

Completion Report on Marine Ecology Enhancement Fund (MEEF) 2025-26

PART I: Project information

- (i) Project Title: Developing a non-invasive method to estimate the age of Chinese White Dolphins and Finless Porpoises using the DNA methylation rate

Project Reference: MEEF2024010

Project Leader: Prof. Masayuki USHIO

Name of the Applicant Organization: The Hong Kong University of Science and Technology

Project Start Date: 01/07/2025

Project End Date: 30/06/ 2026

Project Duration (in months): 12

Reporting Period: 1 July 2025 – 30 June 2026

Brief description of the Project:

This project aimed at developing a non-invasive method to estimate the age of Chinese White Dolphins (CWDs) and Finless Porpoises (FPs) in Hong Kong waters. Age is critical information for the study and conservation of cetaceans. One conventional method of estimating the age of toothed whales is by counting dental growth layer groups, which is both highly invasive and time- and labor-consuming. This project aims to develop a non-invasive method to estimate age based on the DNA methylation rate. The project will be divided into three phases. We will (1) **develop an experimental protocol to measure the DNA methylation rate and estimate the age of CWDs using tissue-derived DNA**, (2) **apply the age estimation method to FPs using tissue-derived DNA**, and (3) **test the possibility of estimating the age of CWDs and FPs based on DNA methylation using environmental samples such as environmental DNA from the water and fecal DNA**. In the first and second phases, we will establish a protocol using the “methylation-sensitive high-resolution melting” method to achieve cost-effective quantification of the DNA methylation rate. The relationship between the DNA methylation rate and the age of CWDs will be analyzed using a machine learning approach, enabling us to estimate the age from the methylation rate. In the third phase, we will determine the technical limitations of the methylation-based age estimation method for environmental samples and apply it. This project will contribute to the development of cost-effective, non-invasive, and efficient methods to gain essential information to enable CWD and FP conservation in Hong Kong waters.

PART II: Executive Summary

In the second phase of the project, our objective was to apply the protocol established in the first phase to the finless porpoise (FP) samples. FP is another resident cetacean in Hong Kong waters, and targeting a different species is a key update for the second phase of this project.

To achieve this goal, we applied an improved version of Methylation-Sensitive High-Resolution Melting (MS-HRM) analysis to FP samples (i.e., we included a pre-amplification step to increase the sensitivity and used *de novo* synthesized DNA to draw a standard curve for the analysis). Using this improved MS-HRM protocol, we amplified three target genetic regions (*GRIA2*, *CDKN2A*, and *TET2*) and measured DNA methylation rates for FP samples. The actual chronological ages of the FP samples were determined using the dental growth layer group (GLG) analysis. We successfully generated a complete set of the methylation rates and actual age for 28 FP individuals.

Then, we constructed two statistical models to predict the actual ages based on the DNA methylation rates: one using only methylation data from the three genetic regions (Model 1), and the other additionally including sex (Model 2). We employed support vector regression (SVR) to ensure robust age predictions and found that the ages predicted by the best model (Model 1) significantly correlated with the actual ages ($R^2 = 0.620$, $P < 0.001$). The mean absolute error was 1.35 years, suggesting that our age prediction model is reasonably accurate.

In summary, we successfully developed an age prediction model for FP using the DNA methylation rates. Based on these achievements, we believe the objectives of the second phase were successfully met. In the third phase, we will test the possibility of predicting the age of cetaceans relevant to Hong Kong waters, including Chinese white dolphins, based on the DNA methylation rate using environmental samples such as environmental DNA from the water and fecal DNA.

PART III: Completed activities against the proposed Work Schedule

Proposed period	Proposed tasks/activities	Achievements	Status
Jul – Aug 2025	Extract DNA from additional tissue samples and prepare standard DNA from FP tissue-derived DNA	Extract DNA from Finless Porpoise tissue samples and prepared <i>de novo</i> synthesized standard DNA.	Completed
Sep – Nov 2025	Determine accurate ages for stranded FP individuals using dental GLGs and prepare the standard curve for MS-HRM analysis using the standard DNA	Prepared the standard curve for MS-HRM analysis using the standard DNA. Thirty-three tooth samples were sectioned, and the chronological ages were determined.	Completed
Dec 2025	Submit a project progress report	The project progress report submitted.	Completed
Dec 2025 – Jan 2026	Measure the DNA methylation rate for FP	Measured the DNA methylation rate of the three genetic regions (<i>GRIA2</i> , <i>CDKN2A</i> , and <i>TET2</i>) for Finless Porpoise.	Completed
Feb – Apr 2026	Analyze the relationship between the DNA methylation rate and the actual age using machine learning	Support Vector Regression (SVR) was applied and the age prediction model with a reasonably high accuracy was successfully constructed.	Completed
May – Jun 2026	Wrap-up 2025/2026 and submit a completion report for the second phase	The completion report for the second phase submitted.	Completed

PART IV: Results/descriptions on the completed activities with appropriate analysis

Key outcomes:

- We utilized the improved protocol developed in the first phase to enhance the analysis sensitivity. Specifically, we performed an additional PCR amplification step before the MS-HRM analysis and utilized *de novo* synthesized DNA to draw standard curves.
- Using the modified protocol, we successfully generated standard curves of DNA methylation rates for three genetic regions for the FP samples (**Figure 2**).
- The DNA methylation rates were determined for three genetic regions: *GRIA2*, *TET2*, and *CDKN2A* (**Figures 3 and 4**).
- A machine learning method, Support Vector Regression (SVR), was employed to construct statistical models to predict the actual ages from the DNA methylation rates (**Figure 5**).
- The ages predicted by the best model significantly correlated with the actual ages ($R^2 = 0.620$, $P < 0.001$), and the mean absolute error was **1.35 years**.

Relationship between body length, age groups, and sex

We first visualized the relationship between body length, age groups, and sex (**Figure 1**). The body lengths increased with the age groups (Adult > Subadult > Juvenile > Calf > Neonate; **Figure 1a**). We did not find statistically significant difference in the body length between female and male individuals (**Figure 1b**).

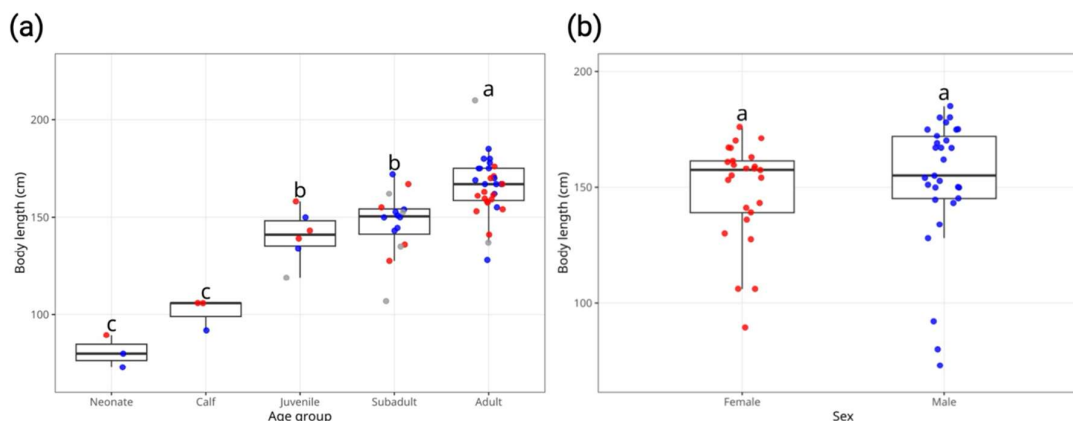


Figure 1. Relationship between body length, age groups, and sex. (a) Body length and age groups and (b) body length and sex. Red, blue, and gray colors indicate female, male, and unknown sex individuals, respectively. Different letters indicate significant differences among different groups ($P < 0.05$, Tukey's HSD).

Standard curves for MS-HRM (Methylation-Sensitive High Resolution Melting) analysis

The following figures are standard curves for the three genetic regions (*GRIA2*, *CDKN2A*, and *TET2*; **Figure 2**). We constructed standard curves using *de novo* synthesized 0% and 100% standard DNA to create a series of methylation ratios (0%, 10%, 20%, 40%, 60%, 80%, and 100%). For all the standards, we tested different regression methods, namely, linear regression, 2nd-order polynomial regression, 3rd-order polynomial regression, 4th-order polynomial regression, and logistic regression, and the 4th-order polynomial model was chosen as the best regression model for the standard curves based on AIC (**Figure 2**). We utilized the best regression method to estimate the methylation rate of our samples.

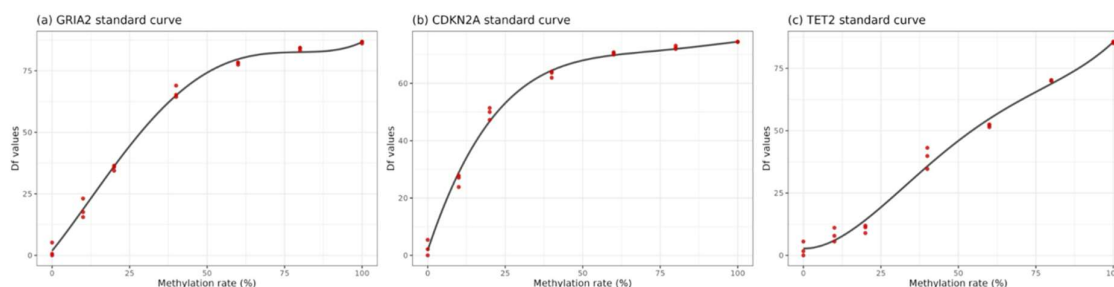


Figure 2. Standard curves for (a) *GRIA2*, (b) *CDKN2A*, and (c) *TET2*. Points represent the Df values (the difference in normalized fluorescence from 0% methylated DNA) for each standard DNA. Lines indicate the best regression methods based on AIC values.

The DNA methylation rates for *GRIA2*, *CDKN2A*, and *TET2*

We measured the DNA methylation rates for tissue samples from the stranded FP individuals and analyzed the relationship among the methylation rates, body length, and actual ages determined by dental GLG analysis.

Figure 3 shows the relationship between the methylation rates and body length. The methylation rate in *TET2* showed a significant linear correlation with the body lengths, but the other two genetic regions did not show a significant linear correlation with the body lengths.

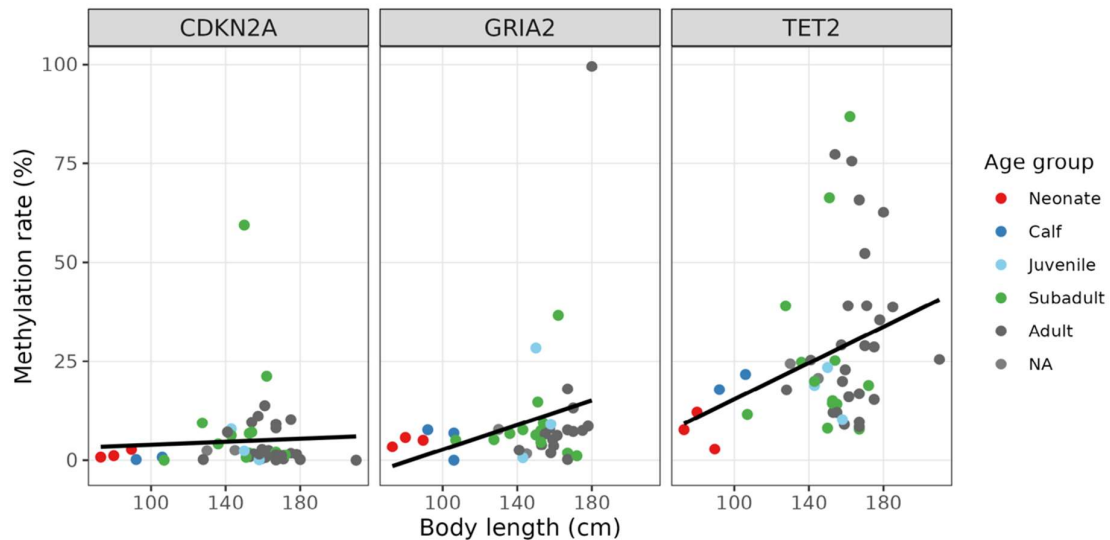


Figure 3. The relationship between the DNA methylation rates and body length. Different panels indicate the results for different genes (*CDKN2A*, *GRIA2*, and *TET2*), and colors represent different age groups (Neonate, Calf, and Adult). Solid lines indicate linear regression.

