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Integrating eDNA and Visual Surveys With Ocean Drift Models to Monitor Marine Mammals in Tropical Waters

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ABSTRACT

Environmental DNA metabarcoding (eDNA) is emerging as a pivotal tool for assessing and monitoring marine biodiversity, exhibiting significant promise for the detection of marine mammals. The primary objective of this study was to evaluate various protocols for eDNA sampling of seawater from a small boat in tropical environment, under conditions devoid of cold chain storage or laboratory facilities and constrained by limited financial resources. Our focus was on optimizing the capture of eDNA and the subsequent detection of marine mammals in a replicable way. This investigation involved a comparative analysis between marine mammal detections via eDNA metabarcoding and traditional visual monitoring. Sampling was primarily conducted in close proximity to marine mammal sightings, off Réunion Island to evaluate the performance of eDNA detections. Réunion Island is located in the tropical western Indian Ocean and serves as a relevant model for this study, where long-term monitoring of cetaceans has been conducted since 2008, thereby enabling a robust comparison between visual sightings and molecular detections. Two sets of primers designed to target the hypervariable regions of mitochondrial 12S rRNA genes for vertebrates and mammals were used. Positive eDNA detections were identified in seven of the nine samples associated with visual sightings of one or more cetacean species. Marine mammal DNA was successfully amplified for three families (Balaenopteridae, Delphinidae, and Kogiidae) and found to be almost ubiquitously present for Delphinidae. Additionally, we investigated the potential influence of particle drift on the dispersal of eDNA. To better understand the spatial dynamics and persistence of eDNA in the marine environment, the Lagrangian model ICHTHYOP was used to simulate particle drift and assess how oceanographic processes might influence eDNA dispersal patterns around Réunion Island. Our study explores the potential of utilizing eDNA for monitoring cetaceans in tropical regions offering a valuable comparison to traditional visual surveys, and provides recommendations for further enhancements in future eDNA studies.

1 | Introduction

In Réunion Island, a French overseas territory located in the southwest Indian Ocean, the surge in human activities and its subsequent impact on coastal marine habitats have intensified alongside population growth. Coastal development poses significant threats to cetaceans, exposing them to chemical pollutants (Dirtu et al. 2016; Mwevura et al. 2010; Pierce et al. 2008), nondegradable debris (Simmonds 2012), and acoustic pollution (Borggaard et al. 1999; Dähne et al. 2013). Furthermore, activities such as whale- and dolphin-watching, along with vessel traffic, have been identified as potential disruptors of animal behavior (Barra et al. 2020; Hoarau et al. 2020; Plot et al. 2025; Parsons 2012; Quintana Martín-Montalvo et al. 2021). Moreover, climate change is amplifying these challenges, with rising sea temperatures affecting the distribution of marine mammals (Albouy et al. 2020).

Efficient monitoring and surveying of marine mammals are crucial for effective management and conservation planning, especially in the context of the European Water Framework Directive (WFD) (European Parliament and Council 2000). The continuous degradation of marine ecosystems emphasizes the urgency to implement advanced monitoring techniques. This directive, applicable to all French departments, whether metropolitan or overseas, underscores the significance of monitoring in regions like Réunion Island. Since 2004, marine mammal monitoring has been ongoing around Réunion Island primarily through visual and acoustic surveys. Among the 28 potential cetacean species in the region (Best 2007), 25 have been documented during visual surveys (Dulau-Drouot et al. 2008; Laran et al. 2017). Resident populations of Indo-Pacific bottlenose dolphins (*Tursiops aduncus*), common bottlenose dolphins (*Tursiops truncatus*), and spinner dolphins (*Stenella longirostris longirostris*) inhabit coastal waters year-round (Dulau et al. 2017; Estrade and Dulau 2020; Condet and Dulau-Drouat 2016). Additionally, the coastal waters of Réunion serve as a breeding area for humpback whales (*Megaptera novaeangliae*) during the austral winter (June–September) (Ceyrac et al. 2018; Dulau-Drouot et al. 2012), while offshore species are observed in close proximity to the shore due to the island's steep topography (Dulau-Drouot et al. 2012; Laran et al. 2017). The increasing anthropogenic pressures on these marine mammal habitats necessitate a comprehensive and integrative monitoring approach.

Environmental DNA (eDNA)-based approaches are becoming a promising alternative tool for biodiversity monitoring (Bohmann et al. 2014; Deiner et al. 2017; Jarman et al. 2018). This nondestructive and efficient method (Bohmann et al. 2014; Rees et al. 2015) has the potential to be less time-consuming and costly than traditional visual monitoring surveys (Sassoubre et al. 2016). eDNA can be isolated from water samples to detect DNA sequences from microorganisms to large vertebrates (Foote et al. 2012; Thomsen, Kielgast, Iversen, Møller, et al. 2012; Kelly et al. 2014; Djurhuus et al. 2017; Closek et al. 2019). In marine environments, eDNA surveys have proven useful for detecting fish (Thomsen, Kielgast, Iversen, Møller, et al. 2012; Thomsen, Kielgast, Iversen, Wiuf, et al. 2012; Kelly et al. 2014; Sassoubre et al. 2016; Sigsgaard et al. 2016; Lafferty et al. 2018; Gold et al. 2021) and vertebrate species (Andruszkiewicz et al. 2017; Closek et al. 2019), assessing the diet of marine species (Deagle et al. 2014; McInnes et al. 2017; Peters et al. 2015), determining the presence/absence of invasive species (Rishan et al. 2023), estimating population genetic diversity (Sigsgaard et al. 2016), and comparing eDNA assessments to visual surveys (Thomsen, Kielgast, Iversen, Wiuf, et al. 2012; Thomsen et al. 2016; Port et al. 2016; Kelly et al. 2017; Yamamoto et al. 2017; Boussarie et al. 2018; Stat et al. 2019; Hsu et al. 2023). The integration of eDNA into marine mammal monitoring aligns with deciphering ecological patterns and facilitates the development of comprehensive conservation strategies.

However, despite its growing potential, the application of eDNA for monitoring cetaceans in tropical marine systems, particularly in resource-limited contexts such as Réunion Island, remains underexplored. The main objective of this study is to evaluate the effectiveness of eDNA metabarcoding as a tool to detect and monitor cetacean species in tropical waters, using the oceanic island of Réunion as a model site. We aim to (1) test and adapt an eDNA sampling protocol suitable for at-sea collection without cold-chain storage or laboratory access, (2) compare eDNA-based detections to visual sightings of cetaceans, and (3) assess how oceanographic drift may influence the spatial interpretation of eDNA signals. We hypothesize that eDNA will detect a broader range of species than visual sightings conducted during the same sampling periods, and that the detected eDNA signals will be consistent with species distribution patterns known from long-term visual monitoring. We also expect that particle drift simulations will help refine the spatial interpretation of positive eDNA detections. To operationalize these objectives, we first developed and tested a field-adapted eDNA sampling protocol tailored to tropical offshore conditions with limited logistical support. We then applied this protocol during a 1-year survey around Réunion Island and compared the eDNA detections with cetacean sightings and species distribution maps. Particle drift simulations were also conducted to better interpret the spatial reliability of eDNA signals.

2 | Materials and Methods

2.1 | Samples

Surface seawater samples were collected from May 2018 to June 2019 to cover the period with and without the presence of migratory baleen whales around Réunion Island. Samples were collected from a boat with no options for cold storage or laboratory facilities using an eDNA protocol that was applicable and replicable with limited financial, material and human resources. To limit cross contamination, personnel onboard were wearing protective gowns and nitrile gloves during sample collection. Sampling was carried out at the front of the vessel to avoid contact with the hull, and samples were collected at the sea surface interface using two 5 L sterile bottles per sample. All sampling was conducted in the morning, between 08:30 and 10:00. This bottle was then placed in a sterile container to avoid U.V., and water was filtered from the bottle using a peristaltic pump and a sterile silicone tubing (internal diameter of 6 mm) connected directly to the encapsulated filter (Merck and Sterivex filtration capsule, see Appendix S1 and video on https://www.youtube.com/watch?v=cu84-o3H_KY&t=56s). Filtration was performed on a surface cleaned with bleach. After filtration, air was injected

into the filters to expel any liquid. A sterile solution of RNAlater (for all final samples) or 1 M Longmire's buffer (Longmire et al. 1997) (used during the testing phase protocol) was pushed through the capsule using a new sterile syringe to preserve any DNA captured. The outlets were capped using the appropriate caps for the Sterlitech encapsulated filters and silicon caps were used for the other filtration capsules. The filtration capsules were stored in a cooler with ice packs during transport (due to the high ambient tropical conditions), and immediately stored in a freezer (−20°C) at the end of the survey day, until extraction and amplification could be carried out. One field blank was processed using the same sterile materials and precautions as the samples (collected at the bow of the boat, using gloves, gown, mask, and hairnet). This blank was assessed for contamination using both Nanodrop and Qubit fluorometers, which detected no measurable DNA. In addition, it was tested via qPCR assays developed in this study and visualized by gel electrophoresis for some targets, with no amplification observed.

