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

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

97 Targeted and incidental fisheries, driven in part by increasing demand for mobulid gill plates
98 (*21*, *34*), have led to widespread population declines in manta rays (*35–39*). Currently, both
99 species are managed through a patchwork of local, regional and international measures with
100 varying levels of implementation and enforcement (*40–42*). To determine the appropriateness
101 of management measures and assess population vulnerability, a global assessment of
102 management units is urgently required (*40*, *43*). Here, we undertake a comparative genomic
103 analysis of manta ray populations from across their global distribution to investigate
104 connectivity, genetic diversity, and historical relationships with an aim to guide effective
105 fisheries management.

106 **Results**

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

112 = 3), Mexico Caribbean (n = 4), Mexico Pacific (n = 9) and Peru (n = 7). Quality controlled
113 sequencing reads were *de novo* assembled using the STACKS v2.64 pipeline and a total of
114 15,312 high-quality SNPs were called across both species. See Materials and Methods and
115 Supplementary Information for details.

116 **Contrasting patterns of population structure at a global scale**

117 To investigate population differentiation within each species we used four complementary
118 approaches: discriminant analysis of principal components (DAPC), admixture, pairwise F_{ST}
119 and isolation by distance analysis. In *M. alfredi*, all methods supported the presence of strong
120 population structure at both global and regional scales. Populations inhabiting different ocean
121 basins displayed the highest degree of differentiation in the DAPC, with Pacific and Indian
122 Ocean populations forming distinct clusters along PC1 (Figure 1B). Regional differentiation
123 was also detected, with Seychelles, Chagos and the Maldives clustering apart along PC2, and
124 Hawaii separating from Australia Pacific and Fiji along PC3 (Figure 1B and Figure S1A). These
125 patterns were reinforced in the admixture analysis which highlighted two major ancestral
126 source populations, inferred an optimal value of $K = 4$ and resolved hierarchical structure up
127 to $K = 7$ (Figure S2 and Figure S3A). Pairwise F_{ST} estimates between ocean basins were on
128 average over two times higher than those within (mean pairwise F_{ST} between ocean basins =
129 0.30, mean pairwise F_{ST} within ocean basins = 0.13, Figure 2A) yet all population comparisons
130 were found to be significant (Figure S4A, mean = 0.23, min = 0.08, max = 0.43). Finally, we
131 detected a significant relationship between pairwise F_{ST} and geographic distance (Mantel's r
132 = 0.84, $P = 0.02$) indicating a strong effect of isolation by distance (Figure 2B).

