Phylogenomics and species delimitation of mobulid rays reveals cryptic diversity and a new species of manta ray

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Abstract

Practical biodiversity conservation relies on delineation of meaningful units, particularly with respect to global conventions and regulatory frameworks. Species delimitation methods have been revolutionised with the advent of next-generation sequencing approaches, allowing diversity within species radiations to be assessed with genome-wide data. Manta and devil rays (*Mobula* spp.) are threatened globally primarily from targeted and bycatch fishing pressure, resulting in recent protective measures under several global conventions and frameworks. However, a collective lack of representative global samples, ongoing taxonomic ambiguity, and ineffectual traceability measures combine to constrain the development and implementation of a coherent and enforceable conservation strategy for these species. Here we generate genome-wide Single Nucleotide Polymorphism (SNP) data from a globally and taxonomically comprehensive set of mobulid tissue samples, producing the most extensive phylogeny for the Mobulidae to date. We assess patterns of monophyly and combine this with species delimitation based on the multispecies coalescent. We find robust evidence for an undescribed species of manta ray in the Gulf of Mexico, and for the resurrection of a recently synonymised species, *Mobula eregoodootenkee*. Further resolution is achieved at the population level, where geographic population structure is identified in *Mobula* species. In addition, we estimate the optimal species tree for the group and identify substantial incomplete lineage sorting, where standing variation in extinct ancestral populations is hypothesised to drive taxonomic uncertainty. Our results provide genome-wide data to support a taxonomic review of the Mobulidae, and generate a robust taxonomic framework to support effective management, conservation and law enforcement strategies.

Introduction

The Anthropocene has been characterised by unprecedented human exploitation of natural resources, resulting in global threats to biodiversity and extinction events within many taxa (Dirzo *et al.*, 2014; McGill *et al.*, 2015). Effective measures for the conservation of biodiversity require an understanding and characterisation of diversity within and among species. The field of conservation genetics provides opportunities for quantifying diversity across space and time (Allendorf *et al.,* 2010) and such approaches are increasingly powerful with the growing incorporation of genome-wide data. Species delimitation, the process by which populations of individuals are grouped into reproductively isolated and separately evolving units, is a fundamental application of genomic data to biodiversity conservation.

Accordingly, species delimitation has received increasing attention recently, with numerous methods now available (Carstens *et al.*, 2013; Zhang *et al.*, 2013; Grummer *et al.,* 2014; Leache *et al.*, 2014; Rannala 2015; Yang 2015). Traditional approaches typically relied upon morphological observation, often resulting in artificially broad delineations arising from difficulties in detecting and identifying cryptic species (Frankham *et al.*, 2012). More recently, DNA sequencing has allowed genetic data to be utilised for species delimitation, although early approaches were limited to information from only a few genes or markers. These early approaches therefore left interpretation challenging, particularly in recently diverged groups with substantial incomplete lineage sorting (Maddison 1997; Maddison and Knowles, 2006). Genome-wide multi-locus approaches have increased the resolution of species delimitation studies and have been used to clarify contentious relationships and phylogenies (eg. Leache *et al.*, 2014; Herrera and Shank, 2016), and disclose previously unknown diversity (eg. Pante et al. 2014). Species delimitation remains constrained by the lack of a single universal species concept (De Queiroz, 2007; Frankham *et al.*, 2012). The delineation of monophyletic assemblages underpins the phylogenetic species concept, and the biological species concept where species occur in sympatry (Frankham *et al.*, 2012). This has application in characterisation of both Conservation Units and Evolutionary Significant Units for the purposes of effective conservation (Funk *et al.*, 2012).

Globally, biodiversity conservation is enacted through conventions and regulatory frameworks, including the Convention on the International Trade in Endangered Species of Wild Fauna and Flora (CITES), and the Convention on the Conservation of Migratory Species of Wild Animals (CMS). These conventions are implemented through national legislation acting at the species level (Vincent *et al.*, 2014), and effective wildlife protection, management and law enforcement therefore depends on unambiguous species classification. Recent examples of proposed taxonomic revisions having far-reaching consequences for biodiversity conservation include giraffe (Fennessy *et al.*, 2016; Bercovitch *et al.*, 2017; Fennessey *et al.,* 2017) and African elephant (Roca *et al.*, 2001). In these cases, genetic research has led to possible reclassification and consequent changes to the legal status of these threatened megafauna.

In the marine realm, manta and devil rays (*Mobula* spp.), are circumglobally distributed megafauna of high conservation priority (Lawson *et al.*, 2017) that also carry substantial economic value for tourism (O'Malley *et al.,* 2013). Despite the economic benefits provided through the non-consumptive use of these species (family Mobulidae; collectively, mobulids), this vulnerable group is threatened primarily by intense targeted and bycatch fishing pressure, in part driven by demand for their gill plates, which are utilised in Asian medicines (Couturier *et al.*, 2012; Croll *et al.*, 2016; Lawson *et al.*, 2017; O'Malley *et al.*, 2017). Exploitation of mobulid rays for human consumption is considered unsustainable due to their life history traits; late maturation, low reproductive rates and long generation times, hindering

their ability to recover from fishing impacts (Dulvy *et al.*, 2014). To alleviate these threats, all species of mobulid ray have recently been listed on the CITES Appendix II to regulate international trade and to the CMS Appendices I and II for governments to coordinate efforts to protect and conserve these species. Additionally, several species are regulated under national jurisdictions, with varying levels of protection and enforcement. Unfortunately, a collective lack of representative global samples, ongoing taxonomic ambiguity, and ineffectual traceability measures has constrained the development and implementation of a coherent and enforceable conservation strategy (Stewart, 2018a).

Recently, White et al. (2017) conducted an evaluation of genetic and morphological datasets for 11 previously recognised species of mobulid ray across two genera. Eight species were recognised, and the authors called for the genus *Manta* (consisting of two species; *Manta alfredi* and *Manta birostris*) to be subsumed into *Mobula* (devil rays); a recommendation that is yet to be reviewed by the International Commission on Zoological Nomenclature (ICZN) at the time of writing. For the purposes of this study, we use the common name 'manta ray' to refer to individuals of the species *M. alfredi* and *M. birostris* or species identified therein. Although multi-locus genetic datasets were used in the study by White et al. (2017), only a single sample was included per putative species, thereby preventing delineation of monophyletic species groups. Furthermore, the conclusion that *M. rochebrunei* is a junior synonym of *M. hypostoma* was based entirely on mitogenome data (White *et al.*, 2017), which is considered unsuitable for species delimitation or phylogenetics when used in isolation (Petit and Excoffier, 2009; Herrera and Shank, 2016). Prior to this study, the most recent major taxonomic change for the Mobulidae came with the resurrection of species status for *Manta alfredi,* resulting in recognition of two species of manta ray (Marshall *et al.,* 2009). Whilst the validity of this split has been confirmed with genetic data (Kashiwagi *et al.*, 2012), there remains evidence of both historic (Kashiwagi *et al.*, 2012) and modern (Walter *et al.*, 2014) hybridisation between the two species. In addition, a third putative species of manta ray is hypothesised to occur in the Caribbean (Marshall *et al.,* 2009; Hinojosa-Alvarez *et al.*, 2016).

The Mobulidae is a group characterised by recent divergence times, estimated to have diverged from Rhinoptera only 30 million years ago (MYA), and having undergone relatively short bursts of speciation associated with periods of decreased ocean productivity (Poortvliet *et al.*, 2015), of which the most recent known is only 0.5MYA (Kashiwagi *et al.*, 2012). The age of these divergences implies that secondary contact and introgression between separately evolving species is likely to be widespread within the group, further encumbering efforts to define species boundaries.