Based on the assessment of the different criteria (feasibility, filtration time, DNA concentration, and cost), a protocol was established (see Supporting Information S1) to sample 20 sites around Réunion Island. At each site, we collected one 10 L seawater sample (2 × 5 L) from the sea surface interface using a Sterlitech filter, RNAlater solution, a peristaltic pump and sterile tubing. We favored samples with observations of marine mammals in order to compare eDNA detections with the marine mammal sightings recorded at the time of sampling. Of the 20 samples, 14 were collected in close proximity (10–20 m) to marine mammals sighting locations. Sea surface temperature (SST, °C), wind speed (kts) (https://www.windy.com/) and the quantitative hydrogen potential (pH) (from a pH-Fix strip) at the sea surface interface were also collected at each sampling site.

2.2 | Genetic Process for eDNA Samples

2.2.1 | Primers

Among several primer sets tested for cetacean detection (see Supporting Information S2), the MiMammal primers (Ushio et al. 2017) were selected for subsequent analyses.

2.2.2 | Extraction

The filters were dried and lysed with ATL buffer and proteinase K (Qiagen). Lysis was facilitated with an overnight incubation at 56°C in a hybridization oven. The lysate from each sample was extracted using a DNeasy Blood & Tissue Kit (Qiagen) using the manufacturer's protocol. To increase DNA yield, 3× more lysate was processed per sample from Merck filter (15 mL of lysate processed per sample instead of 5 mL) using higher capacity spin columns (Epoch Life Science) and Qiagen reagents.

2.2.3 | Amplification and Sequencing

Given the broad nature of the mammal primers and potential loss of sequences to human amplification, we deemed it necessary to design and test human blocking primers to reduce the amount of amplifiable human DNA and therefore increase the efficiency of the sequencing process and increase the chances of detecting rarer mammal DNAs, which might otherwise have been overwhelmed by nontarget DNA.

Following the results of the tests on primers, ~230 bp hypervariable region of the 12S rRNA gene (MiMammal) was amplified. DNA amplifications were performed with 12 PCR replicates in a final volume of 10 μL. The amplification mixture contained 1× Phusion Green Hot Start II High-Fidelity PCR Master Mix (Thermo Scientific), 0.4 μM of each of the tailed primers, 2 μM of our human blocking primer, 0.8 μg/μL bovine serum albumin (BSA—Thermo Scientific), 3% of DMSO (Thermo Scientific), 1.5 mM of $MgCl_2$ (Invitrogen), and topped up with PCR grade water (Thermo Scientific). A human blocking primer was designed to bind specifically to the human 12S gene and modified with a C3 spacer to restrict amplification after binding (5′—TAA GCT ATA CTA ACC CCA GGG TTG GTC AAT T—3′). The human blocking primers were added in a 5× concentration relative to the mammal primers. PCR conditions consisted of an initial denaturation at 98°C for 3 min, followed 45 cycles of 20 s at 98°C, 15 s at 69°C, and 15 s at 72°C, and a final elongation step at 72°C for 5 min. To monitor potential contaminants and validate the performance of the amplification and sequencing processes, a total of three negative extraction controls, three negative PCR controls (ultrapure water, 12 replicates), and three positive control samples (a mock community with a known composition) were amplified and sequenced in parallel to the samples. Amplification success was determined by gel electrophoresis. DNA was purified to remove PCR inhibitors using a DNeasy PowerClean Pro Cleanup Kit (Qiagen). Purified DNA extracts were quantified using a Qubit dsDNA HS Assay Kit on a Qubit 3.0 fluorometer (Thermo Scientific). We followed an optimized protocol aligned with Illumina's specifications for metagenomic sequencing. The full protocol can be found at: https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf. This protocol includes steps for cleaning (using AMPure XP beads) the final library prior to quantification (using dsDNA binding dyes), normalization and pooling of PCR replicates. In preparation for sequencing, the pooled libraries were denatured with NaOH, diluted and enriched with 10% PhiX to improve sequence diversity for low-complexity libraries, following the Illumina MiSeq sample loading protocol. The final library was sequenced using a 15 pM Illumina MiSeq V2 kit. Internal validation of the sequencing workflow under the same laboratory conditions indicated that index hopping (tag-jumping) events were rare, with an average of 2.5 reads per 100,000 and a median of 2 reads per sample. Since our bioinformatic pipeline applies a minimum read threshold of 10, as well as dynamic filtering, these low-level artifacts were effectively excluded from the final dataset. The risk of false positives due to tag-jumps was therefore considered negligible.

2.2.4 | Data Processing

Sequence data were processed using a NatureMetrics custom bioinformatics pipeline for quality filtering, dereplication, and taxonomic assignment. Samples were demultiplexed based on the combination of the i5 and i7 index tags. Paired-end reads

for each sample were merged with USEARCH with a minimum overlap of 20% of the total read length. Forward and reverse primers were trimmed from the merged sequences with CUTADAPT and retained if the trimmed length was between 140 bp and 200 bp. These sequences were quality filtered with USEARCH to retain only those with an expected error rate per base of 0.05 or below and dereplicated by sample, retaining singletons. Unique reads from all samples were denoised in a single analysis with UNOISE, requiring retained sequences to have a minimum abundance of 8 in at least one sample. After filtering, taxa were identified by comparing those sequences to the GenBank reference database of the National Center for Biotechnology Information (NCBI—https://www.ncbi.nlm.nih.gov). A taxon-by-sample table was generated by mapping all dereplicated reads for each sample to the denoised sequences with USEARCH at an identity threshold of 97%. Denoised sequences were identified via BLAST against the nucleotide (*nt*) database from GenBank. Identifications to species level were based on the highest available percentage identity ≥ 99%, with an e-score of 1e−20 and a hit length of at least 80% of the query sequence. In cases where multiple reference sequences match equally to the query sequence then a more conservative higher taxonomic classification is given. Only sequences with species- or genus-level identifications were included in the final results. Where a species is represented by multiple Operational Taxonomic Units (OTUs), the sequence with the highest percentage match to that species was taken as the representative. Typically, the other sequences have the same occurrence pattern and the lower sequence similarity can be attributed to PCR or sequencing errors. The number of raw reads (over 4.7 million), merged pairs, pairs clipped, quality filtered and dereplicated used in the final analyses are provided in the detailed data for each sample (https://doi.org/10.57745/U0PDJW).

2.3 | Genetic Process for Tissue Samples—Additional 12S Fragment Sequences

Two individuals of the species *Tursiops aduncus* (Ta2 and Ta21) and 2 individuals of the species *Stenella longirostris longirostris* (SL7 and SL9) were sequenced on the 12S portion of the mitochondrial DNA (see Supporting Information S3 and data repository in the data archiving section). These samples were available from skin biopsies collected during dedicated surveys as part of other programs and dedicated research permit obtained by GLOBICE. Samples were extracted using the Macherey Nagel NucleoSpin tissue Kit with < 25 mg of tissue and nighttime lysis. We obtained a DNA concentration yield of between 52.5–94.6 (ng/μL). To amplify the 12S mitochondrial sequence, we developed three pairs of primers based on a comparison of the representative sequences of the two species (see details in Supporting Information S3):

Primers PCR1: 12S_F1 (Forward 5′–3′) ATCCGCATCCCAGTGAGAAT

12S_R1 (Reverse 5′–3′) ACCGCCAAGTCCTTTGAGTTT

Primers PCR2: 12S_F2 (Forward 5′–3′) GGACTTGGCGGTGCTTCATA

12S_R2 (Reverse 5′–3′) GCACACCTTCCGGTATGCTT

Primers PCR3: 12S_F3 (Forward 5′–3′) CCACCGCGGTCATACGATT

12S_R3 (Reverse 5′–3′) GCCCATTTCTTCCCAATCCA

DNA amplifications were carried out in a final volume of 25 μL. The amplification mix contained FastStart Taq Roche 1 U, buffer 1× FastStart, dNTP 0.24 mM, 0.4 μM of each of the primers (10 pmol each), 1.5 mM $MgCl_2$ (Invitrogen), 5 μL of DNA extract (minimum 10 ng), and supplemented with PCR-grade water (Thermo Scientific). PCR conditions included initial denaturation at 95°C for 5 min, followed by 35 cycles of 1 min at 95°C, 1 min at 55°C, 1.30 min at 72°C and a final elongation step at 72°C for 10 min. All reactions were performed in the presence of a negative control and the success of amplification was determined by gel electrophoresis. The efficiency and specificity of each reaction was validated by migration on QIAxcel. The PCR products were then purified on membrane before being assayed by fluorimetry and sequenced in Forward/Reverse primers on the Applied Biosystems (ABI) 3730XL sequencer. These reference sequences were added to complete our database. These sequences were 100% identical to those already available on NCBI for these two species.