Figure 4 shows the relationship between the methylation rates and actual ages. Although *GRIA2* and *TET2* exhibited positive trends, none of the three candidate genes showed a statistically significant linear relationship with the actual ages ($P > 0.05$).

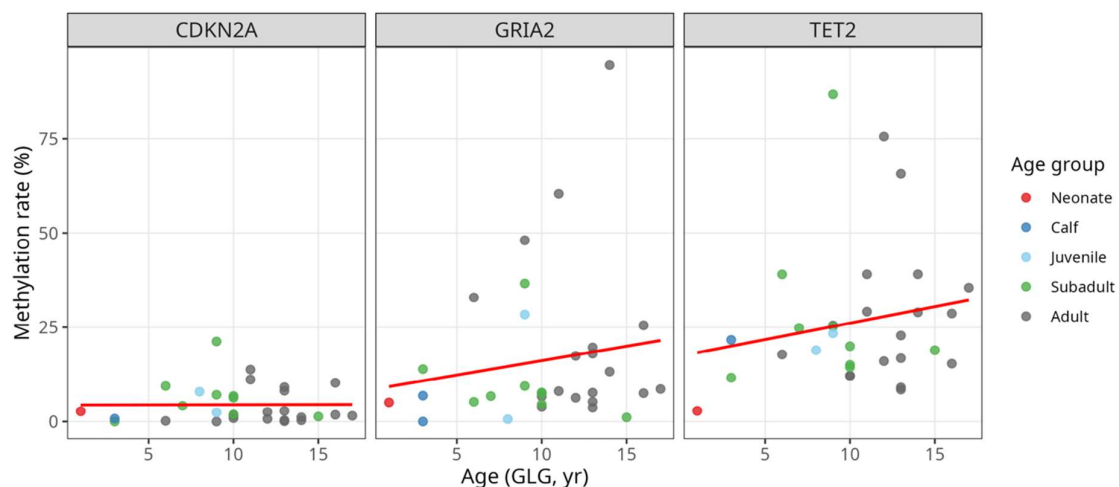


Figure 4. The relationship between the DNA methylation rates and actual ages. Different panels indicate the results for different genes (*CDKN2A*, *GRIA2*, and *TET2*), and colors represent different age groups (Neonate, Calf, and Adult). Solid lines indicate linear regression.

The age prediction model using *GRIA2*, *CDKN2A*, and *TET2* and Support Vector Regression

Although each genetic region did not show any clear linear relationship with the actual ages, we successfully constructed the age prediction model using Support Vector Regression (SVR). We tested two SVR models: one using only methylation data from the three genetic regions (“Model 1”), and the other additionally including sex (“Model 2”). As shown in **Figure 5**, we found a clear positive correlation between chronological age (actual age, x-axis) and predicted age based on DNA methylation rate (y-axis) for both models. Model 1 performed better, with R^2 of 0.620 ($P < 0.001$) and the mean absolute error (MAE) including all individuals was 1.35 years (1.87 years for juveniles, 1.82 years for subadults, and 1.09 years for adults). Model 2 performed slightly worse than Model 1, with R^2 of 0.566 ($P < 0.001$) and the MAE was 1.52 years for all individuals (0.19 years for juveniles, 1.66 years for subadults, and 1.63 years for adults). For both models, the predicted ages were generally unbiased for juveniles and adults, but they tended to be overestimated for subadults (+1.05 years for Model 1 and +1.31 years for Model 2). The maximum prediction error also occurred for a subadult individual: 4.13 years for Model 1 and 3.91 years for Model 2.

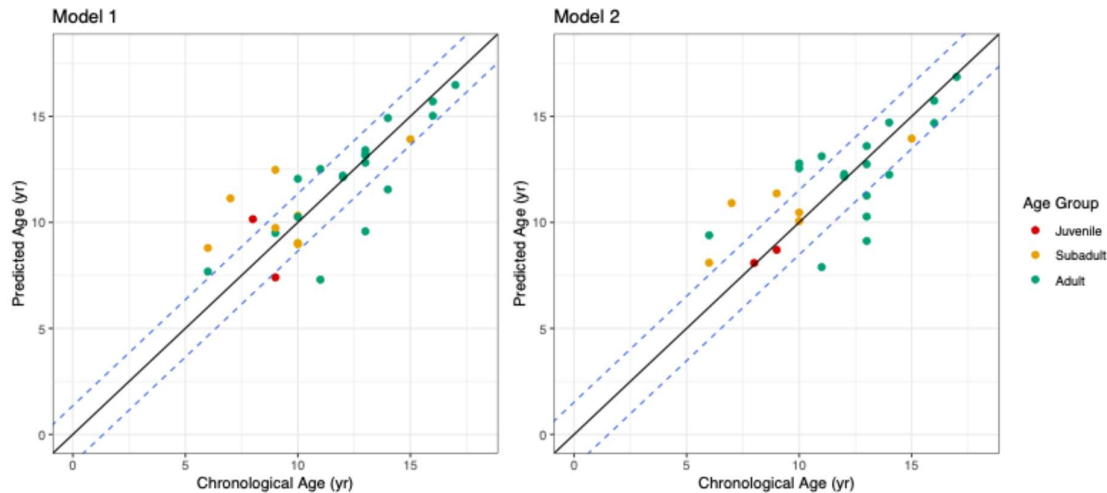


Figure 5. The relationship between the actual ages (x-axis) and that predicted based on the DNA methylation rate (y-axis) using Support Vector Regression (SVR). “Model 1” represents the SVR model with methylation data only, and “Model 2” represents the SVR model with methylation data plus sex. Solid lines indicate the 1:1 line (i.e., perfect prediction). Blue dashed lines indicate the interval of $\pm 1 \times$ MAE (Mean Absolute Error). Colors indicate age groups.

Cross-Species Method Transferability

Our age prediction models rely on the specific relationship between DNA methylation and chronological aging, which can vary across species. Consequently, accurate age estimation requires species-specific calibration, meaning our finless porpoise model cannot be directly applied to Chinese white dolphins. However, the genetic markers (*GRIA2*, *CDKN2A*, and *TET2*) are transferable. In fact, during Phase 1 of this project, we successfully utilized the exact same markers to develop an age estimation model for Chinese white dolphins. While it is statistically possible to input Chinese white dolphin methylation data into the finless porpoise model to predict ages, the predictive performance and biological validity of such a cross-species application are currently unknown.

Summary information for finless porpoise individuals used for this analysis

For transparency and reproducibility, we include the summary information table for finless porpoise individuals used for constructing the age prediction models (Table 1).

Table 1. List of finless porpoise individuals used for constructing the age prediction models.

ID	Sex	Length (cm)	Age (yr)	Age group	GRIA2 (%)	TET2 (%)	CDKN2A (%)	Model 1: Predicted age (yr)	Model 2: Predicted age (yr)
NP24-0701U4	Female	143	8	Juvenile	0.63	18.92	7.93	10.2	8.1
NP20-2611U4C	Male	150	9	Juvenile	28.37	23.39	2.4	7.4	8.7
NP22-0701F4	Female	136	7	Subadult	6.75	24.82	4.17	11.1	10.9
NP22-1202U4	Unknown	152.6	10	Subadult	7.47	14.39	1.9	10.3	NA
NP21-0501F4	Female	127.4	6	Subadult	5.23	39.01	9.43	8.8	8.1
NP23-1203M4	Male	174	15	Subadult	1.1	18.91	1.33	13.9	14.0
NP20-0211	Unknown	162	9	Subadult	36.6	86.86	21.23	12.5	NA
NP20-0912M4	Male	147	9	Subadult	9.43	25.19	7.1	9.7	11.4
NP20-2611A	Male	156	10	Subadult	4.57	15.04	6.8	9.0	10.5
NP20-2611B	Male	155	10	Subadult	7.7	19.96	6.3	9.0	10.1
NP21-2509M4	Male	178	17	Adult	8.63	35.49	1.53	16.5	16.9
NP22-1603	Female	153	10	Adult	3.87	12.11	0.9	12.1	12.6
NP24-1601U4	Female	169	13	Adult	3.67	22.82	2.8	12.8	9.1
NP22-0501F4	Female	163	12	Adult	17.41	75.59	2.53	12.1	12.3
NP21-1112F4	Female	161	11	Adult	60.41	39.03	13.77	12.5	13.1
NP21-0307U5	Unknown	151	9	Adult	48.08	25.47	0	9.5	NA
NP21-0102M4	Male	155	10	Adult	6.67	12.11	1.63	10.3	12.8
NP22-2503U4	Male	175	16	Adult	7.53	28.64	1.8	15.0	14.7
NP21-0701M4	Male	175	16	Adult	25.51	15.36	10.27	15.7	15.7
NP21-2011U4	Female	171	14	Adult	94.62	39.04	0.33	11.6	12.2
NP21-3001M4	Male	167	13	Adult	7.67	8.48	9.13	13.2	13.6
NP21-1902U4	Female	161.4	12	Adult	6.3	16.03	0.7	12.2	12.2
NP21-3001bF4	Female	157.4	11	Adult	8.07	29.14	11.13	7.3	7.9
NP20-0809M4	Male	170	14	Adult	13.23	28.95	1.13	14.9	14.7
NP20-0504	Male	127	6	Adult	32.9	17.78	0.17	7.7	9.4
NP20-2612F4	Female	167	13	Adult	5.23	9.06	0.33	9.6	10.3
NP21-2904M5	Male	167	13	Adult	18.01	16.81	8.17	13.4	11.3
NP20-0410F4	Female	167	13	Adult	19.64	65.76	0.07	13.2	12.7

Promoting activities including scientific publications, conferences, and press release

We introduced our DNA methylation project (together with some other related projects) in an external, invited seminar held on 16 June 2026 at National Cheng Kung University, Taiwan. The presentation title was “*Towards DNA-based comprehensive understanding of resident cetaceans in Hong Kong waters,*” and there were approximately 20 audience there. We were also invited to give a talk in an online, international seminar (an international seminar series named “*PopBio on the Dark Side*”) on 25 June 2026. The presentation title was “*Understanding cetacean ecology from water samples: Cetacean environmental DNA studies in Hong Kong waters,*” and there were approximately 20–30 audience there.

In addition to these invited talks, we published a perspective paper summarizing the current status and application of the DNA methylation technique in June 2026. The paper, titled “*The potential of methylation signal detection in eDNA toward functional ecological monitoring,*” was published in *Communications Biology*, a high reputation, open-access journal in general biology (IF = 5.8; Ranked 9 in 106 biology journals). The paper information is here: Liu C*, Xia F, Hirayama IT, Yagi G, Ushio M* (2026) *Communications Biology* 9:848 (<https://doi.org/10.1038/s42003-026-10496-2>). *corresponding authors

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

In the second phase of the project, we achieved the major objectives of the project: we successfully constructed an age prediction model for the Indo-Pacific finless porpoise based on the DNA methylation rates of the three genetic regions, with a mean absolute error of 1.35 years. Based on these accomplishments, **we believe the objectives of the second phase were successfully met.**

The cost and handling time of the age estimation methods

In this project, we established a method to estimate the age of FP based on DNA methylation patterns using the MS-HRM method. Once the estimation model is established, the cost for age estimation is approximately HK\$200 per sample, including DNA extraction, bisulfite conversion, and MS-HRM analysis (note that this is a rough estimate and may depend on how we control the detailed conditions of the experiment). An experienced researcher can analyze 20–30 samples within 2–3 days. In contrast, the dental GLG analysis typically costs approximately HK\$50–100 per sample, which includes manual cleaning of surface tissue, epoxy resin embedding, sectioning with a slowly rotating diamond saw, hematoxylin staining, and microscopic examination. An experienced researcher can prepare and analyze about 20 samples over 4–5 days. Importantly, it takes a long time to train an experienced researcher for the dental GLG. At present, MS-HRM is more costly but more time-efficient than dental GLG analysis.

PART VI: Summary and Way Forward

In the second phase of the project, we successfully constructed an age prediction model for FP, which showed a significant positive correlation between actual and predicted ages. We will write a manuscript for a scientific journal using the results. In the third year, we will test the possibility of predicting the age of cetaceans relevant to Hong Kong waters, including Chinese white dolphins, based on the DNA methylation rate using environmental samples such as environmental DNA from the water and fecal DNA.

PART VII: Financial statement of the project (enclose as an appendix to the completion report) in the suggested format as set out in Appendix 2

Financial statement is not disclosed due to confidentiality reasons.

PART VIII: Staff attendance record in accordance with the attendance monitoring plan (enclosed as an appendix) (see Section 5.17)

Staff attendance records are 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 authorization has been obtained in respect of information owned by third parties.