Such ongoing uncertainties within the Mobulidae demonstrate a requirement for genomic approaches to enable robust species delimitation. Here, we generate double-digest Restriction-site Associated

DNA sequence (ddRAD) data (Peterson *et al.*, 2012) from the largest and most comprehensive geographic sampling of mobulid species (Figure 1), inclusive of taxon replicates within sampling sites to: (1) delimit mobulid species, resulting in identification of cryptic diversity and an undescribed species of manta ray, (2) estimate the optimal species tree for the group, and (3) identify the extent of incomplete lineage sorting.

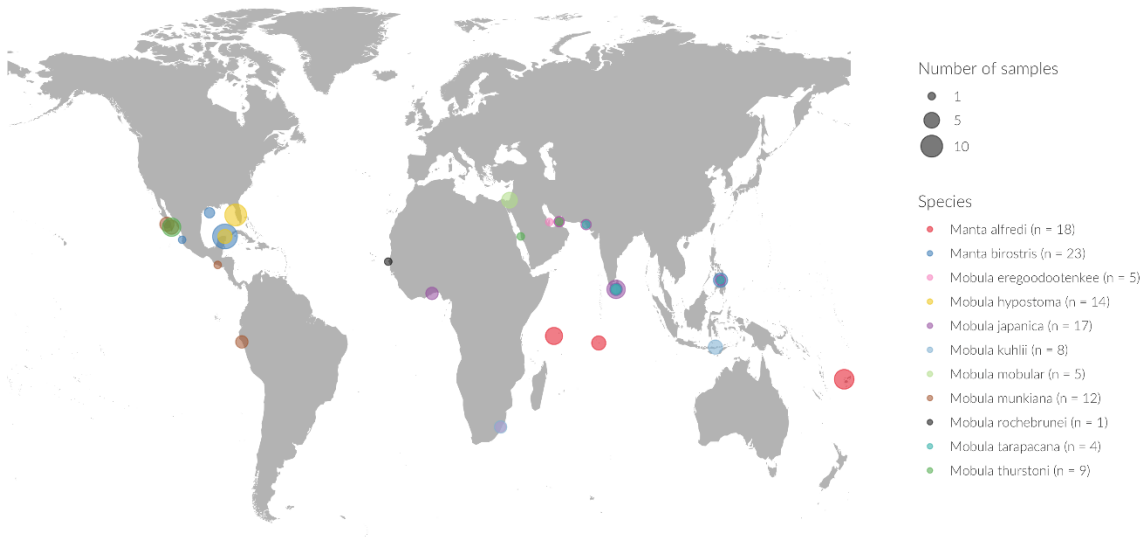

Figure 1: Map of mobulid sampling locations. Species are denoted by different colour points, scaled for sample size. Total numbers of samples for each species are given in the legend. See Supplementary Table 1 for further details. Note that we use species names that were assigned to samples at the time of collection, some of which are now invalid (White *et al.*, 2017).

Results

Monophyly and clustering

Maximum Likelihood phylogenetic trees based on two genome-wide SNP data matrices (hereafter referred to as datasets p10 and p90, see Supplementary Table 2 for details) of varying size displayed highly congruent patterns (Figure 2 and Supplementary Figure 1). These trees represent the most comprehensive phylogenetic trees in terms of numbers of individuals and geographic coverage for mobulid rays published to date. Putative species fall into reciprocally monophyletic groups with high bootstrap support, and these species groups fall into well supported clades separated by long branch lengths. *Mobula japanica* and *Mobula mobular* form a single monophyletic group with 100% bootstrap support. In contrast, *Mobula kuhlii* and *Mobula eregoodootenkee* were resolved into two distinct

monophyletic groups, each with 100% bootstrap support. Furthermore, two distinct monophyletic groups are reported within *M. kuhlii*; each with 100% bootstrap support (based on dataset p10) corresponding to individuals sampled in the West (South Africa) and East (Sri Lanka eastwards) Indian Ocean. Finally, the manta rays can be resolved into distinct monophyletic groups corresponding to *M. alfredi* and *M. birostris*. Within *M. alfredi*, two well supported groups that correspond to Indian and Pacific Ocean populations are observed, whilst *M. birostris* is split into two groups; an Atlantic and a global group. One individual (sampled in Flower Garden Banks National Marine Sanctuary) was noted to switch between *M. birostris* clades depending on the data matrix used and was placed outside each main group with low bootstrap support (69% for dataset p10).

Principal Components Analyses (PCA) were carried out on each of the clades referred to above using dataset p10 (Figure 3; see Supplementary Table 3 for details of SNPs retained following division of data into clades). For the manta rays (Figure 3A & B), the first principal component (hereafter PC) separates *M. alfredi* from *M. birostris*, whilst the second PC distinguishes between *M. birostris*, and a possible third species of manta ray. The third PC provides clear distinction between *M. alfredi* from the Indian and Pacific Oceans (F_{ST} = 0.162). The screeplot shows a steep decline in the amount of variation shown by each axis (Supplementary Figure 2A-B). For *M. mobular* and *M. japanica* (Figure 3C & D), there is no clear separation between the two putative species, although the first PC does provide some evidence to suggest a clustering of individuals into Indo-Pacific and Atlantic (including Mediterranean individuals) groups (F_{ST} = 0.061). The screeplot for this clade shows a much shallower decline, and the amount of variation explained by each axis is much lower than for other clades (Supplementary Figure 2C-D). For the *M. thurstoni*, *M. kuhlii* and *M. eregoodootenkee* group (Figure 3E & F), these three species are very clearly differentiated on the first and second PCs, and this variation is reflected in the corresponding screeplot (Supplementary Figure 2E-F). The third PC reflects the geographic separation of *M. kuhlii* referred to above (F_{ST} = 0.319). For *M. hypostoma* and *M. munkiana* (Figure 3G & H), only the first PC was found to represent a large portion of the variation in the data (Supplementary Figure 2G-H), which corresponds to the separation of individuals into *M. hypostoma* and *M. munkiana*.

COI gene phylogeny

A Maximum Likelihood tree of mobulid species built using COI sequences is presented in Supplementary Figure 3. COI sequencing was unable to resolve the two manta ray species (*M. alfredi* and *M. birostris),* into monophyletic groups, and failed to resolve *M. kuhlii* and *M. eregoodootenkee*. Several species were resolved into reciprocally monophyletic groups with high bootstrap support (*M.*

194 *tarapacana, M. mobular, M. hypostoma* and *M. munkiana*), but several multifurcating nodes within
195 the tree indicate poor resolution achieved with this dataset.