2.4 | Design and Testing of Species-Specific qPCR Primers

To evaluate the potential of a more targeted molecular approach for cetacean detection, we developed and tested qPCR primers for two focal species commonly observed in the region: *Stenella longirostris longirostris* and *Tursiops aduncus*. These primers were designed based on comparative alignments of 12S rRNA gene sequences from tissue-derived mitochondrial DNA, including sequences from local individuals obtained during biopsy campaigns (see Supporting Information S3).

We first validated these primers through qPCR amplification on serial dilutions of purified DNA extracts from reference tissue samples. We then applied this qPCR assay to the first ten eDNA samples collected at sea, using triplicate reactions per sample and 4 μL of eDNA extract per reaction. The results, however, revealed limited concordance with visual surveys and eDNA metabarcoding outcomes. Only one sample (Area 2) showed agreement across all three methods for *S. longirostris*. In contrast, several visually or metabarcoding-confirmed detections were not recovered by qPCR. These results suggest that, although the primers were functional with high-quality DNA, they were not sufficiently reliable for use with complex and degraded eDNA samples in this context. Possible explanations include primer sensitivity, DNA degradation, stochastic effects in low-concentration samples, or spatial mismatch between sampling and species presence. Given the limited overlap with other detection methods, the targeted approach was not pursued further in this study. Full details of primer design, validation, and testing outcomes are provided in Supporting Information S3.

Moreover, the primary objective of the study was to explore the feasibility of developing a broad-scale monitoring approach applicable to all marine mammal species around Réunion Island, which includes 28 potential cetacean species. As such, the use of

universal primers in metabarcoding provided a more inclusive strategy for capturing the full diversity of species in the region, beyond the scope of a species-specific qPCR assay.

2.5 | Spatial Analysis of Sightings and Particle Drift Modeling

In order to assess the level of detectability of cetaceans from seawater samples, and to compare the eDNA results with visual observations, eDNA sampling was mostly conducted in close proximity to cetaceans. Visual monitoring was applied during the surveys at sea and upon sighting, information on the encountered animals including species identification, GPS position and the estimated number of individuals, were collected concurrently with seawater sampling. When no cetaceans were observed, water samples were collected at random locations. The numbers of cetacean species and sightings were compared to the number of species detected from eDNA to assess the efficiency of the eDNA methodology. Both visual and eDNA detections of cetacean species were also mapped in QGIS. To assess the consistency of eDNA results with the habitat of each detected species, eDNA detections at sampling sites were compared to the spatial distribution of cetacean sightings collected during dedicated surveys over a 12-year period (2008–2019) around Réunion (Dulau-Drouot et al. 2008, 2012; Dulau et al. 2017; Estrade and Dulau 2020). To account for uneven survey effort, sighting rates were calculated for each species as the number of cetacean groups sighted divided by survey effort (in km) conducted from 2018 to 2019.

To better understand the spatial origin of the eDNA detected at sea and assess whether detections could be attributed to local cetacean presence or drifting DNA, we simulated reverse particle drift from each sampling site. Given that small (100-bp) eDNA fragments can remain in the marine environment for one to several (0.9–6.7) days (Thomsen, Kielgast, Iversen, Møller, et al. 2012), we simulated reverse particle drift between 1 and 7 days from the sampling time (exact date and coordinates of water collection—Table 1). All releases were made at the sea surface (0 m depth), consistent with the depth of eDNA sampling. To this end, the Lagrangian tool ICHTHYOP designed to simulate larval dispersal and study the effects of physical and biological factors on ichthyoplankton dynamics (Lett et al. 2008) was used. For the model, ten thousand particles were released at each sampling site, a number that was shown to be large enough to provide precise estimates of connectivity values (Andrello et al. 2013). The Ichthyop model was forced by current fields provided by the GLORYS12V1 product, interpolated linearly at the location and time of each particle, and their movement was solved using the Runge Kutta 4th order scheme. No random walk component was included in the simulations, ensuring that particle trajectories were driven solely by advection. The GLORYS12V1 product is the CMEMS (Copernicus Marine Environment Monitoring Service) global ocean eddy-resolving reanalysis covering the altimetry era 1993–2018. The model component is the NEMO platform driven at the surface by ECMWF ERA-Interim reanalysis. Observations are assimilated by means of a reduced-order Kalman filter. Along track, altimeter data (Sea Level Anomaly), satellite Sea Surface Temperature, Sea Ice Concentration and in situ temperature and salinity vertical profiles are jointly assimilated. Moreover, a 3D-VAR scheme provides a correction for the slowly-evolving large-scale biases in temperature and salinity. The global ocean output files are displayed on a standard regular grid at 1/12° (approximately 8 km) and on 50 standard levels. While this spatial resolution is relatively coarse, it was considered adequate for the scale and objectives of this study, which aimed to characterize general particle drift trajectories over short temporal and spatial scales. We used the ocean current data for Réunion Island over the years 2018 and 2019, downloaded from the CMEMS data center website (https://resources.marine.copernicus.eu/?option=com_csw&task=results?option=com_csw&view=details&product_id=GLOBAL_REANALYSIS_PHY_001_030). We plotted maps of trajectories between 1 and 7 days. Analyses were carried out using the software Spyder (Python 2.7). Hence, the reverse drift model was run for a maximum of 7 days.

3 | Results

3.1 | Filtration Capsules

Among the filters tested, the Sterlitech model offered the best compromise between volume, cost, and contamination risk (see Supporting Information S1).

3.2 | Test of Primers

The MiMammal primers yielded higher DNA yields and greater taxonomic resolution (see Supporting Information S2), and were thus used for the main study. The cetacean detections from MiMammal primers accounted for 9.8% of the total DNA sequence reads (Figure 1), with the remainder made up of contaminant mammal sequences. Despite adding human blocking primers to the PCR reactions, the sequence reads predominantly comprised of human DNA, which accounted for 81.7% of the total sequence reads (Figure 1). Other contaminant mammal sequences known to proliferate within PCR reagents included cow, pig, sheep, dog, and cat DNA, together, these nonhuman contaminant sequences made up the remaining 8.5% of the total sequence reads (Figure 1).

3.3 | Sea Surface Temperature (SST) and pH

The SST and wind speed associated with the samples are presented in Table 1. The mean pH was 8.2 for all samples. No relationship was found between SST, wind speed and the number of cetacean DNA sequence detected. The highest values for the number of sequences were found with winds greater than 5 kt (i.e., 9.3 km/h). Sea surface temperature is generally high around Réunion Island and was between 25.3°C and 29°C (mean 27.5°C) during our sampling.

3.4 | Detection of Marine Mammals From eDNA

Here, we only present the results for the samples stored in RNAlater buffer and filtered using the Sterlitech filters at 20 sampling sites around Réunion Island (one sample per site, see Table 1,

TABLE 1 | Number of individuals observed by visual monitoring (Ind.) and number of DNA sequence reads sequenced via eDNA (Seq.) per boat surveys (sampling location #).

Sampling site #	Date	Wind (kt)	SST (°C)	*Megaptera novaeangliae*		*Stenella attenuata*		*Stenella longirostris*		*Tursiops aduncus*		*Tursiops truncatus*		*Kogia breviceps*		Total
				Ind.	Seq.	Ind.	Seq.	Ind.	Seq.	Ind.	Seq.	Ind.	Seq.	Ind.	Seq.	Seq
1	10/06/2018	15.5	28.2			**0**	1739									1739
2	15/06/2018	8	28			**0**	885	12	77			**0**	29,862			30,824
3	30/06/2018	10	26	3	0											
4	01/07/2018	14	26					**0**	115	**0**	31,414					31,529
5	01/07/2018	10	27	2	5544											5544
6	02/07/2018	12	25.3	1	10,034											10,034
7	03/07/2018	5	25.5					13	97	**0**	2350					2447
8	04/07/2018	5	28	2	0							1	60			60
9	04/07/2018	5	28							2	2533					2533
10	13/10/2018	5	24	3	0	**0**	1306			**0**	49					1355
11	23/01/2019	5	28.6			**0**	13,589	**0**	383			5	4744			18,716
12	05/02/2019	5	27.8					**0**	297	1	20,666					20,963
13	05/02/2019	4	27.9							**0**	1424					1424
14	06/02/2019	4	29			**0**	4072	**0**	43					**0**	11,200	15,315
15	06/02/2019	3	29			**0**	272									272
16	07/03/2019	3	29			10	0									
17	09/03/2019	4	28.9			**0**	124	50	0	**0**	173					297
18	09/03/2019	9	28.9			**0**	4491	**0**	132	**0**	866					5489
19	18/03/2019	9	28.3													
20	17/05/2019	0	26.2							8	1028					1028

Note: (0 in red) Detected by visual surveys but not by eDNA. (**0** in bold) detected by eDNA but not visual monitoring. The sample date, wind and sea surface temperature (SST) are also provided. The number of PCR-positive replicates per sample for marine mammal detection ranged from 9 to 12 out of 12. This indicates a consistent and robust detection across replicates.