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.

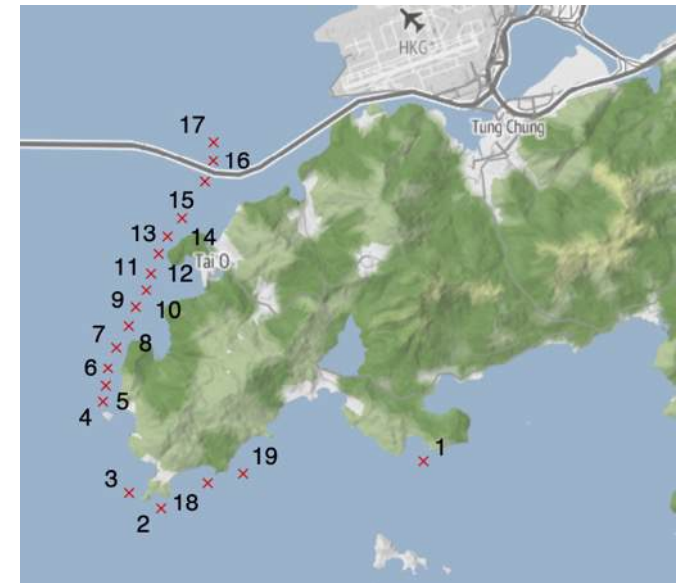
Signature of Project Leader:

A handwritten signature in black ink, reading "Masayuki Ushio", is centered within a rectangular box with a thin black border.

Masayuki USHIO

Department of Ocean Science, HKUST

Towards DNA-based comprehensive understanding of resident **cetaceans** in Hong Kong waters



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OCES, The Hong Kong University of Science and Technology (HKUST)



Cetacean eDNA studies in Hong Kong

Cetaceans and their roles in ecosystems

- **Cetaceans** are large aquatic mammals, which are often iconic and flagship species in marine ecosystems.
- **Cetaceans** play essential roles in maintaining the marine ecosystem functions and services, such as **nutrient cycling**, **trophic cascades**, and **eco-tourism** (Roman & McCarthy 2010; Estes et al. 2011; Kiszka et al. 2022).



Cetaceans are highly threatened

- Megafauna in coastal ecosystems are highly threatened.
- However, conservation of these cetaceans and other megafauna is often neglected as a key component in the conservation scheme, which is referred to as the “**megafauna gap**” (Li et al. 2023 *Science Advances*).
- Thus, efficient and effective cetacean monitoring methods are needed.

SCIENCE ADVANCES | RESEARCH ARTICLE

APPLIED ECOLOGY

A wide megafauna gap undermines China's expanding coastal ecosystem conservation

Xincheng Li¹, Hanchen Wang¹, Douglas J. McCauley², Andrew H. Altieri³, Brian R. Silliman⁴, Jonathan S. Lefcheck^{5,6}, Jihua Wu⁷, Bo Li⁸, Qiang He^{1*}

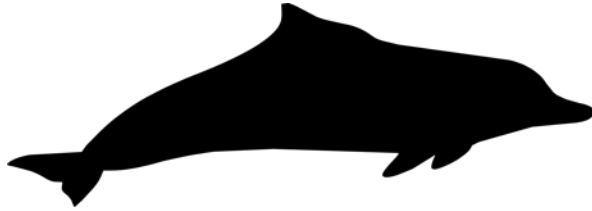
HK resident cetaceans

Two resident cetaceans in HK waters
(they are in HK waters all year around)



HK resident cetaceans: Visual monitoring survey

Chris huh (CC BY-SA 3.0)

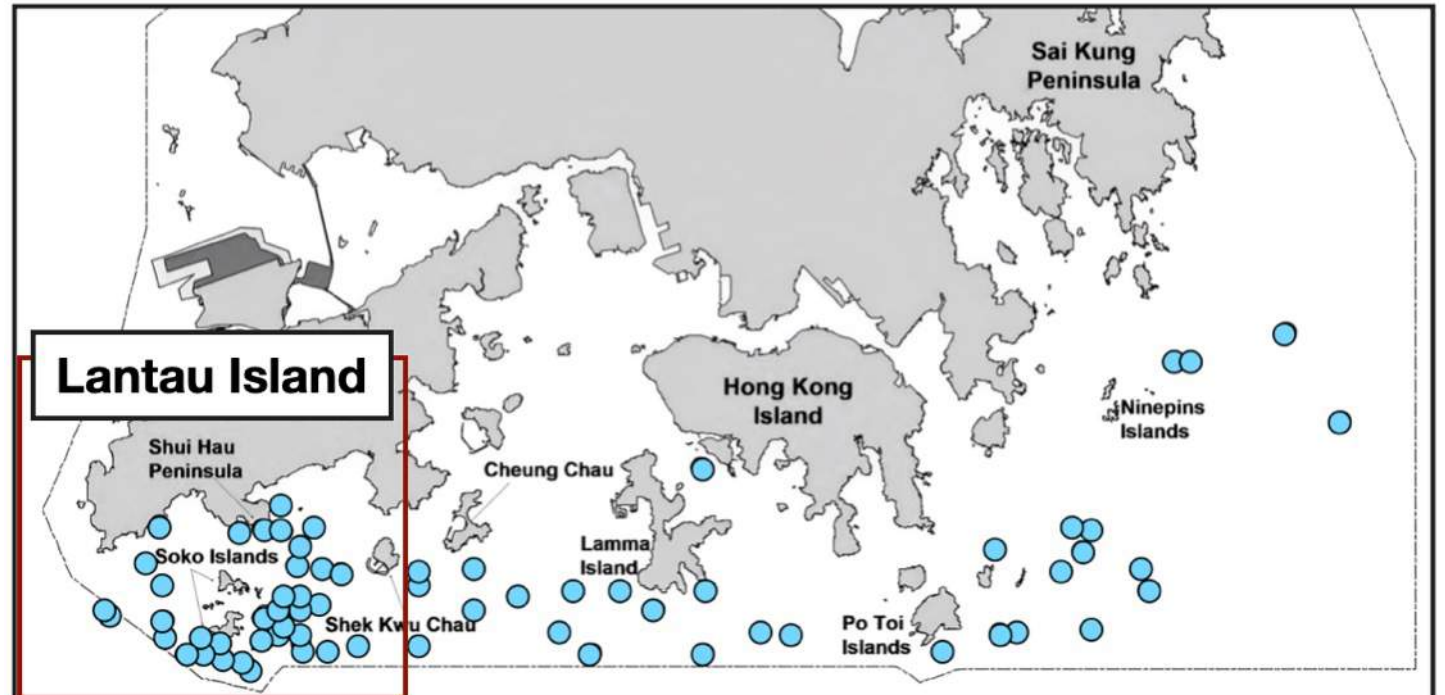
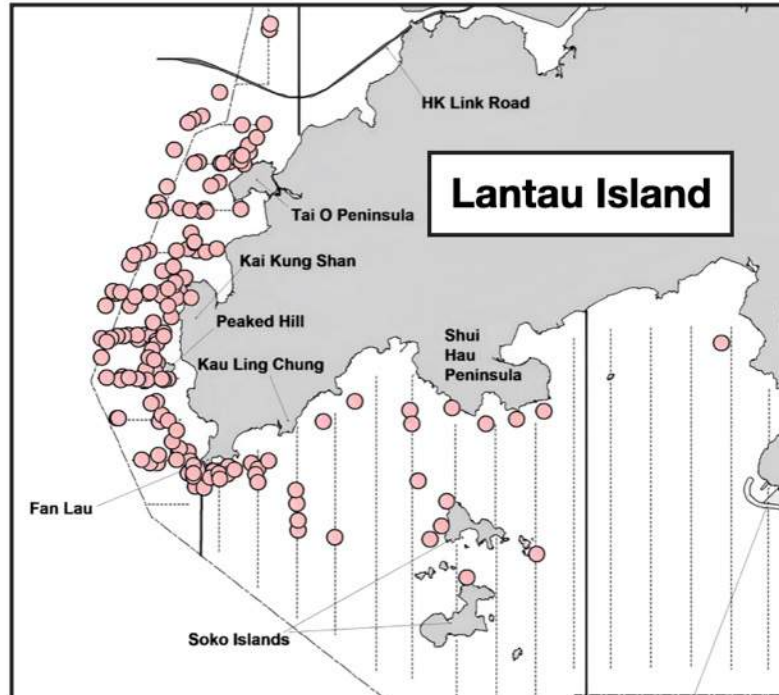


Chinese White Dolphin
(*Sousa chinensis*)

Chris huh (CC BY-SA 3.0)



Finless porpoise
(*Neophocaena phocaenoides*)



HK residential cetaceans

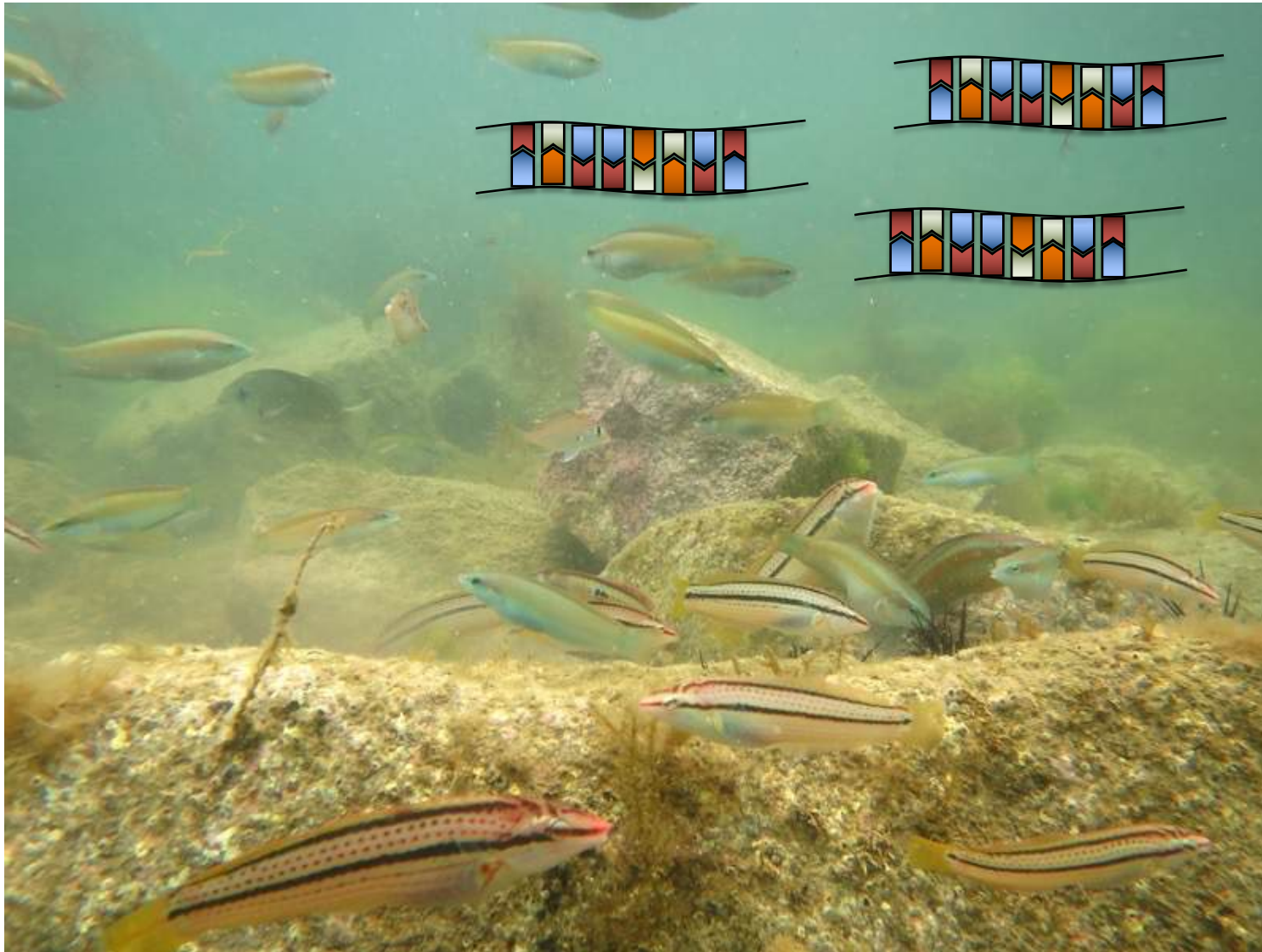


- In Tai O village, there are dolphin-watching boat (40HKD/each for 20 min).
- Chinese White Dolphin is important for eco-tourism, too.



