Final Evaluation Report

Your Details	
Full Name	Chanikarn Chaorattana
Project Title	Potential use of environmental DNA for genetic and ecological monitoring of dolphins, whales, and dugongs in Thailand: Implications for conservation planning
Application ID	38210-1
Date of this Report	2 July 2024

1. Indicate the level of achievement of the project's original objectives and include any relevant comments on factors affecting this.

Objective	Not achieved	Partially achieved	Fully achieved	Comments
Develop eDNA-based surveys and molecular techniques to detect marine mammals in Thai coastal and freshwater ecosystems.				Trials and errors in developing eDNA assays (e.g., laboratory optimization experiments), and validation of their efficiency to assure detections of target species required a lot of time, leading to delay in achieving other objectives.
Apply occupancy model to investigate factors affecting eDNA persistence under varying environmental conditions and estimate probability of occurrence across study sites.				Non-detections in eDNA metabarcoding results of positive control samples (water samples collected while visually detected target animals) led to further investigation of possible errors in our developed assays before proceeding to occupancy models. This objective requires refinement of the methods to successfully detect target animals in positive samples first before investing in costly laboratory expenses (technical replicates) to be used in occupancy modelling.
Determine potentially high-prioritized areas to support management decisions focusing on where conservation interventions should be targeted.				Similar to the 2nd objective's comments.

2. Describe the three most important outcomes of your project.

a). MarVer3 (Valsecchi et al., 2020) provided highest species-level differentiation of whales, dolphins and dugongs in Thailand.

eDNA metabarcoding-based approach allows multiple species detections from a single water sample using universal primers. The primers are used during laboratory process (polymerase chain reaction, PCR) to amplify DNA of chosen genetic markers targeting specific group of animals (e.g., vertebrates, mammals, or fish). Different genetic markers contain varying number of species-specific informative sites used to identify animals to the species level or higher (genus, family, etc.). As we aimed to obtain species-level detections of whales, dolphins, and dugongs for future occupancy modelling, we tested four universal primers (Vert01/12SV5, Mamm01, MarVer1, and MarVer3) to assess whether they had sufficient variable sites to distinguish our target animals to the species level or not.

By observing species-specific clades in a phylogenetic tree reconstructed from each marker, we found that the MarVer3 (Valsecchi et al., 2020), targeting marine vertebrates, provided highest number of species distinguishable to the species level compared to others. Taxonomic ambiguity was presented within the Family Delphinidae in concordance with prior study by Valsecchi et al. (2020). In vitro testing was done with water samples collected from a pool containing an Irrawaddy dolphin and showed a positive detection based on eDNA metabarcoding approach.

We conclude that MarVer3 primer is appropriate for species-level cetaceans and sirenian detection and eDNA metabarcoding using this primer has potential to amplify target species' DNA when it is highly concentrated and contained in a closed water system.

b). Newly curated genetic database of MarVer3 hypervariable region for taxonomic assignment of eDNA metabarcoding results.

To obtain sequence of DNA used to identify species presented in environmental samples, we used next-generation sequencing technique (NGS) with the amplified DNA of chosen genetic marker. Sequencing results would be compared with the genetic reference database to classify the unknown DNA sequence and determine species richness from water samples. However, the database of extracted genetic marker (MarVer3) has not been provided yet. In this study, we used QIIME2 platform (Bolyen et al., 2019) coupled with Python programming to construct a novel MarVer3 database curated from both global genetic information from the National Center for Biotechnology Information (NCBI) and local genetic data from cetaceans and sirenian specimens in Thailand.

The database consisted of all vertebrate records that could be amplified by the MarVer3 primer (in silico PCR simulation). Thus, it enabled us to detect both

target species (whales, dolphins, and dugongs) and non-target species (e.g., fish, domestic animals, and humans), whose DNA was contaminated in the seawater samples. This information allowed us to not only detect and filter out the contamination before proceeding to diversity analysis, but also obtain DNA sequence of all non-target species being amplified by MarVer3 primer in seawater samples. This prior dataset can be used as a template to design blocking primers or qPCR probes to eliminate non-target species' DNA during upstream laboratory processes as they may cause false negative results (target animals are directly sighted but failed to detect from eDNA approach).

c). eDNA metabarcoding has lower efficiency in whales, dolphins, and dugongs detection compared to conventional methods.