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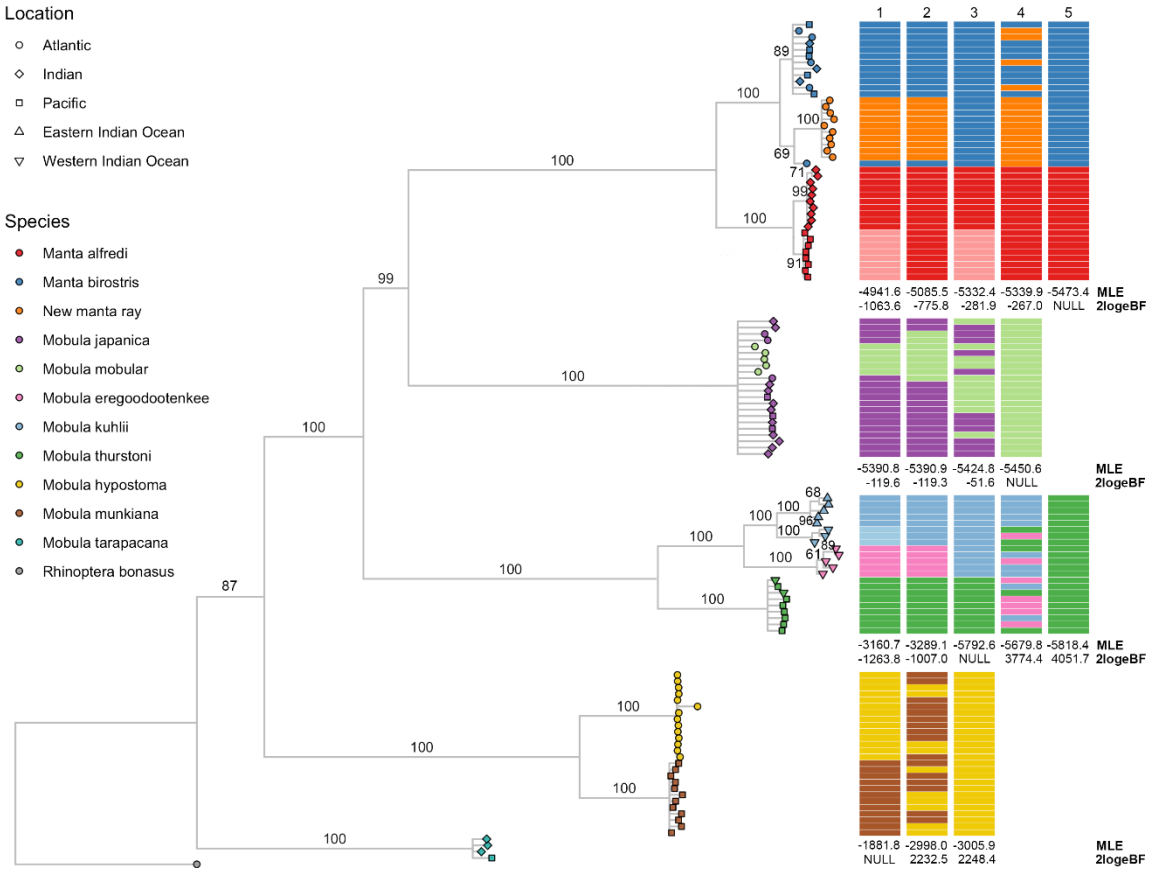

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198 Figure 2: Maximum Likelihood Phylogenetic Tree of mobulid species based on dataset p10 (left). Tips
199 represent individuals, with colour indicating species, and shape the geographic origin of the sample as
200 indicated in the legend. Bootstrap values are shown on the branches. Nodes with less than 50%
201 bootstrap support are collapsed. Bayes Factor Delimitation (BFD*) models are also presented (right)
202 where individuals are assigned to species as indicated by coloured bars. Models are ranked in order
203 of performance from left to right. Marginal Likelihood Estimates (MLE) and Bayes Factors ($2\log_e BF$) for
204 runs with a gamma prior on lambda, relative to the null model are shown beneath each model. See
205 Supplementary Table 4 for MLEs and $2\log_e BF$ for runs with alternative prior combinations. Models that
206 included individuals from the sister clade are not shown, as these consistently performed poorly. Note
207 that we use species names that were assigned to samples at the time of collection, some of which are
208 now invalid (White *et al.*, 2017).

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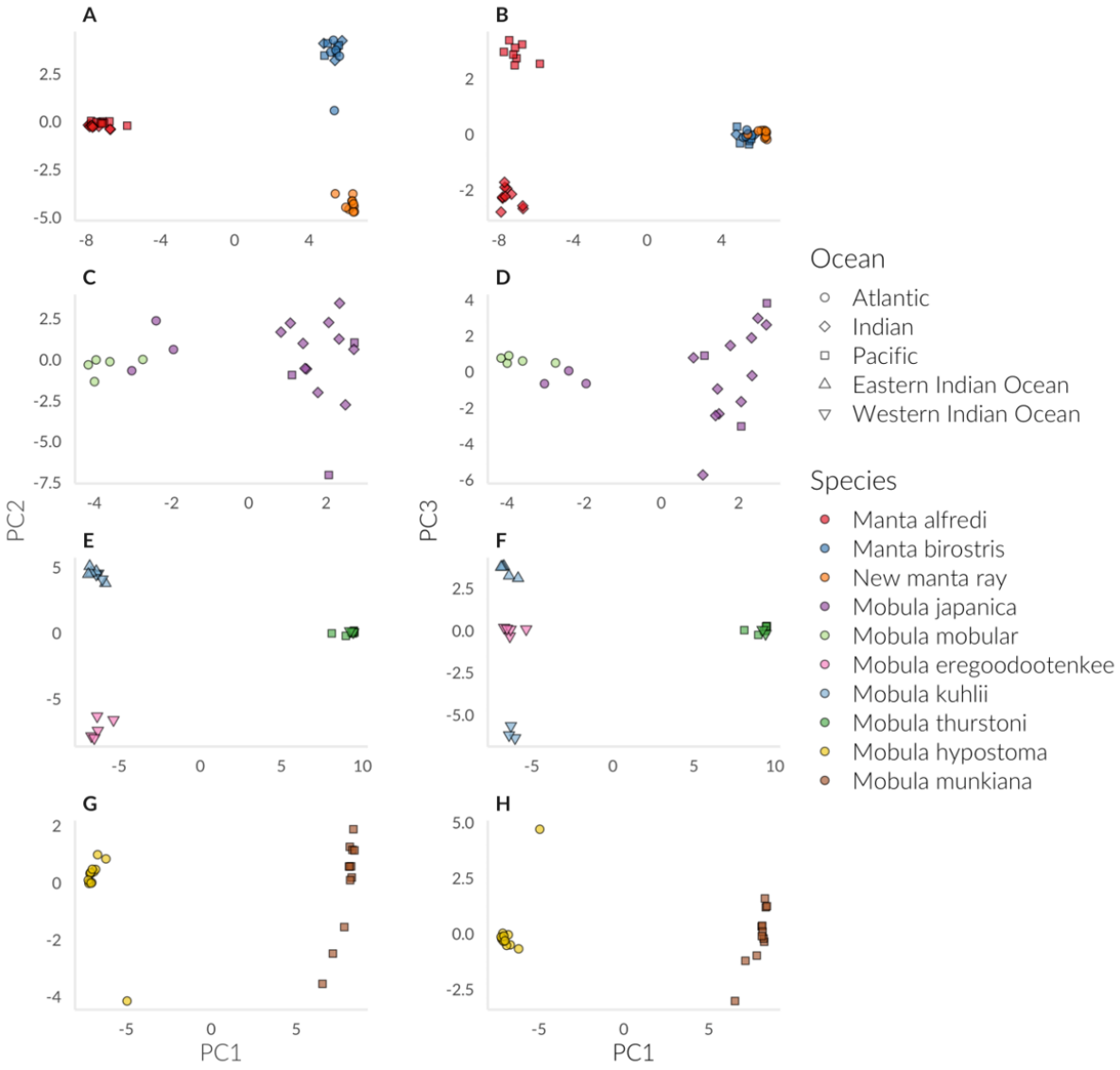


Figure 3: Principle Components 1-3 plotted for each mobulid clade. Putative species are represented with colours, and shape represents the geographical location of sampling as indicated in the legend. A) manta rays, PC1 and 2, B) manta rays, PC1 and 3, C) *M. mobular* and *M. japanica*, PC1 and 2, D) *M. mobular* and *M. japanica*, PC1 and 3, E) *M. thurstoni*, *M. kuhlii* and *M. eregoodootenkee*, PC1 and 2, F) *M. thurstoni, M. kuhlii* and *M. eregoodootenkee*, PC1 and 3, G) *M. hypostoma* and *M. munkiana*, PC1 and 2, H) *M. hypostoma* and *M. munkiana*, PC1 and 3. Note that we use species names that were assigned to samples at the time of collection, some of which are now invalid (White *et al.*, 2017).

Species Delimitation

Species models (see Supplementary Table 4 for details) were tested following the Bayes Factor Delimitation with genomic data (BFD*) method of Leache et al. (2014), and Bayes Factors calculated relative to a null model of mobulid species as defined by White et al. (2017). Marginal Likelihood estimates did not differ considerably between chains with different priors on lambda (Supplementary Table 4). For the manta rays (Figure 2), we find decisive support for models that recognise the Gulf of Mexico and global *M. birostris* clades referred to above as two separate species ($2\log_e BF = -775.82$), and that recognise geographically separated populations of *M. alfredi* as separate species ($2\log_e BF = -1063.58$).