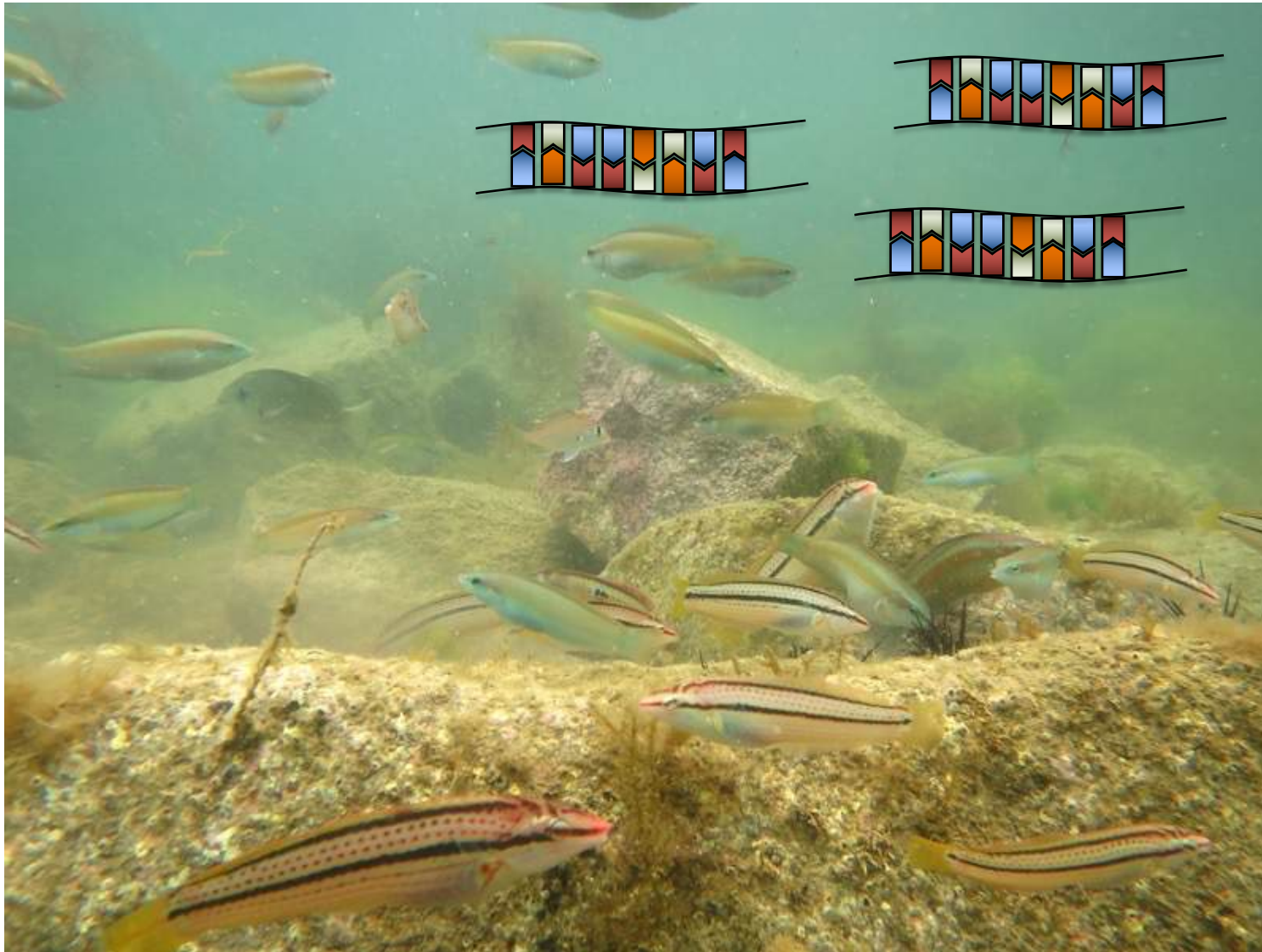
Environmental DNA analysis to complement traditional survey methods

Environmental DNA analysis



- **Definition:** “Environmental DNA (eDNA) is a complex mixture of genetic DNA from many different organisms found in an environmental sample” (Taberlet et al. 2012, 2018)
- eDNA can be extracted from soil, sediment, water, air, feces, and so on.
- Some studies include intracellular DNA (e.g., microbes) (= broad definition) and others do not (narrow definition)

Environmental DNA analysis



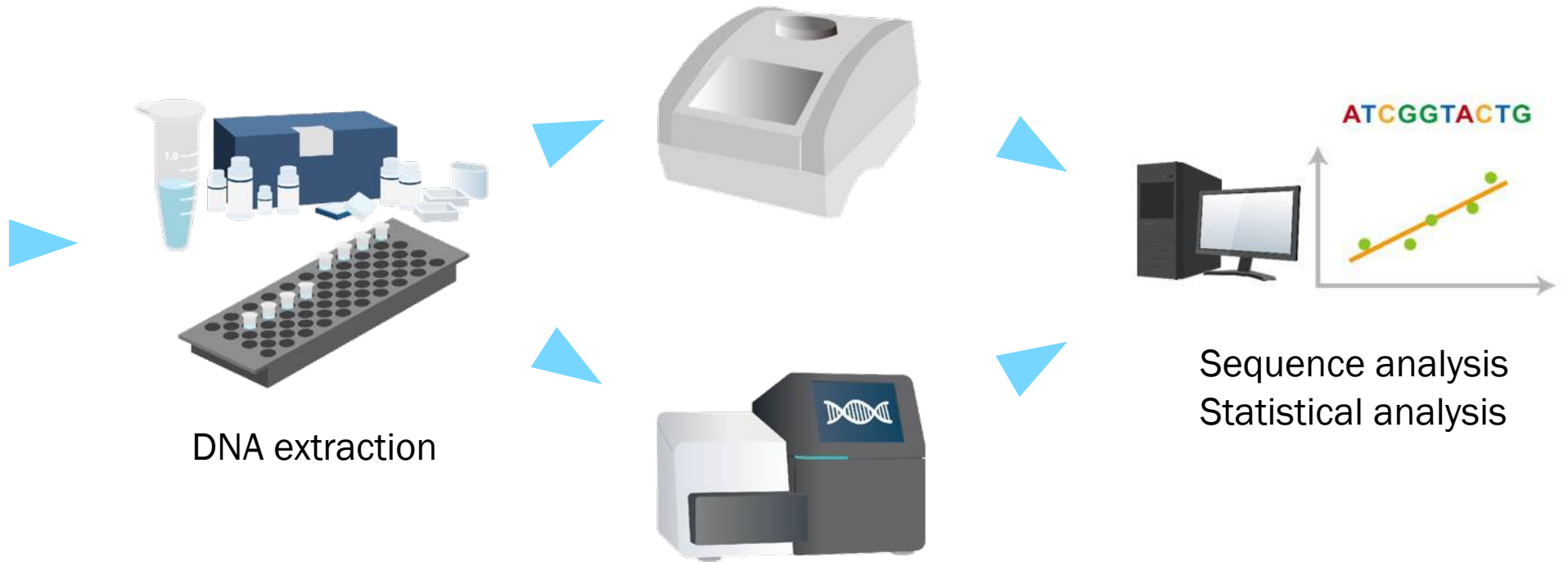
- **eDNA analysis:**
- Highly sensitive
- Low time- and labor-cost in field (e.g., we just need to collect water samples)
- We do not need taxonomic identification skill

Environmental DNA analysis

Two major methods



Collect samples
(e.g., water filtration)



Quantitative PCR

Analyze **single or a few species**, but can quantify DNA concentrations

eDNA metabarcoding

Analyze **many species**, but lower quantitative capacity

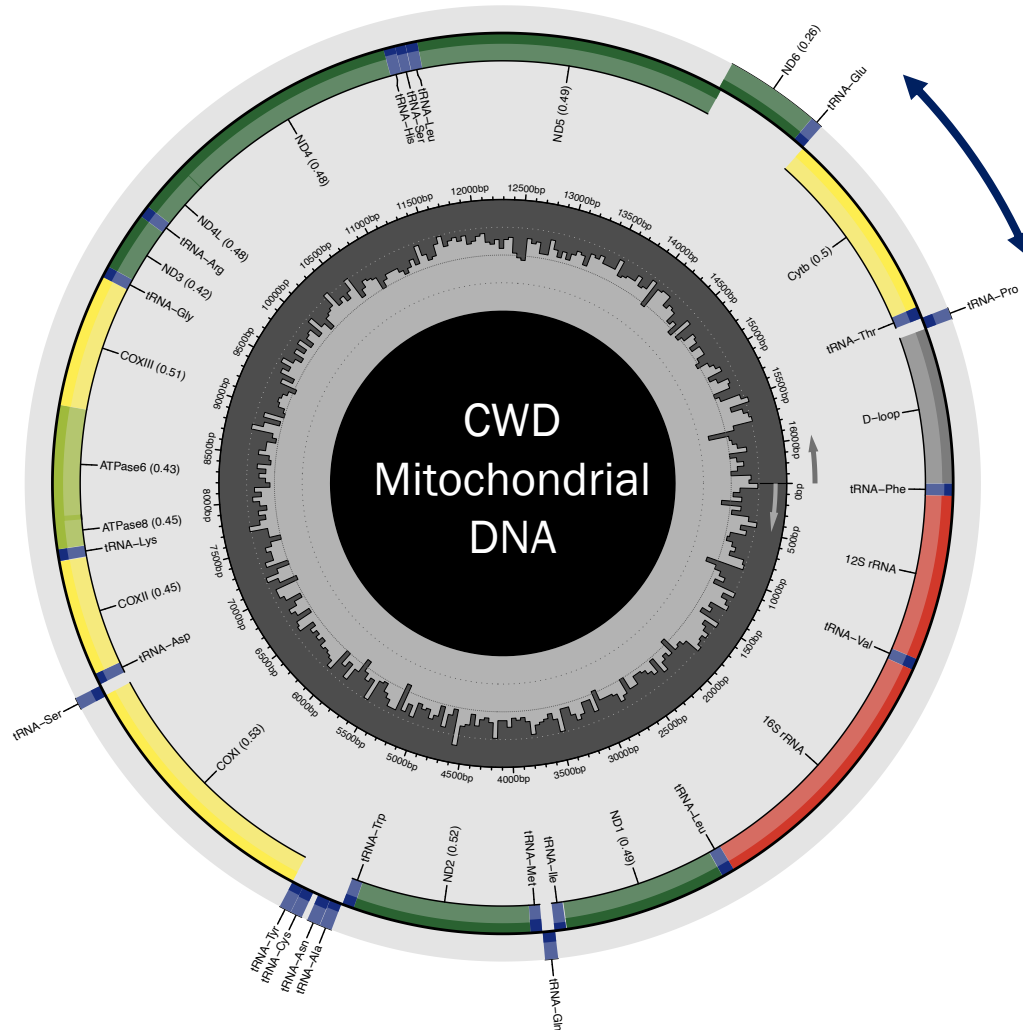
Quantitative PCR

Analyze **single or a few species**, but can quantify DNA concentrations



qPCR-based detection of HK cetaceans

qPCR primers: Chinese white dolphins and Finless porpoises



qCWD-F: TCGCTTTCCACTTTATCCTTCCC

qCWD-R: GATGTCTTTGATTGTATAATAGGGGTGA

qCWD-P: AGCAGCCGTTACCTGCTATTCTACAT

LOD = 3.64 copies/reaction (95% detection prob.)

LOQ = 69 copies/reaction (< 35% C.V.)



Kiser et al. (2025) *bioRxiv*

<https://doi.org/10.1101/2025.10.30.685690>

Nas-Cytb-F: TACGTTCTACCCTGAGGGCAAA Hashimoto et al.

Nas-Cytb-R: GCTTTGTCTACAGAGAACCCGC (2024)

Nas-Cytb-P: TGTCAGCAATCCCTTATATTGGCAGCACAC

LOD = 3.0 copies/reaction (95% detection prob.)

LOQ = 34 copies/reaction (< 35% C.V.)



