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Elasmobranch diversity across a remote coral reef atoll revealed through environmental DNA metabarcoding

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As elasmobranchs are becoming increasingly threatened, efficient methods for monitoring the distribution and diversity of elasmobranch populations are required. Environmental DNA (eDNA) metabarcoding is a progressively applied technique that enables mass identification of entire communities and is an effective method for the detection of rare and elusive species. We performed an eDNA metabarcoding survey for fish communities around a coral reef atoll in the Chagos Archipelago (Central Indian Ocean) and assessed the diversity and distribution of elasmobranch species detected within these communities. Our eDNA survey detected 353 amplicon sequence variants (ASVs) attributed to fishes, 12 of which were elasmobranchs. There were no differences in fish communities based on the presence and absence of ASVs between sample depth (surface and 40 m) or sampling habitat, but communities based on read abundance were significantly different between habitats. The dominant elasmobranch species were grey reef (*Carcharhinus amblyrhynchos*) and silvertip (*C. albimarginatus*) sharks, and elasmobranch communities were significantly different between sampling depth and habitat. Overall, we find that eDNA metabarcoding can be used to reveal the diversity of elasmobranchs within broader taxonomic assays, but further research and development of targeted metabarcoding primers may be required before it can be integrated into a toolkit for monitoring these species.

ADDITIONAL KEYWORDS: barcoding – conservation genetics – eDNA metabarcoding – fish – marine – sharks.

INTRODUCTION

It is estimated that one-third of elasmobranch species (sharks, rays and skates) are threatened with extinction (Dulvy *et al.*, 2021). As a result of continued overfishing and overexploitation, reef sharks are now functionally extinct in several nations (MacNeil *et al.*, 2020) and since 1970, the abundance of oceanic sharks and rays has declined by over 70% (Pacoureau *et al.*, 2021). As elasmobranchs are often high-level predators in marine ecosystems, global declines in their populations threaten to destabilize trophic networks and precipitate cascading effects on marine food webs (Heithaus *et al.*, 2008; McCauley *et al.*, 2010). The conservation of elasmobranchs is crucial to protecting ecosystem function and centres around reducing the impact of fishing through industry regulation (Shiffman & Hammerschlag, 2016) and the establishment of marine protected areas (MPAs) to limit both targeted fishing and incidental by-catch from industrial fishing activities (Koldewey *et al.*, 2010; Knip *et al.*, 2012; Davidson & Dulvy, 2017). However, insufficient monitoring within many existing MPAs currently limits our ability to assess the effectiveness of protected areas for conserving elasmobranch species (MacKeracher *et al.*, 2019).

Established techniques used to monitor elasmobranchs suffer from a number of limitations that can lead to underestimations of biodiversity, both spatially and temporally. For example, populations of pelagic species are commonly assessed by tracking catch and by-catch records from commercial fisheries

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(Walsh *et al.*, 2002; Nakano & Clarke, 2006). However, these data are often taxonomically biased by gear selectivity (Filmalter *et al.*, 2013) and tend to cease, or be significantly reduced, once MPAs are established. Non-destructive methods, such as underwater visual census (UVC) surveys (Robbins *et al.*, 2006) and Baited Remote Underwater Video System (BRUVS) surveys (Santana-Garcon *et al.*, 2014), have proven to be effective for long-term elasmobranch monitoring in MPAs. However, the former tends to be limited to accessible areas located within diver depth, while the latter disproportionately samples scavenging species attracted by bait (Watson & Harvey, 2007; Wraith *et al.*, 2013). Both methods are also time-consuming to implement in the field, limiting the sample sizes and survey coverage that can be achieved.

The detection of species from environmental DNA (eDNA) combined with metabarcoding methods (i.e. the mass amplification and high-throughput sequencing of DNA from multiple species using universal primers) is seen as a major advance for ecosystem monitoring in marine conservation (Ruppert *et al.*, 2019). Advantages of sampling for eDNA include the non-invasive detection of rare and elusive species (e.g. Simpfendorfer *et al.*, 2016) and the ability to provide information on entire communities from single samples (e.g. Stat *et al.*, 2017) that can often be collected quickly by non-specialists with limited equipment (Biggs *et al.*, 2015). Numerous studies have shown that eDNA metabarcoding can detect a greater number of fish species than traditional methods (e.g. Valentini *et al.*, 2016) and estimates of relative abundance, either through site occupancy (Hänfling *et al.*, 2016) or read abundance (Li *et al.*, 2018; Mariani *et al.*, 2021), are starting to be used to reveal snapshots of distributions and biomass hotspots for certain species. However, biases in the extraction and amplification methods involved and lack of knowledge on shedding and degradation rates mean that true abundance estimates cannot currently be generated (Nichols *et al.*, 2018; Ushio *et al.*, 2018).

Small amounts of eDNA from elasmobranch taxa are often detected in marine metabarcoding studies (Thomsen *et al.*, 2016; Andruszkiewicz *et al.*, 2017; Closek *et al.*, 2019; Afzali *et al.*, 2020; Fraija-Fernández *et al.*, 2020; West *et al.*, 2020, 2021; Polanco Fernández *et al.*, 2021) and these are often highlighted as species of interest in the assemblage (Fraija-Fernández *et al.*, 2020; West *et al.*, 2020). One of the first metabarcoding studies specifically targeting sharks found that eDNA samples collected in remote protected areas produced higher shark species richness and more sequence reads per sample than in less remote and unprotected sites (Bakker *et al.*, 2017). A further study showed that eDNA can complement detections from UVC and BRUVS surveys to uncover the hidden diversity of shark species in an area (Boussarie *et al.*, 2018). More recently, Mariani *et al.* (2021) used eDNA metabarcoding to reveal temporal variations in elasmobranch distributions around Réunion Island, and West *et al.* (2021) found that elasmobranch community composition was driven by location and the depth at which eDNA samples were taken from north-western Australia. As a result of these advances, eDNA metabarcoding is now seen as a key method that can be added to the toolkit of techniques [e.g. BRUVS, UVC, population genetics and fisheries surveys (Rigby *et al.*, 2019)] for the long-term monitoring of elasmobranch species.