Figure 2). The presence of negative controls and the success of the amplification were assessed by gel electrophoresis; no bands were observed in the negative controls, and only PCRs that yielded positive results were sequenced. Among the retained reads produced with the MiMammal primers, 149,569 reads were identified to cetacean species. The cetacean species detected belonged to three families (Balaenopteridae, Delphinidae, and Kogiidae), and four genera (*Megaptera*, *Stenella*, *Tursiops, and Kogia*) and represented six species: Humpback whale (*Megaptera novaeangliae* [2]), Pantropical spotted dolphin (*Stenella attenuata* [8]), spinner dolphin (*Stenella (longirostris)* [7]), Indo-Pacific bottlenose dolphin (*Tursiops aduncus* [9]), common bottlenose dolphin (*Tursiops truncatus* [3]) and pygmy sperm whale (*Kogia breviceps* [1]) were detected. All cetacean sequences, except one were 100% matches (percent identity, %ID) to sequences on NCBI GenBank (Table 2). The spinner dolphin (*Stenella longirostris*) exhibited the lowest

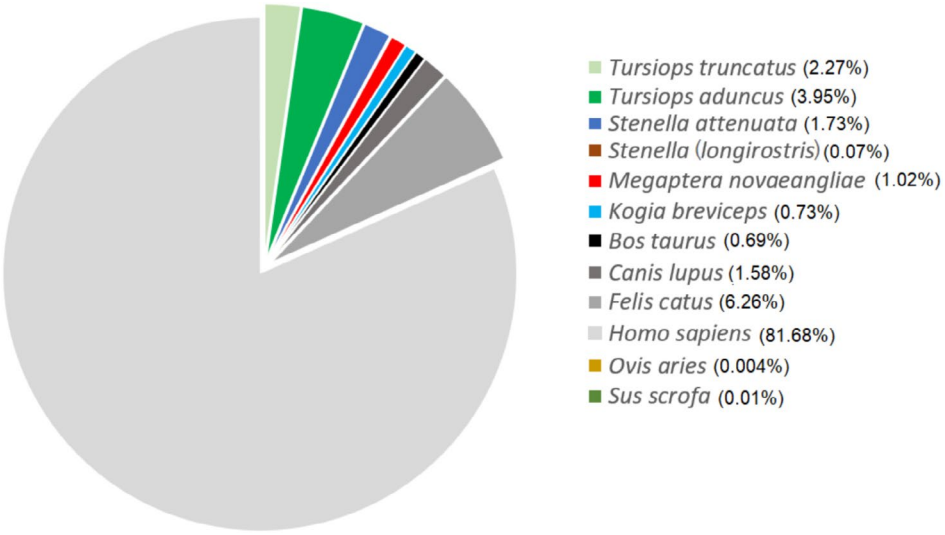

FIGURE 1 | Percentage distribution of sequence reads using MiMammals primers on final samples collected around Réunion Island (20) with human blocking primers. The data included reads from detected mammal species and the known contaminant species.

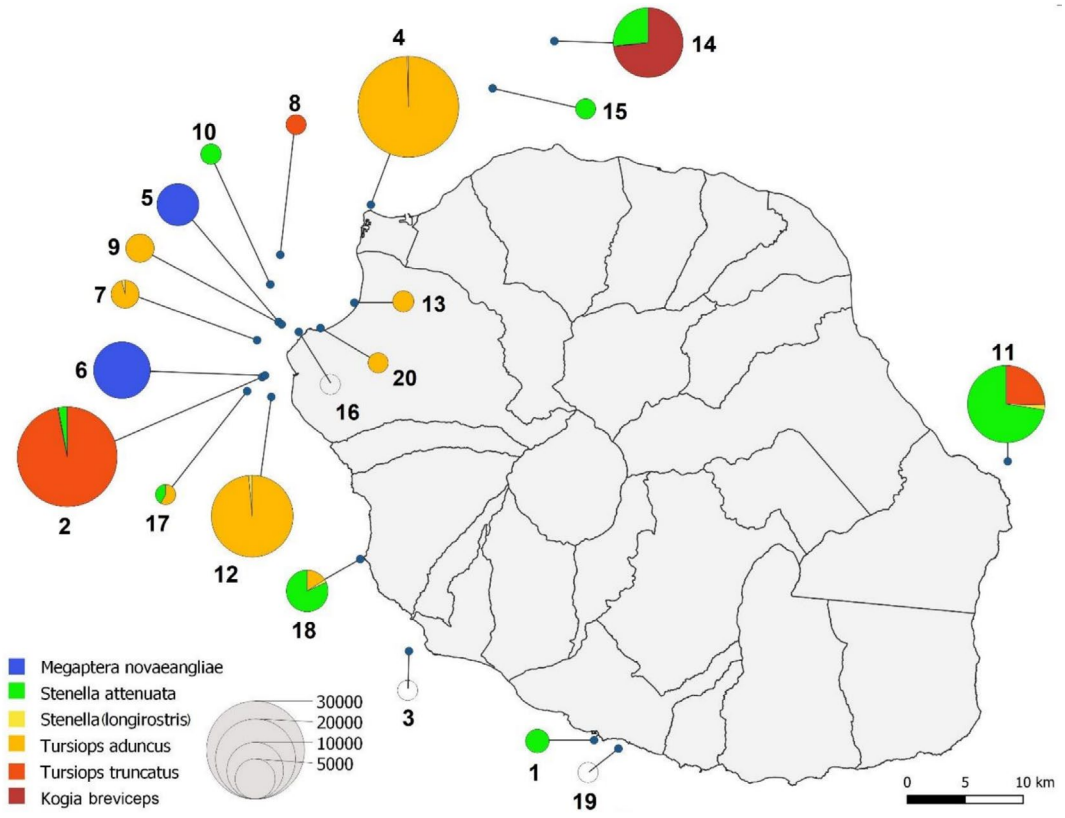

FIGURE 2 | Metabarcoding detection of marine mammals found in seawater samples collected at 20 sampling sites during 20 boat trips around Réunion Island in the southwest Indian Ocean: 30 detections in total (2 times (×) *M. novaeangliae*, 8× *S. attenuata*, 7× *S. (longirostris)*, 9× *T. aduncus*, 3× *T. truncatus*, 1× *K. breviceps*). Numbers represent sampling locations (Table 1). Circles are proportional to the number of DNA sequences detected.

TABLE 2 | Sequences per species detected and their percent identity with NCBI GenBank sequences (%ID), along with the number of matching sequences with the highest %ID.