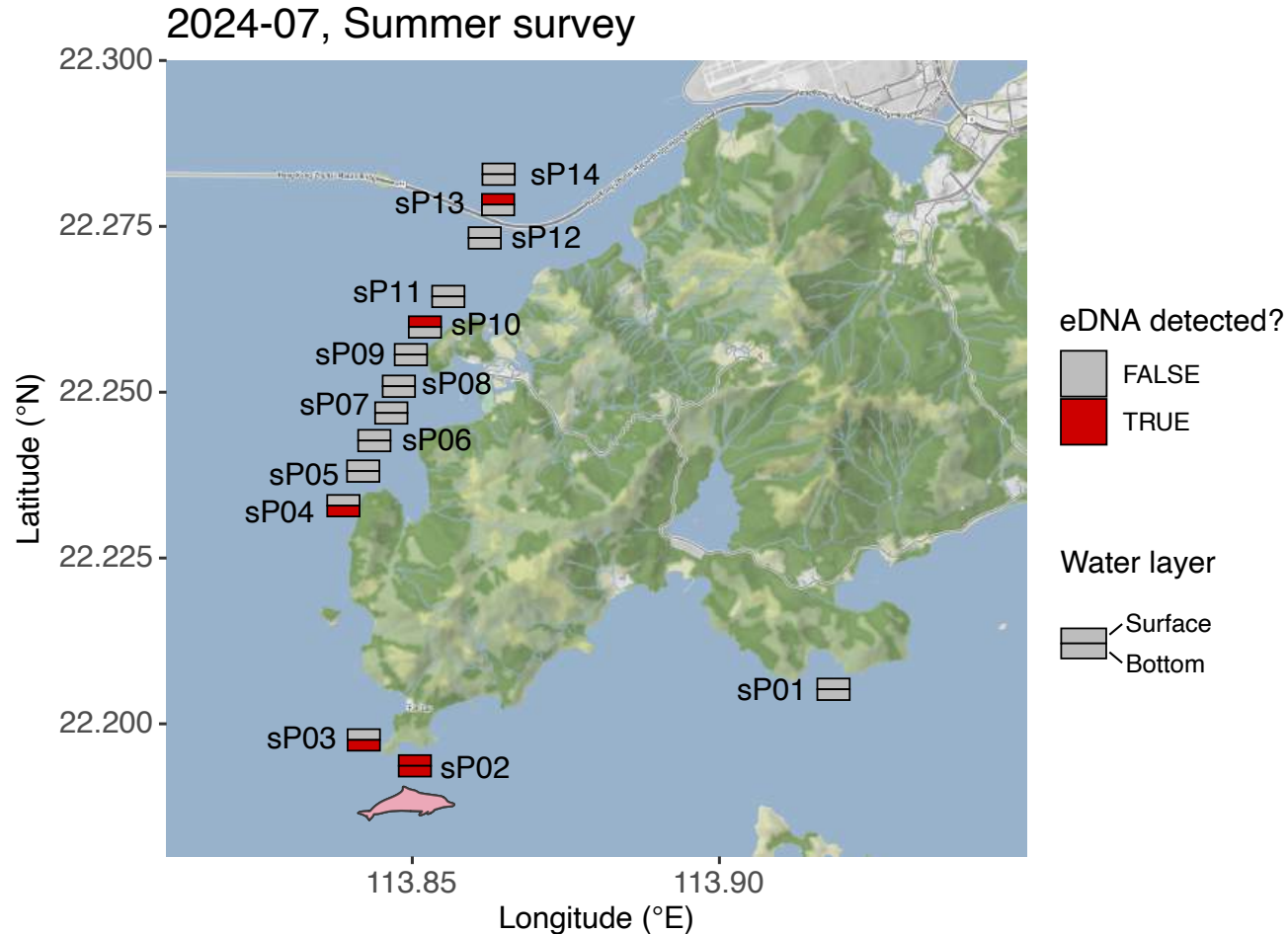
Lin & Ushio (2025) *bioRxiv*

<https://doi.org/10.64898/2025.12.21.695310>

qPCR: Results of Chinese white dolphins

Lantau Island survey: Summer (July 2024) and winter (Jan 2025)

1 L of seawater, gravity filtration, bottom and surface water, eDNA extracted using B&T kit



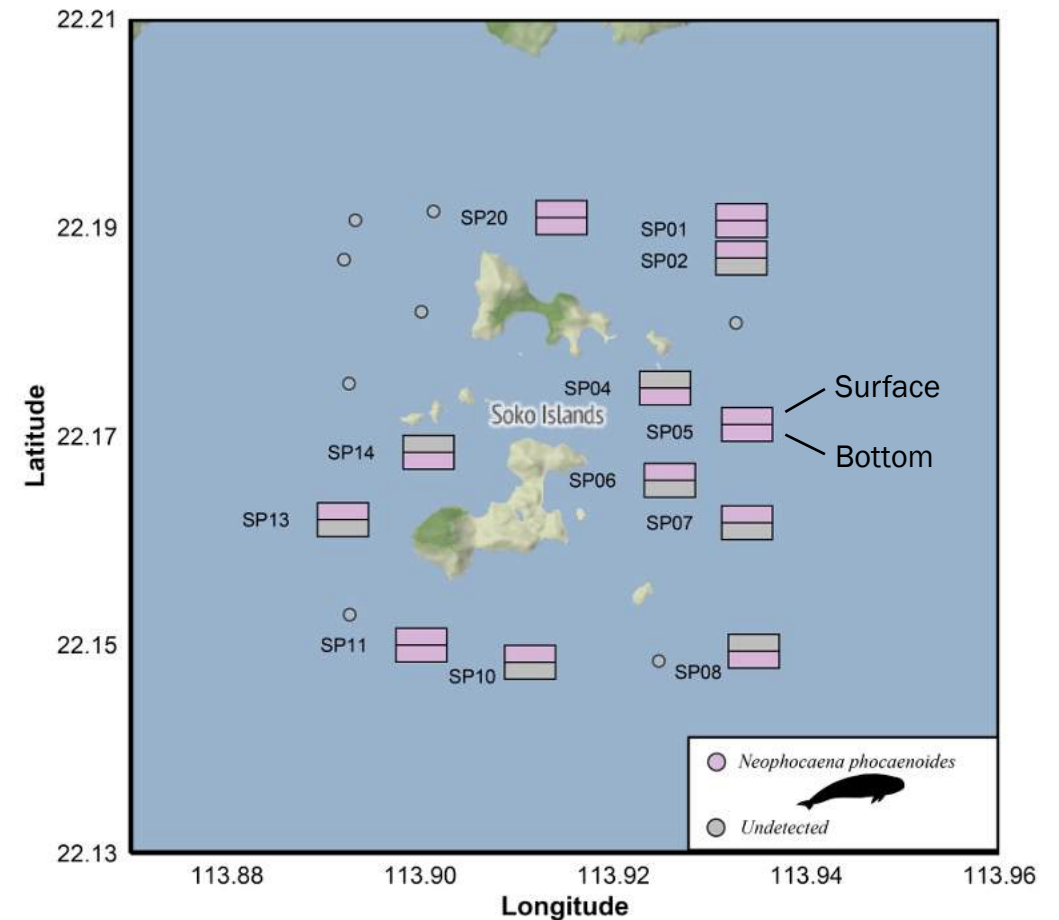
qPCR: Results of Indo-Pacific finless porpoises

Soko Islands survey: Spring survey (Mar 2025)

1 L of seawater, gravity filtration, bottom and surface water, eDNA extracted using B&T kit



Xiaoqi Lin



qPCR surveys

- The primers' performance was evaluated very carefully (specificity, LOD, and LOQ).
- The primer sets successfully detected eDNA of Chinese white dolphins and finless porpoises.
- All detected values were below LOQ (Limit of Quantification), suggesting that cetacean eDNA concentrations in natural environments can be very low (but still detectable).
- We need further refinements to the methods, e.g., water sampling volume, water layer, etc.



eDNA metabarcoding

Analyze **many species**, but lower quantitative capacity

eDNA metabarcoding-based detection

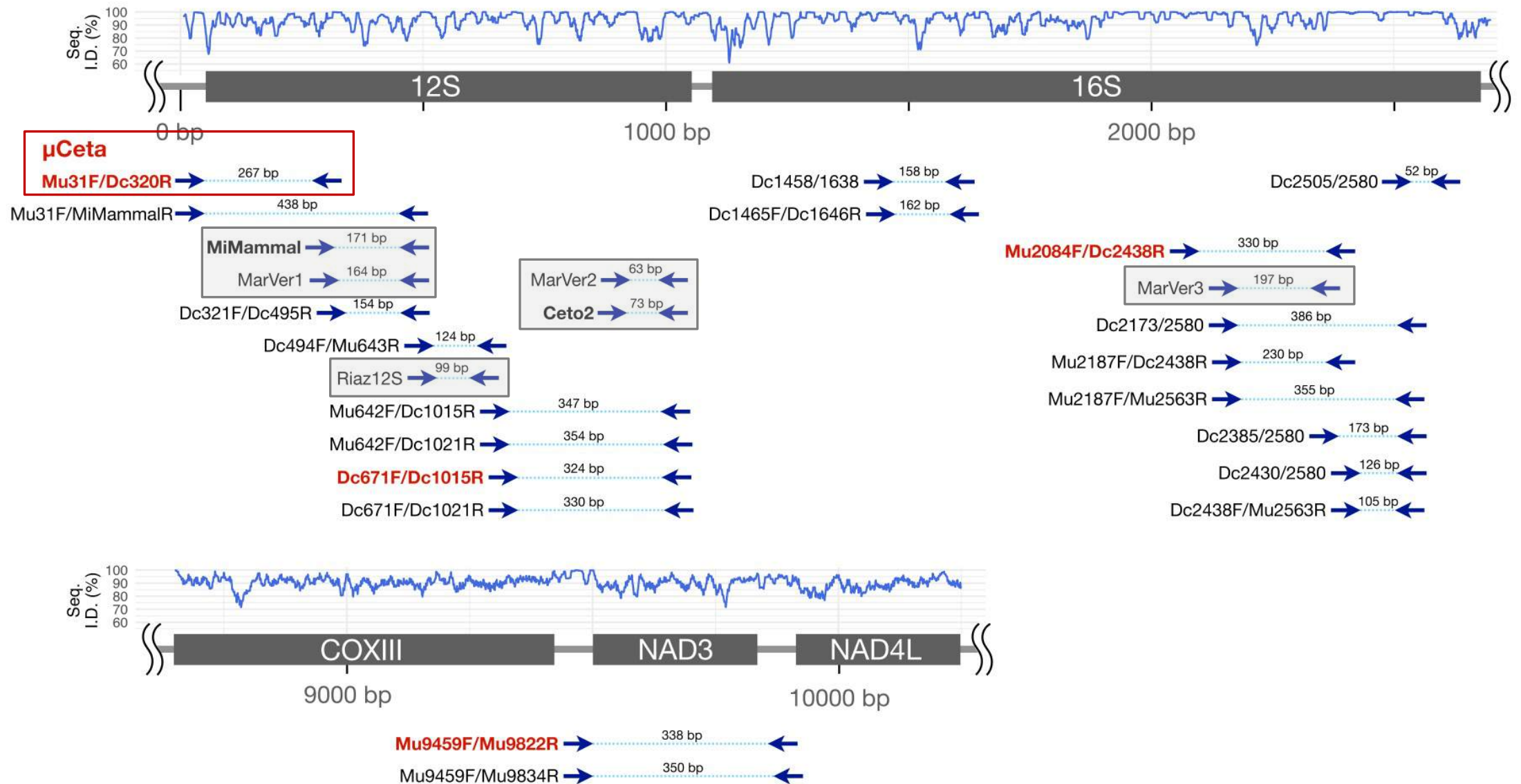


μCeta: A Set of Cetacean-Specific Primers for Environmental DNA Metabarcoding With Minimal Amplification of Non-Target Vertebrates

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¹Department of Ocean Science, The Hong Kong University of Science and Technology, Kowloon, Hong Kong SAR, China | ²Okinawa Churashima Foundation, Motobu-cho, Okinawa, Japan | ³Natural History Museum and Institute, Chiba, Chiba, Japan | ⁴Southeast Asia Marine Mammal Research, Lippo Centre, Queensway, Hong Kong SAR, China | ⁵Ocean Park Hong Kong, Aberdeen, Hong Kong SAR, China

New cetacean-specific primers for eDNA metabarcoding



eDNA metabarcoding primer tips

- **Primer length:** 19–28 bp (best = 21–25 bp)
- **GC%:** 40–60%
- **T_m :** 60 ± 1 °C (T_m of Forward and Reverse primers should match as much as possible) (can be checked with Primer-Blast)
- **Complementarity:** Avoid primers with complementarity of more than 5 bp.
- **3'-end:** C > A > G >>> T. You may avoid T for eDNA primers because T might bind to G (T/G bond). Also avoid GC-rich or repetitive sequence at 3'-end. However, you may intentionally use T or G to cover a broader taxa range (e.g., as in MiFish)