In this study, we assess the diversity and distribution of elasmobranchs detected within fish communities around a remote coral reef atoll, Diego Garcia in the Chagos Archipelago, Central Indian Ocean. The Chagos Archipelago and its Environment Protection and Preservation Zone, encompassing 640 000 km^2 of pelagic and reef habitat, was established in 2010 as one of the largest MPAs in the world (see recent review: Hays *et al.*, 2020). Historical records indicate that the waters around the Chagos Archipelago contain at least 773 fish species, including 21 elasmobranch species (Winterbottom & Anderson, 1997). Ray-finned fish species across the archipelago are relatively well studied from time series of UVC surveys (Samoilys *et al.*, 2018) and in-depth species-specific growth studies (Taylor *et al.*, 2020). However, no recent studies have assessed the biodiversity of elasmobranchs in the region, and past research effort has primarily been directed on the shallow reefs around the atolls in the north of the archipelago. Consequently, the elasmobranch community around Diego Garcia remains relatively understudied and focused on trophic ecology (Curnick *et al.*, 2019). Throughout the wider archipelago, studies have investigated the distributions and abundance of elasmobranch species using BRUVS (Tickler *et al.*, 2017; Letessier *et al.*, 2019), UVC (Graham *et al.*, 2010) and telemetry techniques (Carlisle *et al.*, 2019; Andrzejaczek *et al.*, 2020; Curnick *et al.*, 2020; Jacoby *et al.*, 2020; Williamson *et al.*, 2021). However, the true diversity of elasmobranchs in the region is not fully understood, with several species only known to be in the territory from the historical commercial fishery observer programme and records from arrested illegal, unregulated and unreported (IUU) vessels (Martin *et al.*, 2013; Moir Clark *et al.*, 2015).

Here, we conducted an eDNA metabarcoding from water samples taken at two depths from 20 sites around Diego Garcia and sequenced the extracted eDNA from each sample using fish- and elasmobranch-targeting primers in multiplex. We investigated the biodiversity of fish species recovered from the eDNA surveys and assessed the difference in fish

communities among sampling depths and habitats. We then investigated the sensitivity and reliability of a broad eDNA metabarcoding assay for elasmobranch monitoring by comparing our eDNA results with a new reference list of elasmobranch species in the Chagos Archipelago compiled from historical fisheries data, scientific surveys and IUU catch records.

MATERIAL AND METHODS

eDNA SURVEY

Water sampling and filtration

Water samples were collected over the course of nine days during a research expedition to Diego Garcia in September 2019. Sampling locations around the atoll were selected to systematically survey a representative range of habitats (outer atoll reef crest, shallow lagoon coral reef and shallow sandy-bottom cove), at approximately 5-km intervals. To test whether fish communities differed with depth, wherever possible, sites on the outside of the atoll were sampled at 40 m (hereafter 'deep') and at the surface (< 1 m) following Andruszkiewicz *et al.* (2017). A total of 32 samples were collected, including 12 paired (surface and deep) and three surface samples in open-water habitat beyond the reef crest. Three surface samples were taken over shallow, coral reef habitat in the north of the lagoon and two samples were taken in shallow, sandy-bottom cove habitat to the south of the lagoon. This resulted in 20 discrete sampling sites across three distinct habitats (Fig. 1).

At each paired sampling location on the outside of the atoll, a 5-L Niskin bottle was rinsed with surface water and then deployed to 40 m depth, with the spring-loaded closing mechanism being triggered by a messenger weight to collect the sample. After retrieval of the bottle, the sample was decanted into 5-L containers and stored on ice until processing.

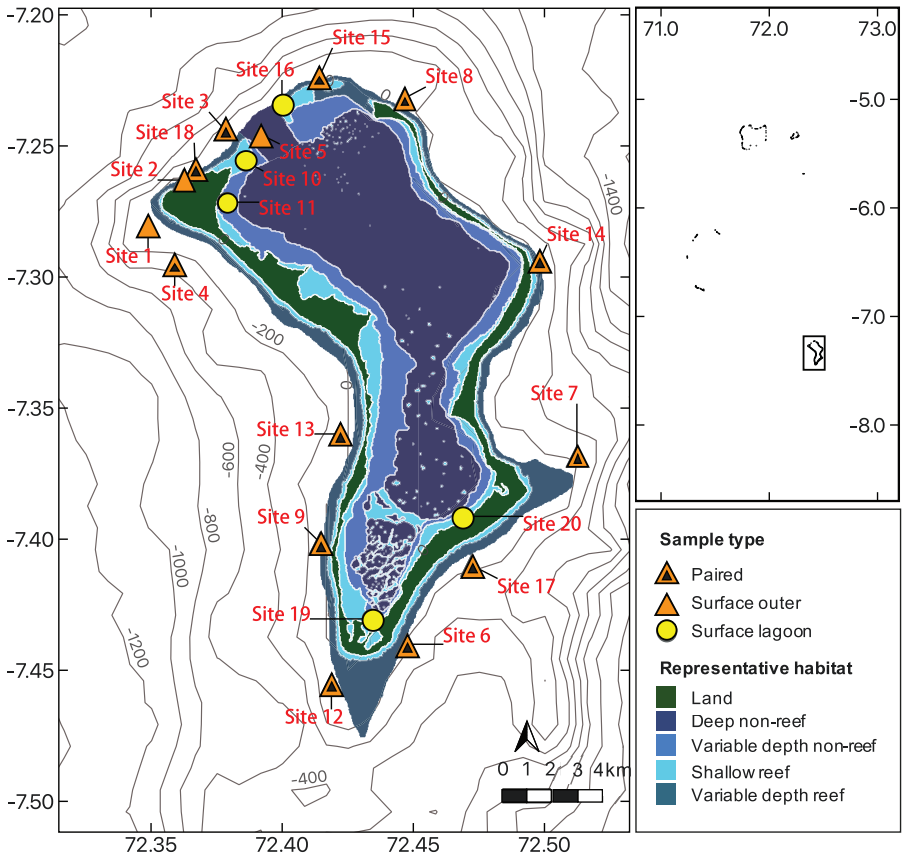

Figure 1. Location of water sampling sites around Diego Garcia. Triangles represent sampling sites on the outside of the atoll ($N = 27$), sites where samples were taken at 40 m and the surface (paired) are shown with a dark triangle inside. Circles represent lagoon samples ($N = 5$). Contour lines show the bottom depth in meters and colours represent the designated habitats around the atoll. Inset shows the location of Diego Garcia with respect to the other atolls in the Chagos Archipelago. Map was made using QGIS v.3.

The Niskin bottle was then re-washed in the surface water, filled with surface water by closing one end and the sample stored in the same manner as the deep sample. For three outer atoll sites and all the lagoon sites, a single 5-L surface sample was taken (Fig. 1). We undertook one sampling trip each morning and one in the afternoon, but as weather conditions prevented sampling trips on some days, there were trips where two samples were taken, and the second sample was stored in a fridge at ~4 °C until filtering. Filtration of all samples took place back on land within three hours of collection.