Species	Sequence	Number sequences on NCBI with higher %ID	%ID
Megaptera novaeangliae	CACCGCGGTCATACGATTGACCCAAATT AATAGGAACACGGCGTAAAGAGTGTTA AGGAGTCACATAAAATAAAGTCAA ACCTTAATTAAGCTGTAAAAAGCCCT AATTAAAATTAAGCCAAACTACGAA AGTGACTTTAACATAACCTGATCACACGACAGCTAAGACC	3	100%
Stenella attenuata	CACCGCGGTCATACGATTGACCCAAA TTAATAGACACCCGGCGTAAAGAGTGTCAAAGAACAATA TAAAAATAAAGTCAAACCTTGA TTAAGCTGTAAAAAGCCATAATTAA AATTAAGTTAAACTACGAAAGTAAC TTTACCATAAACTGAGTACACGACAACTAAGACC	27	100%
Stenella (longirostris)	CACCGCGGTCACACGATTAACCC AAGTCAATAGAAGCCGGCGTAAAGAGTGTCAAAG AACAATATAAAAATAAAGTCAAAC CTTAATTAAGCTGTAAAAAGCCATAA TTAAAATTAAGTTAAACTACGAA AGTAACTTTACCATAAACTGAGTACACGACAACTAAGACC	106	97.08%
Tursiops aduncus	CACCGCGGTCATACGATTGACCCAAG TTAATAGACACCCGGCGTAAAGAGTGTCA AGGAACAATATAAAAATAAAGTCAAAC CTTAATTAAGCTGTAAAAAGCCATAATTA AAATTAAGTTAAACTACGAAAGTAACTTTA CCATAAACTGAGTACACGACAACTAAGACC	25	100%
Tursiops truncatus	CACCGCGGTCATACGATTGACCCA AACTAATAGACACCCGGCGTAAAG AGTGTCAAAGAACAATATAAAAATAAAGT CAAACCTTAATTAAGCTGTAAAAAGCCAT AATTAAAATTAAGTTAAACTACGAAAGTA ACTTTACCATAAACTGAATACACGACAACTAAGACC	> 100	100%
Kogia breviceps	CACCGCGGTCATACGATTGACCCAAG CTAATAAGCATACGGCGTAAAGAGTG TCTAGGAACCACACAAAATAAAGCCAAGCT TTGACTAAGCTGTAAAAAGCCATAGTCAAA ACCAAGATAGACTACGAAAGTGACTTTAA TACAGTCTGACTACACGACAGCTAAGACC	1	100%

Note: Stenella longirostris is enclosed in brackets to indicate uncertainty due to low identity match to the references.

number of reads and a match of only 97.1% to 106 distinct *Stenella longirostris* accessions (NCBI) with four differing nucleotides observed within a sequence of 170 base pairs, including our two sequences of *Stenella longirostris longirostris* sequenced in this study (Supporting Information S3). This sequence is unlikely to be misidentified as *Stenella attenuata*, as we have successfully obtained several other Operational Taxonomic Units (OTUs) corresponding to this specific species. Additionally, the BLAST analysis using NCBI data reveals a lower percentage identity with other species of *Stenella* (*S. coeruleoalba* and *S. attenuata*). Nevertheless, due to the low detection threshold and the presence of four differing nucleotides, we exercise caution with regards to this result and denote this species in parentheses as '*Stenella (longirostris)*'. The number of DNA sequence reads obtained via eDNA, that was attributed to each species per sampling site, is presented in Table 1.

3.5 | Comparison of DNA Detection vs. Cetacean Sighting

During the 20 boat surveys dedicated to eDNA seawater sampling (one eDNA sample per boat trip and a mean of 1 h of visual survey), 14 sightings of five cetacean species were recorded; *Megaptera novaeangliae* [$n = 5$], *Stenella attenuata* [$n = 1$], *Stenella (longirostris)* [$n = 3$], *Tursiops aduncus* [$n = 3$], and *Tursiops truncatus* [$n = 2$] (Figure 3). From the 20 eDNA sampling locations, 30 genetic detections of cetaceans were found (Figure 2). eDNA metabarcoding detected more species (including the elusive *Kogia breviceps*) and more instances of cetaceans than onboard observers (Figure 2 vs. Figure 3): 26% (9 sightings) were shared, 60% (21) were unique to eDNA and 14% (5) were unique to visual surveys. Among the 14 visual sightings, eDNA detected cetaceans in 9 of

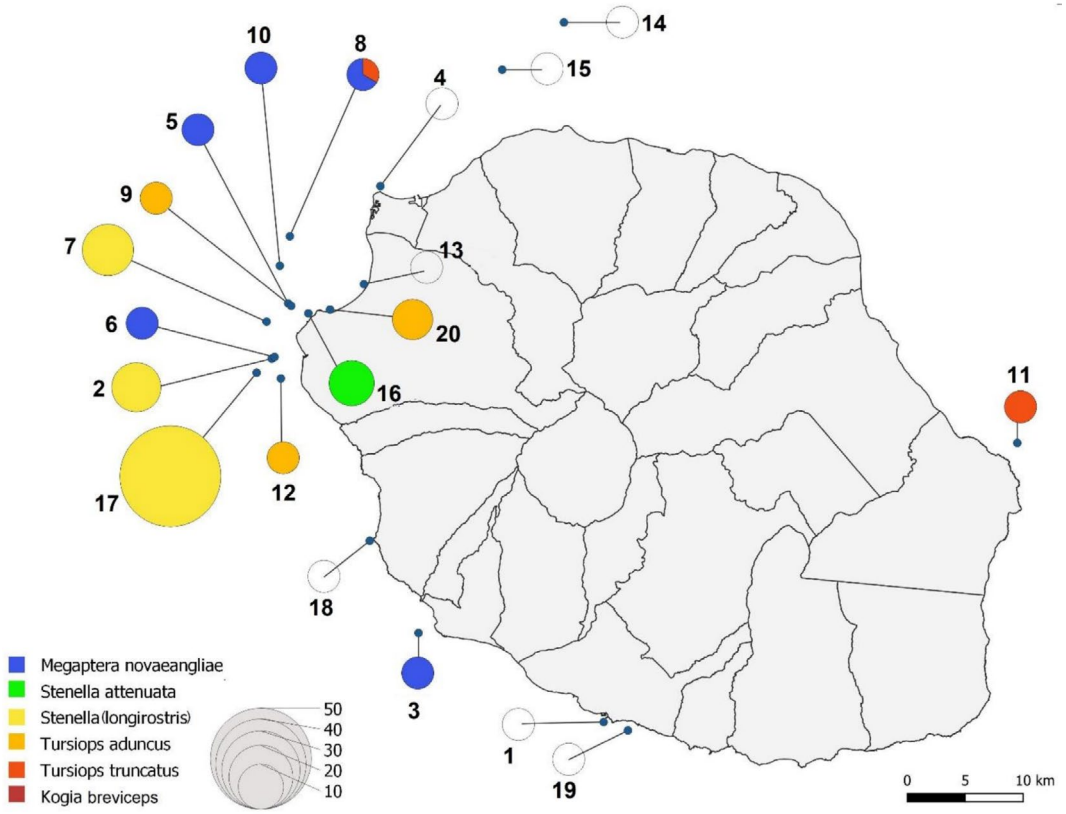

FIGURE 3 | Visual sightings of cetaceans recorded at each sampling site during 20 boat trips around Réunion Island, southwest Indian Ocean: 14 sightings of 6 species: (5 sightings of *M. novaeangliae*, 1 *S. attenuata*, 3 *S. (longirostris)*, 3 *T. aduncus*, 2 *T. truncatus*). Numbers represent sampling location (Table 1). Circles are proportional to the number of individuals observed. White circles represent samples without marine mammal sightings ($n = 6$).

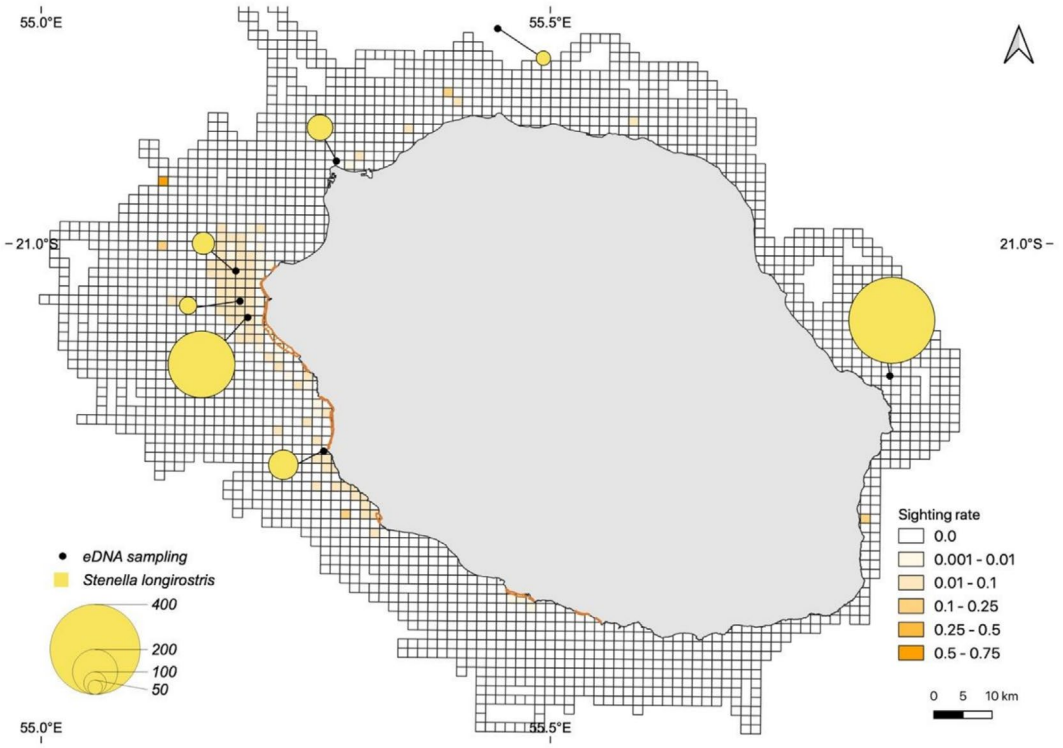

FIGURE 4 | Number of DNA sequences of *Stenella (longirostris)* detected by eDNA around Réunion Island, along with sighting rate of spinner dolphins in 2008–2019. In addition, sighting rate for other species is presented in Supporting Information S4 (Appendices S8–S12).

