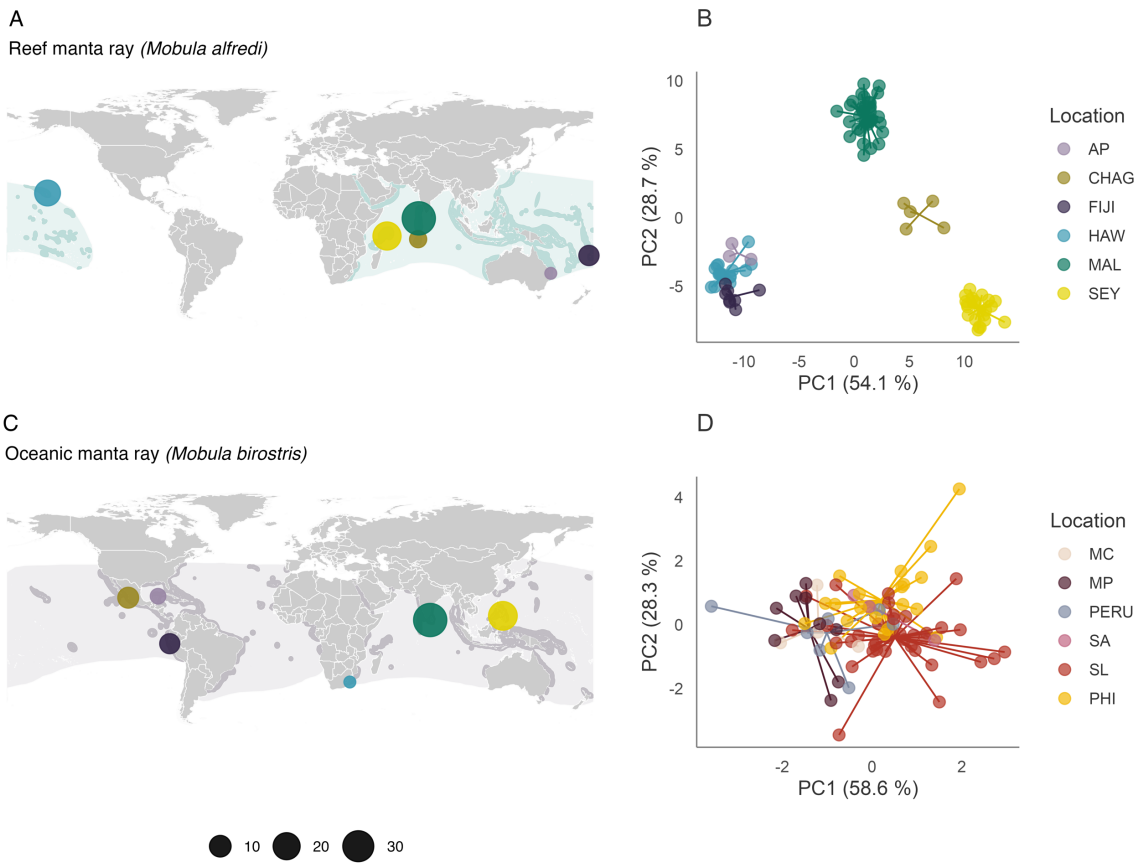

Figure 1. Contrasting patterns of population structure in manta rays. (A, C) Geographic distributions of (A) *Mobula alfredi* and (C) *Mobula birostris* visualised together with the locations of samples used in this study. Dark shaded distributions denote the confirmed species range and light shaded distributions denote the expected species range. Sampling location points are distinguished by colour and scaled by the number of samples. Further details are provided in the Supplementary Material. (B, D) Scatterplots showing individual variation in principal components (PC) one and two derived from discriminant analysis of principal components analysis for (B) *M. alfredi* and (D) *M. birostris* individuals. The amount of variance explained by each PC is shown in parentheses. Population abbreviations: AP = Australia Pacific, CHAG = Chagos, FIJI = Fiji, HAW = Hawaii, MAL = Maldives, SEY = Seychelles, MC = Mexico Caribbean, MP = Mexico Pacific, PERU = Peru, SA = South Africa, SL = Sri Lanka and PHI = the Philippines.