Three 1-L subsamples from each sample were vacuum filtered using 0.22 µm Sterivex (Merck-Millipore) filters. Once a litre had been filtered (*c*. 10 min), the filter was run dry and then removed from the vacuum tubing. The capsule was then filled with 2.5 mL of Longmire's buffer (Renshaw *et al.*, 2015). Capsules were sealed and placed into individual 100-mL Whirlpak bags for storage and transport. After sample processing, containers and vacuum tubing were washed with 20% bleach and rinsed thoroughly to remove any residue. A negative control of 250 mL sterile water was filtered each sampling day to test for any contamination between samples.

DNA extraction

The Sterivex capsules preserved in Longmire's buffer were stored at room temperature for seven weeks prior to DNA extraction following the protocol described by Spens *et al.* (2017) using the DNeasy Blood & Tissue Kit (Qiagen). The lysis solutions from the capsule and buffer components were combined before the addition of buffer AL and the continuation of the Blood & Tissue Kit protocol. Negative extraction controls were performed with distilled water. Final DNA elution occurred with 100 µL nuclease-free water. Extracted DNA was quantified with a Qubit 2.0 fluorometer (Life Technologies) and stored at 4 °C. During the extraction process, a microcentrifuge fault resulted in the loss of one replicate from each sample depth from sites 8 and 9 and, consequently, these samples were amplified in duplicate rather than triplicate.

Metabarcoding

The protocol for DNA amplification and library preparation followed a two-step amplification process outlined in the Illumina 16S protocol (Illumina Inc., 2013) with modifications for amplification using the MiFish primers as described by Miya *et al.* (2015), which targets a 163–185 bp hypervariable region on the mitochondrial 12S gene. DNA was amplified in multiplex using the recommended overhang adapter sequences on the end of the universal fish-targeting primer pair (hereafter MiFish-U) and elasmobranch-targeting primer pair (MiFish-E) (Table 1). The polymerase chain reaction (PCR) mix consisted of 6 µL KAPA 2 × HotStart HiFi Master Mix (Roche), 0.7 µL of each primer, 2.5 µL extracted DNA and 1.2 µL H_2O to make the mixture up to 12.5 µL. Thermal conditions consisted of an initial denaturation step of 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s, 65 °C for 30 s and 72 °C for 30 s, with a final extension of 72 °C for 5 min.

The negative field and extraction controls were included, and non-template PCR controls (NTC) were also run. A PCR clean-up using AMPure beads (Beckman Coulter) was performed and the clean PCR products were visualized on a 1% agarose gel. Products were then diluted with nuclease-free water 1:10 before being used as the template for a second PCR. Here, the three replicates from each sample were pooled to result in a single library for each water sample taken. One negative field control was amplified during the first PCR, so was treated as a sample and included in the second PCR run. The second PCR added Illumina index adapters to the amplified products using the Nextera Index kit (set A). The PCR mix contained 12 µL KAPA HiFi HotStart Master Mix (Roche), 2.5 µL of each index primer, 3 µL template and 5 µL H_2O. Thermal and cycling conditions were 95 °C for 2 min followed by 12 cycles of 55 °C for 30 s and 72 °C for 30 s, with a final extension step of 72 °C for 2 min.

Products from the second PCR were cleaned using AMPure beads and run on a 1% agarose gel alongside a product from the first PCR as a length reference to confirm the Illumina indexes had been added successfully. Each library was then quantified using a

Table 1. Overhang and locus-specific sequences for the MiFish primers (Miya *et al.*, 2015) used in this study. The full sequence used for amplification in the first PCR consisted of the overhang and locus-specific sequence as a single oligonucleotide

Primer name	Direction	Overhang adapter sequencer (5′–3′)	MiFish locus-specific sequence (5′–3′)
MiFish-U	Forward	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	GTCGGTAAAACTCGTGCCAGC
MiFish-U	Reverse	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG	CATAGTGGGGTATCTAATCCCAGTTTG
MiFish-E	Forward	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	GTTGGTAAATCTCGTGCCAGC
MiFish-E	Reverse	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG	CATAGTGGGGTATCTAATCCTAGTTTG

Qubit 2.0 fluorometer (Invitrogen) and ten randomly selected libraries were quantified using the KAPA qPCR library quantification kit (Roche) to confirm the quantified value. Libraries were diluted to 4 nmol/L and then pooled and denatured following the MiSeq library preparation protocol, a 30% PhiX control was added to the pooled library. The libraries were sequenced using 300 bp paired-end sequencing on an Illumina MiSeq with V3 reagents.

Bioinformatics and statistics

The raw sequences were filtered and processed using a DADA2 (Callahan *et al.*, 2016) bioinformatics pipeline in R (R Core Development team, 2020). Sequences longer than the 225 base pairs were removed from the processed sequence files before taxonomic assignment of the amplicon sequence variants [ASVs: unique sequences with at least one nucleotide difference to other sequences of the amplicon (Callahan *et al.*, 2017)], using the web-based BLASTn megablast program (Altschul *et al.*, 1990; Zhang *et al.*, 2000) and the nucleotide collection database. Ten matches per ASV were downloaded as a hit table and the top hit based on Bit-score with a sequence identity greater than or equal to 97%, and an E-value less than $1e^{-20}$ was applied to species assignment for each sequence. This threshold for taxonomic assignment was designated by Miya *et al.* (2015) in the development of the primers and if the top hit had less than 97% identity, all assigned taxonomy below class level (genera and species) was removed to avoid erroneous taxonomic assignment, but the class assignment was retained if all ten matches in the hit table derived from the same class, which was the case in all instances. If two taxonomic assignments matched on every criterion, the lowest common taxonomic classification was retained for the ASV. Using sequences amplified in the negative control, any potential contamination was removed using the *decontam* package (Davis *et al.*, 2018) and the negative controls were then removed from the analysis dataset. The LULU package (Frøslev *et al.*, 2017) was used to curate the dataset by removing potentially erroneous ASVs with a 96% minimum match parameter.

The number of sequencing reads per sample was normalized based on the median read number per sample using the *Phyloseq* package (McMurdie & Holmes, 2013). Read numbers were square-root transformed following Mariani *et al.* (2021) and a distance matrix of each sample based on Bray–Curtis dissimilarity was produced using *vegan* and visualized using a principal component analysis in *ggplot2* (Wickham, 2016). Non-metric multidimensional scaling (NMDS) was also used to test for community differences between the presence of ASVs using the Jaccard dissimilarity matrix. The differences in fish and elasmobranch communities between sampling depth, location (lagoon or outer atoll) and habitat (open water, shallow reef, sandy-bottom cove) were investigated using a permutational multivariate analysis of variance (PERMANOVA) using both the Bray–Curtis and Jaccard dissimilarity matrices. Pairwise analysis of similarity (ANOSIM) tests in the package *pairwiseAdonis* (Martinez Arbizu, 2017) were also carried out between habitat types.

