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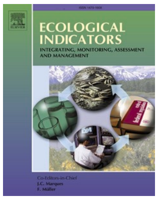

Original Articles

Assessing the potential use of environmental DNA for multifaceted genetic monitoring of cetaceans: Example of a wandering whale in a highly disturbed bay area

Shuai Zhang [a,d], Yiting Cao [a,b], Bingyao Chen [c], Peiwen Jiang [a], Liang Fang [a], Hongting Li [a], Zuozhi Chen [a,d], Shannan Xu [a,d], Min Li [a,d,*]

[a] *South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou 510300, China*
[b] *College of Fisheries and Life Science, Dalian Ocean University, Dalian 116023, China*
[c] *College of Life Sciences, Nanjing Normal University, Nanjing 210023, China*
[d] *Key Laboratory for Sustainable Utilization of Open-sea Fishery, Ministry of Agriculture and Rural Affairs, Guangzhou 510300, China*

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ABSTRACT

Environmental DNA (eDNA) sampling of water is a powerful method for comprehensive and noninvasive monitoring of aquatic animal species. However, there have been few reports of its application to cetacean species. On June 29, 2021, a whale (nicknamed Xiaobu) appeared in Dapeng Bay off Guangdong Province, China. We used eDNA technology to obtain information related to this whale (e.g., species identification and food resources) and to trace its possible origin. Fragments of four whale mitochondrial sequences (12S rDNA, 16S rDNA, cytochrome *c* oxidase subunit 1, and control region) were obtained from amplicons of eDNA collected in Dapeng Bay; sequence barcoding showed that this was an Eden's whale (*Balaenoptera edeni edeni* Anderson 1879). Analysis of potential prey species (PPS) suggested that this whale might enter Dapeng Bay while tracking prey, mainly sardines (*Sardinella lemuru*, *Sardinella gibbosa* and *Sardinella jussieui*) and anchovies (*Thryssa dussumieri*, *Thryssa vitrirostris* and *Thryssa kammalensis*). Retrieval of eDNA metabarcoding data from samples collected in waters adjacent to Dapeng Bay (i.e. Lingding Bay and Daya Bay) revealed that Eden's whale had appeared outside Lingding Bay up to 2 months prior to the appearance of this whale in Dapeng Bay (early April 2021). Overall, this study showed that eDNA is a highly effective noninvasive survey method for the accurate identification of target cetacean species and prey composition; it can be used to monitor megafauna that are under strict legal protection or to monitor megafauna with unknown conditions.

1. Introduction

Cetaceans are found in oceans around the world, and they play key roles in marine ecosystems. However, they are among the most threatened vertebrates worldwide, such that 37% of species are classified as threatened and requiring conservation (IUCN, 2021). With oceans under increasing pressure from human activities (e.g., overfishing, habitat loss, pollution, and climate change), distributions of cetaceans may shift because of their dependence on prey availability, safe environments, or specific water temperatures (Poloczanska et al., 2013; Pecl et al., 2017; Bonebrake et al., 2018; van Weelden et al., 2021). Because of the potential for rapid changes in cetacean geographical ranges, there is a clear need for more rapid and effective biomonitoring methods that can be used in management and conservation.

However, efforts to monitor the presence of cetaceans remain challenging, generally because of their elusiveness, low abundance, and capacity to move over long distances (Hays et al., 2016; Juhel et al., 2021). Most studies focused on the distributions of common marine animals rely on passive acoustic surveys or visual observations from the coast during aerial or boat-based surveys (e.g., Li et al., 2019; Fang et al., 2022). Therefore, limited information has been accumulated regarding the distributions of many species, which limits the capacity to establish effective conservation measures. Accordingly, there is a need to develop complementary and effective tools for the detection and monitoring of threatened, rare, or elusive marine cetacean species; such tools will facilitate conservation efforts.

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* Corresponding author at: South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou, China.
E-mail address: limin@scsfri.ac.cn (M. Li).

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Cetacean species evolve through unique pathways and usually diverge along a continuum, which makes the delimitation of species boundaries a fascinating and difficult process because there is a lack of sufficient morphological data (Taylor et al., 2017a). Subspecies delimitation is more difficult because continuous gene flow is expected, and therefore a clear biological threshold has not been established (Taylor et al., 2017a). An estimated 32% of species have a high likelihood of underclassification errors; an accurate taxonomy may contain twofold more subspecies than currently recognized (Taylor et al., 2017b). Conventional nonlethal methods of genetic sampling from cetaceans (e.g., biopsy, fecal, and blowhole exhalation sampling (Noren and Mocklin, 2012; Borowska et al., 2014; Günther et al., 2022) are also limited because of their invasiveness and/or need for close approach to animals, which increase the risks to both animals and researchers and require a high level of skill.

The analysis of environmental DNA (eDNA) is a sensitive, accurate, and reliable approach to collect genetic information regarding aquatic animals. It is particularly attractive for analysis of endangered species because it is a noninvasive and nondestructive method that poses negligible risk to the target species or the environment (Bohmann et al., 2014; Hunter et al., 2018; Beng and Corlett, 2020). Aquatic animal eDNA refers to genetic material that can be extracted from water samples in a partially degraded form; it can be derived from feces, urine, shed skin, carcasses, or other aquatic animal-related biological processes (Gillett et al., 2010; Borowska et al., 2014; Thomsen and Willerslev, 2015). eDNA sampling offers multiple benefits, such as simplicity and minimal disturbance of the target organisms (Lear et al., 2018; Kumar et al., 2020; Bessey et al., 2021), and it has been used as a biomonitoring tool for various aquatic animals (Tang et al., 2019; Valsecchi et al., 2020; Zou et al., 2020).

eDNA techniques involve the use of polymerase chain reaction (PCR) to amplify informative genetic tags via species-specific or universal primers, usually in commonly amplified mitochondrial sequences (Miya et al., 2015; Qu and Stewart, 2019; Eble et al., 2020). Thus far, eDNA techniques have been successfully established for the detection of single species with conventional PCR or community constituents via metabarcoding (Thomsen and Willerslev, 2015; Compson et al., 2020; Juhel et al., 2021). They have also been used to quantify the absolute abundances of DNA sequences (as a proxy for the relative abundances of species) via quantitative PCR or droplet digital PCR (Baker et al., 2018; Horiuchi et al., 2019; Brys et al., 2021). eDNA-based techniques can extract species or community information regarding distinct biomes from a single sample through the amplification of target DNA fragments; thus, they provide effective and efficient methods to conduct biomonitoring of the whole aquatic ecosystem (Closek et al., 2019; Valsecchi et al., 2020; Zhang et al., 2020).

On June 29, 2021, a whale was spotted by a local fisherman in Dapeng Bay (DPB) off Shenzhen, Guangdong Province. It subsequently attracted considerable interest from local citizens, netizens, conservation experts, and official managers (Huaxia, 2021; Pei, 2021). The subadult whale in DPB with a body length of ~ 8 m was preliminarily identified as a Bryde's whale (*Balaenoptera edeni* ssp., nicknamed Xiaobu) based on external morphology through visual observation (i.e., number of ridges in front of the blowhole and colors of the back and abdomen) (Huaxia, 2021). Multiple conservation measures were implemented to protect this whale in DPB, including limiting boat speed, restricting whale watching activities, monitoring its behavior, removing debris, monitoring water quality, and establishing a no-fishing zone (Shenzhen-Government-Online., 2021a; Shenzhen-Government-Online, 2021b). Furthermore, local officials established a special joint working group focused on whale and dolphin protection in Dapeng New Area, Shenzhen (Zhang, 2021).

Bryde's-like whales are a composite of members in the genus *Balaenoptera*, including eight validated species that are difficult to visually distinguish based on external morphology (Wada et al., 2003; Rosel et al., 2021; Perrin, 2022). The exact taxonomic status of the whale in DPB was unclear, and its origin and reason for its appearance were unknown, although some experts speculated that food resources could explain its movement (Pei, 2021). Baleen whales are under first-class protection in China based on the latest version of the List of State Key Protected Wild Animals (2021). Adequate knowledge regarding this rare Bryde's-like whale is needed to ensure effective conservation actions and fulfill the curiosity of the public. Therefore, this study used noninvasive eDNA techniques to determine the taxonomic identity of this whale through analysis of multiple mitochondrial DNA (mtDNA) markers; it also investigated the prey composition and relative biomass of this whale, then explored its possible origin.

