

Project Number: MEEF2023011

Executive Summary

Ardeids are vital components of the marine biodiversity in Hong Kong Special Administrative Region (HKSAR). Recent data indicates a concerning decline in ardeid nest numbers, particularly in the Mai Po Mangrove, where nests dropped by over 79% in 2022. Given the lack of clear causes for this decline, understanding the ecology of local ardeids, for instances, predation habits and dietary composition, build foundation for conservative efforts. This project aims to investigate the dietary diversity and composition of common ardeid species, e.g., the Little Egret (*Egretta garzetta*), using DNA metabarcoding techniques on fecal samples collected from their colonies.

The project has successfully collected 66 fresh fecal samples from four sites (Mai Po, Mai Po Lung, Hung Shui Kiu, and Kam Sheung Road) in the northwestern region of Hong Kong during breeding season of ardeids. Fresh fecal samples were first analyzed to identify the host species, followed by prey composition. We adopted a well-established protocol to extract high quality DNA from fecal samples and validated a highly sensitive primer in the cytochrome c oxidase subunit I (COI) region to identify host species. We successfully identified 21 (31.8%) samples from *E. garzetta* and 45 (68.2%) samples from the Chinese Pond Heron (*Ardeola bacchus*). Most samples were from Mai Po Lung (46 samples; 69.7%) and Tam Kong Chau Road, Mai Po (21 samples; 31.8%).

We have optimized DNA metabarcoding protocols, using customized and validated primer pairs amplifying two gene regions – 18S ribosomal RNA (18S rRNA) to capture a comprehensive overview of major prey taxa, and 12S ribosomal RNA (12S rRNA) to reveal a detailed composition of fish prey items. The metabarcoding protocol will help uncovering critical food resources that sustain local ardeid populations. This research is expected to yield significant conservation benefits by informing targeted protection measures for ardeid foraging habitats and enhancing public awareness about the importance of preserving these ecosystems. Ultimately, this project seeks to contribute to biodiversity conservation, environmental protection, and the improvement of local marine habitats through informed scientific data and future community engagement.

Project Title: Conservation of ardeids by understanding their foraging ecology through dietary investigation

Brief Description of the Project:

Ardeids (herons, egrets, and bitterns) are one of the most important groups of waterbirds found locally and they form a part of the marine biodiversity in HKSAR. Common ardeid species include the great egret (*Ardea alba*) and the little egret (*Egretta garzetta*). It is concerning that the recent numbers of ardeid nests recorded in HKSAR and in the Deep Bay during their breeding seasons have been declining since 2020, with the number in the Mai Po Mangrove dropping most drastically (>79%) in 2022. Despite no obvious cause for the decline, it is critical to understand more on the ecology of local ardeids, especially their predation habits and dietary composition. The knowledge on their prey species identities and sources will be crucial to the designation of more effective conservation actions, such as more focused monitoring and protection on their prey species and foraging habitats. With the advent of biotechnology, we use DNA metabarcoding to investigate the dietary spectra of local ardeid species using their fecal samples collected underneath their colonies in e.g., Mai Po wetland.

Completed activities against the proposed Work Schedule

The time period of the first phase is extended, and the work schedule is updated as below:

Task No.	Activity (including Planning, Recruitment)	Proposed Date	Venue	Content	Actual Date	Progress of the task
1	Staff recruitment and project planning	Jul 2023	The University of Hong Kong	One staff was recruited.	Sep 2023	Completed
2	Sample collection	Aug 2023- Jul 2024	Sampling sites at the western part of HKSAR	Fecal samples was collected from the ground below the trees of ardeid roosts or egretries in the western part of HKSAR.	Sep 2023 – Sep 2024	Completed

3	Progress report submission	Dec 2023	The University of Hong Kong	Progress of the project was reported to the MEEF.	Dec 2023	Completed
4	Laboratory work	Apr 2024- Oct 2024	The University of Hong Kong	DNA was extracted from the collected fecal samples. DNA metabarcoding library preparation protocol was optimized.	Apr 2024 – Dec 2024	Completed
5	Progress report submission	NA	The University of Hong Kong	Progress of the project was reported to the MEEF.	Jun 2024	Completed
7	Completion report submission	Jun 2024	The University of Hong Kong	Completion report was submitted to the MEEF (This completion report).	Dec 2024	Completed

Objectives

In this study, we focus to examine two common ardeid species that inhabits coastal areas at the west of HKSAR as the water bodies in the west and pearl river estuary can be their important foraging ground. We are particularly interested in investigating the ardeids that occurred in the big colonies in the north-western part where the Mai Po is located. The objectives of this study are:

- 1) To investigate the dietary diversity of two common ardeid species by using DNA metabarcoding to identify the species of food items based on fecal samples collected from western HKSAR.
- 2) To estimate the dietary composition of two common ardeid species by using DNA metabarcoding to quantify the proportion of different food items based on fecal samples collected from western HKSAR.