Sequences from all elasmobranch taxa detected in the samples were individually checked in BLAST and taxonomic assignment was confirmed. The non-parametric Wilcoxon rank sum test was used to compare the difference between reads produced from surface and deep samples. The spatial distribution and copy number abundance of each species at both sampling depths was investigated and visualized using QGIS (v.3.12).

After preliminary analyses of the data, we performed a post hoc run of the DADA2 bioinformatic pipeline without the clipping of primer sequences to investigate the relative contribution of each primer set to the ASV dataset. We ran the pipeline up to the stage of producing ASV sequences and downloaded the ASVs produced. Within these, we searched for each primer sequence, noting the number of sequences that contained each individual primer sequence, and the primer pairs that amplified the sequence.

Elasmobranch species reference list

In order to validate species detections from eDNA, we compiled a comprehensive inventory of elasmobranch species recorded around the Chagos Archipelago by updating a previous checklist of fishes in the archipelago (Winterbottom & Anderson, 1997) with more recent fisheries' observer data, IUU seizure records and UVC and BRUVS surveys. Independent observer records of 41 offshore longline sets deployed between November 2000 and January 2003 were obtained from the Marine Resources Assessment Group (MRAG) who managed the commercial fisheries around the Chagos Archipelago before it was established as an MPA (Mees *et al.*, 2009). From this offshore hook survey, we calculated the relative abundance of each elasmobranch species as the mean catch per unit effort (CPUE – number of individuals caught divided by the number of hooks deployed, multiplied by 1000) following Curnick *et al.* (2020). These data included the pelagic species such as the bigeye thresher shark (*Alopias superciliosus* Lowe, 1841), pelagic thresher shark (*A. pelagicus* Nakamura, 1935), cookiecutter shark (*Isistius brasiliensis* Quoy &

Gaimard, 1824) and the blue shark (*Prionace glauca* Linnaeus, 1758), which made up 22% of the records.

The relative abundances of species recorded in IUU seizures between January 2006 and January 2020 ($N = 77$) were calculated as the proportional contribution of each species to the total number of individuals caught. Data from individuals that were not recorded to species level were discarded. When only total species weight was recorded in a given catch, the average total length of that species in the region was obtained from the IUU catch records and converted to an average weight for the species using established length–weight relationships obtained from FishBase (fishbase.org, see Supporting Information, Table S1 for calculations and references). The recorded weight was then divided by the average species weight to give an estimate of the number of individuals represented by the record. These records identified 19 elasmobranch species, with silvertip shark (*Carcharhinus albimarginatus* Rüppell, 1837) and grey reef shark (*C. amblyrhynchos* Bleeker, 1856) together making up just under half of the catch, at 26% and 22% of the total number of individuals caught, respectively (Supporting Information, Table S2).

We also included the mean number of individuals from the five reef shark species observed per dive in UVC surveys performed during 134 dives in 1996, 2001 and 2006 at depths between 5 and 25 m at three northern atolls in the Chagos Archipelago (Graham *et al.*, 2010) and the frequency of occurrence of the eight shark species observed in 138 BRUVS surveys at 35 sites around the Chagos Archipelago in February and March 2012 (Tickler *et al.*, 2017).

RESULTS

Metabarcoding

From the eDNA metabarcoding, a total of 10 204 919 reads were produced. After clustering, filtering, denoising, merging and the removal of bimeric sequences in DADA2, the dataset contained 3 828 593 reads. The median number of reads per sample used for normalization was 166 427. The majority of reads (83.9%) were attributed to Actinopterygii fishes, followed by mammals (12.5%; humans being the most abundant of the resolved taxa) and elasmobranchs (3.3%), with the remaining reads (0.3%) originating from either birds, reptiles, fungi or bacteria (Fig. 2A). One ASV for *Homo sapiens* Linnaeus, 1758 was removed after analysis with the *decontam* package, and the resulting dataset was reduced from 947 ASVs to 426 final ASVs after curation in LULU.

In the post hoc analysis of primer contribution, a total of 724 ASVs were produced. This differed from the original dataset due to the addition of the primer sequences altering the sequence filtration and error rate calculations. Of these, 620 (85.6%) appear to be amplified by the MiFish-E primer pair and two sequences (0.3%) were amplified by the MiFish-U pair. There were 50 sequences (6.9%) amplified by the forward MiFish-U primer in combination with the reverse MiFish-E primer, and ten sequences (1.4%) were amplified by the forward MiFish-E primer in combination with the reverse MiFish-U primer; 42 sequences (5.8%) appeared to be a hybrid of the two primer sets (Supporting Information, Fig. S1).

Fish biodiversity and community analysis

The fish community dataset contained 353 ASVs, 88 of these sequences failed to meet the threshold criteria to be assigned taxonomy below class and 34 sequences were only resolved to genus due to matches for two species assignments based on the assignment criteria. The remaining 231 ASVs were attributed to a total of 166 species from 136 genera and 56 families (Supporting Information, Table S3). The majority (82%) of ASV sequences directly resolved to a single species and the maximum number of unique sequences attributed to a single species was six. The maximum number of ASVs detected in a single sample was 82, with the mean being 29.47. Although mean diversity was higher in surface samples than samples collected at 40 m (32.6 and 24.25 ASVs, respectively), this difference was not significant.

The dataset was dominated in terms of read abundance by kawakawa (*Euthynnus affinis* Cantor 1849; 19.5% of fish reads) and rainbow runner (*Elagatis bipinnulata* Quoy & Gaimard, 1825; 17.6%), followed by skipjack tuna (*Katsuwonus pelamis* Linnaeus, 1758; 12.7%). Community clusters were driven by the read abundance of ASVs from these three species with *Eu. affinis* and *El. bipinnulata* driving differences on axis 1 and *K. pelamis* driving separation on axis 2. There was no clustering by depth or location in the principal coordinate analysis (Fig. 2B) but sites 19 and 20, taken from the cove habitat, clustered separately and there were significant differences between the read abundances of communities from different sampling habitats ($R^2 = 0.096$, $F = 1.53$, $P < 0.005$) driven by a pairwise difference between the cove and outer atoll samples ($R^2 = 0.124$, $F = 3.83$, $P < 0.005$). Communities based on a Jaccard dissimilarity matrix did not significantly group to sampling depth, location or habitat.