2. Materials and methods

2.1. Study area

Dapeng Bay (DPB, also known as Mirs Bay), where a Bryde's-like whale (nicknamed Xiaobu) was first spotted on June 29, 2021, is a semi-enclosed bay surrounded by two international metropolises in South China: Shenzhen City and Hong Kong Special Administrative Region (Fig. 1). It is an area of approximately 390 km^2 with water depth between 10 and 20 m (mean 18 m) The water temperature ranges from 16.9 °C to 30.9 °C throughout the year (Xu et al., 2021). DPB is one of the world's busiest sea areas with various ships engaged in maritime activities, including cargo ships (such as containers and liquefied natural gas tankers), tourist vessels, and fishing boats. Additionally, the bay contains Yantian Port, which has one of the largest container throughputs worldwide (YICT, 2021).

There are two bays adjacent to DPB: Lingding Bay (LDB) and Daya Bay (DYB) (Fig. 1). LDB is a funnel-shaped subaqueous delta with water depth between 2 and 10 m (mean 5 m), and its channels are deeper than 20 m. Daya Bay is a semi-closed bay with water depth between 6 and 16 m (mean 10 m) (Wang et al., 2008). Regular eDNA metabarcoding monitoring of fish community via MiFish-U/E universal primers (Miya et al., 2015) has been conducted in these two bays since 2021. Because some vertebrates (e.g., cetaceans) can also be detected by the MiFish- U/E primer set, the metabarcoding operational taxonomic unit (OTU) data generated from eDNA off LDB and DYB, which had been sampled and analyzed in April 2021 (i.e., sampling dates nearest to the DPB sampling date) were re-examined for evidence of the presence of large whales. The fish community structures of these two bays were also compared with the structures within the DPB.

2.2. Field sampling

A speedboat ~ 4 m in length was used for whale monitoring and water sampling. Water samples were collected using a 5-L hydrophore from six sites in the hotspot area where the whale Xiaobu had been sighted on July 2–3, 2021, in DPB (Figs. 1 and 2) (Dong et al., 2022). Because the sampling site depths were < 10 m, water samples were collected from 1 m beneath the surface and 1 m above the bottom, then mixed in sterile plastic bottles (Fig. 2). 1-L water samples were filtered using cellulose acetate microfiber membranes (diameter, 47 mm; nominal pore size, 0.45 μm; Jinjing, Shanghai, China) through a vacuum peristaltic pump (Auto-Science, Shenyang, China). Pure ethanol was sprayed onto the filter membrane at the end of filtration to stabilize the eDNA. The same protocol was applied to 1 L of deionized water as a negative control at each station. The filters were immediately placed into sterile 2-mL plastic scintillation tubes, preserved in liquid nitrogen, and transported back to the laboratory where they were stored at − 80 °C until further eDNA extraction.

During the period of whale searching, the speedboat was operated at a low speed (<5 km/h). When the whale was sighted, the speedboat was maintained at a safe distance from the animal (>100 m) and lower speed to 5 km/h (Fig. 2). If the whale foraged, floating fish carcasses were collected after its departure and identified based on morphology (Wu

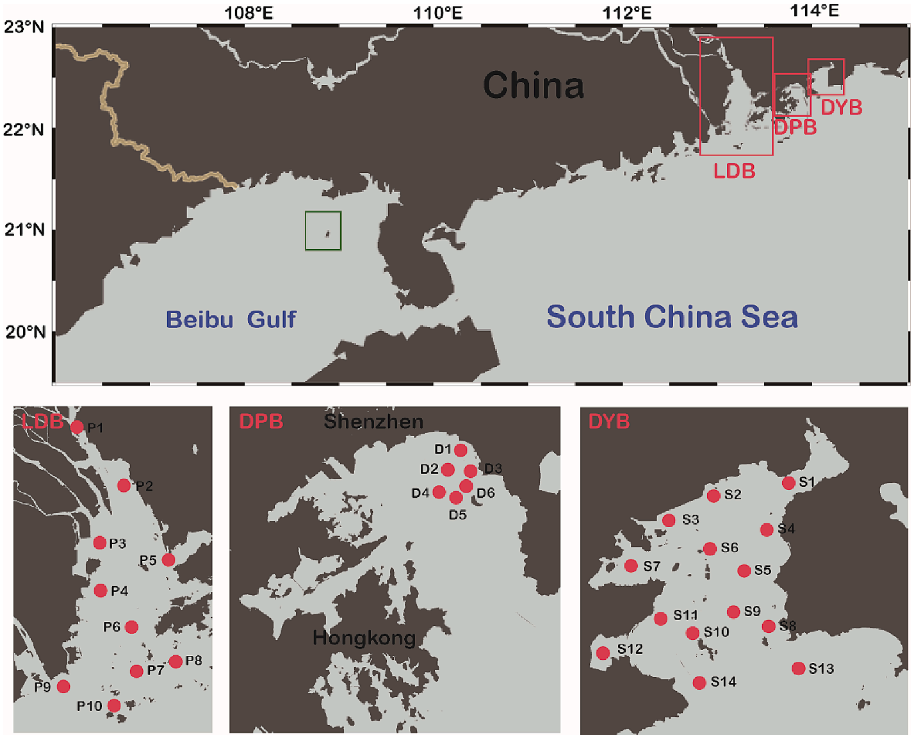

Fig. 1. Map of sampling sites in this study. Water samples were collected from Dapeng Bay (DPB) in July 2021. P1–P10 in Lingding Bay (LDB) and S1–S14 in Daya Bay (DYB) were sampled in April 2021. Green box indicates an area where a population of Eden's whales was reported around Weizhou Island in Beibu Gulf (Chen et al., 2019a; Chen et al., 2019b). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. Whale watching and eDNA sampling in Dapeng Bay. (a) Xiaobu was preying in Dapeng Bay; (b) On-board observation; (c) Environmental DNA sampling.

and Zhong, 2021) for subsequent food composition analyses.

2.3. eDNA extraction

The extraction of eDNA from all samples was performed using a modified DNeasy Blood & Tissue Kit protocol, which was previously described by Zou et al. (2020). In briefly, membranes were cut into small pieces, soaked in 600 μL of 2 × lysis buffer and 60 μL of proteinase K, incubated at 56 °C until membranes were completely lysed, and extracted eDNA was eluted into a volume of 100 μL AE buffer. To eliminate possible eDNA contaminations, disposable experimental materials were preferred and purification procedures were conducted before and after all manipulations, such as sampling and filtering, eDNA extraction and amplification. The eDNA extraction and amplification experiments were conducted in a dedicated laboratory at South China Sea Fisheries Research Institute where all utensils were routinely decontaminated with UV and bleach sterilized. The concentration and purity of DNA were determined using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) and NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Qualified eDNA extracts were preserved at − 24 °C until PCR amplification.

2.4. Cetacean and fish community metabarcoding

For the rapid scanning of cetacean species in DPB waters, two universal primer pairs were used to amplify partial mitochondrial sequences of marine mammals. Specifically, a mitochondrial 12S rDNA primer pair (MarVer1) was used for sampling sites D1, D2, and D3; a mitochondrial 16S rDNA primer pair (MarVer3) was used for sampling sites D4, D5, and D6 (Tables 1 and 2). Due to sampling sites were limited in a small sea area in DPB, which can be considered as a singular sampling unit, not all samples were analyzed using the two universal mammalian primers. Twelve random nucleotides were added to the 5′ end of the reverse primer as a barcode to distinguish among samples in bioinformatics analyses. PCR amplification was performed in a volume of 50 μL, comprising 1 μL of the forward and reverse primers (10 μM), 3 μL of DNA template, 5 μL of 10 × ExTaq PCR Buffer (Mg^{2+} plus), 4 μL of dNTP mix (10 mM), and 0.2 μL of ExTaq (5 U/μL) (Takara, Japan), with molecular biology-grade water for the remaining volume. The PCR protocol consisted of an initial denaturation step of 5 min at 94 °C; 30 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 55 °C (for both 12S and 16S sequences), and extension for 30 s at 72 °C; and a final extension step of 5 min at 72 °C.