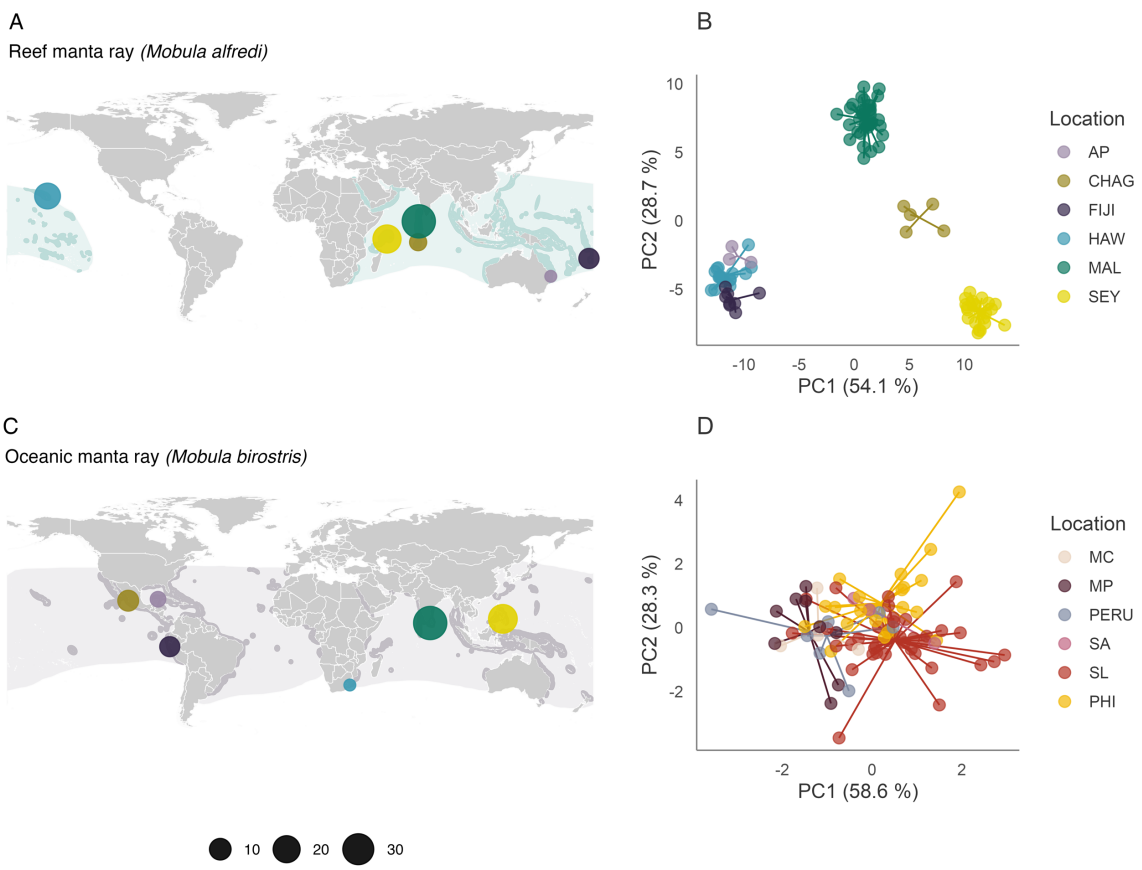
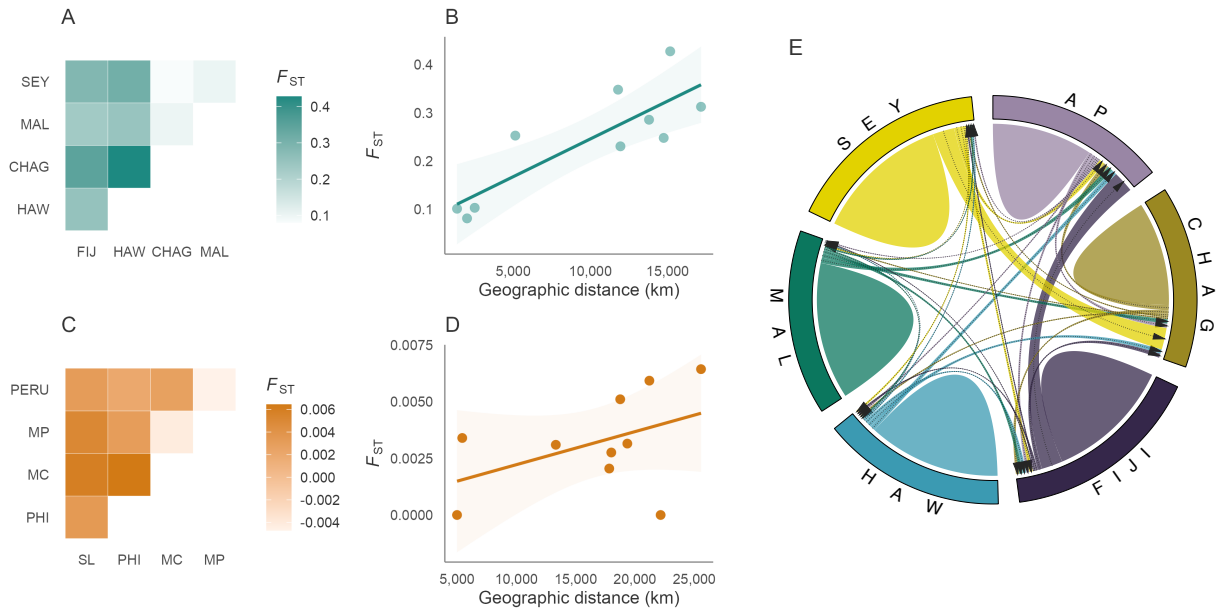