Results/ descriptions on the completed activities with appropriate analysis, with the support of photos, videos, social media platform, etc., if any

Progress to date:

Samples Collection

We successfully collected 66 fresh fecal samples from four sites (Mai Po, Mai Po Lung, Hung Shui Kiu, and Kam Sheung Road) in the northwestern region of Hong

Kong during the breeding season of ardeids. The samples were collected from the ground underneath the egretty and roost of ardeids. The samples were collected from the ground underneath the egretty and roost of ardeids (Fig. 1-7).



Figure 1. Sampling site of ardeids in the north-western region of Hong Kong, pinned in red.

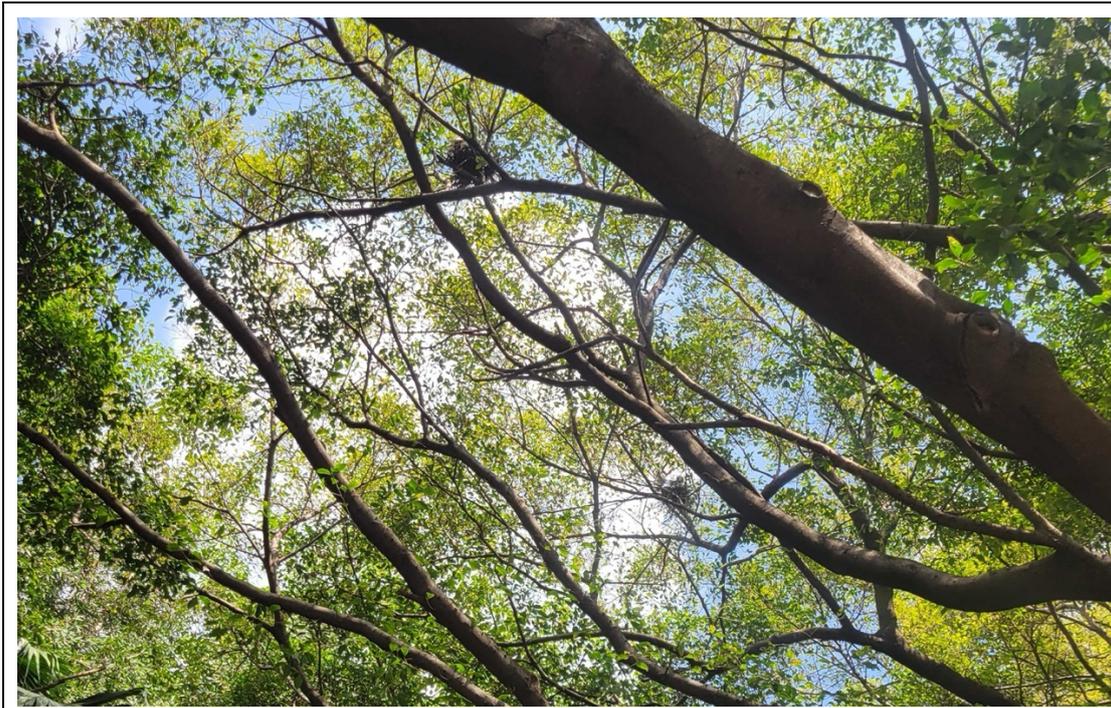


Figure 2. Egretry of *Egretta garzetta* and *Ardea alba* in Hung Shui Kiu.



Figure 3. Feces underneath the colonies of *Egretta garzetta* and *Ardea alba* in Hung Shui Kiu.



Figure 4. Roosting site of *Egretta garzetta* and *Ardea alba* roost near Tam Kon Chau Road, Mai Po.



Figure 5. Egretty at Tam Kon Chau Road, Mai Po.



Figure 6. Egretry at Mai Po Lung, Mai Po.