To determine the composition of fish in DPB, a slightly modified MiFish primer pair (combination of primers MiFish-U and MiFish-E) was used for the amplification of fish mitochondrial 12S rDNA from all six sampling sites (D1–D6) (Table 1). A 12-bp barcode was added at the 5′ end of the reverse primer. PCR amplification was performed in a volume of 50 μL, comprising 1 μL of each primer (10 μM), 3 μL of DNA template, and 25 μL of 2 × ProTaq Master Mix (Accurate Biology, Hunan, China), with molecular biology-grade water for the remaining volume. The PCR protocol consisted of an initial denaturation step of 2 min at 98 °C; 25 cycles of denaturation for 10 s at 98 °C, annealing for 30 s at 60 °C, and extension for 20 s at 72 °C; and a final extension step of 5 min at 72 °C. PCR products were quantified using a Qubit 2.0 Fluorometer (Invitrogen) and NanoDrop One spectrophotometer (Thermo Fisher Scientific), and quality was determined by 1% agarose gel electrophoresis. No amplification bands were observed for the filtration blanks or negative controls. Therefore, the blanks and negative controls were omitted from subsequent analyses.

PCR products were purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), in accordance with the manufacturer's instructions. The purified PCR amplification products were used to construct sequencing libraries (NEBNext Ultra DNA Library Prep Kit; New England Biolabs, Ipswich, MA, USA) in accordance with the manufacturer's recommendations. Index codes were added during library construction. Library quantification and quality assessment were conducted using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific) and Agilent Bioanalyzer 2100 system (Agilent Technologies, Atlanta, GA, USA). Finally, the libraries for locus MarVer1, MarVer3, and MiFish were subjected to 250-bp paired-end sequencing using the Illumina HiSeq2500 platform (Illumina, San Diego, CA, USA) by MAGIGENE Biological Technology Co., Ltd (Guangzhou, China).

Table 2
Positive and negative amplicons for Eden's whale sequences from six sampling sites in Dapeng Bay.

Primer sets	Whale sequences	Fragmen length (bp)	Sampling sites in Dapeng Bay					
			D1	D2	D3	D4	D5	D6
MarVer1	12S rDNA	163	√	√	√	–	–	–
MarVer3	16S rDNA	197	–	–	–	√	√	√
MiFish-U/E	12S rDNA	170	√	√	√	√	√	√
eBed-COI2	COI	222	√	√	×	√	√	√
eBed-CR4	CR	193	√	√	√	√	√	×

√, positive amplification; ×, negative amplification; -, not used for amplification.

2.5. Metabarcoding bioinformatics

To obtain clean paired-end reads, the raw sequences were filtered using TRIMMOMATIC v0.33 (Bolger et al., 2014); barcodes and primers were removed. Clean paired-end reads were merged as raw tags using FLASH v1.2.11 (Magoč and Salzberg, 2011). Sequences were clustered using the USEARCH v8.0.1517 (Rognes et al., 2016) pipeline to identify OTUs based on a 97% similarity threshold. Taxonomic annotation of OTUs was performed in National Center for Biotechnology Information (NCBI) and MitoFish (only for sequences yielded by MiFish primers) databases (Miya et al., 2015). OTUs were designated as belonging to a species if there was ≥ 98.3% sequence identity, based on a 0.017 maximum intraspecific genetic distance of fish in LDB (Jiang et al., 2022). The criteria of sequences assigned to each species followed Zou et al. (2020).

2.6. Subspecies-specific amplification and barcoding

The whale in DPB was preliminarily identified as an Eden's whale based on the results of metabarcoding using MarVer1 and MarVer3 primers. For further identification to the subspecies level, two commonly used barcodes were designed for Eden's whale subspecies identification based on the complete mtDNA sequence (GenBank: AB201258.1): cytochrome *c* oxidase subunit 1 (COI) primer pair (eBed-COI2) and control region (CR) primer pair (eBed-CR4) (Table 1). DNA extracted from sampling sites D1–D6 in DPB was used for amplification.

PCR was performed in a volume of 50 μL using components described above for amplification of cetacean sequences, with the exception of modified primers. The PCR protocol was similar to the protocols described above for amplifying 12S and 16S sequences, with the exception of annealing temperature modification to 58 °C for both COI and CR sequences. PCR products were qualified by spectrophotometry (NanoDrop One; Thermo Fisher Scientific) and 1% agarose gel

Table 1
Primer sequences for amplification of target genes from fish and Eden's whale.

Primer sets	Orientation	Sequences (5′-3′)	Locus	Target species	Sequencing approach	Study area	Reference
MarVer1	F	CGTGCCAGCCACCGCG	12S rDNA	Mammals	High-throughput sequencing	DPB	Valsecchi et al. (2020)
	R	GGGTATCTAATCCYAGTTTG					
MarVer3	F	AGACGAGAAGACCCTRTG	16S rDNA	Mammals	High-throughput sequencing	DPB	Valsecchi et al. (2020)
	R	GGATTGCGCTGTTATCCC					
MiFish-U/E	F	GTCGGTAAAWCTCGTGCCAGC	12S rDNA	Fish and mammals	High-throughput sequencing	DPB, LDB, DYB	Miya et al. (2015)
	R	CATAGTGGGGTATCTAATCCYAGTTTG					
eBed-COI2	F	CTGACTATTCTCAACCAACCACAAA	COI	*E. edeni edeni*	Sanger sequencing	DPB	This study
	R	GGGGGACTAGTCAGTTTCCGAAT					
eBed-CR4	F	CATGCTATGTATAACTGTGCATTC	CR	*E. edeni edeni*	Sanger sequencing	DPB	This study
	R	CATGGTGATTAAGCTCGTGATCTA					

*To combine MiFish-U and -E primers, several base pairs of the MiFish primer were slightly altered from the original sequences. Y, C/T; W, A/T. The underlined sequence was similar between MarVer1 and MiFish primers.

electrophoresis, then subjected to Sanger sequencing with the same primers used for amplification (ABI 3730xl DNA Analyzer; Applied Biosystems, Foster City, CA, USA).

2.7. Subspecies identification

Sequences of COI and CR genes from the Eden's whale were manually assembled using SeqMan (Swindell and Plasterer, 1997) in DNAStar software (DNAStar, Madison, WI, USA). Subspecies identification was performed based on 12S, 16S, COI, and CR sequences. Other sequences previously reported for *Balaenoptera* spp. were downloaded from the NCBI database. The humpback whale (*Megaptera novaeangliae*) and fin whale (*Balaenoptera physalus physalus*) were included as outgroups.

Sequences were aligned using ClustalW with the default parameters (Thompson et al., 1994), and ModelFinder was used to identify the best model for phylogenetic estimation. Phylogenetic tree construction was performed using the neighbor-joining method with 1000 bootstrap replicates (Saitou and Nei, 1987). All of these analyses were performed in MEGA 6.06 software (Tamura et al., 2013).

2.8. Potential prey species (PPS) analysis

Eden's whales generally prey on pelagic species that form large schools (Alberto Nino-Torres et al., 2014; Kato and Perrin, 2018). Field observations in DPB suggested that Xiaobu also exhibited this tendency. To explore the Eden's whale prey species composition, we defined the fish species with pelagic habitats, schooling behavior, and proportion of total eDNA reads $\geq$ 1% as potential prey species (PPS) based on eDNA metabarcoding data. The ecological characteristics of fish were defined based on the classification systems established by Froese and Pauly (2022) and Wu and Zhong (2021). It should be noted that PPS was not the fish actually consumed by Xiaobu, and the species collected during the field observation (the floating fish carcasses collected after its predation) were also used for further confirmation.

2.9. Comparison of metabarcoding data with data from adjacent waters

OTU sequences of eDNA metabarcoding data from LDB and DYB samples collected in April 2021 were re-examined to determine whether a large whale could be detected in this period. Samples were collected from 10 sites in LDB (P1–P10) sampled during April 15–18, 2021, and 14 sites in DYB (S1–S14) sampled during April 7–9, 2021 (Fig. 1). These sampling sites covered the entire area of the two bays. Water sampling, eDNA extraction, amplification, sequencing, and bioinformatics analyses were conducted in a manner identical to the methods described above for DPB, and the analyses were completed in May 2021.

To explore differences in fish community composition among bays, non-metric multidimensional scaling was performed based on Bray-Curtis similarity of eDNA metabarcoding data from the sampling sites. The results were statistically analyzed by permutational multivariate analysis of variance (PERMANOVA) in the vegan package in R v.4.1.2 (R Core Team, 2022). The threshold for statistical significance was regarded as $\alpha = 0.05$.