Power of eDNA metabarcoding



@Okinawa Churaumi Aquarium, Japan



Collection of water samples from aquarium tanks



@Okinawa Churaumi Aquarium, Japan
Aquarium pools with a “known” species composition



Churaumi pool samples



Common bottlenose dolphin
Tursiops truncatus



Indo-Pacific bottlenose dolphin
Tursiops aduncus



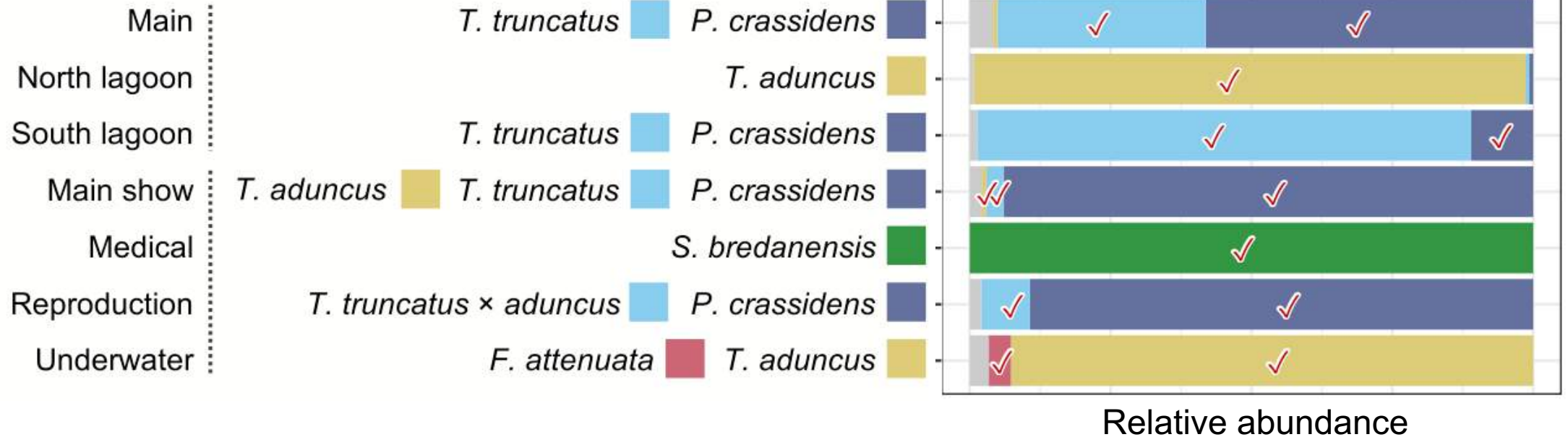
False Killer Whale
Pseudorca crassidens



Pygmy Killer Whale
Feresa attenuata



Fough-toothed dolphin
Steno bredanensis



Test with natural seawater samples

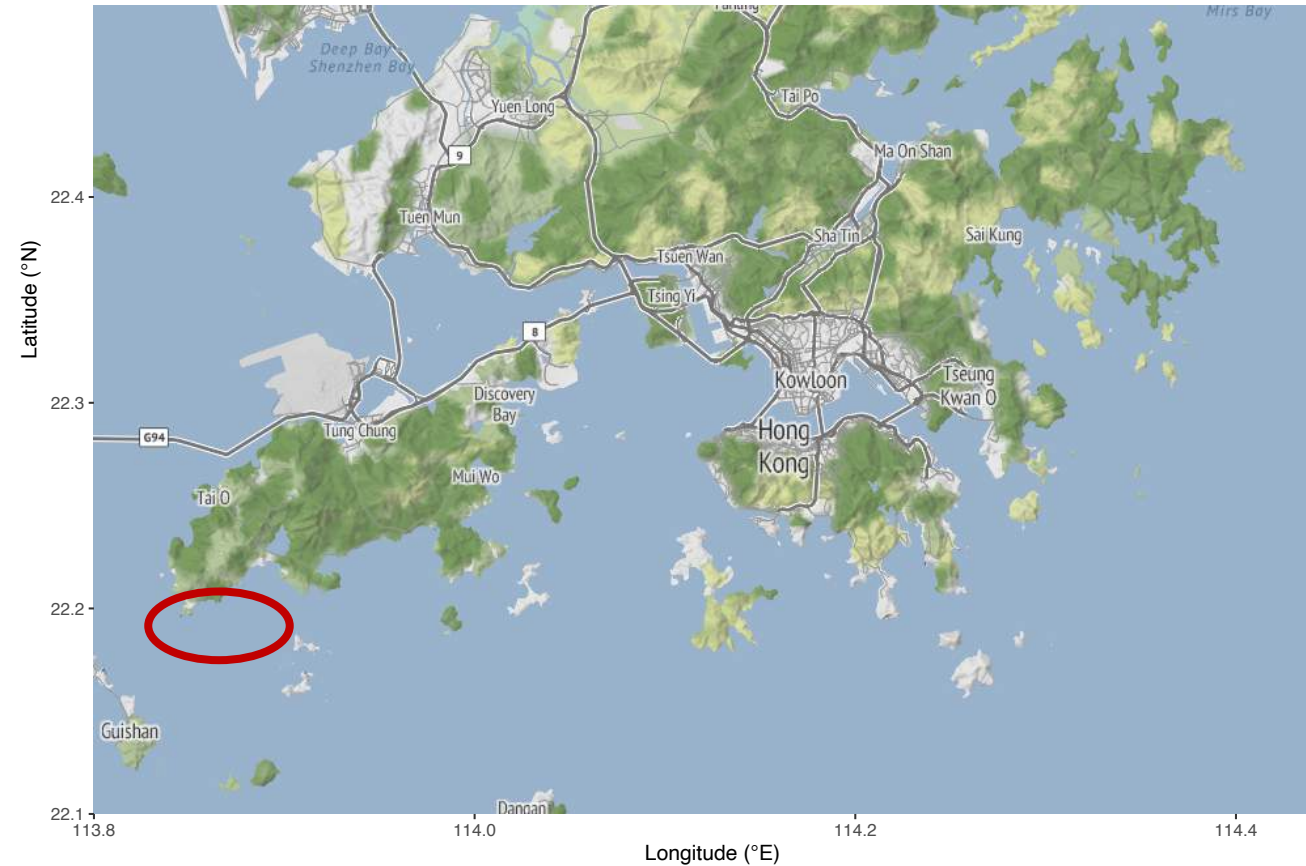
16 July 2024, 500–1,500 ml seawater
0.45- μ m Sterivex, 30–50 m apart from CWD
Metabarcoding: Detected CWD seqs and
almost **NO non-target sequences.**
qPCR: 1100–1700 copies/L water



© E. Matrai



μ Ceta-F: GACACTGAAAATGTCTAGATGG
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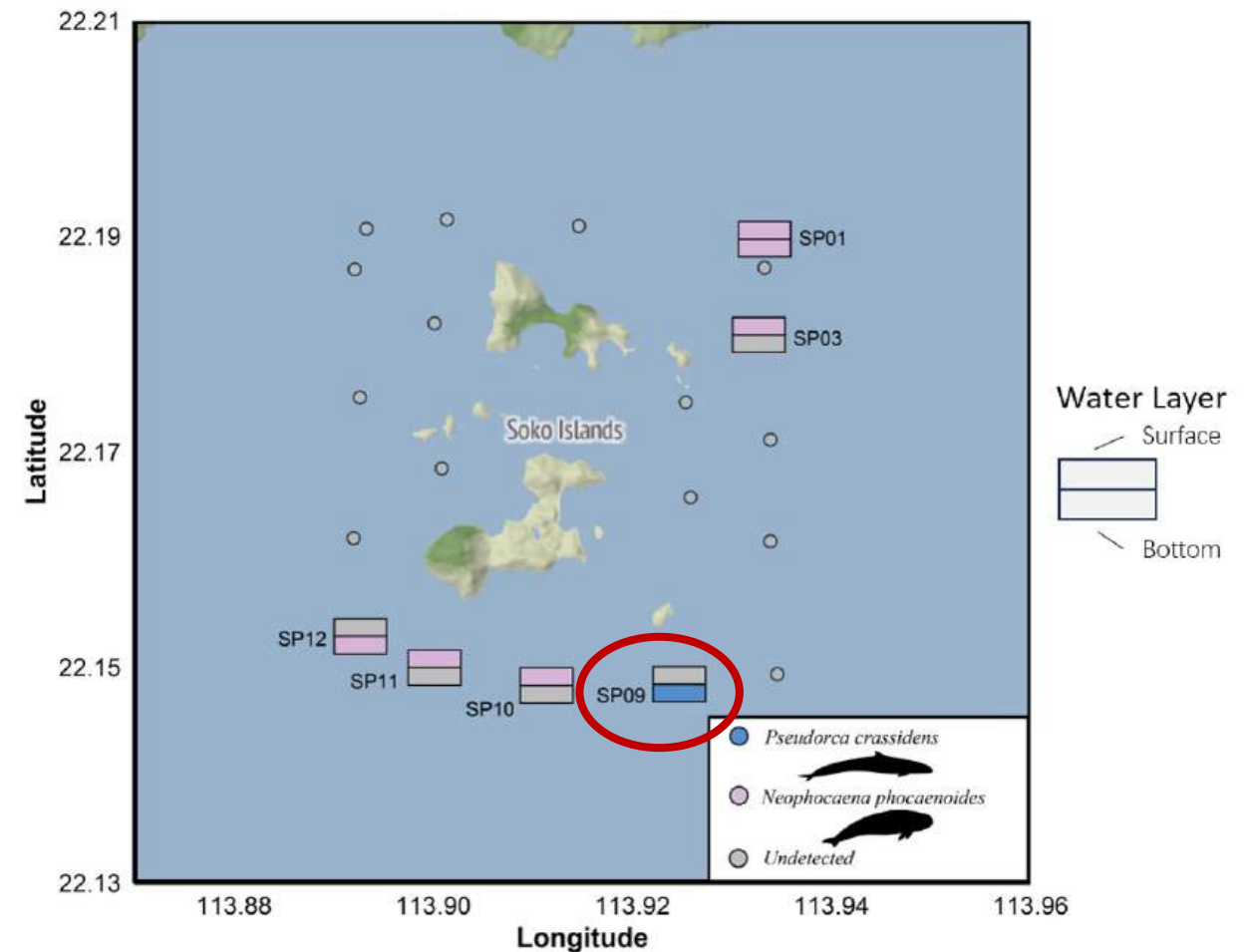
Detection of multiple cetacean species from HK waters

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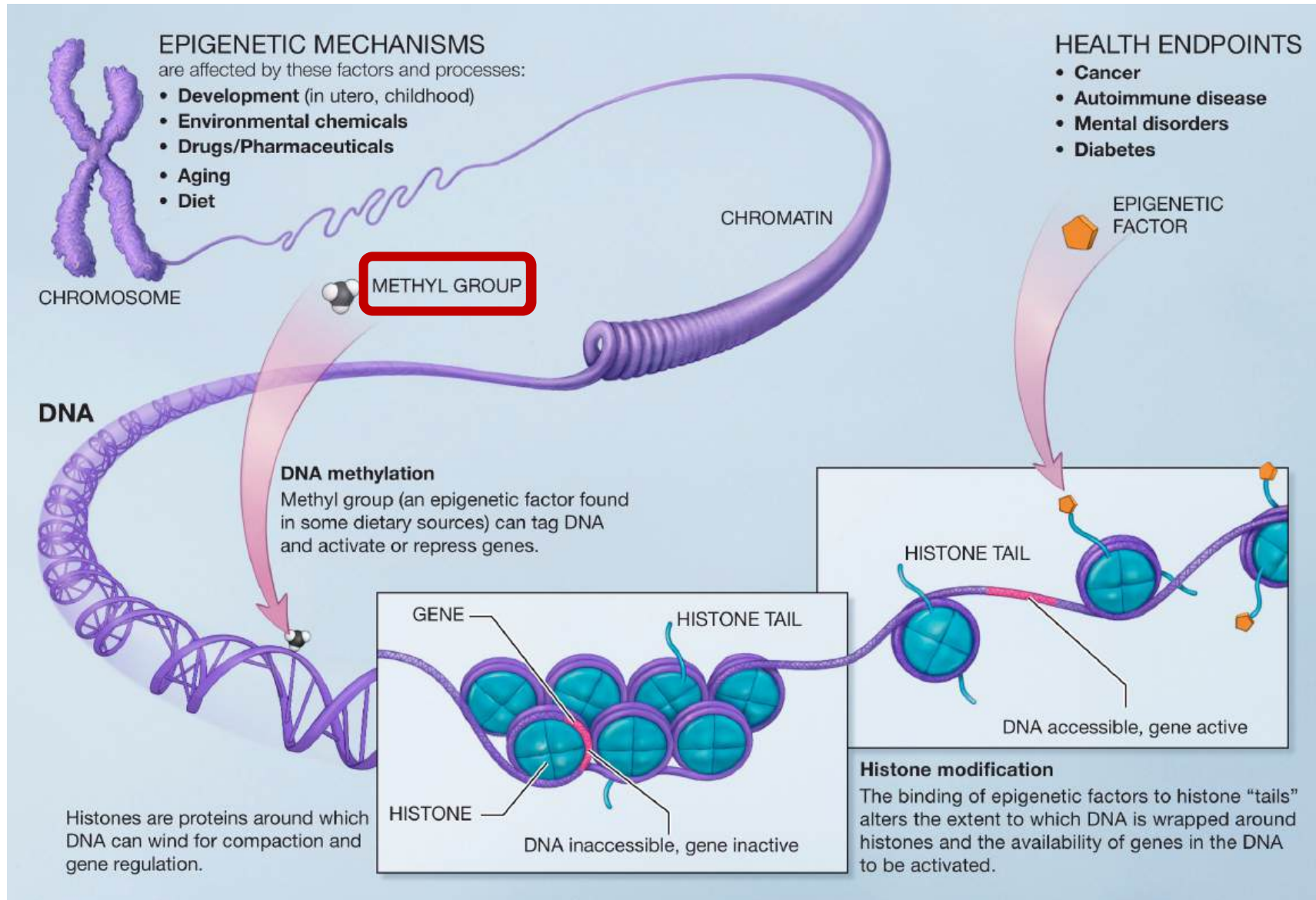