The *M. mobular* and *M. japanica* clade was best described by models that were more similar in their performance (Figure 2). The null model performed poorly in comparison to three models that split individuals based on geographical information (indeed, prior to White et al. (2017); *M. mobular* was considered to be restricted to the Mediterranean Sea, whilst *M. japanica* was considered circumglobal). The model that split individuals into these two previously recognised species performed best ($2\log_e BF = -119.58$ relative to null model) but was only marginally better than a model that split individuals into Atlantic (including the Mediterranean) and Indo-Pacific groups ($2\log_e BF = -119.34$ relative to null model).

Decisive support was found for the *M. thurstoni*, *M. kuhlii* and *M. eregoodootenkee* clade (Figure 2), in models that resurrect *M. eregoodootenkee* as a valid species, and that further split *M. kuhlii* based on geographical information ($2\log_e BF = -1007.04$ and -1263.8 respectively).

Finally, within the *M. hypostoma* and *M. munkiana* clade, we find decisive support for the null model, that recognises *M. hypostoma* and *M. munkiana* as distinct species (Figure 2).

In all clades, models assessing support for interaction from higher up the tree, as well as models testing random assignment of individuals to species, perform comparatively poorly (Supplementary Table 4).

Relationships among the Mobulidae

Maximum Likelihood trees using the two species level data matrices containing varying amounts of missing data were highly congruent (Figure 4 and Supplementary Figure 4). Both data matrices support the findings of White et al. (2017); that manta rays are nested within *Mobula*, and sister to *M. mobular* (≥95% bootstrap support) and hereafter all species of manta ray are referred to with genus name *Mobula*. In addition, these trees strongly suggest that the undescribed third species of manta ray is

most closely related to *M. birostris* (100% bootstrap support). Finally, *M. tarapacana* is tentatively placed on the first lineage to diverge from the remaining Mobulidae (84% bootstrap support with dataset p10).

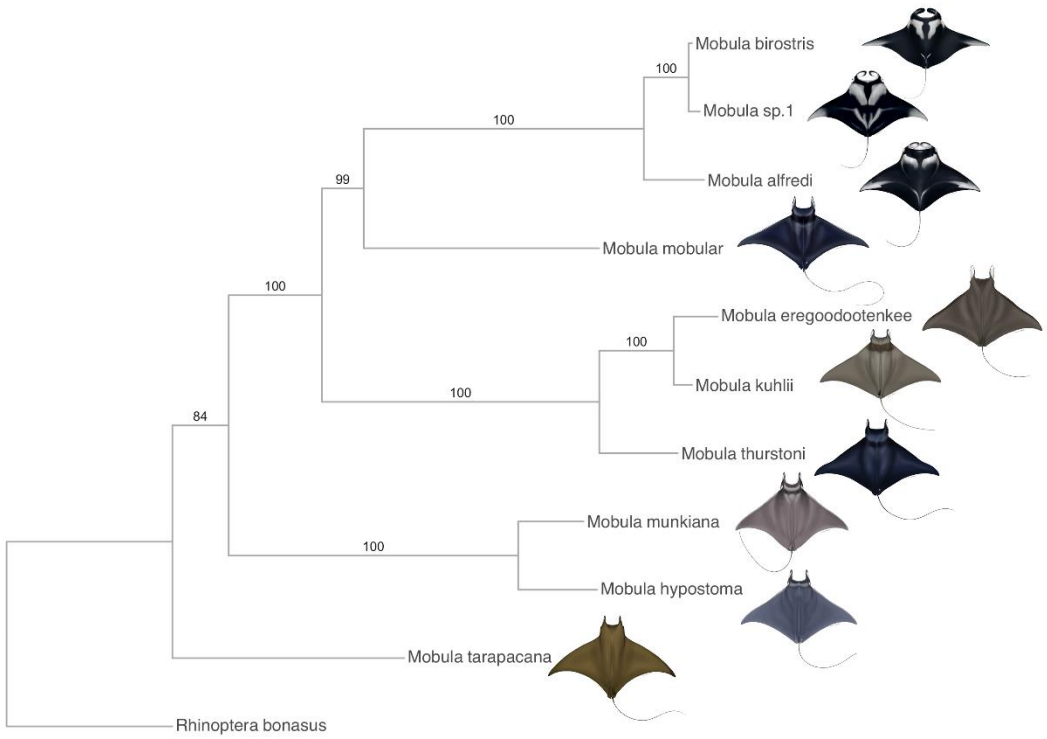
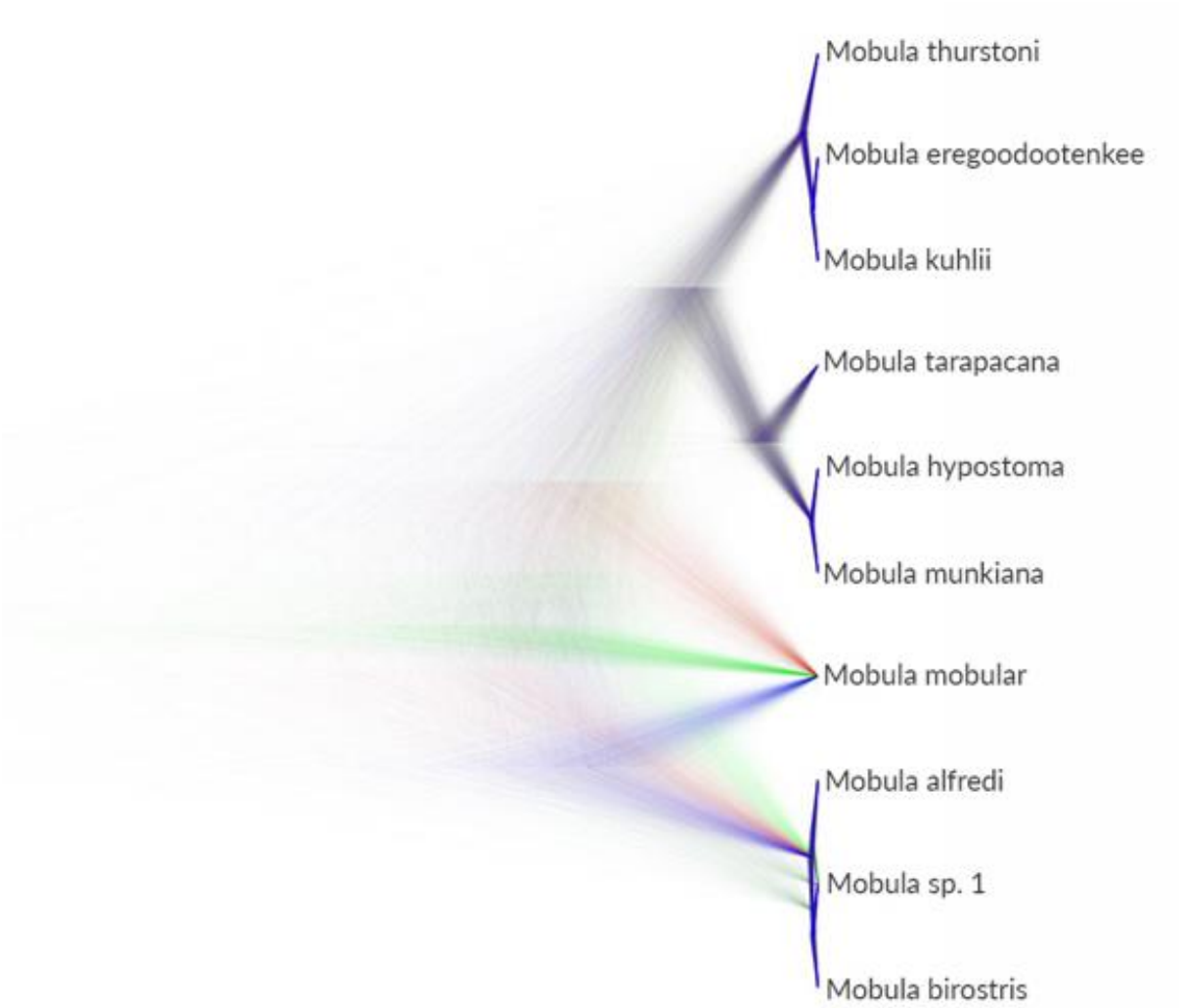


Figure 4: Maximum Likelihood tree of mobulid species based on dataset p10. Bootstrap values are shown on the branches. Illustrations © Marc Dando. The drawing of *Mobula* sp. 1 is based on images of dozens of individuals off the Yucatan Peninsula, Gulf of Mexico.