Elasmobranch reads

The number of reads attributed to elasmobranch species per sample was highly variable, ranging from no reads in the surface sample at site 18, to 23 613 reads in the surface sample at site 9. The mean number

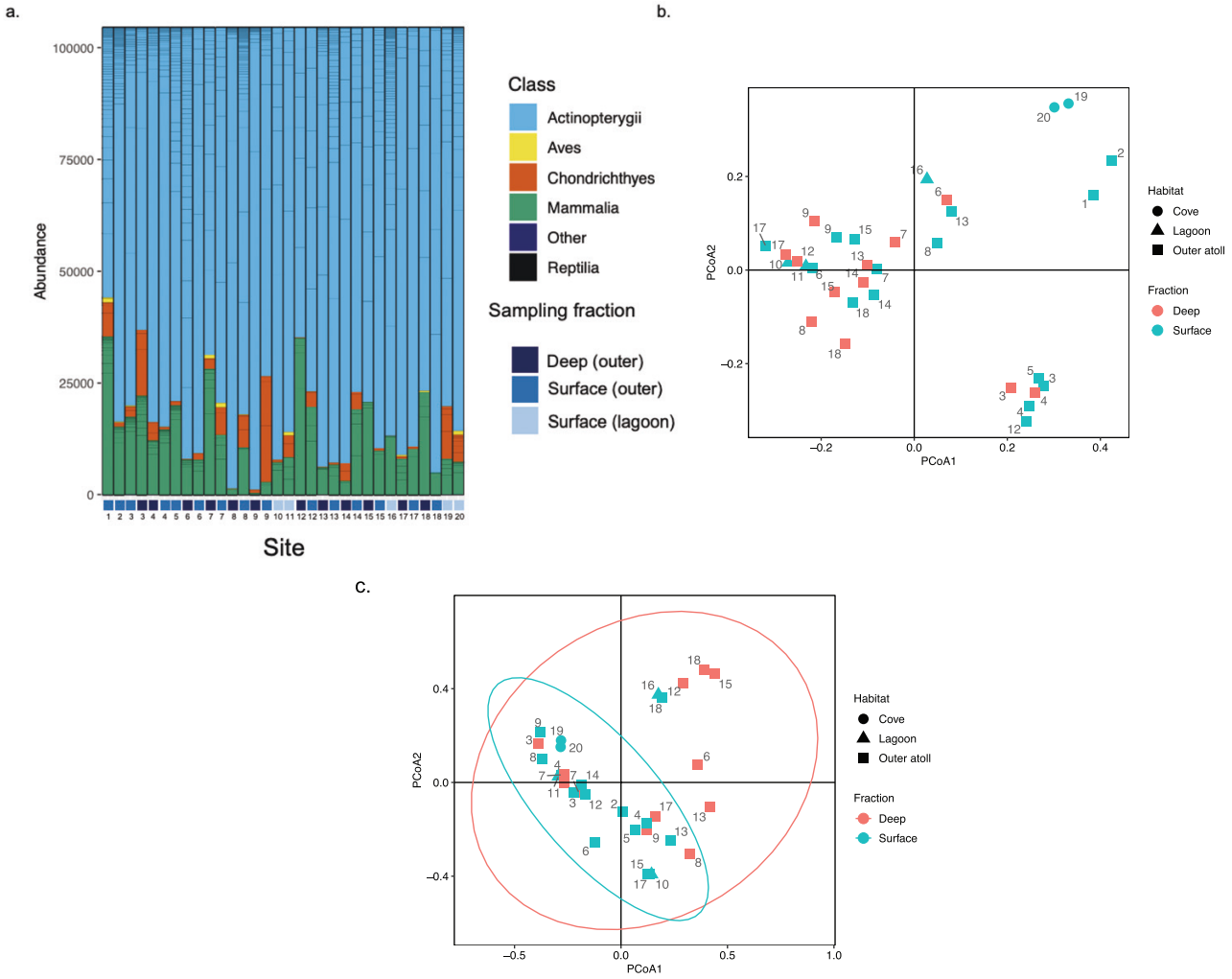

Figure 2. A, taxonomic breakdown of eDNA reads by class in water samples collected around Diego Garcia atoll in September 2019. In each case, the fraction of the water column sampled is denoted by the colour key displayed below each bar. B, principal coordinate analysis (PCoA) of read abundance of all fish and elasmobranch ASVs per sample based on Bray–Curtis similarity. C, PCoA of read abundance of elasmobranch ASVs per sample based on Bray–Curtis similarity. Site numbers refer to the sites described in Figure 1.

of reads was 4158 ± 1256 (SE) in surface samples and 2258 ± 1215 in deep samples. Yet this difference was not significant, even when the two highest records from each fraction were removed as outliers (Supporting Information, Fig. S2).

Elasmobranch communities based on Bray–Curtis dissimilarity were significantly different between surface and deep fractions (Fig. 2C; $R^2 = 0.082$, $F = 2.68$, $P < 0.05$) and between samples taken from outer atoll sites compared to cove habitat ($R^2 = 0.081$, $F = 2.39$, $P < 0.05$). There were insufficient elasmobranch species to robustly investigate differences in the presence or absence of species between sites. However, the two samples collected in sandy cove habitats suggested a distinct species assemblage with the highest overall elasmobranch diversity and *c*. 40% of reads from stingrays, which were rare or absent at other sites (Fig. 3A).

Twelve ASVs were assigned to elasmobranch species with > 97% confidence (Table 2). *Carcharhinus albimarginatus* and *C. amblyrhynchos* were both assigned two ASVs differing by one nucleotide each, which were combined into their respective species for the analyses resulting in a total of ten elasmobranch species recorded. The most abundant species was *C. albimarginatus*, accounting for 51.4% of the total sequences, followed by *C. amblyrhynchos* with 39.1% of the total reads (Fig. 3).