In stark contrast, *M. birostris* displayed little evidence for strong population structure across all methods. Individuals from different ocean basins clustered closely together along each axis in the DAPC (Figure 1D and Figure S1B). Admixture identified $K = 1$ as the optimal number of clusters, with increasing values of K merely introducing additional mixing (Figure S2 and Figure S3B). Pairwise F_{ST} estimates were two-fold lower than in *M. alfredi*, with no pairwise comparison falling above 0.007 (mean = 0.002, min = -0.005, max = 0.006, Figure 2C). Nevertheless, despite these broad patterns, several lines of evidence indicate the presence

of subtle geographic differentiation in this species. First, individuals from Mexico Pacific, Peru, and Mexico Caribbean clustered apart from those sampled in South Africa, Sri Lanka, and the Philippines along PC1 (Figure 1D). Second, despite pairwise F_{ST} estimates being low, comparisons between Eastern-Pacific and Indo-Pacific populations, and between Sri Lanka and the Philippines were statistically significant (Figure S4B). Small F_{ST} values are expected when minor allele frequencies are low and therefore do not necessarily reflect an absence of differentiation (*44*). Finally, while no significant relationship was observed between pairwise F_{ST} and geographic distance (Mantel's $r = 0.45$, $P = 0.10$), there was a tendency for populations separated by greater distances to display higher differentiation (Figure 2D).

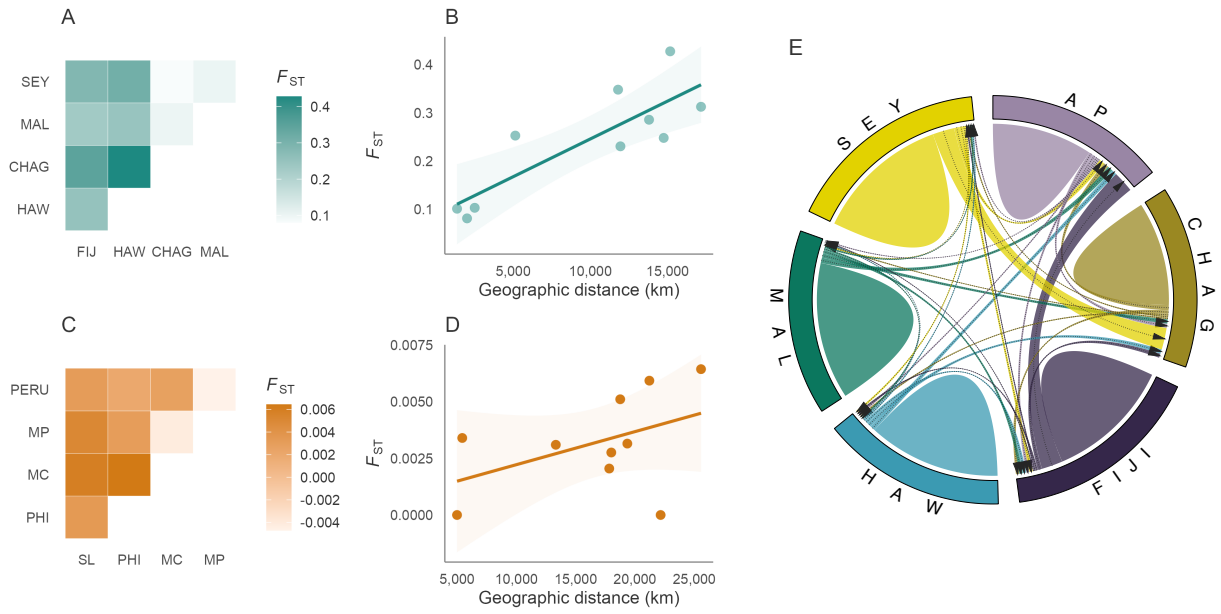

Figure 2. Population genetic differentiation, isolation by distance and contemporary migration in manta rays. (A, C) Pairwise F_{ST} estimates between sampling locations for (A) *M. alfredi* and (C) *M. birostris*. Samples from Australia Pacific and South Africa were excluded from this analysis due to low sample sizes. (B, D) Relationship between genetic (F_{ST}) and geographic distance as calculated by least-cost path analysis for all pairwise population comparisons in (B) *M. alfredi* and (D) *M. birostris*. Solid lines and shaded areas reflect the regression slopes and standard errors respectively. (E) Contemporary gene flow estimates between populations of *M. alfredi*. The direction of each arrow represents the direction of gene flow, and the width of each ribbon reflects the relative amount of gene flow.