With respect to Bayesian tree estimation under the multispecies coalescent, the consensus tree topology and estimates of theta were relatively consistent across independent runs that included different individuals from each species (Supplementary Table 5). This suggests that there was no major effect of subsampling on topology of the species trees inferred with SNAPP. In trees inferred with SNAPP, *M. tarapacana* was consistently placed within a clade separate to the ingroup of *M. hypostoma* and *M. munkiana* (highest posterior density (HPD) = 1.0). Other nodes within the tree were generally poorly supported. This topological uncertainty is apparent when visualised as a cloudogram of gene trees sampled from the posterior distribution (Figure 5 and Supplementary Figures 5-7). The number of alternative topologies inferred per subsampling and within the 95% HPD ranged from 9-25 (Supplementary Table 6). In all inferred topologies within the 95% HPD, the topology within the clades

272 separated by long branches, previously discussed, remains the same, and the main difference was the
273 placement of *M. mobular* relative to the other clades.



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275 Figure 5: SNP phylogeny of 30 individuals assigned to ten species based on dataset p90, individual
276 subsample 1 (see Supplementary Table 5 for details). Tree cloud produced using DENSITREE of
277 sampled trees (representing samples taken every 1000 MCMC steps from 5,000,0000 iterations) from
278 SNAPP analysis to visualise the range of alternative topologies.

279

280 TreeMix inferred an admixture graph with the same topology as that inferred with RAxML (see
281 Supplementary Figure 8). This model was found to explain 99.86% of the variance in the data,
282 indicating that species placement is unaffected by admixture, where species may be more closely
283 related than the tree suggests, or where species may be forced closer together due to unmodeled
284 migration (Pickrell and Pritchard, 2012). Furthermore, three-population tests were all positive

(Supplementary Table 7). We therefore found no evidence of introgression between clades containing *M. alfredi, M. mobular* and *M. thurstoni*.

Discussion

Our analyses of a globally and taxonomically comprehensive set of mobulid tissue samples produced the most extensive phylogeny for the Mobulidae to date. Genome-wide SNP data provided a high degree of resolution compared to analysis of a single gene. Combined with results from analyses based on the multispecies coalescent, our findings provide robust support for several changes to be made to mobulid taxonomy, including the recognition of a new species of manta ray, and have implications for management, conservation and law enforcement.

It is important to recognise speciation as a continuous process, where lineage splitting does not necessarily correspond to speciation events. When this is explicitly modelled, the multispecies coalescent has been shown to overestimate species numbers, recovering all structure both at the level of the species and the population (Sukumaran and Knowles, 2017). In contrast to previous studies of mobulid taxonomy, the global nature of our dataset allows for this conflict to be resolved, where in many cases, individuals from pairs of putative species are sampled within sites, thereby allowing this distinction to be made.

We find strong evidence supporting the existence of a third, undescribed species of manta ray in the Gulf of Mexico (hereafter referred to as '*Mobula* sp. 1'). Samples were collected at two sites within the Gulf of Mexico; offshore of the Yucatan Peninsula and Flower Garden Banks National Marine Sanctuary, and were provisionally identified as *M. birostris*. When these Gulf of Mexico samples were analysed alongside *M. birostris* samples collected elsewhere (Sri Lanka, Philippines and Mexico Pacific), individuals were found to fall within two distinct groups; one containing only individuals from the Gulf of Mexico sites, and the other containing additional individuals from the same Gulf of Mexico sites as well as *M. birostris* individuals sampled elsewhere. In addition, we find decisive support for two models which recognise these groups as distinct species through Bayes Factor Delimitation (BFD*; Figure 2). Given that samples from both groups were collected within Gulf of Mexico sites, *M. birostris* can be considered to occur in sympatry with *Mobula* sp. 1, constituting separately evolving lineages (De Queiroz, 2007). Monophyly of groups supports these as separate species under the phylogenetic species concept (Frankham *et al.*, 2012). Furthermore, sympatry of populations suggests reproductive isolation driven either by a factor other than geographical separation, or historical separation followed by modern secondary contact (as hypothesised by Hinojosa-Alvarez et al. (2016)), and these species

317 are therefore further supported under the Biological Species concept (Frankham *et al.*, 2012). In
318 addition, we report on a single individual which could be considered as genetically intermediate
319 between the two groups (Figures 2 and 3), indicating that hybridisation may occur between the two
320 species, as between *M. alfredi* and *M. birostris* (Walter *et al.*, 2014).

321 Novel mtDNA haplotypes have previously been reported from manta rays off the Yucatan Peninsula,
322 and a speciation event hypothesised (Hinojosa-Alvarez *et al.*, 2016), in addition to previous
323 morphological observations (Marshall *et al.,* 2009). Our study is the first analyses of genome-wide
324 data to suggest that there are two species of manta ray present in the Gulf of Mexico; a finding that
325 is consistent with previous studies (Hinojosa-Alvarez *et al.*, 2016; Stewart *et al.*, 2018b). Monophyly
326 of groups indicate that some *M. birostris* individuals using sites in the Gulf of Mexico are more closely
327 related to *M. birostris* in Sri Lanka and the Philippines than to individuals of *Mobula* sp. 1 using those
328 same Gulf of Mexico sites. It is likely that these species occur in a state of mosaic sympatry, as with *M.*
329 *alfredi* and *M. birostris* elsewhere (Kashiwagi *et al.*, 2011). For effective conservation it will be
330 necessary to formally describe this new species and determine the extent of its range.

331 A recent taxonomic review concluded that *M. eregoodootenkee* is a junior synonym of *M. kuhlii* based
332 on mitogenome and nuclear data for a single sample per putative species (White *et al.*, 2017). In direct
333 contrast, our phylogenetic analysis of genome-wide SNPs which included multiple individuals per
334 species from multiple geographic locations, placed individuals of *M. kuhlii* and *M. eregoodootenkee*
335 into discrete monophyletic clades with very high bootstrap support (Figure 2). This pattern was also
336 mirrored in the results of our Principal Components Analysis (Figure 3). In addition, BFD* models that
337 recognised *M. eregoodootenkee* as a distinct species from *M. kuhlii* are consistently favoured over the
338 null model (Figure 2). Given that both species groups included samples that were collected within the
339 same ~120km stretch of South African coastline, the divergence reported here between *M. kuhlii* and
340 *M. eregoodootenkee* cannot be attributed to geographic population structure (Sukumaran and
341 Knowles, 2017). There is evidence to suggest that periods of speciation within the Mobulidae
342 correspond to episodes of global warming and associated changes in upwelling intensity and
343 productivity, and it is hypothesized that this led to fragmentation and subsequent divergence with
344 respect to feeding strategies (Poortvliet *et al.*, 2015). Differences in morphology between *M. kuhlii*
345 and *M. eregoodootenkee* (Notarbartolo Di Sciara, 1987; Notarbartolo di Sciara *et al.*, 2017), and
346 particularly the suggestion of differences in the length of the cephalic fins and gill plate morphology
347 (Paig-Tran *et al.,* 2013), that relate directly to the filter feeding strategy of mobulid rays, may lend
348 support to this hypothesis. Notwithstanding, the present study provides the best available evidence
349 regarding the species status of this group, and as such we resurrect *Mobula eregoodootenkee* as a
350 distinct species.