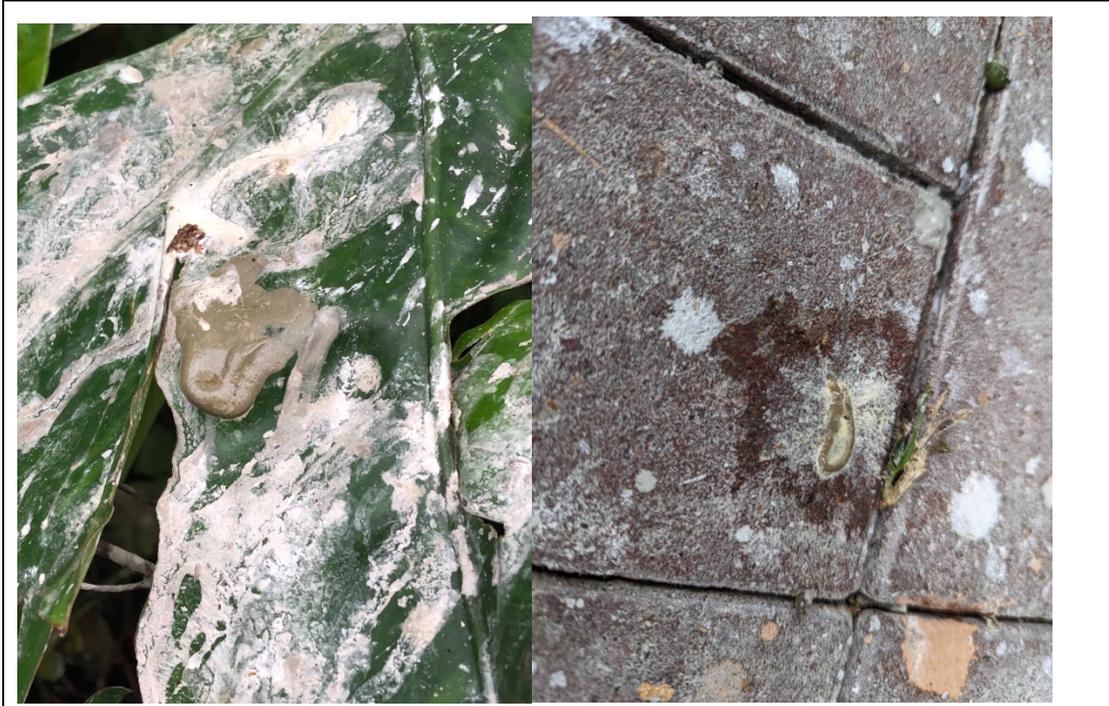


Figure 7. Examples of fresh ardeid fecal samples.

Sampling Methodology

Samples were collected from the northwestern region of Hong Kong. We maximized sampling efficiency by targeting egretty and roost exclusive to ardeids. Three sites were selected for sampling: (1) Tam Kon Chau Road, Mai Po, (2) Mai Po Lung, Mai Po, and (3) Hung Shui Kiu. Fecal samples were collected using a sterilized spatula on various substrates (muddy, grassy, concrete, and brick grounds). To ensure sample quality, we only collected samples that were freshly defecated. To minimize contamination from the environment, only a fragment of the non-uric acid portions of the fecal samples were collected, which were immediately stored in absolute ethanol. The fecal samples were then preserved at -80°C until transportation to the laboratory for DNA extraction.

DNA barcoding methodology and host identification

We identified the host of the collected fecal sample by DNA barcoding of the cytochrome c oxidase subunit I (COI) gene region with a 196 base pair amplicon. We customized primers targeting the cytochrome c oxidase subunit I (COI) region to amplify host DNA from freshly collected fecal samples. The amplicon was designed to be short (150–300 bp) to accommodate the fragmented DNA typically found in fecal material. We first downloaded COI sequences of ardeid species available from public genetic database, GenBank. The sequences were aligned by MUSCLE alignment in the software Geneious Prime (version 2024.0.5). We manually screened the alignment and selected potential primer site based on the following criteria: (1) primer binding site

must be conserved across ardeid species; (2) primer binding site should avoid conserved region of common prey items (e.g. fish, crustacean, etc.); (3) amplicon should be variable enough to distinguish between host species identity, and (4) adherence to common practices in primer design [primer length (between 18-24 base pair); optimised GC content (between 40-60%); primer melting temperature (between 55-65°C)]. A selection of primer candidates were validated *in vitro*. The primer pair of the best performance is: COI_ARD_F 5'-CCGCYCA YGCCTTYGTAATAA-3'; COI_ARD_R 5'-GTACCTGCYCCTGCTTC-3'.