Contemporary gene flow

To characterise the strength and direction of gene flow between populations we used the program BA3-SNPs (*45*) to estimate recent migration. As this method assumes low levels of connectivity and imposes an upper-bound on the proportion of non-migrants in a population,

we restricted the analysis to *M. alfredi* (see Materials and Methods for details). As expected, contemporary gene flow was low (Figure 2E); the average migration rate between populations, measured as the estimated number of migrants per generation, was 0.029 (min = 0.008, max = 0.15), with this figure falling to 0.018 (min = 0.008 , max = 0.03) when considering gene flow between populations in different ocean basins. Migration into both Hawaii and the Maldives was lowest, indicating these populations are the most isolated of those sampled (Table S1). Migration rates were only deemed significant between Seychelles and Chagos (0.15) and between Fiji and Australia Pacific (0.15), in line with these populations being last to separate in the admixture analysis. These patterns highlight that while *M. alfredi* have the propensity to travel over large distances, restricted movement likely dominates.

Historical relationships among populations

To place patterns of genetic differentiation into a historical context, we investigated population origins and colonisation patterns using TreeMix (*46*). This program uses allele frequency data to infer patterns of population splits and admixture events through the construction of a maximum likelihood tree. In *M. alfredi*, internal branch lengths were relatively long, with an initial split clearly separating populations in the Indian and Pacific Oceans (Figure 3A). The Maldives and Australia Pacific were the first to separate within each locality and displayed the lowest levels of genetic drift overall. Hawaii was among the last populations to split and displayed the highest amount of drift, in line with its geographic isolation. The best supported model inferred two migration events (Figure S5A); one from the *M. alfredi* population in Seychelles into *M. birostris*, and one from Chagos into Hawaii. However, because not all geographic regions are represented in our data set, the true sources and sinks of these admixture events may originate from related ghost populations. In contrast to *M. alfredi*, the addition of migration events led to no substantial improvement in the model for *M. birostris* (Figure S5B) and therefore the tree without migration is presented here. Interestingly, internal branch lengths were considerably shorter in *M. birostris*, indicating rapid radiation from a shared ancestral source population (Figure 3B). External branch lengths were also short, consistent with larger populations displaying marginal drift and low divergence. Nevertheless, despite these patterns, geographic signal could be detected in the *M. birostris* tree, with populations from the Eastern Pacific and the Caribbean (Peru, Mexico Caribbean, and Mexico Pacific) grouping separately from those in the Atlantic and Indo-Pacific (South Africa, the Philippines, and Sri Lanka).