In agreement with the conclusion of White et al. (2017), we find no evidence to support *M. japanica* as a distinct species to *M. mobular*. Individuals provisionally identified as *M. mobular* as it was formerly recognised (with a distribution that was restricted to the Mediterranean Sea), do not form a reciprocally monophyletic group to the exclusion of individuals belonging to *M. japanica* (a species previously considered to be circumglobally distributed with the exception of the Mediterranean Sea), and instead these individuals form a single clade, with high bootstrap support (Figure 2). Clustering analyses indicate a degree of population structure, with some modest differentiation between Indo-Pacific and Atlantic (including Mediterranean) groups (F_{ST} = 0.06). Results from BFD* are far less conclusive than those for other clades (Figure 2), and support for split models being driven by geographic segregation of populations cannot be ruled out (Sukumaran and Knowles, 2017). We therefore uphold *M. mobular* as a single species, with *M. japanica* considered a junior synonym of the same.

With respect to species delimitation of the final clade examined, we find strong evidence to support *M. hypostoma* and *M. munkiana* as distinct species (Figures 2 and 3). Whilst these species are geographically segregated in the Atlantic and Eastern Pacific Oceans respectively, the divergence is of a similar magnitude to that of other species groups within the Mobulidae (Figures 2 and 3, Supplementary Figure 2) and morphological differences between the two species are considered sufficient to recognise two species (Notarbartolo Di Sciara 1987; Stevens et al. 2018). As such we find no evidence to support any modification to the taxonomy of this clade.

Previous studies found morphological differences sufficient to consider *M. rochebrunei* (a pygmy devil ray species described off the coast of West Africa) a distinct species (Cadenat, 1960); summarised in Notarbartolo Di Sciara (1987). In this study, we were unable to generate molecular data representing *M. rochebrunei* (now considered to be a junior synonym of *M. hypostoma* (White *et al.*, 2017)). However, the revision published by White et al. (2017) is based on low mitochondrial sequence divergence between single representative samples of the two putative species, and is consistent with sequence divergence estimates for other mobulid groups where further study has resolved separate species status: *M. alfredi* and *M. birostris* (Marshall *et al.,* 2009; Kashiwagi *et al.,* 2012; this study), and *M. kuhlii* and *M. eregoodootenkee* (this study). Therefore, given the high vulnerability to extinction which exists for any mobulid species with a restricted range in this region (Atta-Mills *et al.*, 2004; Doumbouya 2009) efforts to resolve this taxonomic uncertainly should be given a high priority (see Stewart 2018a).

Through phylogenetic and clustering analyses, we identify substantial geographically-mediated population structure within *M. kuhlii* and *M. alfredi*. In both cases, individuals fall into monophyletic

groups corresponding to the East and West Indian Ocean (F_{ST} = 0.32), and Indian and Pacific Oceans (F_{ST} = 0.16), respectively, with high bootstrap support. This pattern is consistent in our clustering analysis, and BFD* supports models that recognise these populations as distinct species. Indeed, there are anecdotal suggestions of morphological differences occurring in *M. kuhlii* across the Indian Ocean (Stevens *et al.,* 2018). However, given that we cannot rule out a geographic driver of these patterns, *M. kuhlii* and *M. alfredi* must currently be maintained as singular species. Further study is required to investigate this pattern, and to assess the population genetic structure of both species to support effective management.

The inference of relationships within the Mobulidae provided largely congruent results across Maximum Likelihood and Bayesian analyses, with an exception of the placement of *M. tarapacana*. Our ML analysis placed *M. tarapacana* on the oldest mobulid lineage, as result consistent with similar ML analysis based on nuclear data (White *et al.*, 2017). Yet our Bayesian analyses consistently placed *M. tarapacana* as sister species to *M. hypostoma* and *M. munkiana*. Analyses employing mitochondrial data support *M. tarapacana* as a sister species to the manta rays and *M. mobular* (Poortvliet *et al.*, 2015; White *et al.*, 2017), an observation that we were unable to reproduce with our data. Discordant trees in phylogenomic studies may be attributed to a small number of genes or loci, either driven by positive selection resulting in convergent evolution, or by evolutionary processes such as incomplete lineage sorting or hybridisation (Shen *et al.,* 2017). Coalescent-based approaches, such as the independent analysis of unlinked SNPs completed here, account for each gene trees history, and are therefore less likely to be influenced by single genes (Shen *et al.,* 2017), lending support to the hypothesis that *M. tarapacana* is sister to *M. hypostoma* and *M. munkiana*.

Application of a multispecies coalescent-based approach to our data allowed visualisation of the uncertainty in species tree topology and incomplete lineage sorting. Our Maximum Likelihood phylogenetic analysis indicates that the previously recognised genus *Manta* is nested within *Mobula*, and provides further justification for the associated change in nomenclature implemented by White et al. (2017). However, concatenated approaches can be prone to converge to an incorrect phylogeny (Kubatko and Degnan, 2007), whilst ignoring heterozygous sites can effect estimates of divergence times (Lischer *et al.,* 2014). Whilst our Bayesian multispecies coalescent analyses do not specifically refute the observation that *Manta* is nested within *Mobula*, we find substantial uncertainty in the placement of *M. mobular*. Trees within the 95% HPD that place *M. mobular* with the manta rays are present in approximately equal proportions to trees placing the species with the remaining devil rays (Supplementary Table 6), thereby producing trees where the two formerly recognised genera are reciprocally monophyletic. In groups that have undergone a rapid speciation process and had large ancestral effective population size, the effects of incomplete lineage sorting on species tree estimation

418 are particularly prominent (Flouri *et al.*, 2018). The Mobulidae are known to have undergone recent
419 rapid bursts of speciation (Poortvliet *et al.*, 2015), and our estimates of theta (mutation-scaled
420 effective population size), were larger on the deeper branches of the tree reflecting the large effective
421 population size of the extinct shared ancestral species of the contentious extant taxa (Supplementary
422 Figure 9). Thus, standing variation in ancestral populations of mobulid rays is likely to drive taxonomic
423 uncertainty with respect to the validity of *Manta* as a genus. Since there is no evidence of admixture
424 driving these patterns (Supplementary Table 7), this uncertainty can be attributed to incomplete
425 lineage sorting. Given that recently separated populations or species will pass through stages of
426 polyphyly and paraphyly before becoming reciprocally monophyletic in the absence of additional
427 introgression (Avise 1990; Patton and Smith, 1994), it is reasonable to hypothesise we are observing
428 this process here. Based on current information however, we support *Mobula alfredi* and *Mobula*
429 *birostris* as being taxonomically valid (White *et al.*, 2017).

430 Our proposed changes to the taxonomy of the mobulid rays will have profound implications for
431 practical conservation of the Mobulidae on an international scale, as conventions designed to regulate
432 and effect conservation measures rely on systematic review at the species level (Shafer *et al.*, 2015).
433 Furthermore, many of these administrations rely on experts to evaluate the literature and assess
434 priorities for species conservation, for example, under the IUCN's Red List framework. Of particular
435 importance from this study is the distinction of *M. eregoodootenkee* from *M. kuhlii*, given that they
436 share a similar geographic range across a region with intensive fishing pressures (Notarbartolo di
437 Sciara *et al.*, 2017). Although each species is still treated as a single stock across the Indo-Pacific due
438 to limited data available on their population structure, inference from related species suggest that
439 their low reproductive output likely results in population numbers that will not withstand heavy fishing
440 pressure (Dulvy *et al.*, 2014; Croll *et al.*, 2016). As such, their conservation status would be considered
441 quite critical, requiring very specific management measures. In contrast, species such as *M. mobular*
442 will now likely face lower conservation concerns given that *M. japanica* is a junior synonym. However
443 as with other mobulid species, further investigations into population structure are warranted in order
444 to conduct clear stock assessments for fisheries management.