Figure 1. Contrasting patterns of population structure in manta rays. (A, C) Geographic distributions of (A) *Mobula alfredi* and (C) *Mobula birostris* visualised together with the locations of samples used in this study. Dark shaded distributions denote the confirmed species range and light shaded 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

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



161

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

170 **Contemporary gene flow**

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

174 we restricted the analysis to *M. alfredi* (see Materials and Methods for details). As expected,
175 contemporary gene flow was low (Figure 2E); the average migration rate between populations,
176 measured as the estimated number of migrants per generation, was 0.029 (min = 0.008, max
177 = 0.15), with this figure falling to 0.018 (min = 0.008 , max = 0.03) when considering gene flow
178 between populations in different ocean basins. Migration into both Hawaii and the Maldives
179 was lowest, indicating these populations are the most isolated of those sampled (Table S1).
180 Migration rates were only deemed significant between Seychelles and Chagos (0.15) and
181 between Fiji and Australia Pacific (0.15), in line with these populations being last to separate
182 in the admixture analysis. These patterns highlight that while *M. alfredi* have the propensity to
183 travel over large distances, restricted movement likely dominates.

184 **Historical relationships among populations**

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

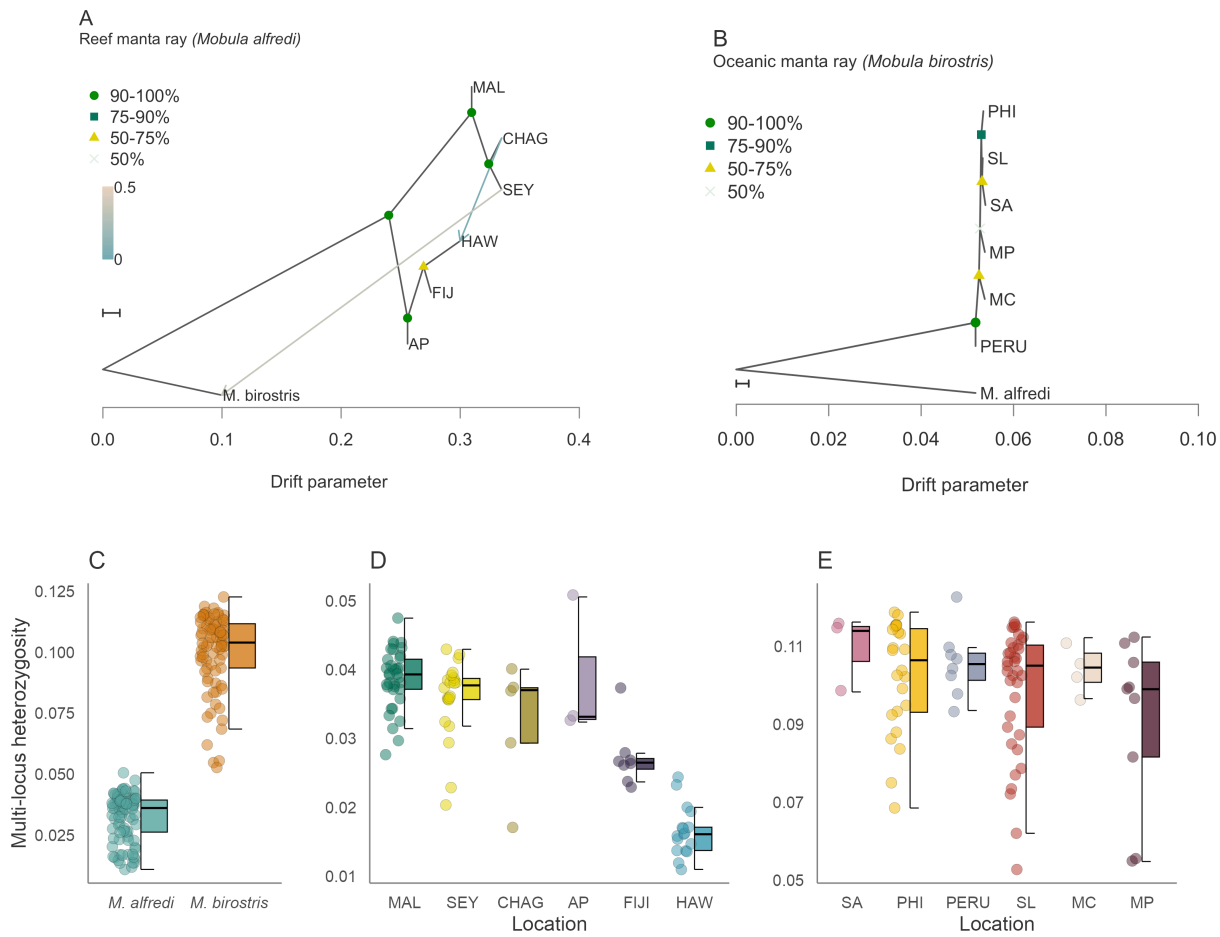


Figure 3. Historical relationships and genetic diversity in manta rays. (A–B) TreeMix maximum likelihood consensus tree displaying the historical relationships among (A) *M. alfredi* and (B) *M. birostris* populations. Horizontal branch lengths reflect the amount of genetic drift that has occurred along 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

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

236 **Discussion**

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

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

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

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

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

289 aggregations of adult individuals over relatively short timeframes (*32*, *67*, *69*, *70*). Such studies
290 have a tendency to capture seasonal migrations as opposed to dispersal events and may
291 explain why very few long-distance (~1,000 km) movements have been recorded in the
292 species (*23*, *24*). Indeed, with only a few migrants per generation required to obscure strong
293 population structure when N_e is large (*71*), it is possible that the patterns we observe translate
294 to infrequent dispersal events. Furthermore, dispersal could be segregated by age and/or sex
295 (*72*, *73*), and may vary among individuals (*74–76*). While challenging, there is benefit in