To assess the efficiency of eDNA technique, we compared between detections of target species from observation-based surveys and eDNA approach. During inshore boat surveys, five species of cetaceans and sirenian were directly sighted from their hotspots (Figure 1A), including Bryde's whale (*Balaenoptera edeni*), Indo-Pacific humpback dolphins (*Sousa chinensis*), Irrawaddy dolphins (*Orcaella brevirostris*), Indo-Pacific finless porpoise (*Neophocaena phocaenoides*), and dugongs (*Dugong dugon*). Water samples were collected from every station we directly sighted target animals within 500 m from the boat and used metabarcoding assays described in Table 1 to obtain species detections from eDNA analysis. We successfully detected three marine mammals; *Balaenoptera edeni*, *Sousa chinensis*, and *Stenella longirostris* (spinner dolphin) from four out of 40 samples processed (Figure 1C, 1D, and 1E). Three samples yielded species detections in concordance with direct sightings, but the one with spinner dolphin's detection (eDNA) while directly sighted Indo-Pacific humpback dolphins was suspected to be false positive as there has been no record of spinner dolphin in the sampling area before. 7.5% of successful eDNA detections suggested that this approach is less efficient compared to direct sightings, and still requires further development and validation.

During surveys, we observed that both Bryde's whales and Indo-Pacific humpback dolphins, species presenting positive eDNA detection, had highly active behaviours, such as frequent spraying from blowhole, lunge feeding (Bryde's whales), and playing (humpback dolphins), while Irrawaddy dolphins, Indo-Pacific finless porpoises, and dugongs (negative eDNA detection), presented more subtle breathing, diving, and feeding behaviours. Highly active behaviours of whales and dolphins might lead to higher shedding of mucus, skin, and faeces (genetic materials) in the water in a sufficient amount that can be detected through eDNA analysis. However, no statistical analysis could be done to confirm this hypothesis yet due to inadequate positive eDNA detections.

During laboratory processes, use of universal primer can cause PCR bias heightening amplification of most abundant DNA of certain taxa in a sample, while

taxa with rarer DNA got overshadowed (Bylemans et al., 2018). In our results, human DNA was highly abundant showing more than half of all samples processed had relative human DNA reads of more than 50%. We suspected that preferable amplification of more abundant human DNA may cause false negative detections of target species.

qPCR (single-species detection) is a promising approach to specifically amplify only target species' DNA. However, qPCR can only detect one species at a time. If multiple species detection approach (metabarcoding) is still desirable (e.g., scope of study extends to co-occurrence prey detections), assays from this study coupled with human blocking primer, used to reduce human DNA amplification, can be further developed and applied. We preliminarily tested newly designed human blocking primers, but they failed to reduce human DNA. We suspected that there were mismatches between human DNA sequence retrieved from global NCBI database and the actual sequence found in Thai coasts. As a result, the blocking primer may fail to attach to human DNA in a sample and eliminate them efficiently. Local human DNA sequences obtained from this metabarcoding results can be used for better blocking primer designs in the future.

eDNA assays could still be further developed and tested as mentioned above. However, investing in comprehensive boat and aerial observation-based surveys would be more practical and efficient for cetaceans and sirenian monitoring in Thai coasts if time and budget on laboratory and bioinformatics analysis required for eDNA approach are limited.

Table 1 eDNA metabarcoding essays used in this study.