445 Similarly, for conservation conventions such as CITES and CMS, and fisheries management bodies,
446 management plans are drafted and approved at a species level and can severely impact anthropogenic
447 pressures on a species. It is therefore imperative that decisions on species status are based upon the
448 best available evidence.

449

Conclusions

This study represents the most comprehensive phylogenomic study in terms of numbers of individuals and geographic coverage for mobulid rays published to date and makes use of genome-wide SNP data to evaluate the taxonomy of the group and relationships between species. We present genome-wide evidence to support ten species within the Mobulidae: *Mobula alfredi, Mobula birostris, Mobula mobular, Mobula thurstoni, Mobula kuhlii, Mobula eregoodootenkee, Mobula hypostoma, Mobula munkiana, Mobula tarapacana* and a currently undescribed species of manta ray (*Mobula* sp. 1) in the Gulf of Mexico. In addition, we advocate the recognition of *Mobula rochebrunei* for conservation purposes until more data is available. We emphatically urge policy-makers, particularly the large conventions (such as the CITES and CMS) and the relevant specialist group within the IUCN to evaluate these as separate units in their assessments and when implementing conservation policy.

Future work in this area will necessarily involve formal description of the third species of manta ray (*Mobula* sp. 1), shown here to be present in the Gulf of Mexico. In addition, population level studies on individual species will allow more informed management by delineating conservation units. In the case of the Mobulidae, a group known to be vulnerable to overexploitation, assessment of stock structure within fisheries will allow for effective management.

This significant increase in the resolution of species diversity within the global evolutionary radiation of the Mobulidae was achieved through an international collaboration of researchers, contributing to a global collection of representative samples, combining multiple genome-wide markers with a combinatorial approach to data analysis. As such, the study provides a framework for molecular genetic species delimitation which is relevant to other wide-ranging taxa of conservation concern and highlights the potential for applied research to support conservation, management and law enforcement.

Materials and Methods

Sample collection, DNA extraction and Sanger sequencing

Tissue samples were collected representing all described species of mobulid ray, including the recently invalid species' *Mobula japanica, Mobula eregoodootenkee* and *Mobula rochebrunei,* currently considered to be junior synonyms of *Mobula mobular*, *Mobula kuhlii* and *Mobula hypostoma* respectively (White *et al.*, 2017), and an outgroup species, *Rhinoptera bonasus*. Where possible, samples were collected from a broad geographical range, and with multiple samples per site. Samples

were identified to species level based on morphological characters described in Stevens et al. (2018). Samples included in the analyses described below (those yielding high quality DNA), totalling 20 countries and 31 sites, are shown in Figure 1, and details given in Supplementary Table 1. We use the original species names that were assigned to samples at the time of collection, some of which are now considered invalid following White et al. (2017).

Genomic DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit following the manufacturer's instructions and eluted in nuclease-free water. DNA yield was measured using a Qubit 3.0 Broad Range Assay, and quality assessed on a 1% agarose gel stained with SafeView. The single sample of *Mobula rochebrunei*, from the Musee de la Mer, Goree, Senegal, had been stored in formalin, yielded no detectable DNA, and was therefore not sequenced.

To investigate the utility of traditional markers for mobulid species delimitation, PCR amplification of an approximately 650bp portion of the COI gene was carried out using universal Fish primers (Ward *et al.*, 2005) or, where these primers failed to amplify, as was the case for *M. munkiana* and *M. hypostoma* samples, primers MunkF1 (GGGATAGTGGGTACTGGCCT) and MunkR1 (AGGCGACTACGTGGGAGATT) were designed in-house using Primer-BLAST (Ye *et al.*, 2012). PCR was carried out in 15µl reactions, consisting of: 5.6µl nuclease-free water, 7.5µl of ReddyMix PCR Master Mix (ThermoFisher), 0.45µl of each primer, and 1µl DNA. PCR cycling conditions consisted of: 95°C for 2 min, followed by 35 cycles of 94°C for 30s, 54°C for 30s and 72°C for 1 min, with a final extension of 72°C for 10 mins. Sanger sequencing was carried out by Macrogen Europe, and raw sequences edited using the software Chromas Lite, yielding 110 high quality sequences (see Supplementary Table 1). Data was imported into MEGA7 (Kumar *et al.,* 2016), aligned using ClustalW, and the alignment checked for stop codons. The HKY+G model was identified as most suitable for this dataset using the Find Best Model option in MEGA7, and a Maximum Likelihood tree built with 1000 bootstrap replicates.

ddRAD library preparation and sequencing

ddRAD libraries were prepared in-house using a modified version of the protocol published by Peterson *et al.* (2012), and fully described in Palaiokostas *et al.* (2015). For each sample, 21ng of genomic DNA was digested with the restriction enzymes *SbfI* and *SphI* (NEB). Unique P1 and P2 barcode combinations were ligated to the resulting fragments for individual identification before samples were pooled. DNA fragments between 400 and 700bp were size-selected using gel

electrophoresis and PCR amplified. Individual sample replicates within and among libraries were included to assess error rates following the method described by Mastretta-Yanes *et al.* 2015.

A pilot ddRAD library was sequenced on the Illumina MiSeq at the Institute of Aquaculture, University of Stirling. Subsequent ddRAD libraries were sequenced by Edinburgh Genomics, University of Edinburgh on Illumina HiSeq High Output v4, with the 2 x 125PE read module.

Data quality control and filtering

Data quality was assessed with FastQC software (Andrews 2010) with particular interest in the per base sequence quality module for SNP calling and the overrepresented sequences module to check for adapter contamination. Stacks (version 1.46; (Catchen *et al.*, 2011)) was used for demultiplexing, quality filtering and assembling raw read data. Data were demultiplexed using the process_radtags.pl module and due to an indication of adapter contamination, adapter sequences were filtered out at this stage, with two mismatches allowed in the adapter sequence. In addition, the score limit was raised to 20 (99% probability) within the process_radtags sliding window to remove low quality sequence reads. Reads with an uncalled base were also discarded at this stage.

To minimise the level of linkage in our SNP data, only forward reads were included in the next stages of analysis. To remove any short fragments that were not successfully filtered out at the size-selection stage of the wet-lab protocol, a custom bash script was used to remove any sequence reads that contained a cut site for the *SphI* enzyme. This amounted to 8.5% of reads across samples.

In order to assemble loci and call SNPs, the denovomap.pl program was executed in Stacks (Catchen *et al.*, 2011). The three main parameters for assembly were set as those that generated the largest number of new polymorphic loci shared across 80% of individuals, following the method for parameter testing described by Paris *et al.* (2017). Four identical reads were required to build a stack (-m), stacks that differed by up to four nucleotides were merged into putative loci (-M) and putative loci across individuals that differed by up to five nucleotides were written to the catalog (-n). This resulted in an average coverage of 105x across loci and samples. Allele and SNP error rates, as defined by Mastretta-Yanes *et al.* (2015), were below 6% and 2.5% respectively.