296 extending future tagging efforts to transient individuals away from known aggregation sites
297 (*27*), as well as previously underrepresented age classes – such as juveniles – to capture
298 what may be infrequent yet evolutionarily relevant movements.

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

313 On the basis of these considerations, we propose that a combination of large historical
314 population size and contemporary gene flow have contributed to the comparatively high levels
315 of diversity and genetic homogeneity in *M. birostris*. The subtle population differentiation we
316 observe between the Indian Ocean, South-East Asia and the Eastern Pacific is likely best
317 explained by the geographic limits of dispersal as opposed to complete geographic isolation.
318 Yet, unlike in *M. alfredi* where genetic clusters almost certainly reflect discrete demographic
319 units relevant for conservation management, the extent to which genetic connectivity in *M.*
320 *birostris* reflects demographic connectivity is less clear. For example, in extreme cases, the
321 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

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

359 **Materials and Methods**

360 **Sample collection**

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

371 **DNA extraction and ddRAD sequencing**

372 Genomic DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit and quantified
373 using a Qubit 3.0 Broad Range Assay. Double digest restriction-site associated DNA (ddRAD)
374 libraries were prepared following the Peterson *et al.* (2012) protocol with modifications
375 described in Palaiokostas *et al.* (2015) and were 125 bp paired-end sequenced on an Illumina
376 HiSeq. Sequencing reads from both species were assessed for quality using FastQC and
377 processed together using the Stacks v2.54 pipeline (*87*). The three main assembly parameters
378 were chosen following the optimisation procedure outlined in Rochette and Catchen (2017)
379 (Figure S7). Initial quality filters were applied to the resulting genotypes before generating
380 three high-quality datasets for use in downstream analysis: two species-specific datasets; and
381 one dataset comprising both species. For the species-specific datasets, we extracted either
382 *M. birostris* or *M. alfredi* individuals, removed individuals with high relatedness coefficients
383 (*89*, *90*) and filtered out SNPs with a minor allele count of less than 3, a genotyping rate less
384 than 90% and that were in linkage disequilibrium using PLINK. This left a total of 1,553 SNPs
385 in 91 *M. alfredi* individuals, and 6,278 SNPs in 82 *M. birostris* individuals. For the dataset
386 comprising both species, we first removed closely related individuals and then filtered out
387 SNPs with a minor allele count of less than 3 and a genotyping rate less than 90%. This left a
388 total of 15,312 SNPs called in 91 *M. alfredi* and 82 *M. birostris* individuals. See Supplementary
389 Material for further information on library preparation, read processing and SNP and individual
390 filtering.