2.10. Ethics statement

Eden's whale surveys were conducted with the permission and approval of the South China Sea Fisheries Research Institute Animal Welfare Committee. Surveys for whale watching and water sampling were performed in accordance with best practice guidelines and conformed to the National Wildlife Conservation Law in China.

3. Results

3.1. Whale sequences obtained from amplicons by multiple eDNA markers

eDNA metabarcoding generated 369499 reads for 12S rDNA (using the MarVer1 primer set) from three sampling sites (D1–D3), and 311164 reads for 16S rDNA (using the MarVer3 primer set) from the other three samples (D4–D6) in DPB. The results revealed 310 and 143 OTUs in analyses of 12S and 16S rDNA, respectively. Among these sequences, OTU_ID121 (12S) and OTU_ID95 (16S) with lengths of 163 bp and 197 bp, respectively, were identified as Eden's whale (Tables 2 and S1). MtDNA COI and CR sequences were successfully amplified and sequenced using two species-specific eDNA primer sets in five of the six sampling sites, with lengths of 222 bp and 193 bp, respectively (Tables 2 and S1).

Additionally, analyses of nontarget species from MiFish metabarcoding data of fish successfully amplified Eden's whale 12S rDNA (OTU_ID44) in all sampling sites from DPB (Table 2), with a sequence 7 bp longer than the segment amplified by MarVer1 metabarcoding (170 bp *vs.* 163 bp, respectively, Table S1). Surprisingly, analyses of MiFish metabarcoding data from the other two bays (LDB and DYB) adjacent to DPB revealed the presence of Eden's whale 12S rDNA sequences in some of the samples collected from LDB in April 2021, approximately 70 days earlier than the date of the whale's appearance in DPB. However, no whale sequences were obtained through MiFish metabarcoding in DYB.

3.2. Subspecies identification of target living whale

Two mammalian mtDNA markers were first amplified from DPB through MarVer1 and MarVer3 metabarcoding. The resulting sequences (12S and 16S) were analyzed by BLASTn in the NCBI database, with 100% query cover and identity with Eden's whale. The neighbor-joining tree could be discriminated from other species in the genus *Balaenoptera*, particularly for the genetically close subspecies, Bryde's whale (*Balaenoptera edeni brydei*) (Fig. 3a and b).

To confirm the accuracy of subspecies identification, two commonly used markers (mtDNA COI and CR) for whale species identification were also included in analyses of single-species sequences. The BLASTn results showed 100% query cover and identity with Eden's whale for these two markers. The neighbor-joining tree also permitted discrimination from other species in the genus *Balaenoptera*, particularly for the genetically close subspecies, Bryde's whale (*B. edeni brydei*) (Fig. 3c and d).

3.3. Estimated composition of Eden's whale diet in DPB

During the period of whale observation and water sampling in DPB, we also collected prey fish if the whale was observed foraging. Fourteen fish carcass specimens floating on the sea surface were collected after the whale had left and nine species were identified based on morphology: *Sardinella lemuru*, *Nuchequula nuchalis*, *Konosirus punctatus*, *Thryssa kammalensis*, *Caranx kalla*, *Mugil cephalus*, *Thryssa* sp1, *Thryssa* sp2, and *Anchoviella* sp1 (Fig. 4a).

For eDNA metabarcoding, 326255 reads of mitochondrial 12S rDNA sequences were obtained from six samples in DPB. In total, 189 fish species from 25 orders, 69 families, and 137 genera were identified (Table S2). The proportions of reads for 15 species were $\geq$ 1% based on the total number of sequence reads (Table 3). The 15 most abundant fish species constituted 84.4% of the total reads. Eight PPS were included: *S. lemuru*, *Stolephorus insularis*, *S. gibbosa*, *Encrasicholina heteroloba*, *T. dussumieri*, *T. vitrirostris*, *S. jussieui*, and *T. kammalensis* (Table 3). Among them, sardines (*S. lemuru*, *S. gibbosa*, and *S. jussieui*) were the predominant group, constituting 71% of PPS, followed by anchovies (*S. insularis*, *E. heteroloba*, *T. dussumieri*, *T. vitrirostris*, and *T. kammalensis*), which constituted 29% of PPS based on metabarcoding

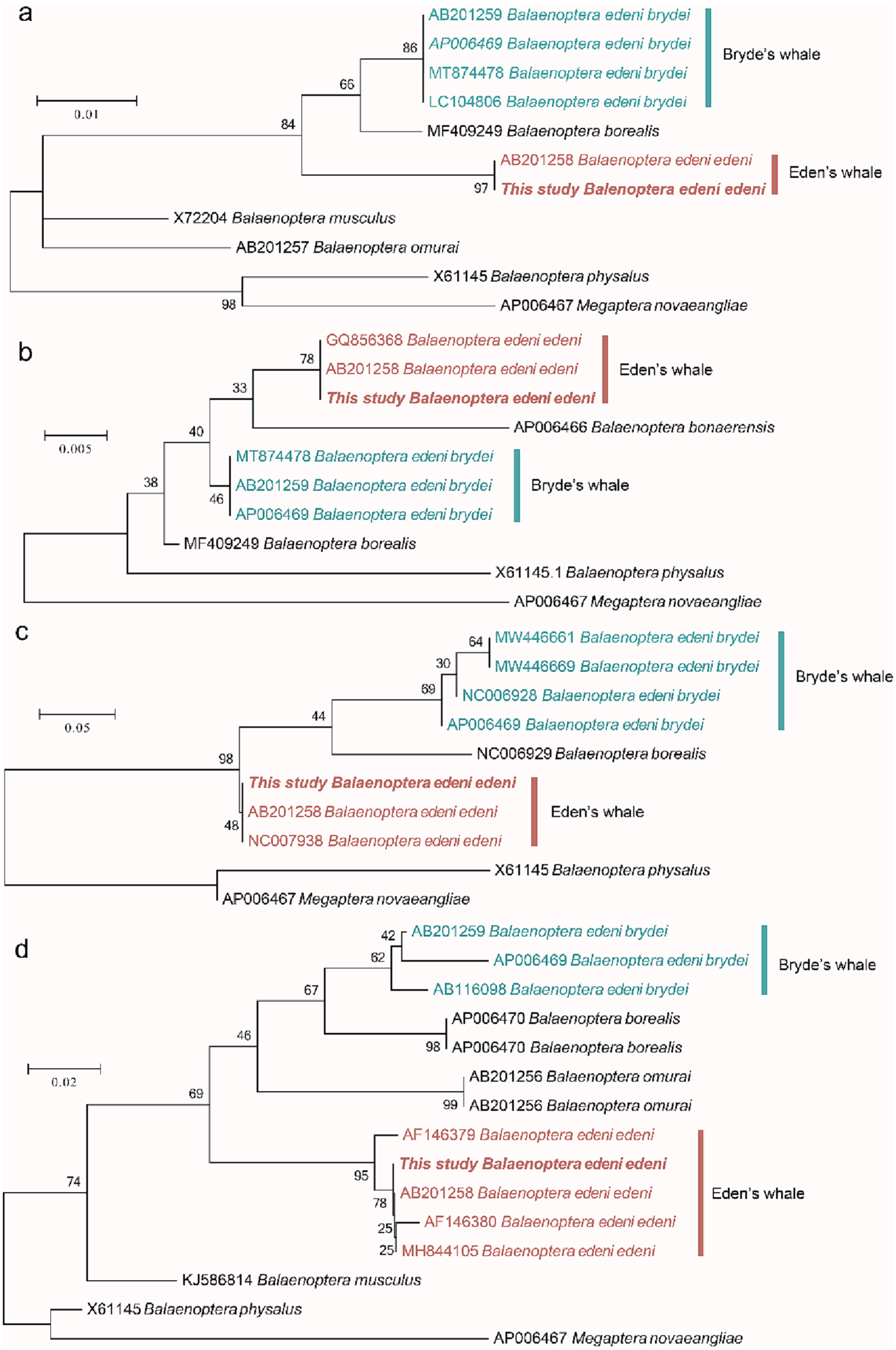

Fig. 3. Subspecies identification and phylogenetic tree of Eden's whales using neighbor-joining tree method. (a) 12S rDNA with model Tamura 3-parameter; (b) 16S rDNA with model Tamura 3-parameter; (c) COI with model Kimura 2-parameter ($G = 0.06$); (d) CR with model Tamura 3-parameter ($G = 0.23$).

data (Fig. 4b). Furthermore, *S. lemuru* was the most abundant species, constituting 58.6% of these eight PPS.