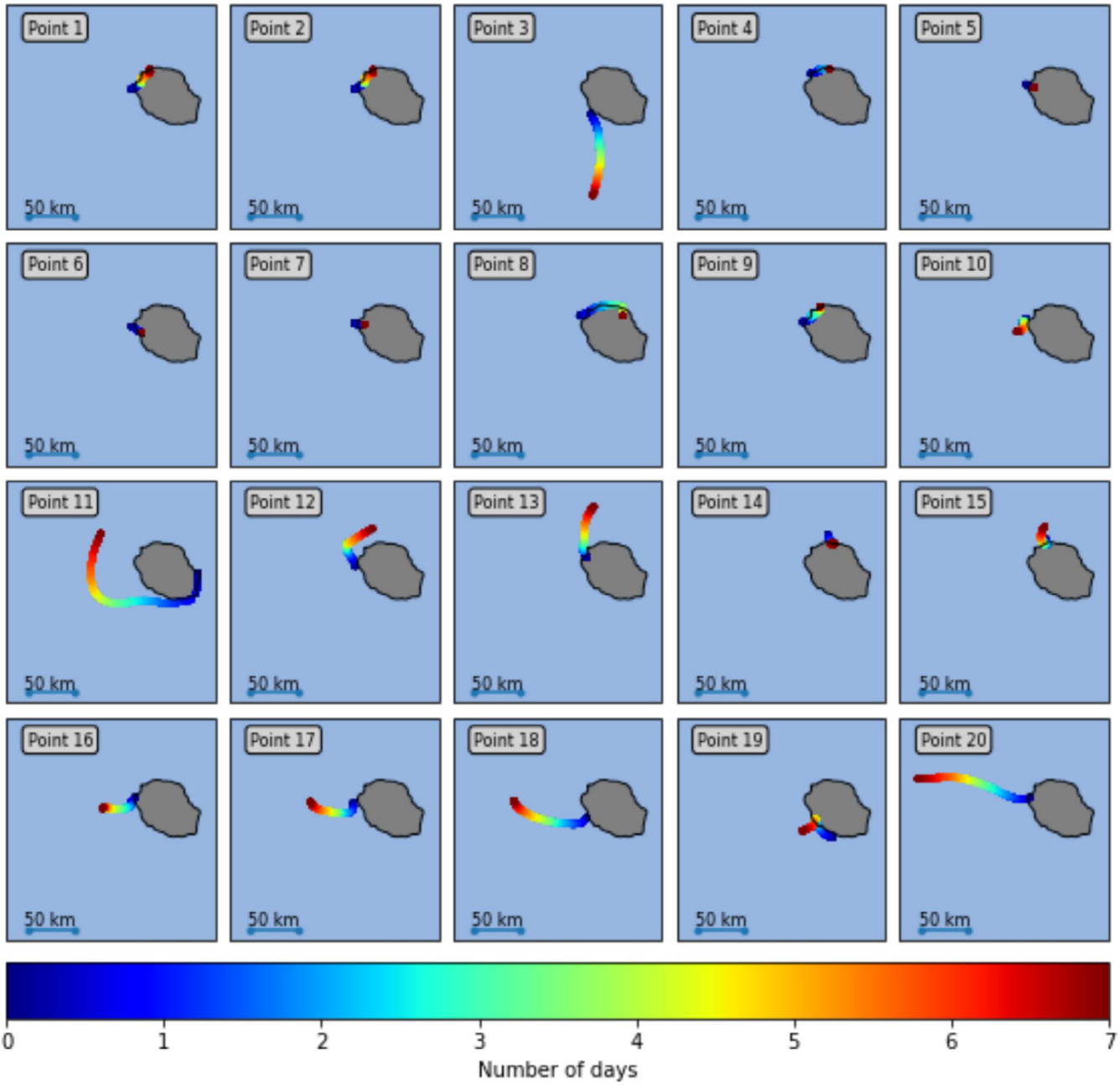

FIGURE 5 | Maps of simulated reverse particle drift trajectories from 20 eDNA sampling locations, based on the exact date of water collection: 1-day and 7-day backtracking trajectories. Some trajectories may appear to overlap land due to the spatial resolution of the oceanographic model.

those cases, corresponding to 64.3% overlap. Conversely, eDNA revealed additional detections in 11 samples where no animals had been observed visually, highlighting its capacity to capture recent presence beyond immediate sighting conditions. On only five occasions, eDNA did not detect a species when visual monitoring had recorded the presence of cetaceans (*Megaptera novaeangliae* (at sampling location #3, 8, and 10), *Stenella attenuata* at (sampling location #16), and *Stenella (longirostris)* (#17) (Table 1)). Cetacean eDNA positive detections were highly concordant with the distribution of most observed species around Réunion Island (*Megaptera novaeangliae, Tursiops aduncus* and *T. truncatus* and *S. attenuata*) (Appendices S8–S12), with the highest number of sequences detected per species located in areas used by the species (Figure 2). DNA sequences associated with *Stenella (longirostris)*, for which identification was less than 100% occurred mostly in areas where the sightings rate of spinner dolphins is higher (Figure 4), supporting the identification at the species level.

3.6 | Reverse Particle Drift to Redesigned eDNA Map

The results of the reverse particle drifts from the 20 sampling locations (Table 1) over a period of 7 days (Figure 5) suggest that in most cases particles had remained in the vicinity of the sample sites. For 6 sampling location (#3, 11, 13, 17, 18, and 20), the results show that particles could have originated far from the coast, after 7 days of drift, followed by 3-day drift close to the coast. For 4 of them (#3, 11, 18 and 20), the drift ranged over long distances (> 100 km).

4 | Discussion

4.1 | Genetic Detection From eDNA

This study demonstrates the capability of eDNA metabarcoding in detecting cetacean species in tropical marine environments. While previous studies have successfully identified cetaceans through eDNA (e.g., Porpoise: Foote et al. 2012; Orcas: Baker et al. 2018; sperm whale: Juhel et al. 2021), few have targeted multiple cetacean species simultaneously (Valsecchi et al. 2020). Our findings reveal spatial consistency and complementarity between visual sightings and eDNA detections. While direct sample-level overlap was limited, eDNA detected cetaceans in 64% of the cases where visual sightings occurred, but also revealed an additional 60% of detections that were not observed in proximity to the sampling site. This highlights the broader temporal and spatial sensitivity of eDNA, particularly for elusive or submerged individuals that may go unnoticed during short visual surveys. Specifically, we detected six cetacean species and demonstrated that the geographical locations of eDNA detections align with the species range. Notably, these detections exceeded the number observed through visual observations made in this study, providing valuable complementary data for long-term monitoring on the east side of Réunion Island, which is less accessible. The probability of eDNA detection is influenced by factors such as water volume, number of sample replicates, concentration, preservation methods, PCR replicates, sampling timing, and amplification methodologies (Alberdi et al. 2018; Harper et al. 2018; Schultz and Lance 2015; Spens et al. 2017; Stewart 2019). In our protocol using Sterlitech and a peristaltic pump, the filtering time was reduced to 15–20 min, rendering the protocol highly efficient on a small boat. Regarding PCR replicates, a minimum of eight replicates per PCR is recommended to reduce false positives when the occupancy (presence/absence) of a species is unknown (Ficetola et al. 2015). In our study, we employed 12 replicates, justified by the known presence of the main target species from visual monitoring, thereby mitigating concerns about false positives. The inclusion of PCR replicates, sample replicates, and field replicates is also recommended for achieving reliable results (Leray and Knowlton 2015; Piggott 2016; Schultz and Lance 2015; Taberlet et al. 1996; Willoughby et al. 2016). Utilizing 10 L per sample, this can be enhanced by conducting replicates per site of 10 L, analyzed separately. Consequently, future work could explore the maximum threshold of detectability based on the number of sample replicates. eDNA approaches necessitate rigorous standards and controls; without these, the information obtained might not only be noisy but outright misleading (Bohmann et al. 2014). By comparing observations with eDNA detections, this study highlights the concordance between methods for detecting cetaceans in tropical waters. Although only one field blank was collected and not sequenced, it was subjected to DNA quantification (Nanodrop and Qubit fluorometry). While this provides a degree of reassurance against field or laboratory contamination, we acknowledge that sequencing field blanks is increasingly considered standard practice in eDNA studies, as it allows detection of low-level contaminants not captured by fluorometric or qPCR methods. However, in the context of this study, sequencing was not performed in order to optimize project costs and develop a streamlined protocol suitable for long-term monitoring by local conservation stakeholders in the southwestern Indian Ocean. Importantly, the human DNA detected in several samples is interpreted as a genuine environmental signal rather than laboratory contamination. This interpretation is supported by the higher human read counts observed at locations and periods known for intensive recreational water use (e.g., swimming, snorkeling), consistent with anthropogenic DNA input in coastal waters. Therefore, while sequencing the field blank might have confirmed the absence of procedural contamination, the observed human DNA in samples is ecologically relevant and aligns with the environmental context of sampling. Hence, we strongly advocate the continuation of this study to standardize protocols.