391 **Population structure**

392 To investigate population structure we used the species-specific datasets and three
393 complementary approaches. First, we carried out a discriminant analysis of principal
394 components (DAPC) using the R package adegenet (*91*). This approach initially transforms
395 the SNP data using a principal components analysis (PCA) and then performs a discriminant
396 analysis on the retained PCs. This serves to maximise discrimination of individuals between
397 groups while minimising variation within (*92*). Following the recommendations outlined in Thia
398 (2023), the number of PCs retained as predictors was determined based on the *K–1* criterion,
399 where *K* is equal to the number of effective populations. For *M. alfredi*, this was set to 5, under
400 the assumption that each sample site reflects a separate population. For *M. birostris*, this was
401 set to 4 under the assumption that Mexico Pacific and Peru may represent a single population
402 given their close geographic proximity. Second, we estimated admixture proportions for the
403 individuals in each dataset using ADMIXTURE. Admixture runs were performed for ancestry
404 clusters ranging from *K* = 1–8, with 10 runs for each *K*. The optimal *K* was identified based on
405 the lowest cross-validation error. The runs with the highest likelihood were visualised. Third,
406 we estimated pairwise genetic differentiation between populations within each species using
407 the Weir and Cockerham F_{ST} value (*94*) calculated in the R package dartR (*95*). Confidence
408 intervals and *p*-values were estimated based on bootstrap resampling of individuals within
409 each population 1000 times. *Mobula alfredi* samples from Australia Pacific and *M. birostris*
410 samples from South Africa were excluded from this analysis due to low sample sizes.

411 **Isolation by distance**

412 To investigate patterns of isolation by distance, we examined the relationship between genetic
413 and geographic distance between all pairs of populations in each species. Genetic distances
414 were based on pairwise F_{ST} estimates calculated above. Geographic distances were
415 determined based on a least-cost path analysis implemented using the R package marmap
416 (*96*) with a minimum depth constraint of -10 metres in order to prevent paths overland. The
417 significance of associations between genetic and geographic distance matrices was inferred
418 using Mantel tests with the R package ade4 (*97*) .

419 **Contemporary gene flow**

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

426 coefficient) using the autotune function in BA3-SNPs. We than ran BayesAss-3 with
427 10,000,000 iterations, a burn-in of 1,000,000 and a sampling interval of 1000. Mixing
428 parameters were set to dM = 0.21, dA = 0.44 and dF = 0.08. Results were averaged across
429 five replicate runs and migration rates were considered significant if 95% credible sets (mean
430 migration rate ± 1.96 x mean standard deviation) did not overlap zero. Chain convergence
431 was assessed, and migration rates visualised using R (Figure S9).

432 **Historical relationships among populations**

433 To explore historical relationships among populations of *M. alfredi* and *M. birostris* we used
434 the program TreeMix (*46*). TreeMix uses population allele frequencies to estimate a bifurcating
435 maximum likelihood tree with which to infer historical population splits, admixture events and
436 the degree of genetic drift. We first supplemented the *M. alfredi* dataset with one randomly
437 selected *M. birostris* individual and the *M. birostris* dataset with one randomly selected *M.*
438 *alfredi* to act as outgroups when rooting the trees. Both datasets were then filtered for linkage,
439 a minor allele count of less than 3, genotyping rate of less than 90% and related individuals
440 using PLINK v1.9 (*98*). Allele frequencies for each population were then calculated using the
441 –freq and –within arguments in PLINK. For both the *M. birostris* and *M. alfredi* datasets we
442 then performed 10 initial runs of TreeMix for each migration event (M) ranging from 0 to 10.
443 The number of migration edges that explained 99.8% of the variance was selected as the best
444 model for each species (*M. birostris:* M= 0; *M. alfredi:* M = 2, Figure S5). We then re-ran
445 TreeMix 100 times using the optimal number of migration edges. Consensus trees and
446 bootstrap values were estimated and visualised using code modified from the BITE R package
447 (*99*).

448 **Genome-wide heterozygosity**

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

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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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