3.4. eDNA distribution and abundance of Eden's whale in DPB and LDB

In DPB samples, Eden's whale sequences were successfully amplified by three primer pairs (MarVer1, MarVer3, and MiFish) from each station by eDNA metabarcoding. The numbers of 12S rDNA sequences were 1 (D1), 5 (D2), and 8 (D3) for MarVer1 metabarcoding; the numbers of 16S rDNA sequences were 2 (D4), 3 (D5), and 21 (D6) for MarVer3 metabarcoding (Table S3). For MiFish metabarcoding, the numbers of 12S rDNA sequences ranged from 1 to 340; sampling site D4 had the greatest number of reads (Fig. 5). During Xiaobu's stay in DPB, no other whale species/individuals appeared. Therefore these whale sequences

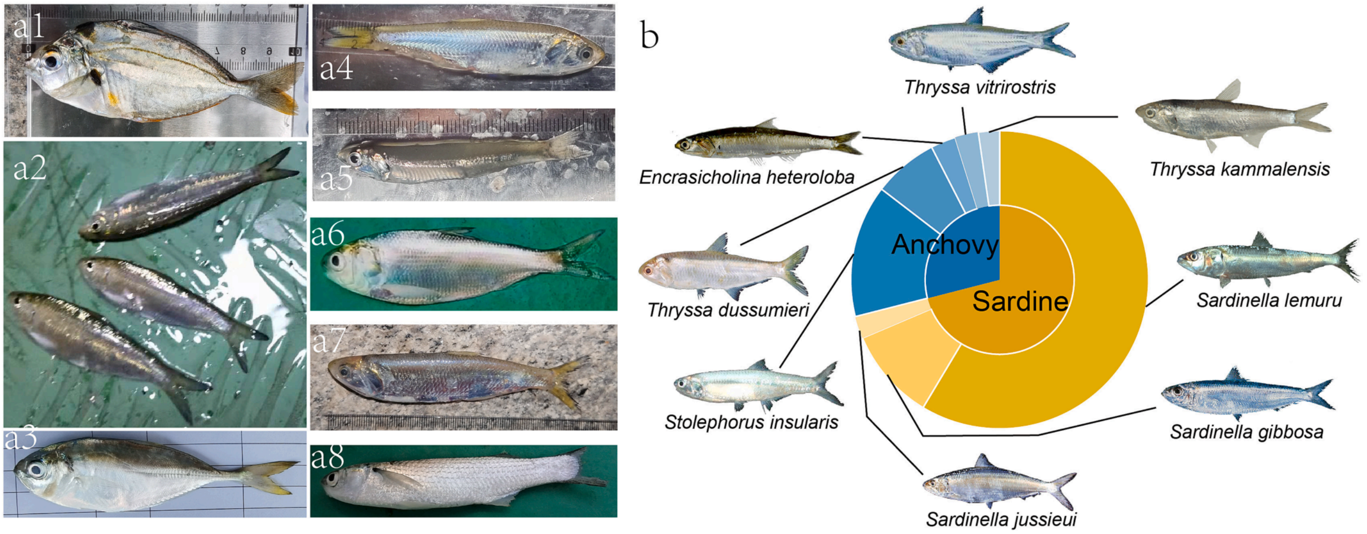

Fig. 4. Fish carcasses collected after departure of the Eden's whale (a) and relative composition of Eden's whale potential prey species (PPS) based on MiFish metabarcoding data (b) from Dapeng Bay. a1, *Nuchequula nuchalis*. a2, *Sardinella lemuru*. a3, *Alepes djedaba*. a4, *Thryssa kammalensis*. a5, *Anchoviella* sp1. a6, *Konosirus punctatus*. a7, *Thryssa* sp1. a8, *Mugil* sp1.

Table 3
Dominant fish species in Dapeng Bay and their ecological characteristics according to fish eDNA metabarcoding data from six sampling sites.

Fish species	Total reads	Portion of total reads/%	Habitat	Behavior
Sardinella lemuru	116658	35.6	Pelagic	Schooling
Stolephorus insularis	28645	8.7	Pelagic	Schooling
Sardinella gibbosa	19942	6.1	Pelagic	Schooling
Collichthys lucidus	18497	5.6	Demersal	Nonschooling
Odontamblyopus rebecca	17061	5.2	Demersal	Nonschooling
Nuchequula nuchalis	16429	5.0	Demersal	Schooling
Thryssa dussumieri	14012	4.3	Pelagic	Schooling
Ambassis gymnocephalus	13774	4.2	Demersal	Schooling
Encrasicholina heteroloba	5428	1.7	Pelagic	Schooling
Thryssa vitrirostris	5040	1.5	Pelagic	Schooling
Sardinella jussieui	4804	1.5	Pelagic	Schooling
Gerres erythrourus	4567	1.4	Demersal	Schooling
Thryssa kammalensis	4561	1.4	Pelagic	Schooling
Hilsa kelee	4082	1.2	Pelagic	Insufficient data
Psenopsis anomala	3383	1.0	Demersal*	Schooling*
Others	51103	15.6		

* Larvae schooling is pelagic, but adult schooling is demersal (Wu and Zhong, 2021). *Hilsa kelee* and *Psenopsis anomala* were not regarded as potential prey species in this study.

were actually from Xiaobu.

In LDB samples, Eden's whale sequences were successfully amplified from nine stations (except P9) by MiFish eDNA metabarcoding (Fig. 6). The numbers of 12S rDNA sequences ranged from 1 to 29; sampling site P10 on the edge of LDB had the greatest number of reads (Fig. 5).

3.5. Comparison of fish communities among LDB, DPB, and DYB

Non-metric multidimensional scaling ordination of the fish community structure showed significant separation among the three bays (PERMANOVA, F = 15.052, $P = 0.001$) (Fig. 6a). The proportion of PPS was higher from DPB (60.8%) than from LDB (15.3%) or DYB (3.7%) (Fig. 6b).

4. Discussion

There have been 16 reports of Eden's whales in the coastal waters of China based on records of stranded specimens (Wang, 2012; Chen et al., 2019a); the last report of a Bryde's whale in DPB was a dead specimen in 2005 from Sha Tau Kok, Hong Kong Special Administrative Region (Fig. 7) (Wang, 2012). Whales are rarely sighted in inshore waters of China, and their appearance is generally a source of great concern. The present study demonstrated the benefits of eDNA technology for the genetic monitoring of cetaceans in situ, which would provide a better understanding of the circumstances affecting these animals and would assist conservation efforts.

4.1. eDNA is a valid method for discrimination among Bryde's whale subspecies

Six species from the genus *Balaenoptera* have been identified near the coast of China according to stranding records: the common minke whale (*Balaenoptera acutorostrata* Lacépède, 1804), sei whale (*Balaenoptera borealis* Lesson, 1828), blue whale (*Balaenoptera musculus* Linnaeus, 1758), Omura's whale (*Balaenoptera omurai* Wada, Oishi & Yamada, 2003), fin whale (*B. physalus* Linnaeus, 1758), and Bryde's whale (*B. edeni* Anderson, 1878) (Wang, 2012; Liu et al., 2022). Moreover, *B. edeni* (a single species of Bryde's whale) is recognized as the composite of two subspecies: Eden's whale, *B. edeni edeni*, and Bryde's whale, *B. edeni brydei* (Olsen, 1913) (Kershaw et al., 2013; Luksenburg et al., 2015; Thomas et al., 2016). Among taxa that are morphologically similar to Bryde's whale, skull morphology data are indispensable for conventional taxonomic identification (Wada et al., 2003; Kato and Perrin, 2018; Rosel et al., 2021); however, such data cannot be obtained for living individuals.