We performed polymerase chain reaction (PCR) to amplify host DNA 25 µL reaction. Each reaction comprise of 5 µL 5X GoTaq Buffer; 3 µL of 25mM MgCl₂, 2.5 µL of 10% dimethyl sulfoxide (DMSO), 0.5 µL of 10mM deoxy-ribonucleoside triphosphate (dNTPs), 1 µL of forward and reverse primer, 0.125 µL of GoTaq DNA polymerase, 0.125 µL of 20 mg/ml bovine serum albumin (BSA), 9.75 µL of UltraPure DNase/RNase-Free distilled water, and 2 µL of DNA template. All PCR products were visualised in gel electrophoresis (1.2 % agarose gel; 120V for 45 min) and sent for Sanger sequencing (BGI, Hong Kong).

The Sanger sequencing data were trimmed to remove low-quality bases. The sequences were then compared with the database in GenBank to identify host species of each fecal samples.

Here summarise the species composition collected at the four sites:

Table 1 Host species identity of the fecal samples

Ardeid species	No. of samples (percentage of total)
Little Egret (<i>Egretta garzetta</i>)	21 (31.8%)
Chinese Pond Heron (<i>Ardeola bacchus</i>)	45 (68.2%)

Table 2 Sampling locations of the fecal samples

Site	No. of samples (percentage of total)
Mai Po	16 (24.2%)
Mai Po Lung	46 (69.7%)
Hung Shui Kiu	2 (3%)
Kam Sheung Road	2 (3%)

Evaluation of the project effectiveness in achieving the proposed objectives as well as the impact (benefits) of the Project

With the approved extension of the project, we are on track with our progress and will continue with optimizing and preparing DNA metabarcoding libraries.

We are making promising progress on this project within the proposed timeline. We successfully identified host species of each fecal sample. In our objectives, we mentioned that we will focus to examine two common ardeid species. After host identification, we found that the two most common species were the Little Egret (*Egretta garzetta*) and the Chinese Pond Heron (*Ardeola bacchus*). We will therefore analyze the diets of these two species. We are in progress of laboratory work, including DNA metabarcoding optimization and library preparation of two regions: 18S rRNA and 12S rRNA. By analyzing the diet of ardeids, we are working towards to achieve the following impact (benefits) as proposed in the first phase of the project:

- **Conserving biodiversity:** Identifying the crucial food resources that sustain ardeid species is essential for effective conservation efforts. Conducting regular, long-term biodiversity surveys in areas like Mai Po and other western waters can help assess the availability of food resources and monitor the animal taxa that form the core components of ardeids' diets. This information will aid the government in implementing specific and effective conservation measures.
- **Environmental protection:** Providing robust scientific data will support the protection of the foraging habitats vital to ardeids, ensuring the diversity and abundance of their essential food sources. This will contribute to safeguarding the local ardeid populations and their habitats.
- **Environmental improvement:** By analyzing the fish species present in ardeids' diets, we can determine the types of habitats that serve as their foraging grounds. For instance, if specific marine fish species are identified, this indicates the presence of specific marine microhabitats near their roosts that serve as feeding grounds. Sharing this information with society will increase public awareness about the conservation of ardeids and the importance of protecting these habitats. It can also be used to promote reducing plastic usage to minimize the consumption of microplastics by fish in marine waters.

Summary and Way Forward

This report highlights the progress made in the first phase of the project, which investigates the diets of ardeid species in Hong Kong. We have successfully collected a large number of fresh fecal samples for subsequent DNA metabarcoding analysis. We

are confident that our optimized DNA metabarcoding protocol can effectively uncover the dietary diversity and composition of local ardeids. We believe that the insights gained from this study will not only enhance our understanding of ardeid diets but also spur further research into foraging behaviors among ardeid species. Furthermore, these findings can provide valuable early scientific data for ecosystem management, informing future conservation strategies and actions.

A list of all project assets with photos enclosed as an appendix to the completion report

List of Project Assets

Project Assets*	Quantity	Date of Purchase	Receipt Reference no.	Location of Item	Person-in-Charge (Name and post)	Photo is / is not Provided
No equipment was purchased using this funding.						

Recruitment record for all project staff employed under the project enclosed as an appendix to the completion report in accordance with the recruitment plan.

Recruitment record is not disclosed due to confidentiality reasons.

Attendance Record of Staff Employed:

Attendance record is not disclosed due to confidentiality reasons.

Declaration:

I hereby irrevocably declare to the MEEF Management Committee and the Steering Committee of the relevant Funds including the Top-up Fund, that all the dataset and information included in the completion report has been properly referenced, and necessary authorisation has been obtained in respect of information owned by third parties.

Disclaimer:

Any opinions, findings, conclusions, or recommendations expressed in this report do not necessarily reflect the views of the Marine Ecology Enhancement Fund or the Trustee.

Project Leader Signature:



Simon Yung Wa Sin

Assistant Professor, School of Biological Sciences, the University of Hong Kong