The cowtail stingray (*Pastinachus sephen* Forsskål, 1775) was the third most abundant taxon in our eDNA samples, responsible for 5.2% of the reads, despite being only detected in the lagoon (Fig. 3A). The fourth

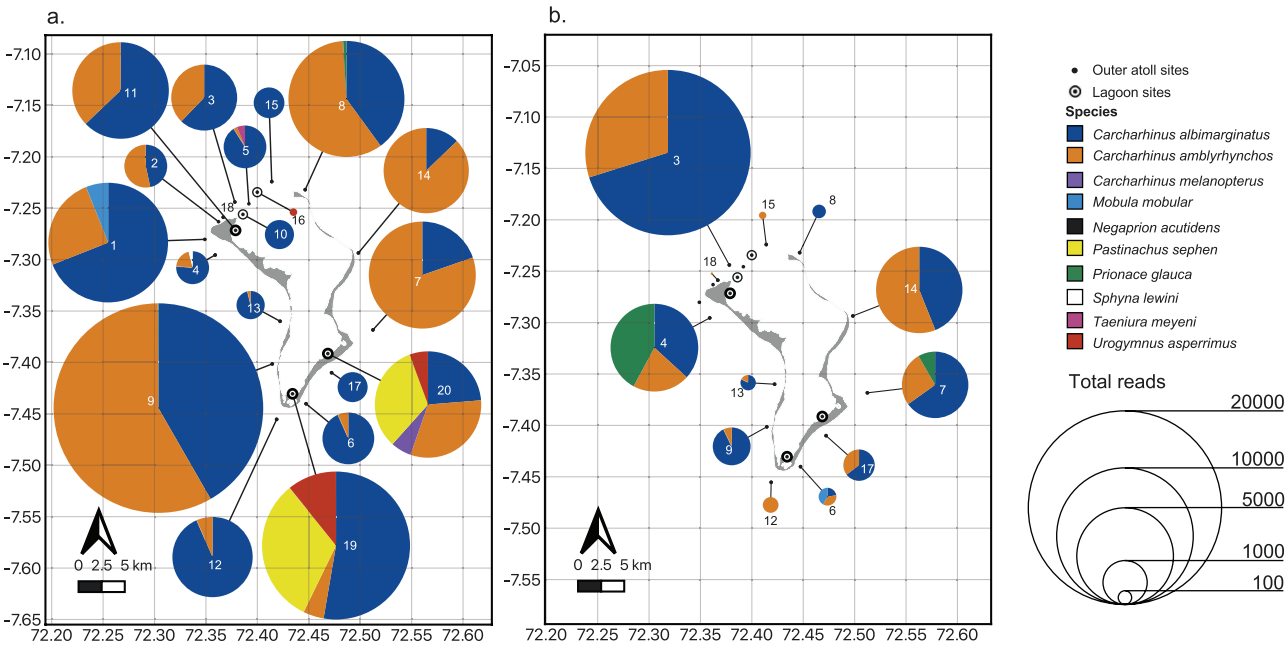

Figure 3. Spatial variation in elasmobranch abundance and diversity inferred from eDNA metabarcoding of surface (A) and deep (40 m) (B) water samples collected around Diego Garcia. *Negaprion acutidens* is not visible in the charts as a result of low copy number, but was detected at site 8 in surface samples. Numbers correspond to the site numbers detailed in Figure 1.

most abundant elasmobranch species in our eDNA samples was *Pr. glauca*, accounting for 1.8% of the sequences across four locations on the outside of the atoll (Fig. 3A, B).

The remaining six taxa accounted for 2.4% of the total elasmobranch eDNA reads and five of these taxa were only detected in a single eDNA sample (Table 2). The spinetail devil ray (*Mobula mobular* Bonnaterre, 1788) was detected at two sites, while the scalloped hammerhead shark (*Sphyrna lewini* Griffith & Smith, 1834) and the sicklefin lemon shark (*Negaprion acutidens* Rüppell, 1837) were each detected in one surface water sample. The roundtail ribbon ray (*Taeniura meyeni* Müller & Henle, 1841) was detected in the north of the lagoon and eDNA from the blacktip reef shark (*C. melanopterus* Quoy & Gaimard, 1824) and porcupine ray (*Urogymnus asperrimus* Bloch & Schneider, 1801) were detected in the south of the lagoon (Fig. 3).

Elasmobranch species reference data

Our compilation of elasmobranch species recorded in the Chagos Archipelago, including species detected in our eDNA samples, produced an inventory of 35 elasmobranch species across 11 families, of which five species are Endangered and four are listed as Critically Endangered in the IUCN Red List (Supporting Information, Table S2). All but one of the species (*M. mobular)* detected within our eDNA survey was present in the reference database.

Just five species in the IUU records were also detected in the eDNA survey around Diego Garcia; the only species detected in both the observer data and eDNA datasets was *Pr. glauca*. Three reef shark species detected in the UVC surveys were also detected in the eDNA samples in this study and four of species identified in BRUVS surveys were detected in the eDNA survey (Fig. 4).

DISCUSSION

The rapid collection of diversity data for long-term monitoring is a vital part of the conservation of coral reefs and efficient methods for surveying and monitoring vulnerable elasmobranch species will be critical to the implementation of successful conservation measures. Our results support the view that eDNA metabarcoding can be used to complement established approaches for determining the diversity of fishes and elasmobranchs in remote ocean ecosystems, and with further development, will become an important tool for monitoring areas where the time and expertise needed to complete more established monitoring techniques may not be available. By combining primers for the amplification of fishes and elasmobranchs, we obtained a large dataset containing

Table 2. Elasmobranch species detected by eDNA metabarcoding at Diego Garcia. For each species, the percent identity assigned to the classification by BLAST is presented along with the number of samples and sampling fraction (deep or surface) and habitat that each taxon was detected in

Common name	Species name	IUCN conservation status	BLAST identity (%)	No. samples detected in (N = 32)	Sampling fraction present in	Habitat present in
Silvertip shark	*Carcharhinus albimarginatus*	VU	100	28	Both	All
Grey reef shark	*Carcharhinus amblyrhynchos*	NT	100	26	Both	All
Blue shark	*Prionace glauca*	NT	100	4	Both	Outer atoll
Cowtail stingray	*Pastinachus sephen*	NT	99.5	2	Surface	Cove
Porcupine ray	*Urogymnus asperrimus*	VU	98.4	3	Surface	Lagoon reef and cove
Spinetail devil ray	*Mobula mobular*	EN	100	2	Both	Outer atoll
Blacktip reef shark	*Carcharhinus melanopterus*	NT	99.5	1	Surface	Cove
Round Ribbontail ray	*Taeniurops meyeni*	VU	99.4	1	Surface	Lagoon reef
Scalloped hammerhead shark	*Sphyrna lewini*	CR	97.8	1	Surface	Outer atoll
Sicklefin lemon shark	*Negaprion acutidens*	VU	100	1	Surface	Outer atoll

Abbreviation: IUCN, International Union for Conservation of Nature; CR, critically endangered; EN, endangered; NT, near threatened; VU, vulnerable.

a total of 353 ASVs, which resulted in 166 resolved fish and elasmobranch species. The species detected here can provide baseline biodiversity data for whole reef fish communities to be monitored over time and complement more in-depth surveys that focus on distinct indicator species (Samoilys *et al.*, 2018).