Both cetacean populations and subspecies reportedly can be discriminated based on genetic data (Sasaki et al., 2006; Kanda et al., 2007; Rosel and Wilcox, 2014; Luksenburg et al., 2015; Rosel et al., 2021). The results of the present study also suggested that marine cetacean can be detected and identified by the amplification of eDNA from seawater samples and through the use of species-specific DNA sequences (e.g., DNA barcodes). This approach has been successfully used for the detection and identification of multiple aquatic mammals, including the harbor porpoise (*Phocoena phocoena*) (Foote et al., 2012), Yangtze finless porpoise (*Neophocaena asiaeorientalis asiaeorientalis*) (Tang et al., 2019), and dwarf sperm whale (*Kogia sima*) (Juhel et al.,

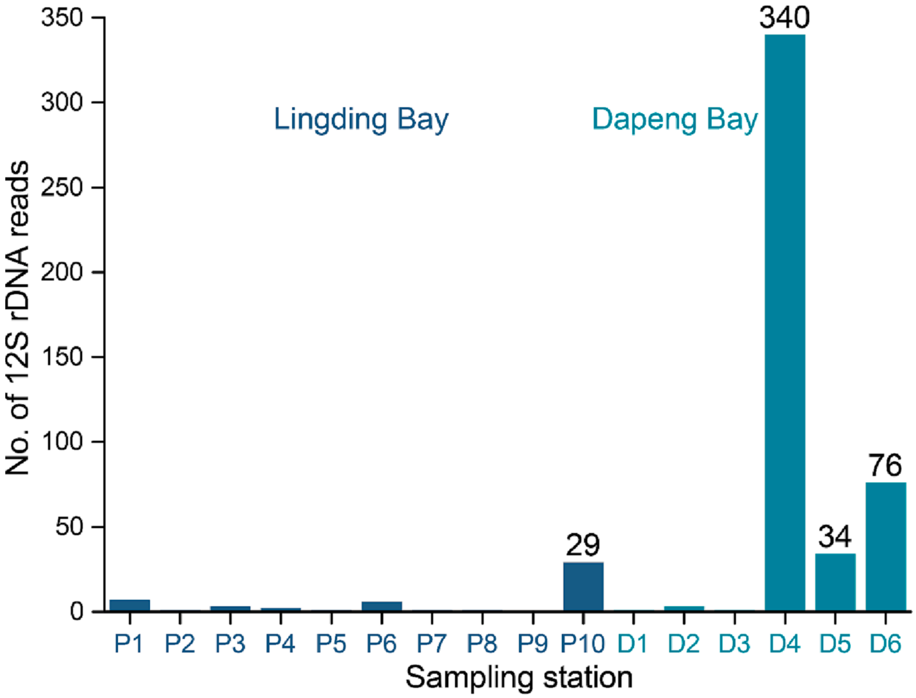

Fig. 5. Distribution of Eden's whale eDNA reads in Lingding Bay (three replicates) and Dapeng Bay (one replicate) based on MiFish metabarcoding data. No Eden's whale sequences were recorded in Daya Bay. eDNA data with < 10 reads are not shown.

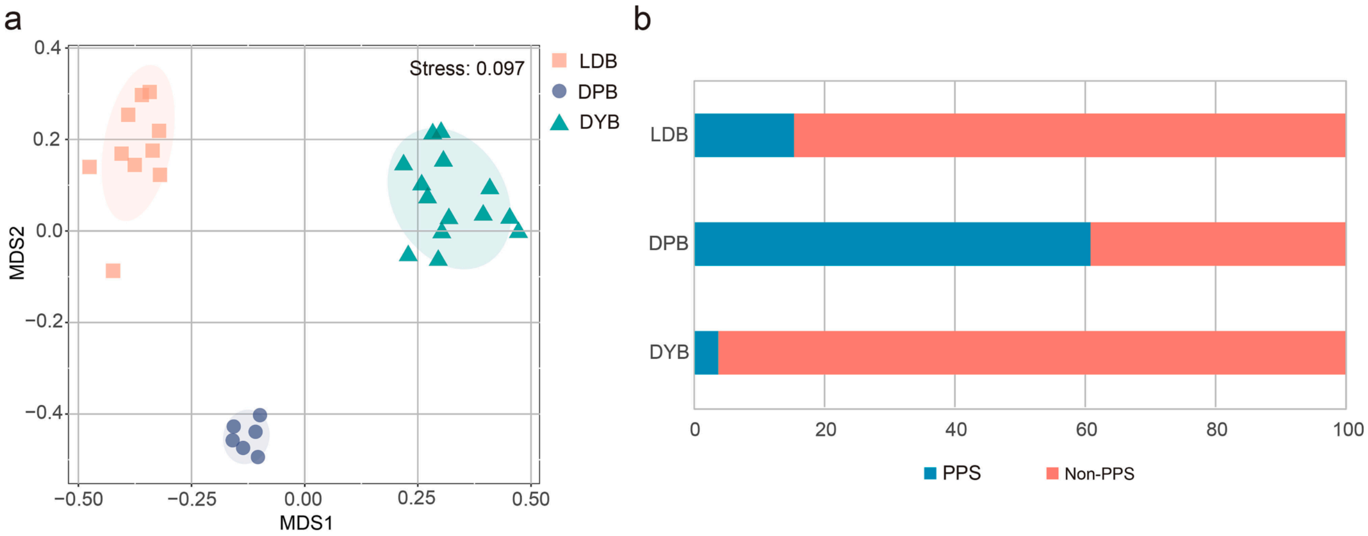

Fig. 6. Fish community structures in three bays based on MiFish metabarcoding data. (a) Non-metric multidimensional scaling analyses. (b) Read proportions of Eden's whale potential prey species (PPS). DPB, Dapeng Bay; DYB, Daya Bay; LDB, Lingding Bay.

2021).

Nearly all previous studies used mtDNA data, typically control region sequences, for the taxonomic evaluation of cetacean species (Taylor et al., 2017b). This marker was obtained for Eden's whale and other mtDNA markers (12S rDNA, 16S rDNA, and COI) have also been validated for subspecies identification among other Bryde's-like whales. Various mtDNA markers can be amplified from eDNA-based materials because single eukaryotic cells contain tens to thousands of copies of mtDNA. All mtDNA fragments can be used for taxonomic identification. For example, the whole mtDNA genome of the whale shark (*Rhincodon typus*) could be determined through eDNA-based methods from multiple individuals (Jensen et al., 2021).

Water-based eDNA technology can be used for the detection of a single species (Foote et al., 2012; Qu et al., 2020) and for community or biodiversity monitoring through metabarcoding in various aquatic mammals (Sales et al., 2020; Lyet et al., 2021; Mena et al., 2021). For multispecies aggregations and overlapping distributions of cetacean species, it may be difficult to visually identify the species composition, and minor species with few individuals may be easily neglected. For example, the dusky dolphin (*Lagenorhynchus obscurus*) is commonly observed with the long-beaked common dolphin (*Delphinus capensis*) in large feeding aggregations of up to several thousand individuals (Van Waerebeek and Würsig, 2018). Additionally, *Tursiops aduncus* has overlapping distributions with many species, including *Tursiops truncatus*, *Sousa* spp., *Neophocaena phocaenoides*, *Stenella longirostris*, *S. attenuata*, and *Orcaella* spp. (Wang, 2018). Moreover, interspecies