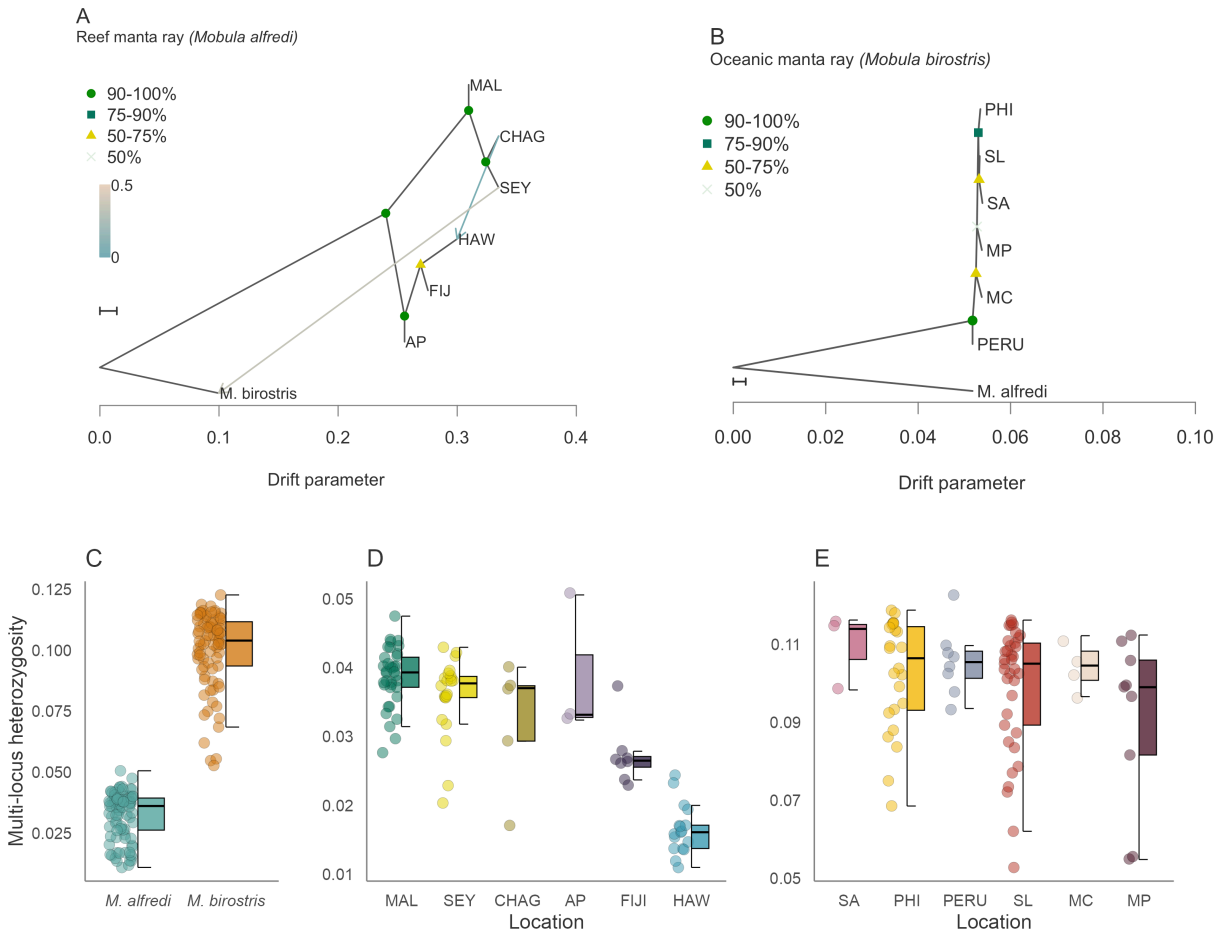

Figure 3. Historical relationships and genetic diversity in manta rays. (A–B) TreeMix maximum likelihood consensus tree displaying the historical relationships among (A) *M. alfredi* and (B) *M. birostris* populations. Horizontal branch lengths reflect the amount of genetic drift that has occurred along each branch. Bootstrap support values for each node are indicated. Migration edges inferred using TreeMix are represented as arrows and coloured according to their migration weight. The scale bar reflects 10 times the average standard error of the entries in the sample covariance matrix. (C–E) Variation in individual multi-locus heterozygosity between (C) species and among populations of (D) *M. alfredi* and (E) *M. birostris*. Centre lines of boxplots reflect the median, bounds of the boxes extend from the first to the third quartiles, and upper and lower whiskers reflect the largest and smallest values but no further than 1.5 * the interquartile range from the hinge.

Heterozygosity landscape across species and populations

To explore how patterns of population structure and colonisation are associated with genome-wide variation, we compared individual multi-locus heterozygosity between species and among populations. Strikingly, heterozygosity was on average over three times higher in *M. birostris* (mean = 0.10, min = 0.053, max = 0.12) than in *M. alfredi* (mean = 0.03, min = 0.01, max = 0.051), with every individual displaying a higher value than any *M. alfredi* (ß = 0.07, 95% CI = 0.06–0.07, $P = <2.2 \times 10^{-16}$, Figure 3C). This finding is in line with the patterns of

population structure and historical splits we observed in each species. Variation in genetic diversity was also observed at a population level (Figure 3D–E). In *M. alfredi*, the Maldives and Australia Pacific had the highest levels of heterozygosity within each ocean basin, in line with these populations being first to split in the TreeMix analysis. Indian Ocean populations displayed higher overall levels of diversity than Pacific populations and while a weak negative trend in variation was observed, mean values were overall similar. A steeper decline in diversity was observed in the Pacific populations, with heterozygosity in Hawaiian individuals being around half that of the Australian animals (Figure 3D), in line with this population being last to split in the TreeMix analysis and displaying the highest amount of drift. In contrast, *M. birostris* populations displayed less extreme variation in heterozygosity, with mean values differing by less than 0.02 (Figure 3E). Furthermore, variance within populations was an order of magnitude greater in *M. birostris* than in *M. alfredi*, and was particularly large in Sri Lanka, the Philippines, and Mexico Pacific populations.

Discussion

Manta rays are iconic megafauna with cultural and socio-economic significance. Due to targeted and bycatch fisheries operating across their broad-ranging distributions, populations are declining worldwide. Elucidating levels of connectivity and genetic variation remains a crucial priority for conservation management. We use reduced-representation sequencing on a global set of samples and reveal striking differences in the population genetic landscape of two recently diverged manta ray species. By considering the influence of both contemporary and historical processes, our study takes a precautionary approach to assessing conservation units, with important implications for management.