Xiaoqi Lin



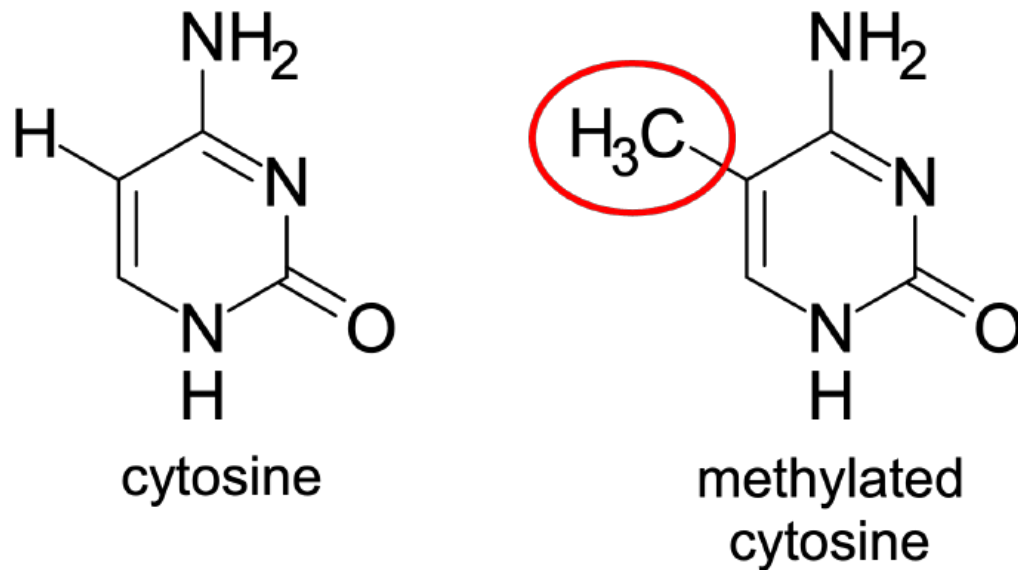
Towards eDNA-based detection of cetacean age

DNA and age estimation



Introduction to ageing and DNA methylation

- DNA methylation
 - Occurs in cytosine
 - In mammals (or animals), cytosine methylation can occur at a **5'-CG-3'** site (or, "**CpG**" site).
 - Enzymes that add a methyl group are called **DNA methyltransferases**.



DNA and age estimation

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nature aging



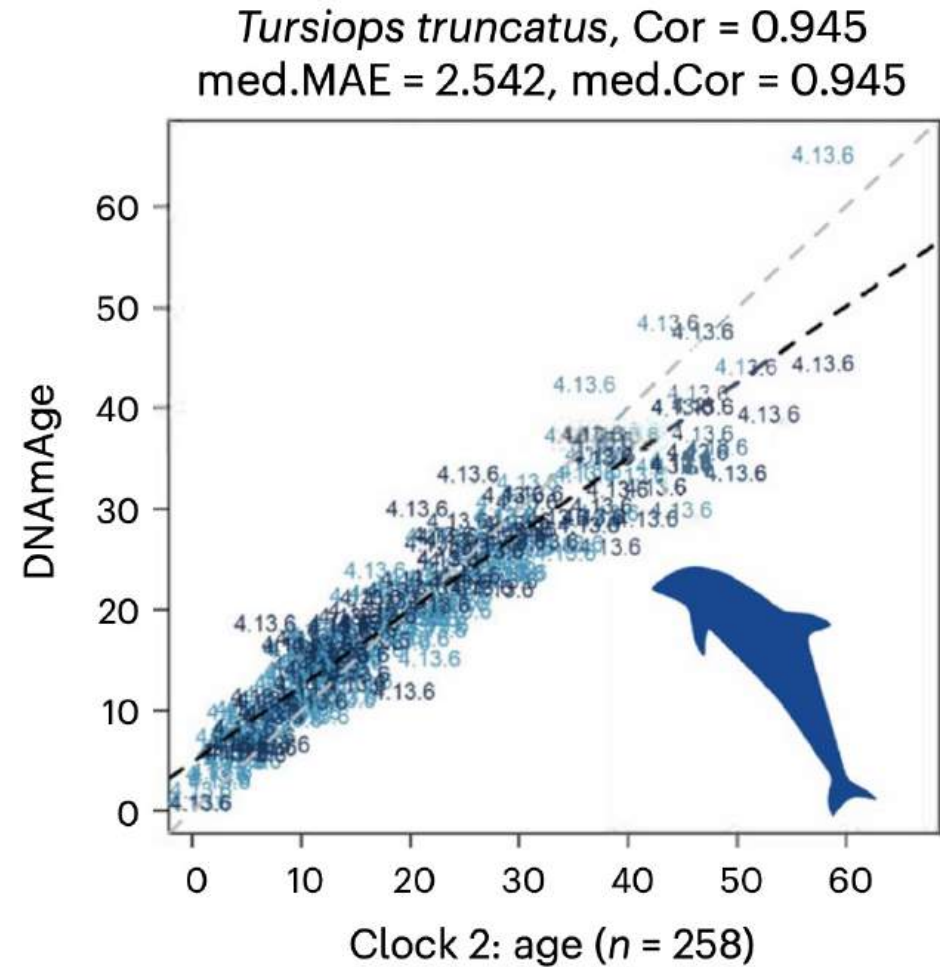
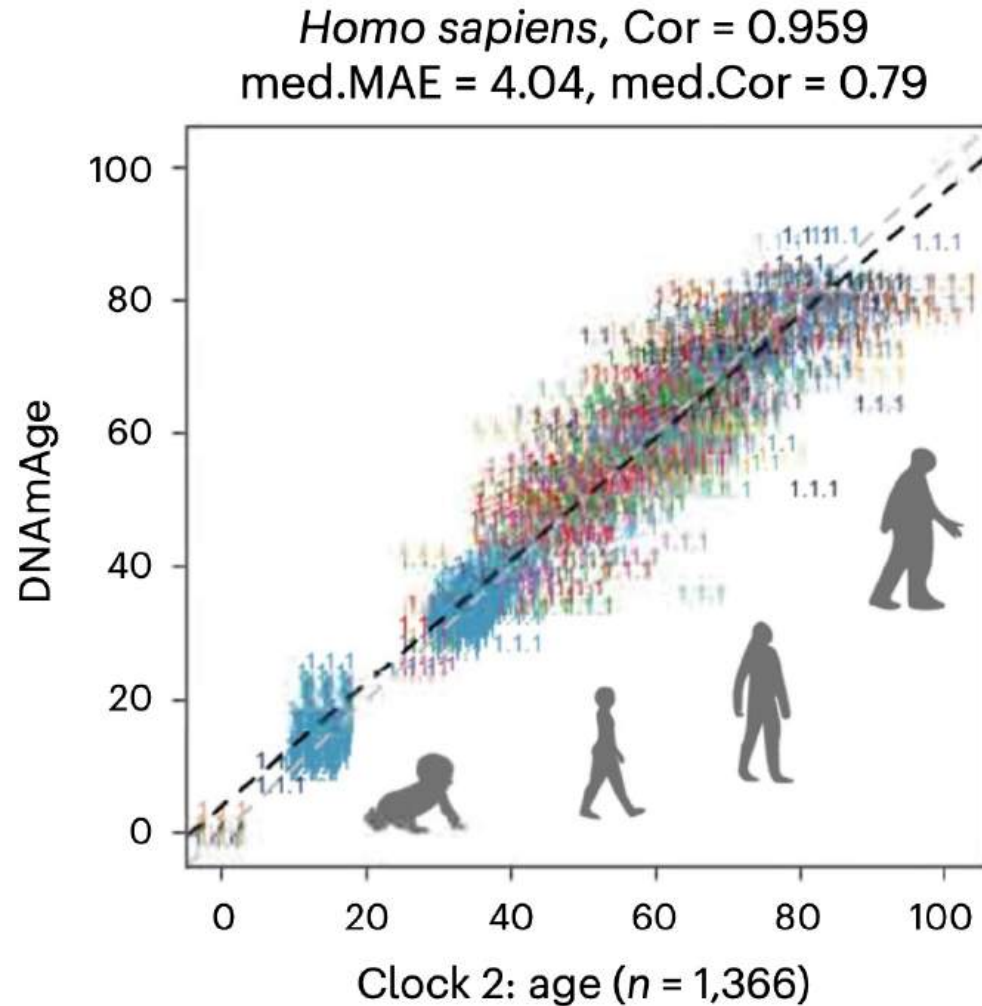
Resource