Activities	Assays	Used in this study
Field	Pore size & type of filter paper	0.2 – 1 µm, cellulose nitrate filter
	Filtration method	In-house developed filtration equipment (Figure 2)
	Water volume	20 to ~ 1,367 L
	Filter paper preservation	95% ethanol
Laboratory	DNA extraction from filter paper	DNeasy Blood & Tissue Kit (Qiagen, Germany; modified protocols)
	eDNA detection method	Metabarcoding (multiple species)
	Genetic marker & metabarcoding primer	*MarVer3* universal primer on 16S rRNA gene (amplicon size ca. 245 bp)
	PCR: Annealing temperature	55°C
	Sequencing technology	Illumina Miseq x300 cycles, paired-end
Bioinformatics	Metabarcoding analysis	Amplicon Sequence Variance-based (ASV); DADA2 denoising algorithm via QIIME2 platform
	Database curation	Curated from global NCBI database (targeted vertebrates' records [TAXID 7742]) and local cetaceans & sirenian genetic data from Thailand
	Taxonomic assignment strategy	Sequence composition-based via QIIME2 platform

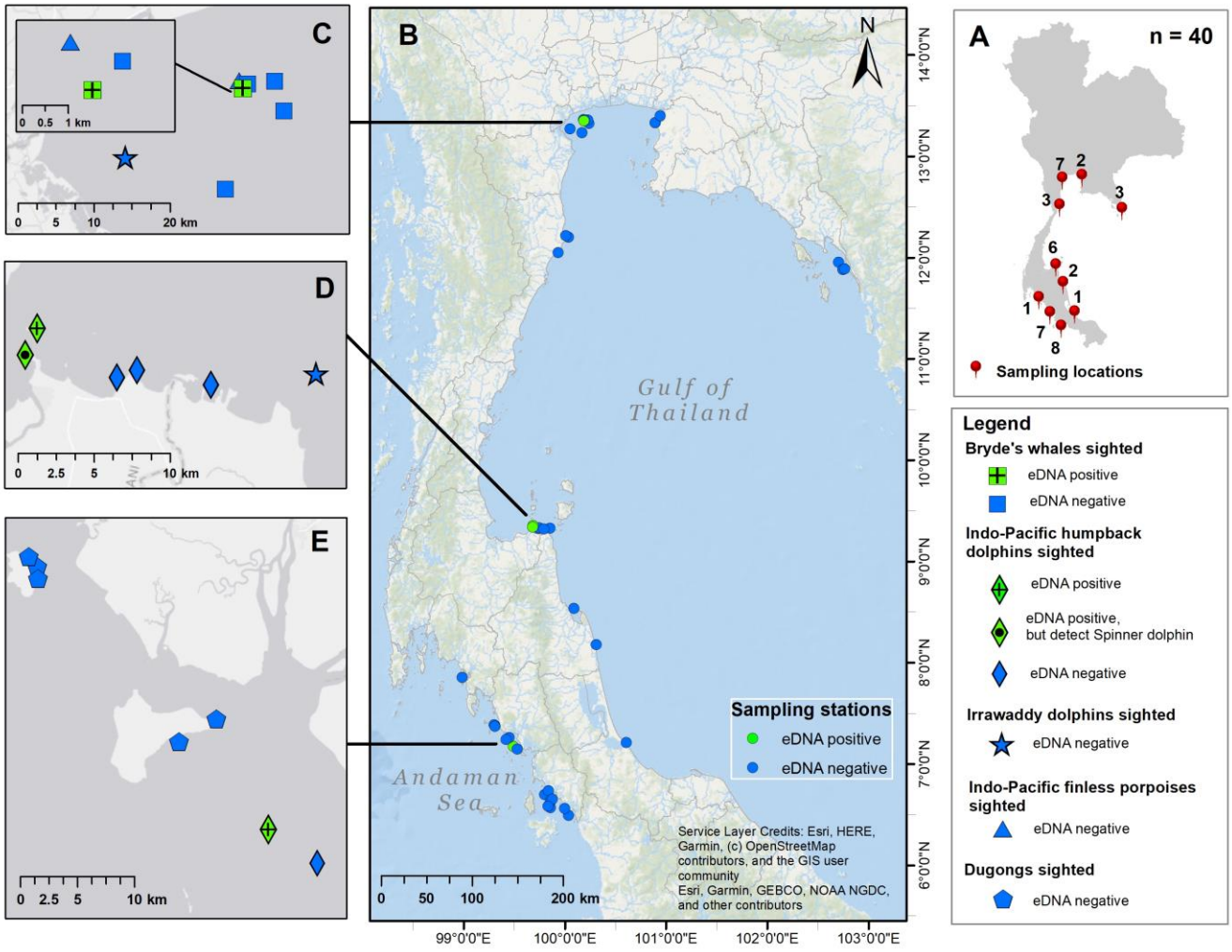

Figure 1 Maps of sampling locations, sampling stations and detections of target species based on eDNA detections and direct sightings, the description of each symbol was shown in the legend; **A)** Designed sampling locations in cetaceans and sirenian hotspots across coastal Gulf of Thailand and Andaman Sea, numbers above and below the pins represent number of samples collected from each location with total of 40 samples; **B)** Sampling stations where water samples were collected while directly sighted target animals, 1 circle represents 1 filter sample; **C)** Sampling stations where Bryde's whales, an Irrawaddy dolphin, and an Indo-Pacific finless porpoise were detected from western Upper Gulf of Thailand; **D)** Sampling stations where Indo-Pacific humpback dolphins and an Irrawaddy dolphin were detected from middle Gulf of Thailand (Donsak district, Surat Thani province); **E)** Sampling stations where dugongs and Indo-Pacific humpback dolphins were detected from lower Andaman Sea (Muk, Libong, and Sukon islands, Trang province).

Figure 2 eDNA sampling equipment, a sterile water dipper and a funnel used with vertical water flow.

3. Explain any unforeseen difficulties that arose during the project and how these were tackled.

During the laboratory methods optimisation, we designed novel blocking primers aiming to reduce amplification of human DNA during laboratory processes. This step was executed based on an assumption that human DNA contamination in water collected from coastal areas near anthropogenic activities would overshadow DNA detections of rarer target marine mammal species DNA, causing false negative detections. We tested variations of blocking primer treatments compared with the controls (no blocking primer was added). Unfortunately, none of the treatments showed significant reduction in human DNA and no target species sighted during water collection were detected based on eDNA approach.