We first demonstrate the presence of strong genetic differentiation in *M. alfredi* at a global and regional scale. From a total of six sampling locations, we found evidence for at least five genetically distinct, and by extension, demographically independent populations. Two of these were separated by a distance of ~1,200 km, which is close to the maximum recorded movement in the species (*47*, *48*), indicating that long distance migrations are likely rare. Indeed, contemporary gene flow was low – especially between geographically distant locations – with only a small proportion of individuals in any population being identified as first- or second-generation migrants. Furthermore, when gene flow was observed, it tended to be unidirectional. These results are in line with recent studies demonstrating population differentiation between Western Australia and Mozambique (*28*) and between Eastern Australia and New Caledonia (*29*), together highlighting how large ocean basins form

significant barriers to dispersal in coastal elasmobranchs (*49*). High site-fidelity has been widely reported in *M. alfredi* based on tagging and mark-recapture studies (*47*, *50–55*). However, the degree of residency has been shown to vary, with movements rarely exceeding a few hundred kilometres in some locations (*50–52*, *54*, *56*) yet reaching over 1,000 km in others (*48*, *57*). Our study presents a comparatively broad-scale analysis relevant for regional and global management planning. Further work on local patterns of population structure will shed light on the nuances and drivers of fine-scale movement patterns in this species (*30*).

To explore the mechanism by which manta rays colonised their distribution, we reconstructed historical relationships and assessed levels of heterozygosity. In *M. alfredi*, we found strong evidence for an initial split between the Indian and Pacific Oceans, followed by range expansion within each. Population splits were associated with increasing genetic drift and reduced heterozygosity, particularly in the Pacific, indicating that *M. alfredi* underwent a stepping-stone pattern of colonisation involving opportunistic long-range movements and associated founder events. This is consistent with a recent observation of a pregnant *M. alfredi* individual at Cocos Island, Costa Rica (*58*), almost 6,000 km east of the nearest confirmed sighting, and the first record of *M. alfredi* in the Eastern Pacific. Range expansion inherently impacts genetic diversity, with a stepping-stone model of colonisation predicted to result in the strongest cumulative effect of founder events (*59*). Among our sampled populations, Hawaii is the most geographically isolated, situated at the edge of the *M. alfredi* distribution. Interestingly, not only was Hawaii the most genetically differentiated from all populations in our study, but it displayed the longest external branch lengths in the TreeMix analysis and the lowest levels of heterozygosity. Genetic variation is fundamental for enabling populations to adapt in response to selection (*9*, *60*, *61*). Our findings therefore expose how isolated *M. alfredi* populations at the periphery of their distribution may be intrinsically more vulnerable to changing environmental conditions and the genetic impacts of population decline.

In stark contrast to the patterns observed in *M. alfredi*, *M. birostris* displayed markedly higher levels of heterozygosity and with only subtle genetic differentiation across ocean basins. Weak population structure is common in highly mobile marine species (*62–64*), yet warrants careful interpretation, particularly considering management recommendations (*65*). On the one hand, these findings may be an indication of high contemporary gene flow and low natal philopatry, in line with the species' occurrence at remote oceanic islands, tendency to range into sub-tropical habitats and lower overall re-sight rates than *M. alfredi* (*66–68*). To date, our understanding of the movement behaviour in *M. birostris* has largely been based on coastal

aggregations of adult individuals over relatively short timeframes (*32*, *67*, *69*, *70*). Such studies have a tendency to capture seasonal migrations as opposed to dispersal events and may explain why very few long-distance (~1,000 km) movements have been recorded in the species (*23*, *24*). Indeed, with only a few migrants per generation required to obscure strong population structure when N_e is large (*71*), it is possible that the patterns we observe translate to infrequent dispersal events. Furthermore, dispersal could be segregated by age and/or sex (*72*, *73*), and may vary among individuals (*74–76*). While challenging, there is benefit in extending future tagging efforts to transient individuals away from known aggregation sites (*27*), as well as previously underrepresented age classes – such as juveniles – to capture what may be infrequent yet evolutionarily relevant movements.

An alternative explanation for the patterns we observe in *M. birostris* is that insufficient time has elapsed to reliably identify recent genetic divergence among localities. In contrast to *M. alfredi*, our TreeMix analysis indicated that *M. birostris* rapidly radiated from a large ancestral source, with only marginal genetic drift occurring between regions. This was further evidenced by substantially higher levels of genetic variation that differed little across sampling locations. In addition, little differentiation was observed between Mexico Pacific and Mexico Caribbean, two regions that have been geographically separated since the emergence of the Isthmus of Panama. These findings are consistent with a recent mark-resight analysis that estimated the population of *M. birostris* in coastal Ecuador to number at least 22,000 individuals (*67*). Large effective population sizes and high genetic variation increase the time taken for populations to diverge due to genetic drift (*14*, *71*, *77*). This is further compounded in species with long and overlapping generations (*78*) as is the case for manta rays (*79*). Taken together, genetic similarities among *M. birostris* localities may be partially confounded by recent shared ancestry and large effective population size.