The primers employed in this study demonstrated efficacy of MiMammal in detecting Delphinidae, with percent identity reaching 100% for most identified 12S sequences. However, an exception was observed for the spinner dolphin (*Stenella longirostris*), which exhibited a lower percent identity (97.08%) despite the large number of 12S sequences available in international databases (> 100 sequences) and including sequences mitochondrial sampled around Réunion Island (from this study). This lower identity score, associated with relatively lower number of reads than other detections (on the order of tens or hundreds, compared to thousands for other species), introduces uncertainties in species identification. While the sequence could be tentatively assigned to the genus '*Stenella*', it falls below the commonly accepted species-level threshold of 99%. No other closely related reference sequences are available to conclusively resolve this discrepancy. Additionally, this sequence forms a distinct OTU composed of multiple reads, lending credibility to its detection but also necessitating caution in interpretation. The presence of four differing nucleotides within this 170-base pair sequence attributed to *Stenella (longirostris)*—based on comparisons with both the NCBI database and sequences from individuals from Réunion—raises questions about the underlying causes of these discrepancies. Potential explanations for the observed discrepancies include incomplete reference databases, sequencing errors, or even the presence of cryptic species. Given the conserved nature of the 12S rRNA gene, the presence of these differences is noteworthy and warrants careful consideration. *Stenella longirostris* comprises four recognized subspecies, but 12S sequences for these subspecies are not available individually in the NCBI database, where only the species-level designation (*S. longirostris*) is present. *S. l. longirostris* (Gray's spinner dolphin) is widely distributed across the Atlantic, Indian, and Pacific Oceans, except the Eastern Tropical Pacific; *S. l. orientalis* (Eastern spinner dolphin) and *S. l. centroamericana* (Central American spinner dolphin) are restricted to the Eastern Tropical Pacific; while *S. l. roseiventris* (Dwarf spinner dolphin) is present in parts of Southeast Asia (e.g., Indonesia, Malaysia) and northern Australia (INPN 2024). Around Réunion Island, *S. l. longirostris* is the subspecies historically recorded from visual surveys and genetic studies (Viricel et al. 2016). The lack of a perfect match in the eDNA sequence to this subspecies, despite its comparison with the 12S sequence from the local population, raises questions about the variability of this sequence, although it is crucial to acknowledge that sequencing errors or biases introduced during the amplification or sequencing process cannot be entirely excluded as contributing factors to the observed discrepancies. However, the generation of additional local genetic data to improve reference databases and further

tests of the eDNA methodology on spinner dolphin is essential to ensure the reliability of the method for detecting the species. This emphasis on local genomic analysis underscores its pivotal role in enhancing the accuracy of reference sequences, particularly in cases where population-specific genetic variations may affect primer efficacy and the subsequent interpretation of eDNA metabarcoding results. Integrating more sequence data to represent the global distribution of a species and subspecies is therefore paramount, not only to enhance the reliability of eDNA-based methodologies but also for broader applications in biodiversity and conservation research.

Six different cetacean species were detected by eDNA analysis (*Megaptera novaeangliae*, *Stenella attenuata*, *Stenella (longirostris)*, *Tursiops aduncus*, *Tursiops truncatus*, *and Kogia breviceps*), which had all been previously reported in Réunion waters from visual surveys and stranding reports (Dulau-Drouot et al. 2008; Dulau et al. 2024) Visual observation during eDNA sampling confirmed five of these species, excluding *Kogia breviceps*. Interestingly, twenty-one time genetic detections of Delphinidae occurred without corresponding visual sightings, suggesting potential persistence of cetacean eDNA at the water surface for several hours. Nevertheless, this study was hampered by the universality of the primers and thus the subsequent inefficiency of the assay owing to potential data loss to nontarget amplification and sequencing particularly human DNA. The issue is compounded by the prevalence of human DNA, which is a common laboratory contaminant but also prevalent throughout reagent manufacturing, sampling, and laboratory processes. While human blocking primers alleviated some of these inefficiencies, more optimisation is required to fully block human amplification. Our study took place on the coast of Réunion Island which is very popular with snorkelers, scuba divers and swimmers during whale watching (swim-with-whales activities very popular and frequent in Réunion Island (Hoarau et al. 2020)). Moreover, the great urbanization on the coast and the high numbers of people swimming in the lagoons is also likely to have led to a high probability of detect human DNA in seawater. This is further compounded by the prevalence of other mammalian DNAs, which are likely to be prevalent in reagent manufacturing (e.g., Bovine Serum Albumin will result in *Bos taurus* detection, gelatin will result in *Sus scrofa* detection). Multiple blocking primers, as suggested by Calvignac-Spencer et al. (2013), could potentially reduce these inefficiencies. While we designed and tested human blocking primers, these did not go far enough to completely block human priming, although they did increase the amount of cetacean detections. Even if these blocking primers worked perfectly, there would still be exogenous sources of other mammal DNA to contend with. An alternative solution involves designing cetacean-specific primers to mitigate nontarget data loss, favoring short and informative (hypervariable) DNA regions. Valsecchi et al.'s (2020) work, validating new primers (Ceto2), allows comprehensive marine vertebrate communities surveys through single HTS metabarcoding assessments, streamlining workflows, reducing costs, and enhancing accessibility. Sequence variation on mitochondrial 12S and 16S regions provides suitable targets for marine mammals, offering good taxonomic resolution for macro-eukaryotes, while also maintaining conserved sites across regions for primers placement (Deagle et al. 2014). We redesigned cetacean-specific 12S rRNA primers based on those designed by Shinoda et al. (2009), which do not amplify human, cow, pig, sheep, cat, or dog DNA. These primers were originally designed to detect illegal use of whale materials mixed with other species typically used in animal feed (bovine, pork, poultry materials). Redesigned Shinoda primers (Cet-F1 and Cet-F2) seem more conservative than the mammal primers used in this study. The newly designed multiplexable set of marine mammal primers yielded promising results. However, while these primers exhibited high specificity and the amplifiability, the discriminatory power of the amplicons is insufficient to confidently differentiate among the Delphinidae species known to occur around Réunion Island. It is likely that longer amplicons will be needed to differentiate these species; however, this would result in lower detection rates. It would be interesting to further explore these amplicon regions using high-throughput sequencing in order to find the best balance between species discrimination and detection efficiency.

The challenge of high-cost and low-efficiency flow cells in eDNA metabarcoding underscores the need for optimized protocols that mitigate nontarget sequence amplification. In our study, we observed an overabundance of nontarget mammalian sequences relative to marine mammals. Initial attempts to implement human-blocking oligonucleotides yielded insufficient specificity in excluding nontarget DNA. This limitation highlights the need for further refinement of blocking strategies and more stringent primer design. Future work should focus on developing and implementing more effective blocking primers or alternative methods such as CRISPR-Cas targeted DNA enrichment (Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and the CRISPR-associated proteins (Cas)). This approach has shown potential in reducing off-target amplification (Kardailsky et al. 2024). Unlike CRISPR depletion, CRISPR enrichment techniques selectively target and enhance specific DNA sequences in eDNA samples (Kardailsky et al. 2024). By using Cas enzymes to treat and isolate DNA in various ways, as detailed by Schultzhaus et al. (2021), CRISPR-Cas-based enrichment becomes a versatile tool for amplifying almost any target sequence before sequencing (Kardailsky et al. 2024). Additionally, optimizing DNA extraction protocols to selectively enrich target DNA may enhance the cost-efficiency of metabarcoding assays, especially for organizations with constrained resources. Improving the specificity of eDNA detection would not only align with the conservation goals for cetacean monitoring around Réunion Island but would also contribute to a broader understanding of marine mammal ecology through more accurate and reliable data.