To design the blocking primers, human DNA sequences retrieved from global NCBI database were required as templates. We expected that the designed blocking primer would effectively attach with human DNA presented in seawater and eliminate them during PCR process. However, failed results suggested that this was not the case. We suspected that there were mismatches between DNA sequence of designed blocking primer and of human in water samples collected from Thai coasts. To deal with these challenges, preliminary results on local human DNA sequences are needed to be used as templates to improve blocking primer designs, followed with rigorous in-vitro testing with synthesised human DNA before applying them to water samples using metabarcoding approach to ensure their

efficiency. Unfortunately, we did not realise the necessity of these validation processes, leading to underestimated other parts of laboratory efforts and experimental designs required for well-supported and successful results (e.g., varying primer concentrations, and *in-vitro* testing). Moreover, as eDNA metabarcoding study aimed to detect marine mammals have not been thoroughly investigated in this region before, it remains to be explicitly tested whether human DNA was a severe issue causing false negative detections and required such robust and costly laboratory optimisation efforts or not.

We shifted our focus on identifying tangible causes of false negatives, while simultaneously archiving data on human DNA sequences that could be later used for human blocking designs (if proven necessary). We screened total of 40 positive control samples collected while visually detecting target animals, and varied in species and number of animals sighted, volume of water collected, environmental conditions (pH, salinity, temperature), extracted DNA concentration, proportions of non-target species DNA read counts. Unfortunately, we detected target species (confirmed with direct sighting only) from only three out of 40 samples, which was inadequate to do statistical analysis and draw well-supported conclusion. Despite inconclusive results, we observed a high abundance of human DNA in most samples and can confirmed that it would be beneficial to try to reduce them through the use of blocking primer if metabarcoding approach is still desirable. Further designs in human blocking primer can be done by using sequencing data from this result as templates to ensure less mismatch of blocking primer and actual human DNA in water samples (see outcome C).