On the basis of these considerations, we propose that a combination of large historical population size and contemporary gene flow have contributed to the comparatively high levels of diversity and genetic homogeneity in *M. birostris*. The subtle population differentiation we observe between the Indian Ocean, South-East Asia and the Eastern Pacific is likely best explained by the geographic limits of dispersal as opposed to complete geographic isolation. Yet, unlike in *M. alfredi* where genetic clusters almost certainly reflect discrete demographic units relevant for conservation management, the extent to which genetic connectivity in *M. birostris* reflects demographic connectivity is less clear. For example, in extreme cases, the number of migrants required to eliminate signals of population structure will not be enough to

322 demographically link populations, and more importantly, replenish those that have been
323 depleted (*11*). Interestingly, while re-sight rates are typically lower in *M. birostris* than *M.*
324 *alfredi*, demographic independence has been implicated in several mark-recapture studies
325 where re-sightings follow predictable patterns (*26*, *70*). Furthermore, a population genetic
326 analysis based on F_{ST} outliers uncovered allele frequency differences between two Mexican
327 locations and Sri Lanka (*32*), suggesting recent divergence against a background of ongoing
328 gene flow. Taken together, we highlight the potential for further work investigating adaptive
329 divergence between *M. birostris* populations and emphasise the need to combine molecular
330 measures of connectivity with empirical demographic data in this species (*3*, *65*, *80*).

331 **Conservation implications**

332 The remarkable differences we observe in the population genetics of manta rays directly
333 inform likely response to continued exploitation and respective conservation measures. At
334 present, *M. alfredi* is among the most protected mobulid species worldwide, with some
335 management frameworks in place at local, national, and international levels (*40*, *81*). Our
336 findings of global population structure underline how local initiatives recognising populations
337 as distinct management units will be most appropriate for this species. However, we also
338 demonstrate the consequence of geographic isolation on genetic variation and reveal how *M.*
339 *alfredi* likely faces a greater risk from local depletion. This is especially true for populations at
340 the edge of the species range and in regions with high coastal fishing pressure. Prioritising
341 these populations in conservation action plans and maintaining local connectivity will therefore
342 be crucial for boosting resilience and preventing local extinction in this vulnerable species.

343 The implications of our findings for *M. birostris* are more nuanced. Despite detecting only
344 subtle population genetic differentiation, we cannot rule out the possibility that historical
345 processes and large effective population size are obscuring a higher degree of contemporary
346 demographic separation. Together with studies reporting high site-fidelity and restricted
347 movement patterns, our findings strongly suggests that local and national management action
348 should be considered essential for protecting resident aggregations of *M. birostris*.
349 Nevertheless, we expect that weak population structure and high genetic variation are
350 simultaneously being driven by some degree of contemporary dispersal. Consequently, any
351 fishing activity taking place along migratory corridors threatens to disrupt a mode of gene flow
352 that is likely fundamental for long-term resilience of the species. Similarly, although we have
353 limited understanding of the number and distribution of breeding and nursery grounds (*24*, *82*,
354 *83*), significant reduction of local stocks may impact long-term recruitment at oceanic and even

355 global scales. We therefore emphasise the escalating need to improve the implementation of
356 regional and international measures that seek to protect taxa in the high seas. Together with
357 local scale management, appropriate evidence-based actions will contribute to maintaining
358 large, connected and genetically diverse populations of manta rays into the future.

Materials and Methods

Sample collection

Tissue samples were opportunistically collected from 12 geographic locations to represent the global distribution of each species (Figure 1A, C). For *M. alfredi* (total *n* = 119), these originated from the Chagos Archipelago (n = 5), the Maldives (n = 48), Seychelles (n = 23), Australia Pacific (n = 4), Fiji (n = 9) and Hawaii (n = 30). For *M. birostris* (total *n* = 111), these originated from Sri Lanka (n = 43), the Philippines (n = 36), South Africa (n = 3), Mexico Caribbean (n = 4), Mexico Pacific (n = 13) and Peru (n = 12). Samples from Mexico Caribbean, where a third putative manta ray species occurs in sympatry (*Mobula cf. birostris,* Hinojosa-Alvarez *et al.* 2016; Hosegood *et al.* 2020), were visually and genetically confirmed as *M. birostris*. For both species, samples were collected from a combination of live animals and fisheries specimens (See Supplementary Files for further information).