The fish communities recorded from the sandy-bottom cove habitat in the south of the lagoon significantly differed from the communities identified from samples taken in open ocean water around the atoll. This is in line with a recent eDNA metabarcoding study in the Cocos (Keeling) Islands, which utilized assays that targeted fishes, elasmobranchs, crustaceans and all eukaryotes, and found that fish and elasmobranch communities from the outer reef terrace clustered apart from samples taken within the low complexity lagoon habitat of the atoll (West *et al.*, 2020). In our study, the difference appears to be driven by the substantially lower read abundance of kawakawa (*Euthynnus affinis*), rainbow runner (*Elagatis bipinnulata*) and skipjack tuna (*Katsuwonus pelamis*) in the shallow cove habitat. The fact that these reef-associated and pelagic species were not dominant in the cove habitat supports the growing view that eDNA can reveal fine-scale community differences across connected but distinct habitats (Fraija-Fernández *et al.*, 2020; West *et al.*, 2020; Oka *et al.*, 2021).

Reads from elasmobranch species accounted for just 3.3% of the total reads in the analysed dataset, yet this identified ten elasmobranch species, which is comparable with the biodiversity uncovered by eDNA metabarcoding studies using the *COI* marker gene (Bakker *et al.*, 2017; Boussarie *et al.*, 2018), as well as with a recent study in the Indian Ocean that targeted elasmobranch communities around Reunion Island using the MiFish-E primers (Mariani *et al.*, 2021). The number of fish and elasmobranch species detected in our study is also comparable to that of a similar study using the 12S gene region in the Arabian Gulf (Sigsgaard *et al.*, 2020), which provides further support for the idea that eDNA metabarcoding can be used to provide essential fisheries-independent data for elasmobranchs in remote regions. Our primer contribution analysis identified that the majority of sequences produced were amplified by the elasmobranch-targeting MiFish-E primers; it has been established that these primers will amplify fish eDNA despite being targeted to elasmobranchs (Mariani *et al.*, 2021), but our analysis shows that the MiFish-E primers appear to outcompete the MiFish-U primers when in multiplex in the conditions used in this study. These results suggest that the MiFish-E primers may be inefficient for the amplification of elasmobranch eDNA from samples taken in areas containing high fish biomass, such as coral reefs, as rarer sequences from elasmobranchs may be swamped out in the

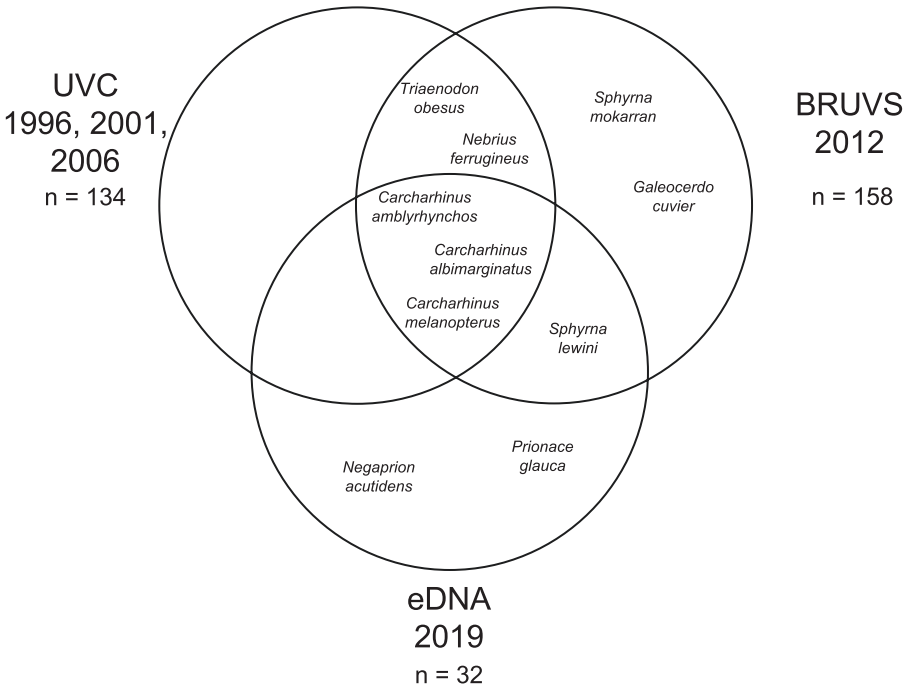

Figure 4. Venn diagram showing the overlap of shark species detected in previous UVC and BRUVS surveys in the MPA and the eDNA samples from around Diego Garcia analysed in this study.

PCR reactions by the relatively more abundant fish eDNA in the samples (Wilcox *et al.*, 2013). The reverse MiFish-U primer appears redundant when in combination with the MiFish-E primers and future analyses should investigate the efficiency of these primers experimentally.

It is clear from the spatial distribution and dominance of eDNA abundance that silvertip sharks (*Carcharhinus albimarginatus*) and grey reef sharks (*C. amblyrhynchos*) were ubiquitous in the sampling environment. This is consistent with previous knowledge of elasmobranchs in the archipelago where UVC surveys and by-catch records have shown that, despite declines in abundance, these two species are among the dominant sharks on the reefs (Graham *et al.*, 2010; Ferretti *et al.*, 2018). In this study, *C. albimarginatus* was detected at higher abundance than *C. amblyrhynchos*, contrasting with the previous UVC and BRUVS surveys from the northern atolls (Graham *et al.*, 2010; Tickler *et al.*, 2017), indicating that Diego Garcia may represent a hotspot for *C. albimarginatus* within the MPA. The consistent level of detection we achieved here indicates that eDNA methods could be used to advance our understanding of the relative abundance of these species across the MPA.

We found that sampling depth significantly impacted elasmobranch eDNA communities based on read abundance and that communities in the outer atoll sites were significantly different to those in the cove habitat. It is likely that these differences are driven by a lesser abundance of eDNA derived from *C. albimarginatus* and *C. amblyrhynchos*, along with a higher overall species richness and a greater proportion of eDNA derived from stingrays in the cove habitat compared to the outer atoll sites. No species were detected exclusively in deep samples, indicating that surface samples may be sufficient to investigate the biodiversity of elasmobranch species using eDNA. However, as most of our samples were taken in water that ranged from 100 to 400 m deep, sampling this deep water environment may result in identifying greater species richness in future studies (e.g. Truelove *et al.*, 2019) and greater differences in species composition may be expected if sampling is undertaken above and below the thermocline (Littlefair *et al.*, 2021).