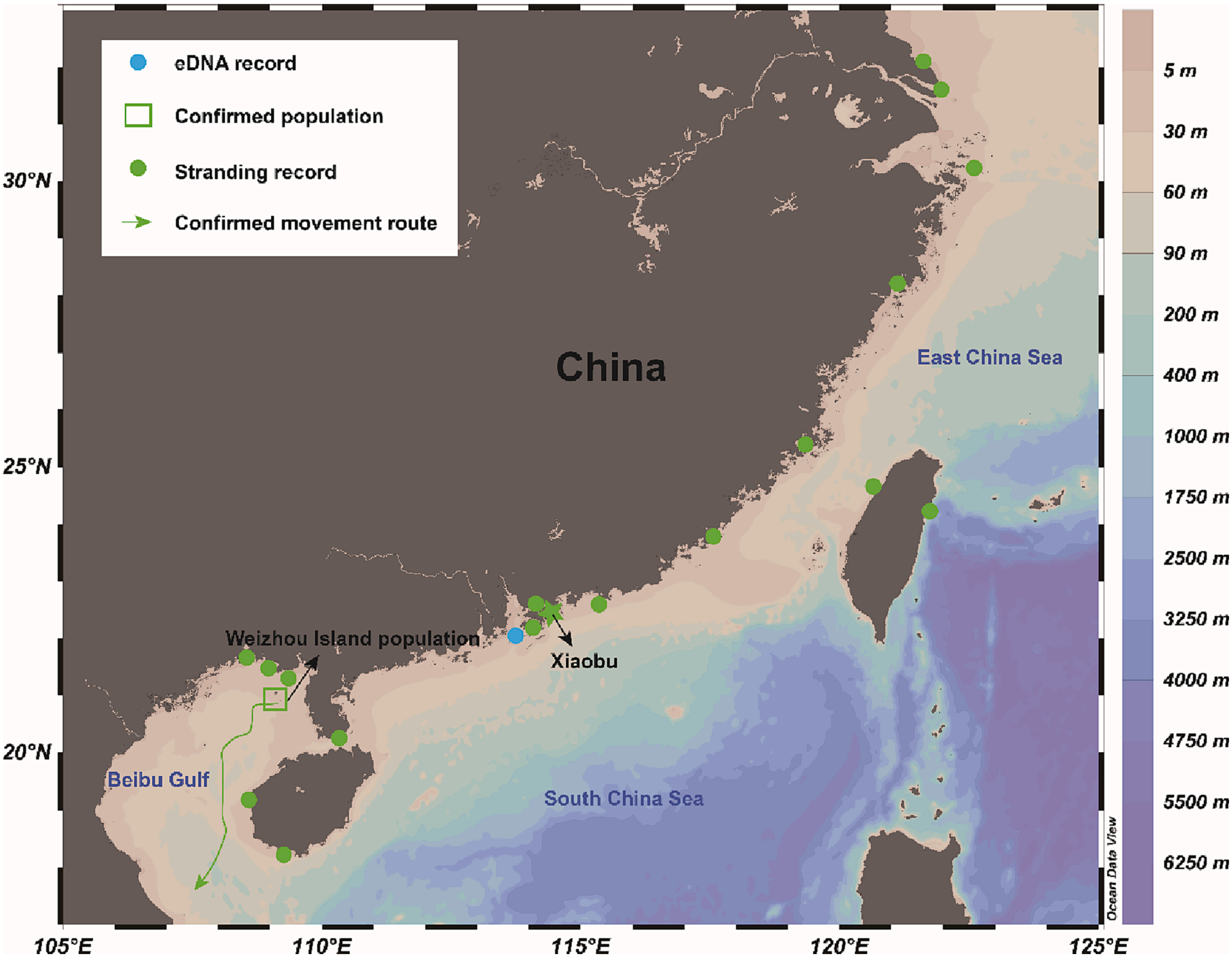

Fig. 7. Records of Eden's whale along the coast of South China. Previous stranding records of Eden's whales were based on Chen et al. (2019a) and the confirmed movement route based on Liu et al. (2021).

activities increase the difficulty of determining the community structures of cetacean species (Elliser and Herzing, 2016).

MiFish primers could also amplify Eden's whale 12S sequences, which generated longer fragments than the fragments achieved using mammalian universal primers (MiFish, 170 bp *vs.* MarVer1, 163 bp). Although the MiFish primers used for eDNA metabarcoding were optimized for fish (Miya et al., 2015), they used a 7-bp longer forward primer combined with the reverse primer used for MarVer1 (see Table 1). The detection of various fauna from a single sample through the use of different or single primers could facilitate the use of eDNA methods for ecosystem monitoring (Garlapati et al., 2019; Zhang et al., 2020).

The fragmented nature of eDNA and the limited read lengths available using Illumina sequencing have restricted eDNA metabarcoding to analyses involving short amplifiable regions of mtDNA. Taylor et al. (2017a) suggested that sequence data with a minimum length of 300 bp could be used to delineate cetacean subspecies or species. For direct organismal tracing, there is a greater likelihood of obtaining longer segments because the newly released genetic material is less degraded than the residual eDNA, which also can be specific to the individual. For example, species-specific primers for longer mtDNA control region sequences successfully amplified a sequence of 310 bp for the Chinese white dolphin (*Sousa chinensis*) in LDB (Li, 2022). This also confirmed the claim by Jensen et al. (2021) that complete mtDNA genome can be obtained from eDNA.

4.2. eDNA is a user- and animal-friendly biomonitoring method

Accurate taxonomic assignment can be performed through analyses of eDNA collected from water samples, which requires less technical expertise than morphological character-based identification, a skill that is currently in rapid decline (Qu and Stewart, 2019). eDNA can be used to detect and accurately identify species both rapidly and efficiently. It allows the identification of more aquatic mammalian taxa than conventional biomonitoring surveys, and it avoids the potential for observer-related subjective recording errors (Handley, 2015; Hunter et al., 2018; Closek et al., 2019). Moreover, the substantial financial and temporal commitments, as well as the requirement for a substantial workforce of qualified observers, have hindered the usefulness of conventional species detection surveys in assessing the global statuses of species and populations (Qu and Stewart, 2019; Broadhurst et al., 2021; Mena et al., 2021).

For Xiaobu, a newly arrived whale of unclear status in DPB, there is a need to avoid any activities that could have a negative effect and cause the animal to experience stress. However, information regarding this whale is important for conservation and management efforts. Because eDNA-based techniques require only water sampling that can be conducted after the whale has left the immediate area, these techniques allow biodiversity assessment in an animal-friendly manner (Sales et al., 2020). Baker et al. (2018) reported the detection of killer whales eDNA in seawater samples collected up to several hours after the whales had passed through the area, thus considerably expanding the spatial and temporal scales of monitoring. Typically, nonlethal genetic sampling from cetaceans has primarily been conducted using biopsy darts projected with a pole, crossbow, or modified veterinary capture rifle (Krützen et al., 2002; Noren and Mocklin, 2012). The conventional biopsy method involves a degree of invasiveness and requires close approach to animals, thus increasing the risks to both animals and researchers; it also requires a high degree of skill (Liu et al., 2019). Moreover, the conventional approach remains challenging because some cetaceans exhibit elusive behavior, rarity, cryptic nature, and/or a sensitivity to disturbance (Noren and Mocklin, 2012; Tang et al., 2019; Garrigue and Derville, 2021). Finally, permission for direct sampling is forbidden or highly restricted in some regions/countries or for specific species, which limits the use of this approach.

4.3. *eDNA shows potential as an early warning system for the presence of cetaceans*

eDNA traces of Eden's whales were recorded from LDB in April 2021, although no large whale sightings had been reported at that time. Analyses of samples from LDB were completed in May 2021, prior to the collection of water samples from DPB in July 2021. Additionally, no other genetic experiments of this species were conducted in the laboratory where the work was performed. Because of the rigorous clean laboratory procedures used, it is unlikely that these results were caused by experimental contamination. Importantly, there are no Eden's whales in marine parks or zoos close to LDB. Therefore, the results indicated the presence of an Eden's whale in the area close to LDB prior to the sighting of Xiaobu in DPB.

Eden's whales were recorded in both LDB and DPB within a short time (approximately 70 days), although stranding and sighting records indicate that such whales are rarely present in this region (Wang, 2012; Chen et al., 2019a; Liu et al., 2022). They are usually observed singly or in small groups of two to three individuals along the coastal waters of China (Kato and Perrin, 2018; Chen et al., 2019a). Thus, the whale found in LDB and DPB may have been the same individual or from the same population, although conclusive identification is not possible.

The numbers of eDNA reads are typically positively correlated with biomass/abundance (Takahara et al., 2012; Evans et al., 2016; Horiuchi et al., 2019). In DPB, a hotspot for the target species where the Eden's whale lingered, the number of eDNA reads reached 340 reads. In LDB, the outermost site P10 had the greatest number of reads (29), suggesting that this site was nearest to the typical-location where the Eden's whale appeared. However, some reads were recorded in the inner LDB, which is not an ideal whale habitat because of the low surface water salinity (<20 psu). Considering the generally long survival time of eDNA in water, combined with the potential for its dispersal over long distances in water currents (particularly in estuarine waters with violent tide exchange and torrential water flow), eDNA may have been released by the whale at substantial distance from the sampling site in LDB. This whale may have preyed on fish in the sea around Wanshan Islands, which is a conventional fishing ground with abundant fishery resources (Tang et al., 2022).

eDNA-based methods use bodies of water that passively aggregate eDNA shed from target species over a wider area than the sampling point alone. eDNA could be detected for hours to a few days after shedding into the water (Harrison et al., 2019; Beng and Corlett, 2020). Moreover, eDNA assays are sufficiently sensitive to detect target DNA at low concentrations via PCR, and they theoretically can detect a single gene copy per reaction (Bohmann et al., 2014; Akre et al., 2019). Therefore, eDNA-based monitoring networks show great promise for providing early warning signals regarding the presence of marine mammals in a more efficient and comprehensive manner than conventional visual methods (e.g., transect line surveys).