DNA extraction and ddRAD sequencing

Genomic DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit and quantified using a Qubit 3.0 Broad Range Assay. Double digest restriction-site associated DNA (ddRAD) libraries were prepared following the Peterson *et al.* (2012) protocol with modifications described in Palaiokostas *et al.* (2015) and were 125 bp paired-end sequenced on an Illumina HiSeq. Sequencing reads from both species were assessed for quality using FastQC and processed together using the Stacks v2.54 pipeline (*87*). The three main assembly parameters were chosen following the optimisation procedure outlined in Rochette and Catchen (2017) (Figure S7). Initial quality filters were applied to the resulting genotypes before generating three high-quality datasets for use in downstream analysis: two species-specific datasets; and one dataset comprising both species. For the species-specific datasets, we extracted either *M. birostris* or *M. alfredi* individuals, removed individuals with high relatedness coefficients (*89*, *90*) and filtered out SNPs with a minor allele count of less than 3, a genotyping rate less than 90% and that were in linkage disequilibrium using PLINK. This left a total of 1,553 SNPs in 91 *M. alfredi* individuals, and 6,278 SNPs in 82 *M. birostris* individuals. For the dataset comprising both species, we first removed closely related individuals and then filtered out SNPs with a minor allele count of less than 3 and a genotyping rate less than 90%. This left a total of 15,312 SNPs called in 91 *M. alfredi* and 82 *M. birostris* individuals. See Supplementary Material for further information on library preparation, read processing and SNP and individual filtering.

Population structure

To investigate population structure we used the species-specific datasets and three complementary approaches. First, we carried out a discriminant analysis of principal components (DAPC) using the R package adegenet (*91*). This approach initially transforms the SNP data using a principal components analysis (PCA) and then performs a discriminant analysis on the retained PCs. This serves to maximise discrimination of individuals between groups while minimising variation within (*92*). Following the recommendations outlined in Thia (2023), the number of PCs retained as predictors was determined based on the *K–1* criterion, where *K* is equal to the number of effective populations. For *M. alfredi*, this was set to 5, under the assumption that each sample site reflects a separate population. For *M. birostris*, this was set to 4 under the assumption that Mexico Pacific and Peru may represent a single population given their close geographic proximity. Second, we estimated admixture proportions for the individuals in each dataset using ADMIXTURE. Admixture runs were performed for ancestry clusters ranging from *K* = 1–8, with 10 runs for each *K*. The optimal *K* was identified based on the lowest cross-validation error. The runs with the highest likelihood were visualised. Third, we estimated pairwise genetic differentiation between populations within each species using the Weir and Cockerham F_{ST} value (*94*) calculated in the R package dartR (*95*). Confidence intervals and *p*-values were estimated based on bootstrap resampling of individuals within each population 1000 times. *Mobula alfredi* samples from Australia Pacific and *M. birostris* samples from South Africa were excluded from this analysis due to low sample sizes.

Isolation by distance

To investigate patterns of isolation by distance, we examined the relationship between genetic and geographic distance between all pairs of populations in each species. Genetic distances were based on pairwise F_{ST} estimates calculated above. Geographic distances were determined based on a least-cost path analysis implemented using the R package marmap (*96*) with a minimum depth constraint of -10 metres in order to prevent paths overland. The significance of associations between genetic and geographic distance matrices was inferred using Mantel tests with the R package ade4 (*97*) .

Contemporary gene flow

To infer the strength and directionality of contemporary gene flow between populations we used the program BA3-SNPs BayesAss v1.1 (*45*) which estimates the proportion of immigrants in a given population using Bayesian inference. This analysis was restricted to *M. alfredi* as it assumes low levels of connectivity and imposes an upper-bound on the proportion of non-migrants in a population. We first performed initial runs of BayesAss to determine optimal mixing parameters (dM = migration rate, dA = allele frequency and dF = inbreeding

coefficient) using the autotune function in BA3-SNPs. We than ran BayesAss-3 with 10,000,000 iterations, a burn-in of 1,000,000 and a sampling interval of 1000. Mixing parameters were set to dM = 0.21, dA = 0.44 and dF = 0.08. Results were averaged across five replicate runs and migration rates were considered significant if 95% credible sets (mean migration rate ± 1.96 x mean standard deviation) did not overlap zero. Chain convergence was assessed, and migration rates visualised using R (Figure S9).