4. Describe the involvement of local communities and how they have benefitted from the project.

We received help from local fishermen and ecotourism groups during surveys. Apart from boat services, they provided insights on local hotspots for marine mammal detections, potential threats upon the populations, and their attitude towards the animals. We assisted in distributing income to the local communities through boat rent funded by The Rufford Foundation.

5. Are there any plans to continue this work?

We plan to test whether a single-species detection approach using qPCR will provide higher detections of target species compared to the multiple-species detection approach used in this work with financial support from co-funding sources.

If we successfully validate and confirm the efficiency our assays, we aim to do occupancy modelling to estimate probability of occupancy across sites and identify high prioritised areas for conservation strategies.

6. How do you plan to share the results of your work with others?

I plan to publish this work on the optimisation methods (objective #1) and present the results at international conferences.

7. Looking ahead, what do you feel are the important next steps?

Developing assays for single-species detections using qPCR is the important step. As metabarcoding approach (multiple species detections) can possibly lead to false negatives due to more abundant non-target species DNA overshadowing the rarer target species during laboratory processes. Specifically achieving detections of target species can be done by selectively amplifying only their DNA through species-specific primer.

8. Did you use The Rufford Foundation logo in any materials produced in relation to this project? Did the Foundation receive any publicity during the course of your work?

No. This is because we have not presented this work in any public conferences yet. As this project is still ongoing, we plan to use the logo once it is finished, concluded, and ready to reach the public eyes.

9. Provide a full list of all the members of your team and their role in the project.

- **Academic staff from King Mongkut's University of Technology Thonburi**
  1. Worata Klinsawat (PhD): Consultant on Genetics and laboratory techniques
  2. Wanlop Chutipong (PhD): Consultant on Ecology, sampling techniques and statistical analysis
  3. Kanthida Kusonmano (Dr. rer. nat.): Consultant on Bioinformatics data analysis
  4. Peerada Prommeenate (PhD): Consultant on high-throughput sequencing
  5. George A. Gale (PhD): Consultant on field techniques and ecology

- **Academic staff from Department of Marine Science, Faculty of Science, Chulalongkorn University**
  1. Sanit Piyapattanakorn (PhD): Consultant on Molecular Ecology in marine ecosystems

- **Academic staff from Faculty of Veterinary Science, Kasetsart University**
  1. Manakorn Sukmak (PhD): Consultant on Molecular Biotechnology and laboratory techniques

- **Staff from government agency: Department of Marine and Coastal Resources**
  Consultants on marine mammals' research and surveys in the coastal areas of Thailand (Andaman Sea and Gulf of Thailand), providing information on animal detections and hotspots from visual surveys and citizen science.

  1. Chalatip Junchompoo
  2. Atichat Intongkham
  3. Ratree Suksuwan
  4. Suthep Jualaong
  5. Santi Ninwat
  6. Thanaphan Chomcheun

7. Oranee Jongkolpath
8. Patcharaporn Yaowasooth
9. Luxkana Munkhetkit
10. Watchara Sakornwimon
11. Patcharaporn Kaewmong
12. Pathompong Jongjit
13. Boontika Intaring
14. Supaphon Aongsara
15. Aittipon Chucherdrat
16. Piyarat Khumraksa
17. Tanuwat Sangpan

10. Any other comments?

No.

References

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F. and Bai, Y., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature biotechnology, 37(8), pp.852-857.

Bylemans, J., Gleeson, D.M., Hardy, C.M. and Furlan, E., 2018. Toward an ecoregion scale evaluation of eDNA metabarcoding primers: A case study for the freshwater fish biodiversity of the Murray–Darling Basin (Australia). Ecology and evolution, 8(17), pp.8697-8712.

Valsecchi, E., Bylemans, J., Goodman, S.J., Lombardi, R., Carr, I., Castellano, L., Galimberti, A. and Galli, P., 2020. Novel universal primers for metabarcoding environmental DNA surveys of marine mammals and other marine vertebrates. Environmental DNA, 2(4), pp.460-476.