4.2 | eDNA and Dispersal Drift

Regarding the potential preservation and dispersal of eDNA in the ocean, results on drift of eDNA around Réunion Island revealed that the DNA collected could not have come far from its source point and is likely to have come from local populations using the waters of Réunion. Simulations over periods of 1–7 days showed mainly coastal movements. Most of the particle drift occurred on short distances.

High temperatures increase the kinetics of many processes responsible for DNA degradation, including: lysis of cells and organelles, hydrolysis and oxidation of DNA molecules, and

degradation by extracellular enzymes (McCartin et al. 2022). eDNA can persist at quantifiable concentrations for more than 2 weeks at low temperatures (≤10°C) and 1 week (or less) at ≥20°C (McCartin et al. 2022). In our study from tropical marine waters, the sea surface temperatures were always above 20°C with a mean of 27.5°C. Regarding the eDNA time persistence until 99.9% degradation from McCartin et al. (2022) at 27.5°C, eDNA in seawater may only persist for a few hours up to 2 days.

According to the particle drift simulations, if we assume that eDNA does not persist for more than 2 days, the eDNA detected should come from the area it was sampled in, which explains the consistency with observations since 2008 and it confirms that eDNA is a good alternative to visual surveys for the species detected around Réunion Island.

4.3 | Limit of eDNA Detection

The type of environment, sea surface temperatures, weather conditions, animal behavior, body size, age, density, along with habitat use/frequency, sampling, and storage techniques are all important factors that are known to influence the production and degradation of eDNA (Pinfield et al. 2019). Enzymes are generally less active at pH 8 and during our sampling the pH was around 8.2. In our study, we did not find a correlation between the concentrations of cetacean DNA and environmental variables such as the SST and wind but the number of samples was insufficient to detect potential correlations. eDNA-based approaches, as a management tool, requires an understanding of its spatio-temporal persistence. eDNA can be detected in aquatic environments for a long time (Strickler et al. 2015; Lance et al. 2017; Tsuji et al. 2017) which means that false positives (the detection of a species that is not actually present in the area, even though its eDNA is detected because the DNA may have traveled from another location and been amplified in this zone) may be present when working with eDNA without considering temporal and spatial persistence. However, determining the persistence of eDNA over time is not sufficient on its own, as eDNA degradation occurs through endonucleases, water (hydrolysis), UV radiation, the action of bacteria and fungi in the environment (Shapiro 2008), as well as temperature, pH, O_2, and salinity (Wang et al. 2021). Thus, eDNA persistence in the marine environment is complex, especially as DNA is found in different states, it is either free or encapsulated, which in turn also determines its degradation rate. The fraction between free and encapsulated DNA is generally not known and this was the case for our study. The extracellular (i.e., free DNA) fraction could be a minor fraction as has been shown in carp fish (Turner et al. 2015) but in the marine environment and in marine mammals these estimates have not been made. To avoid capturing free DNA, it is possible to work with filtration capsules with a porosity greater than 0.45 μm, since measurable concentrations of free DNA in water are found from 0.45 μm (Zaiko 2022). We are currently lacking a solid understanding of how water chemistry and other environmental parameters influence eDNA states in aquatic environments and how they persist (Mauvisseau et al. 2022). To resolve species detection issues during monitoring, we would need to detect the individual within a short time frame. RNA, on the other hand, is much less affected by temperature but degrades faster than DNA in water making it an interesting tool (Qian et al. 2022). At temperatures below 20°C, DNA lasts longer while RNA has its degradation rate unchanged (McCartin et al. 2022). This means that when eRNA is detected in marine water, we could assume that living target organisms could have passed within 24 h as revealed by a recent study on shrimp (Qian et al. 2022). We still have much to understand about the DNA degradation factors and the potential of environmental RNA. To date, it is very complicated to use eDNA data as a monitoring and biomonitoring tool without taking physical parameters of the environment (including temperature) into consideration. Working with filtration capsule for both free and encapsulated DNAs certainly allows a better chance of detecting DNA but the fractionation of these different types of DNAs makes the understanding of the degradation processes more complex to understand.

To conclude, eDNA offers huge potential for monitoring cetaceans' diversity as revealed by the present study (higher detection by eDNA than visual observation during sampling) but additional research is needed to support the interpretation of positive detections with regard to space and time and also into the design of more species-specific primers to limit the number of false negative detections in eDNA studies. Despite the successful detection of several odontocete species, including the elusive Pygmy sperm whale, large baleen whales—such as *Megaptera novaeangliae*—were poorly represented in our metabarcoding dataset. This result aligns with the known challenges of detecting mysticetes via eDNA, likely due to their lower eDNA shedding rate and broader habitat use (Székely et al. 2022). One promising avenue to improve detection of such species is the use of species-specific qPCR combined with high-resolution melting (HRM) assays. This approach was recently developed and validated for humpback whales by Robinson et al. (2024), using newly designed primers targeting the COI mitochondrial region. However, the development and validation of species-specific qPCR assays or HRM approaches require access to high-quality reference DNA from tissue samples—a step that remains logistically challenging for many marine mammal species. Biopsy sampling, although widely used for genetic monitoring, can be time-consuming, costly, and invasive, necessitating close proximity to animals (Clapham and Mattila 1993; Krützen et al. 2002). For numerous cetaceans that are rare, elusive, or sporadically observed, skin biopsy sampling is not only impractical around Réunion Island, but often unattainable across their entire range (Székely et al. 2022). The prospect of designing and validating qPCR assays for all 28 species reported in the region is thus constrained by the inaccessibility of biological material and the significant resources needed for empirical testing. In addition, while mock communities of known DNA mixtures offer a valuable strategy for validating metabarcoding primers and reducing false positives or negatives (Székely et al. 2022), the creation of mock mixtures representative of the full cetacean community in this tropical system would have been unfeasible. As highlighted by Mugnai et al. (2021), the effectiveness of eDNA-based biodiversity assessments remains fundamentally limited by the taxonomic coverage and accuracy of reference sequence databases such as GenBank. Although mitochondrial genomes are now available for many species, substantial gaps persist for Indian Ocean taxa, reducing the reliability of species-level assignment in metabarcoding data. These limitations underscore the necessity of complementary approaches: while

targeted assays may provide superior sensitivity for specific taxa—particularly in the context of monitoring single, threatened species—universal eDNA metabarcoding currently offers one of the operationally feasible strategies for broad-scale biodiversity assessment in species-rich and under-documented ecosystems such as those surrounding Réunion Island. This choice does not imply the inherent superiority of metabarcoding over targeted approaches, but rather reflects practical constraints related to sampling effort, reference database completeness, and access to tissue-derived DNA. Ultimately, these methodologies should be seen as complementary, with targeted qPCR tools, visual surveys, and acoustic monitoring offering valuable specificity and quantification potential, and metabarcoding providing an inclusive first-pass overview of community diversity. Future improvements in both primer design and reference database coverage will further enhance the reliability and interoperability of these approaches for marine mammal monitoring.

Author Contributions

A first draft of the manuscript was prepared by N.N. and revisions by all authors. Data collection was carried out by N.N., E.C., V.D., and V.E. The field sampling protocol and molecular laboratory techniques were devised by N.N. and R.P. Samples were extracted and sequenced by NatureMetrics. N.N., E.C., L.H., N.B., and V.E. performed the analysis of the data.

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Ethics Statement

We confirm that our manuscript has not been submitted elsewhere, and all research conducted adheres to the ethical guidelines of our study country. Our use of environmental DNA entails strict adherence to ethical standards regarding the treatment of animals. Our DNA samples were obtained from water samples, and the two tissue samples for sequencing were collected from stranded individuals in Réunion by GLOBICE, a member of the national stranding network (RNE) with authorization for marine mammal activities (green card) issued by PELAGIS. This ensures that all reasonable steps were taken to ensure the humane treatment of animals, minimizing discomfort, distress, and pain, in compliance with relevant local animal welfare laws, guidelines, and policies. We are prepared to provide a statement of compliance with these regulations to the Editor if necessary. We agree with the 'permission to reproduce material from other sources'.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data, including eDNA data and 12S fragment sequencing data, are available at https://doi.org/10.57745/U0PDJW or through https://entrepot.recherche.data.gouv.fr/dataset.xhtml?persistentId=-doi:10.57745/U0PDJW. Genome sequencing data include four individuals: IDs Ta2 and Ta21 for *Tursiops aduncus*, and IDs SL7 and SL9 for *Stenella longirostris*.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** edn370209-sup-0001-DataS1.docx.