Historical relationships among populations

To explore historical relationships among populations of *M. alfredi* and *M. birostris* we used the program TreeMix (*46*). TreeMix uses population allele frequencies to estimate a bifurcating maximum likelihood tree with which to infer historical population splits, admixture events and the degree of genetic drift. We first supplemented the *M. alfredi* dataset with one randomly selected *M. birostris* individual and the *M. birostris* dataset with one randomly selected *M. alfredi* to act as outgroups when rooting the trees. Both datasets were then filtered for linkage, a minor allele count of less than 3, genotyping rate of less than 90% and related individuals using PLINK v1.9 (*98*). Allele frequencies for each population were then calculated using the –freq and –within arguments in PLINK. For both the *M. birostris* and *M. alfredi* datasets we then performed 10 initial runs of TreeMix for each migration event (M) ranging from 0 to 10. The number of migration edges that explained 99.8% of the variance was selected as the best model for each species (*M. birostris:* M= 0; *M. alfredi:* M = 2, Figure S5). We then re-ran TreeMix 100 times using the optimal number of migration edges. Consensus trees and bootstrap values were estimated and visualised using code modified from the BITE R package (*99*).

Genome-wide heterozygosity

To assess levels of genetic diversity both within and between species we used the high-quality SNP dataset comprising both species. Multi-locus heterozygosity was then calculated for each individual using the R package inbreedR (*100*).

Acknowledgements

We are grateful to the following people and organisations for their support sourcing and collecting tissue samples: Kerstin Forsberg, Jon Slayer, John Nevill, Dan Bowling, Heather Pacey, Karen Fuentes, Manta Caribbean Project, Annie Murray, Tam Sawers, Beth Taylor, Adam Thol'hath, Isabel Ender, Musa Mohamed, the Barefoot Collection, Four Seasons Resorts Maldives, Six Senses Laamu and all Manta Trust and LAMAVE staff, volunteers and field team. We are grateful to Helen Senn and Jennifer Kaden from the Royal Zoological

Society of Scotland for support during library preparation. ddRAD sequencing was carried out by Edinburgh Genomics. Tissue sample collection in Seychelles was approved by the Seychelles Bureau of Standards, the Seychelles Ministry of Environment, Energy and Climate Change, and The University of Western Australia (RA/3/100/1480). Blue Resources Trust (BRT) would like to thank the Department of Wildlife Conservation and the Department of Fisheries and Aquatic Resources for enabling the fieldwork to be conducted in Sri Lanka. Sample collection in the Maldives was made possible thanks to the permission and support provided by the Ministry of Fisheries and Agriculture ([OTHR]30-D/INDIV/ 2016/220) and the Ministry of Environment and Energy (EPA/2016/PSR-M02). Sampling in Mexico Caribbean was made possible thanks to CONAPESCA (PPF/DGOPA-126/16 and PPF/DGOPA/315717) and SEMARNAT (SGPA/DGVS/07618/15 and SGPA/DGVS/10067/17). We would like to acknowledge Richard Coleman for helpful comments on the manuscript. For the purpose of open access, the author has applied a Creative Commons Attribution (CC BY) licence to any Author Accepted Manuscript version arising from this submission.

Funding

We are grateful to the Save Our Seas Foundation and The People's Trust for Endangered Species who provided funding for this work. JH was supported by a Natural Environment Research Council CASE studentship through the ENVISION DTP (case partner: Royal Zoological Society of Scotland) and received additional grants from the Fisheries Society of the British Isles and the Genetics Society. Fieldwork in Seychelles was supported by the SOSF-D'Arros Research Centre. RB was funded by the Save Our Seas Foundation and the Marine Conservation Action Fund of the New England Aquarium. BRT acknowledges the generous support provided by the Save Our Seas Foundation and the Marine Conservation and Action Fund (MCAF) for field work and sample collection.

Author contributions

EH, JH, GC, MdB, SC, GMWS and RO conceived and designed the study. GMWS, AA, RB, MD, DF, NF, LRP, SP, AP, JDS and SW provided samples. JH and JK carried out laboratory work. EH analysed the data with input from JH. EH wrote the paper with input from all other co-authors.

Competing interests

The authors declare no competing interests.

Data and materials availability

Sequencing data will be available on the European Nucleotide Archive and scripts will be available on GitHub.

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