4.4. *Possible composition of Xiaobu's diet in Dapeng Bay*

It is difficult to determine the diets of cetacean species, which spend most of their lives at sea and typically feed away from human observers (Kot et al., 2014). Even when feeding can be observed or fish carcasses can be harvested, the resulting information only represents a small fraction of the overall diet. The abundance of each prey species is difficult to determine. The stomachs of stranded animals are often empty or the contents are severely degraded, hindering analysis (Alberto Nino-Torres et al., 2014). Feces analysis is difficult because the defecation timing of target animals is typically unclear and the sampling time is limited (Günther et al., 2022).

eDNA released into the environment from organisms living in a habitat contains information regarding species identity. It can also be used as an index to estimate biomass (Garlapati et al., 2019; Horiuchi et al., 2019; Ruppert et al., 2019; Jiang et al., 2023). There is reportedly a positive linear relationship between eDNA quantity and organism biomass (Takahara et al., 2012; Evans et al., 2016; Ushio et al., 2018). Therefore, the use of eDNA methods could provide additional information regarding interactions between forage species assemblages and predators at both local and regional scales (Fleming et al., 2016; Closek et al., 2019).

The hunting method commonly used by baleen whales is known as filter feeding (Goldbogen et al., 2017). Eden's whales generally feed on schooling fishes in pelagic coastal waters; they also prey on pelagic euphausiids (Kato and Perrin, 2018; Chen et al., 2019b). Based on field observations in situ and the analysis of collected fish carcasses, we concluded that Xiaobu mainly preyed on pelagic fish in DPB. Eden's whales are regarded as opportunistic feeders (Kato and Perrin, 2018); their diets differ according to location and season (Watanabe et al., 2012; Thongsukdee et al., 2014; Iwata et al., 2017), typically in a manner that corresponds to prey abundance. For example, the consumption of fish (e.g., Japanese anchovy, mackerel, and Pacific saury) by common minke whale, sei whale, and Bryde's whale was equivalent to the catches of fisheries in the western North Pacific (Tamura et al., 2009). Although co-occurring fish could also occasionally be preyed upon by Eden's whales, nontarget prey typically constitute a small fraction of their diet (Tamura et al., 2009; Watanabe et al., 2012; Thongsukdee et al., 2014). Therefore, we speculate that sardines and anchovies might be the main prey of the Eden's whale in DPB because eDNA metabarcoding data indicated that these species were the most abundant PPS in the area. Among them, Bali sardinella (*Sardinella lemuru*) was presumably the primary source of food for Xiaobu, because its abundance was highest in DPB.

Elucidation of the relationship between collected eDNA and organism biomass requires a clear understanding of eDNA generation, persistence, and transport dynamics, which considerably vary depending on eDNA source and environmental conditions (Harrison et al., 2019). However, spatial and temporal variabilities in eDNA concentration and the mechanisms by which these variations may affect the results of eDNA studies have not been addressed in DPB. Thus, the development of emerging technologies (e.g., droplet digital PCR) and species-specific quantitative models to estimate the biomass of aquatic organisms may significantly advance the application of eDNA to ecological research (Eble et al., 2020; Wang et al., 2021).

4.5. *Life history of Xiaobu and what can be learned*

Migration or seasonal displacement is an important component of the life histories of cetaceans, particularly baleen whales, which enables maximal utilization of marine resources. Bryde's whale could undertake seasonal displacements, such as inshore-offshore movements (Kerosky et al., 2012; Bauer and Klaassen, 2013). Sardines and anchovies are typically multiple spawning species, with breeding migration behavior and aggregation in the coastal waters of Pearl River Estuary and the surrounding area (Wu and Zhong, 2021; Froese and Pauly, 2022). For example, spawning of the dominant species, *S. lemuru*, tends to occur in inshore waters; spawning aggregates form at the beginning of the rainy season (Froese and Pauly, 2022). However, although LDB had one of largest fishing and spawning ground in the northern region of the South China Sea, the substantial Pearl River freshwater flow in the rainy season may have decreased the salinity, reducing the likelihood of marine whale residence in the area. Therefore, Xiaobu may have migrated to the DPB in an effort to follow the spawning prey species—this area had a greater proportion of PPS, compared with nearby LDB and DYB.

The only reported Eden's whale population in China is located around Weizhou Island in Beibu Gulf (Fig. 7) (Chen et al., 2019a; Chen et al., 2019b). There are > 60 recognized individuals, and their numbers continue to increase (Wu, 2021). In 2021, the Eden's whale population around Weizhou Island decreased beginning in March. The majority left before April 30 (CBY, observation data). We recorded Eden's whales on April 15–18 in LDB, Xiaobu was reported on June 29 in DPB, and an Eden's whale was stranded in the coast of Zhejiang Province on July 27 (CBY, observation data). Eden's whales generally reside within 20 miles from the coast (Kato and Perrin, 2018). This species has a typical swimming speed of 2–7 km/h (Kato and Perrin, 2018), and individuals from the Weizhou Island population can travel a distance of 464 km in 6 days (Liu et al., 2021). Previous stranding and sighting records indicated the presence of Eden's whales in the coastal waters of South China (Fig. 7). Accordingly, we speculate that Xiaobu might be originated from the Weizhou Island population. However, satellite tagging of Eden's whales in Weizhou Island showed that this population moved from Weizhou Island to the south of Beibu Gulf (Fig. 7) (Liu et al., 2021), this was consistent with Bryde's whale shows inshore-offshore movements (Best, 2001; Kerosky et al., 2012). It also can be deduced that Xiaobu might be originated from the outside of the Pearl River Estuary. The movement pattern and routes of Eden's whale along the coast of south China is far from answer and further confirmation is needed.

The Eden's whale Xiaobu was first sighted in DPB on June 29, 2021. Unfortunately, it disappeared on August 27, and its carcass was found 3 days later in DPB, despite strict conservation management. Although Xiaobu is gone, many of its siblings survive along the coast of South China, and there is a need for further research and scientific management to avoid the loss of these animals. However, there remains insufficient knowledge regarding this species to ensure effective science-based conservation in China. Further research is necessary to track the spatial dimensions of this population, and the distribution patterns should be identified to establish a baseline for future analyses. Moreover, environmental RNA technology is expected to be useful for real-time monitoring of whale health, including assessments of their physiological state and behavior (Yates et al., 2021).

5. Conclusion

As flagship animals of marine ecosystems, cetaceans are the main targets of aquatic research and conservation efforts worldwide. Management decisions for cetacean conservation should be based on sound scientific investigations and rigorous monitoring regimes, particularly for threatened populations. Despite extensive efforts over the past 30 years, there remain many knowledge gaps regarding the biological information of threatened and difficult-to-study species (Juhel et al., 2021). The use of eDNA offers novel insight as a noninvasive tool to augment existing survey methods, including (but not limited to) methods focused on species detection and the monitoring of biodiversity and population genetics (Adams et al., 2019; Ruppert et al., 2019; Eble et al., 2020). The present study demonstrated the applicability of eDNA technology to marine megafauna, including accurate identification, early signals of emergent organisms/population, and assessments of forage species assemblages. Overall, eDNA technology is superior to conventional methods for taxonomic identification and species detection under certain condition. It requires less effort and is user-friendly, causes limited ecosystem disturbance, can be used for simultaneous monitoring of the whole bio-ecosystem, and can be implemented in areas where conventional methods are not permitted or effective. Therefore, eDNA technology holds considerable promise for the acquisition of more accurate data that will allow effective recommendations regarding the management and conservation of aquatic mammals.

CRediT authorship contribution statement

Shuai Zhang: Conceptualization, Data curation, Formal analysis, Visualization, Writing – original draft. **Yiting Cao:** Investigation, Methodology. **Bingyao Chen:** Investigation, Methodology, Writing – review & editing. **Peiwen Jiang:** Investigation, Visualization, Formal analysis. **Liang Fang:** Investigation. **Hongting Li:** Investigation, Methodology. **Zuozhi Chen:** Validation, Writing – review & editing. **Shannan Xu:** Data curation, Writing – review & editing. **Min Li:** Conceptualization, Data curation, Validation, Investigation, Project administration, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2023.110125.

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