The reference list of elasmobranchs compiled for this study indicates that there are at least 35 elasmobranch species that are either resident or migrants in the region. Records from the IUU seizures contributed the most species to the list, highlighting the need for a suite of non-invasive methods to match fisheries' records for elasmobranch monitoring. The use of length–weight relationships to complete the number estimates for a subset of the IUU records for three species assumes that the individuals caught were mature and of average length and weight, and as such should be

treated with caution. However, these records were all below 100 kg and, therefore, resulted in the addition of between one and 14 individuals to the respective records, which did not significantly alter their respective relative abundances. The number of shark species detected from eDNA was greater than the number detected by UVC surveys in the atoll but less than the number detected in BRUVS surveys, potentially due to the low spatial coverage of the eDNA survey. However, identification of eDNA from the spinetail devil ray (*Mobula mobular*) may represent the first scientific record of this species in the MPA, and we also detected the critically endangered scalloped hammerhead shark (*Sphyrna lewini*) in our samples. *Sphyrna lewini* has been recorded previously in the IUU records and in BRUVS surveys above Sandes' seamount, located *c*. 30 km north-west of Diego Garcia (Tickler *et al.*, 2017). As it has been predicted that eDNA can be transported tens of kilometres before it degrades (Andruszkiewicz *et al.*, 2019), it is possible that the eDNA from *S. lewini* detected here was shed at the seamount. We are not currently able to estimate the spatial origin of eDNA particles in the region and research into hydrodynamics and eDNA degradation in the MPA will be required before this will be possible. However, current estimates of degradation rates based on a short amplicon fragment length (< 200 bp) and high seawater temperature (> 25 °C) would suggest that eDNA degrades rapidly in the region (Saito & Doi, 2021) and would, therefore, not be able to travel great distances. Furthermore, as several species were only detected in less than three samples, the reproducibility of these detections is uncertain. Therefore, long-term monitoring will be required to determine the reliability of low copy number and single-sample detections.

Despite the detection of rare and elusive species, the eDNA survey failed to detect two species of reef shark thought to be relatively common in the archipelago from UVC and BRUVS surveys: the whitetip reef shark (*Triaenodon obesus* Rüppell 1837) and the tawny nurse shark (*Nebrius ferrugineus* Lesson, 1831) (Graham *et al.*, 2010; Tickler *et al.*, 2017). Previous research has indicated that eDNA can fail to record species that are known to be present in the sampling environment (Boussarie *et al.*, 2018) and it is possible that our sampling strategy was insufficient to capture eDNA from these two predominantly benthic reef species (Randall, 1977; Dharmadi *et al.*, 2015). Stochasticity during the sampling, amplification and sequencing process may also have resulted in these species going undetected (DiBattista *et al.*, 2017; Boussarie *et al.*, 2018). Furthermore, as the UVC and BRUVS surveys were conducted over ranging temporal scales, we cannot rule out that shifts in community assemblage contributed to variation in species detection between the methods. For example, due to their proximity to the military base and the port of the MPA patrol vessel, elasmobranch communities around Diego Garcia are more protected from IUU fishing pressure than those elsewhere around the archipelago. There, they are targeted by illegal fishers from India and Sri Lanka (Collins *et al.*, 2021), leading to community-level impacts and population declines (Graham *et al.*, 2010; Ferretti *et al.*, 2018; Tickler *et al.*, 2019).

All biological monitoring methods suffer from inherent biases and limitations, and eDNA metabarcoding is no exception, with a lack of information on the spatial origin of eDNA and no information on the size, age or sex of the detected species. Nevertheless, our results suggest that the technique can contribute to a modern toolkit for monitoring elasmobranch diversity and abundance by complementing information gained from established but spatially and taxonomically limited techniques. The speed with which samples can be collected in the field, combined with the limited equipment and expertise required to collect them, makes eDNA well suited for carrying out rapid assessments of remote, inaccessible or unstudied areas, where opportunities for labour-intensive sampling using established methods are limited. The non-destructive and non-invasive nature of the technique also makes it well suited to studying elasmobranch communities in the growing number of highly protected marine areas in remote regions, where fewer monitoring options are available. Many of these, such as the Chagos Archipelago, include large expanses of open-water habitat that are currently challenging to monitor. Ultimately, a comprehensive monitoring system will be needed to fully understand the changes currently affecting our oceans and the effectiveness of conservation measures aimed at protecting species within them. As we show here, eDNA metabarcoding has the potential to form an integral part of such a system but further research into the primers used will be required before elasmobranch communities can be monitored efficiently using these methods.

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AUTHOR CONTRIBUTIONS

ND and DC designed the study and carried out fieldwork sampling with assistance from SA and SW. ND performed the lab work and analysed the data. DC, CC and VS supervized the research. ND wrote the original draft with all authors contributing to subsequent edits.

DATA AVAILABILITY

Amplicon sequence data files are archived on Dryad doi:10.5061/dryad.n8pk0p2tn.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. Contribution of each primer pair [the fish-targeting MiFish-U forward (F) and reverse (R), and the elasmobranch-targeting MiFish-E forward (F) and reverse (E)] to the amplicon sequence variants (ASVs) produced by re-running the bioinformatics pipeline to include primer sequences in the final sequences. Hybrids include primer sequences containing one or more difference nucleotide base in the primer region to the actual primer sequence.

Figure S2. Boxplot of number of reads per sample for deep and surface fractions for all samples (A) and after the removal of one outlier from each fraction (B).

Table S1. Species and their lengths and weights used in calculating numbers from weight records in the IUU catch dataset. The fishbase link to the length-weight calculator for each species is included as hyperlinks to the species common names.

Table S2. The elasmobranch species previously recorded in the BIOT MPA and relative abundance of each species recorded from each data source. Numbers in the BRUVS column relate to the percentage of samples each species was recorded in and numbers in the Diver surveys column relate to the average number of individuals seen during UVC surveys. Numbers in eDNA column relate to the relative copy number abundance in this study. The relative abundance of IUU species was calculated by the number of individuals from each species identified after the removal of non-specific records. *names have changed since 1997 list. ** May be *Odontaspis ferox*.

Table S3. List of ASVs and attributed taxonomy of the fish and elasmobranchs detected in this study. See csv file.