To generate a SNP matrix at the individual level, the populations.pl program in Stacks (Catchen *et al.*, 2011) was used to output a VCF file containing all discovered SNPs across every polymorphic locus that was shared across more than a specified minimum number of individuals (10 or 90). This generated two matrices of varying size and with varying levels of missing data (see Supplementary Table 2). In order to remove possible paralogous loci from these matrices, VCFtools (Danecek *et al.*,

2011) was used to generate information on the average coverage at each locus across individuals. Those loci that were sequenced at more than double the standard deviation of coverage were assumed likely to be paralogous loci and were excluded. In addition, loci that were sequenced at less than one-third the standard deviation of coverage were excluded to mitigate for the effects of allele dropout (Arnold *et al.*, 2013; Gautier *et al.*, 2013). Moreover, loci were assessed for excess heterozygosity due to mapping artefacts, where those loci that were identified as having a high probability of heterozygote excess in one or more species were excluded from the entire dataset. Finally, to exclude erroneous SNPs called due to indels in the sequence, that are not accounted for in Stacks, any SNP in the last five nucleotide positions was excluded. To output final quality controlled SNP matrices for downstream analysis, the remaining loci and SNPs were written to a whitelist, and passed back to the populations.pl program in Stacks (Catchen *et al.*, 2011). The –write_random_snp option was enabled at this stage to output a single random SNP per locus, thereby minimising the risk of genetic linkage, since this is a fundamental assumption of some of our downstream analyses. This resulted in two final matrices, p10 and p90, with 7926 and 1762 SNPs and 47.1% and 14% missing data respectively (summarised in Supplementary Table 2).

At the species level, these same whitelists were passed to populations.pl along with a population map assigning individuals to species based on the best-supported species model. The resultant matrices (summarised in Supplementary Table 2) were used for the species level analyses described below. Reduced numbers of SNPs reported are due to a population (or species in this case) having incompatible loci – those with more than two alleles – which becomes possible when grouping individuals together.

Assessment of monophyly and clustering

To infer relationships among mobulid individuals, Maximum Likelihood (ML) phylogenetic analysis was carried out on concatenated ddRAD loci using RAxML version 8.2.11 (Stamatakis 2014). Analyses were run for both datasets since missing data is known to influence aspects of phylogenetic inference such as branch length (Leaché *et al.*, 2015). The GTRGAMMA model of rate heterogeneity was implemented following assessment of best fit models in jModelTest (Darriba *et al.*, 2015). Support for clades was assessed with 1000 bootstrap replicates and *Rhinoptera bonasus* was used as the outgroup to root the tree.

Once clades had been delimited with RAxML, the data were split into four groups, corresponding to four highly supported clades that were separated by long branch lengths. These four groups

correspond to the manta rays (*M. alfredi* and *M. birostris*), *M. mobular* (including specimens identified as *M. japanica* prior to the taxonomic revision published by (White *et al.*, 2017)), *M. thurstoni* and *M. kuhlii* (including specimens identified as *M. eregoodootenkee* prior to the taxonomic revision published by (White *et al.*, 2017)) and *M. hypostoma* and *M. munkiana*. See Supplementary Table 3 for details of numbers of SNPs sampled within each clade.

To assess how individuals cluster together, Principal Components Analysis (PCA) was performed on dataset p10 using the Adegenet package in R (Jombart 2008). After assessment of up to ten axes, three axes were retained in all cases. The populations.pl program in Stacks (Catchen *et al.*, 2011) was used to calculate pairwise F_{ST} values among inferred clusters.

Bayes Factor Delimitation of species

Species delimitation was carried out using the Bayes Factor Delimitation method with genomic data (BFD*) (Leache *et al.*, 2014), which allows for direct comparison of Marginal Likelihood Estimates (MLE) for alternative species delimitation models under the multispecies coalescent. This analysis was carried out using the modified version of SNAPP (Bryant *et al.*, 2012), implemented as a plug-in to BEAST (version 2.4.8; (Bouckaert *et al.*, 2014)). Path sampling was carried out with 10 steps, (1,000,000 MCMC iterations, 20% burnin), implementing the log-likelihood correction available in the program (Leache *et al.*, 2014). Since marginal likelihood estimates are affected by improper prior distributions, a gamma distribution was implemented on the lambda (tree height) parameter. To ensure that the ranking order of models was not affected by the priors, a second round was carried out retaining the default 1/X distribution on lambda, implementing upper and lower bounds of 10,000 and 0.00001 respectively, so that the prior becomes proper. Bayes Factors ($2\log_e BF$) were calculated from the MLE from each model for comparison (Kass and Raftery, 1995; Leache *et al.*, 2014), using the formula:

$$2\log_e BF = 2*(MLE_{null} - MLE_{test})$$

Where positive $2\log_e BF$ values indicate support for the null model, whilst negative BF values favour the tested model. $2\log_e BF$ values < 10 are considered decisive support (Leache *et al.*, 2014).

Due to the high computational requirements of running SNAPP, this analysis was carried out on the smaller dataset, p90, and the data was split up into clade specific datasets, as described above. For each clade however, four random individuals from the sister clade were included, to assess support for interaction from higher up the tree. See Supplementary Table 4 for details of numbers of SNPs sampled within each clade.

Alternative species delimitation models for each clade were informed both by the literature and by our own phylogenetic and clustering analyses (see Supplementary Table 4 for details). In addition, a model that randomly assigns individuals to two or three species was included for each clade, to assess relative support for other models. In all clades, the null model was considered as those species defined by White et al. (2017), and all Bayes Factors were calculated relative to this null model.

Species tree inference

To estimate relationships among the Mobulidae, phylogenetic analyses of individuals belonging to each of the best supported species was carried out using both Maximum Likelihood and Bayesian methods. Maximum Likelihood phylogenetic analysis was carried out on concatenated ddRAD loci for both species-level datasets, as described above for the individual-level datasets.

To test the tree topology and evaluate uncertainty, for example, due to incomplete lineage sorting, species tree inference was also carried out in SNAPP (Bryant *et al.*, 2012), which allows each SNP to have its own history under the multispecies coalescent whilst bypassing the need to sample each individual gene tree. Due to the computational constraints associated with running SNAPP on a dataset as large as ours, dataset p90 was used, and three individuals per species were randomly selected following (Foote and Morin, 2016), whilst maximising geographical coverage within species. This process was repeated a further three times, randomly sampling individuals with replacement, resulting in four subsampled alignments (individual-specific details of each subsample, as well as details of numbers of SNPs retained with each subsample are provided in Supplementary Table 5). These four independent runs were carried out with an MCMC chain of 5,000,000 iterations, sampling every 1000 and retaining default priors on lambda and theta. Similar runs with different prior combinations produced similar results. Convergence to stationary distributions were assessed by visual inspection after 20% burnin in TRACER (Rambaut *et al.*, 2018). The distribution of trees was visualised after 20% burnin in DensiTree (version 2.2.6; (Bouckaert 2010)). The maximum clade credibility tree was drawn using TreeAnnotator (version 2.4.7; (Bouckaert *et al.*, 2014)).

Multi species coalescent approaches, such as SNAPP used in this study, assume that any discordance of topologies among loci results from incomplete lineage sorting and do not consider introgression as a source of discordance. Therefore, to investigate the extent to which the variation in these data is best explained by a single bifurcating tree, TreeMix (Pickrell and Pritchard, 2012) was used to evaluate whether there is evidence for significant introgression events within the Mobulidae. TreeMix involves building a maximum likelihood tree of user defined groups and calculating how much of the variance

639 in the data this fixed tree model accounts for. TreeMix was run on dataset p10. Given patterns
640 observed using SNAPP with respect to uncertainty in the placement of *M. mobular*, the three-
641 population test (Reich *et al.*, 2009) was additionally used to test for 'treeness' between clades. Similar
642 to TreeMix, the three-population test estimates the covariance of allele frequencies between
643 populations, but is a simple and less parameterised model than TreeMix, and thus can be a more
644 powerful tool for identifying introgression. In addition to *M. mobular, M. alfredi* and *M. thurstoni* were
645 randomly chosen from their respective clades for this test.

646

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Author Contributions

JH, EH, GC, MdB, RO, SC and GS designed and conceived of the study and secured funding for consumables relating to laboratory work. EH, GS, DF, AP, MA, JS, SP, SW, RJ, MP, MM, KBH, RB, JS and LP were responsible for sourcing and collecting samples. JH, HS and JK carried out laboratory work. JH, EH, GC, MdB, RO, SC, HH, AF and HS contributed to analysis of genome-wide SNP data. Figures were designed by EH and JH and produced by EH. All authors contributed to writing and editing the manuscript.

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