<https://doi.org/10.1038/s43587-023-00462-6>

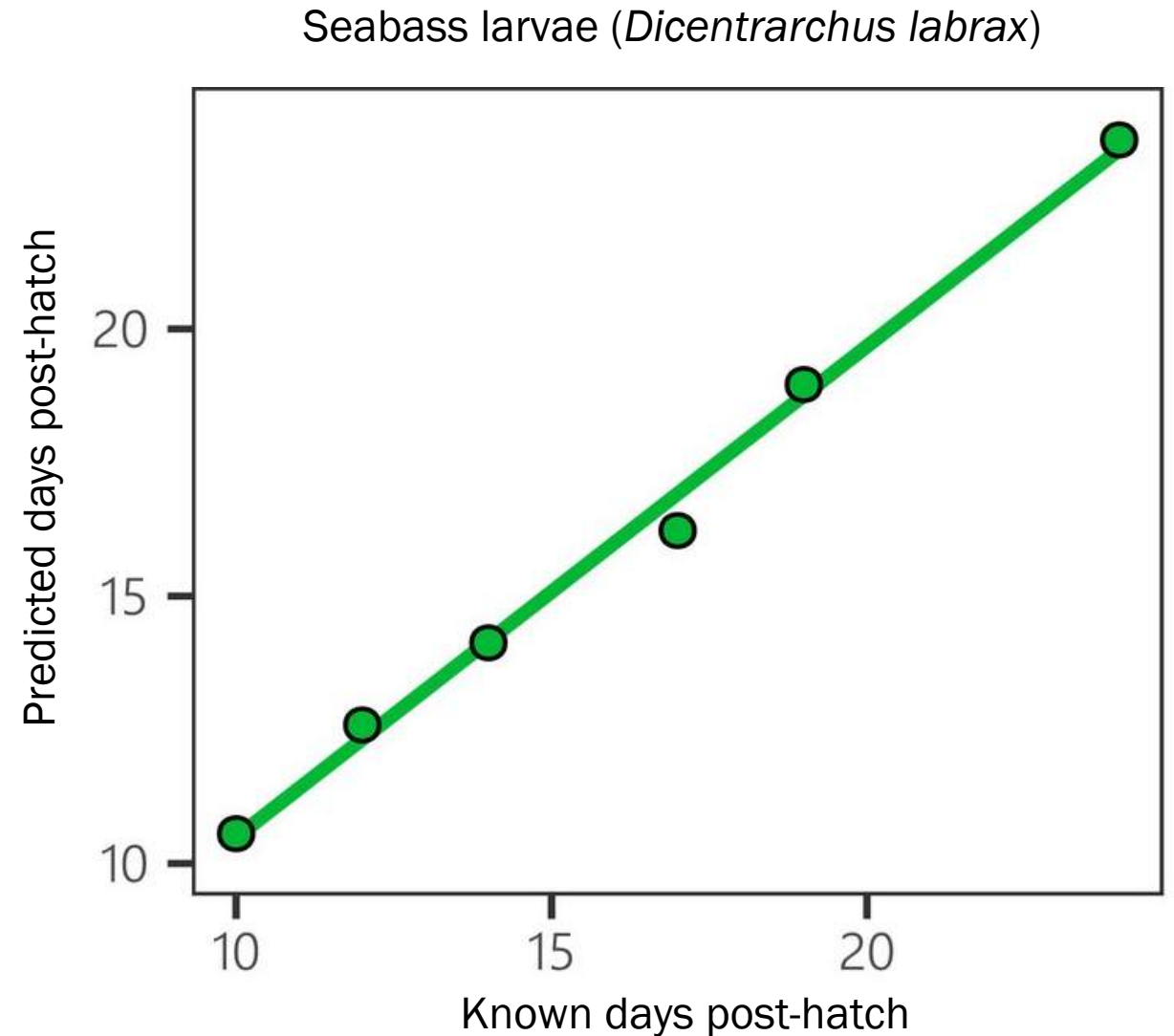
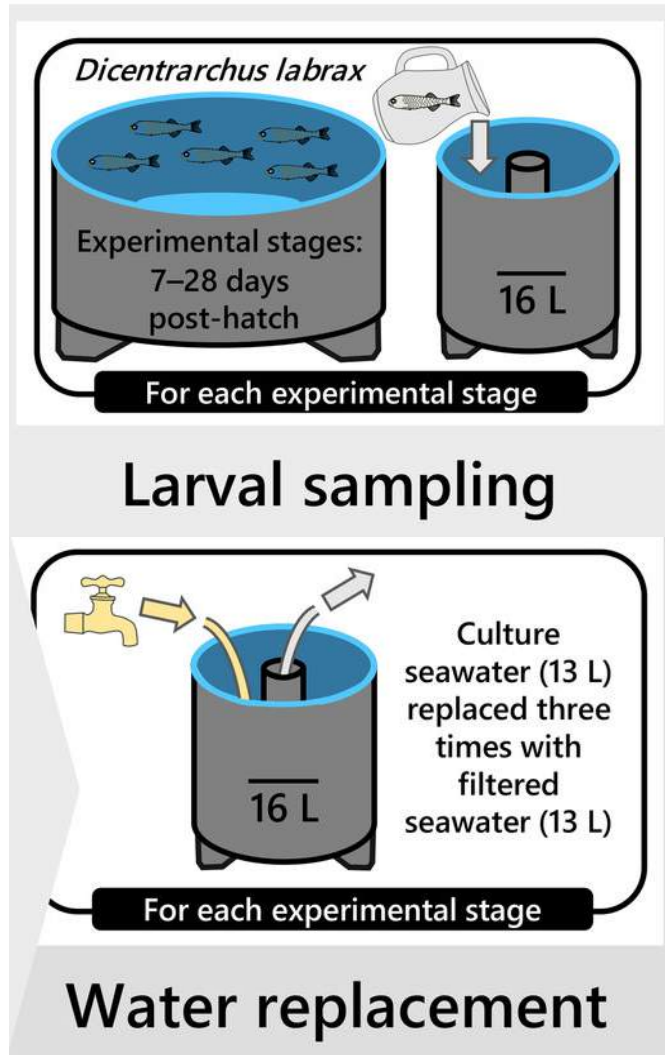
Universal DNA methylation age across mammalian tissues

Lu et al. (2023) *Nature Aging*

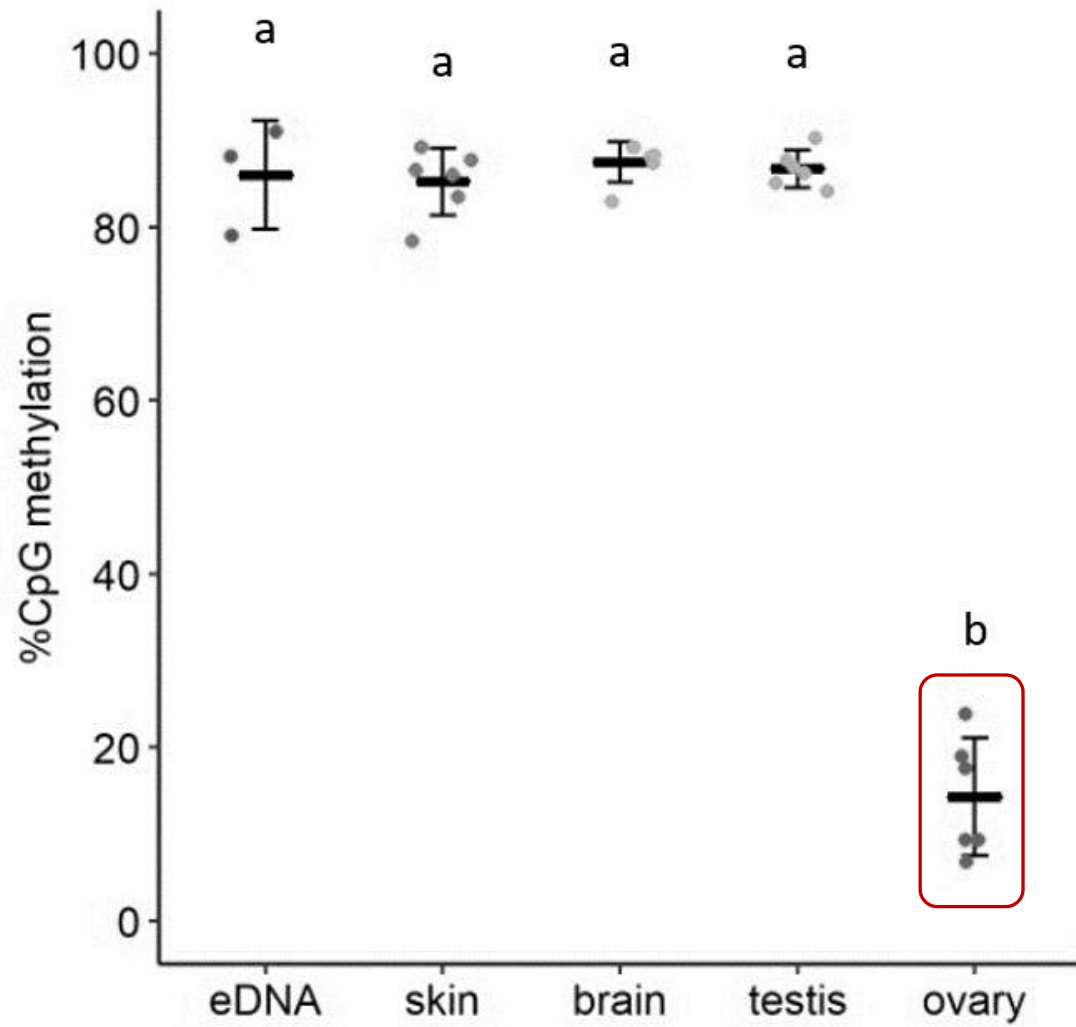
DNA and age estimation



eDNA and age estimation



eDNA and age estimation



Zebrafish (*Danio rerio*)

D. Terver (CC BY-NC 4.0)

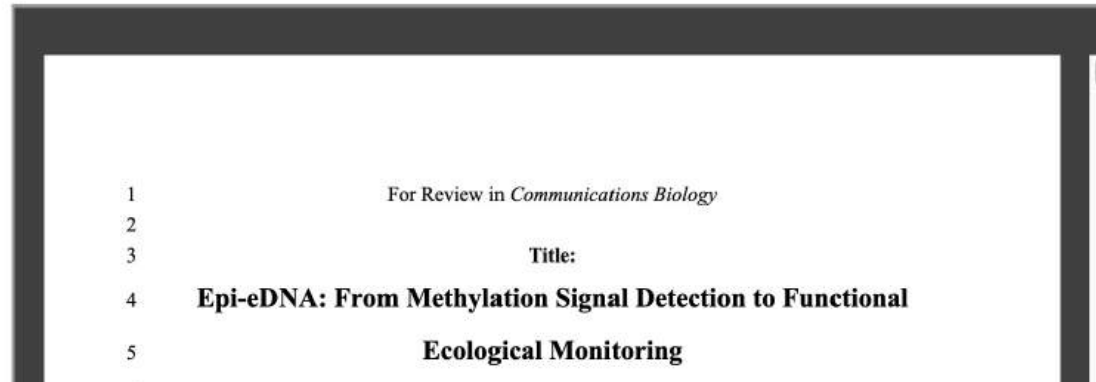
Young tissue has lower DNA methylation rate!

eDNA and age estimation



Epi-eDNA: From Methylation Signal Detection to Functional Ecological Monitoring

This is a Preprint and has not been peer reviewed. This is version 1 of this Preprint.



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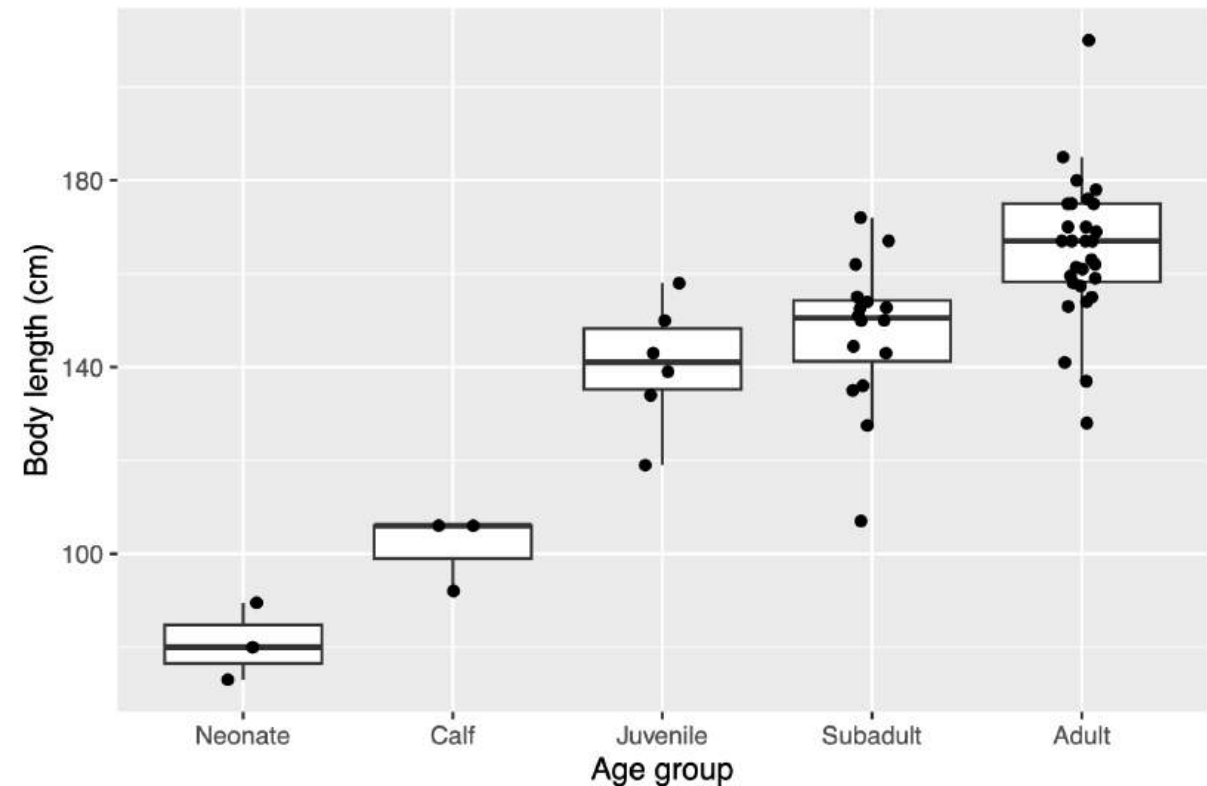
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Authors

Chengbin Liu, Fei Xia, Itsuki T. Hirayama, Genfu Yagi, Masayuki Ushio

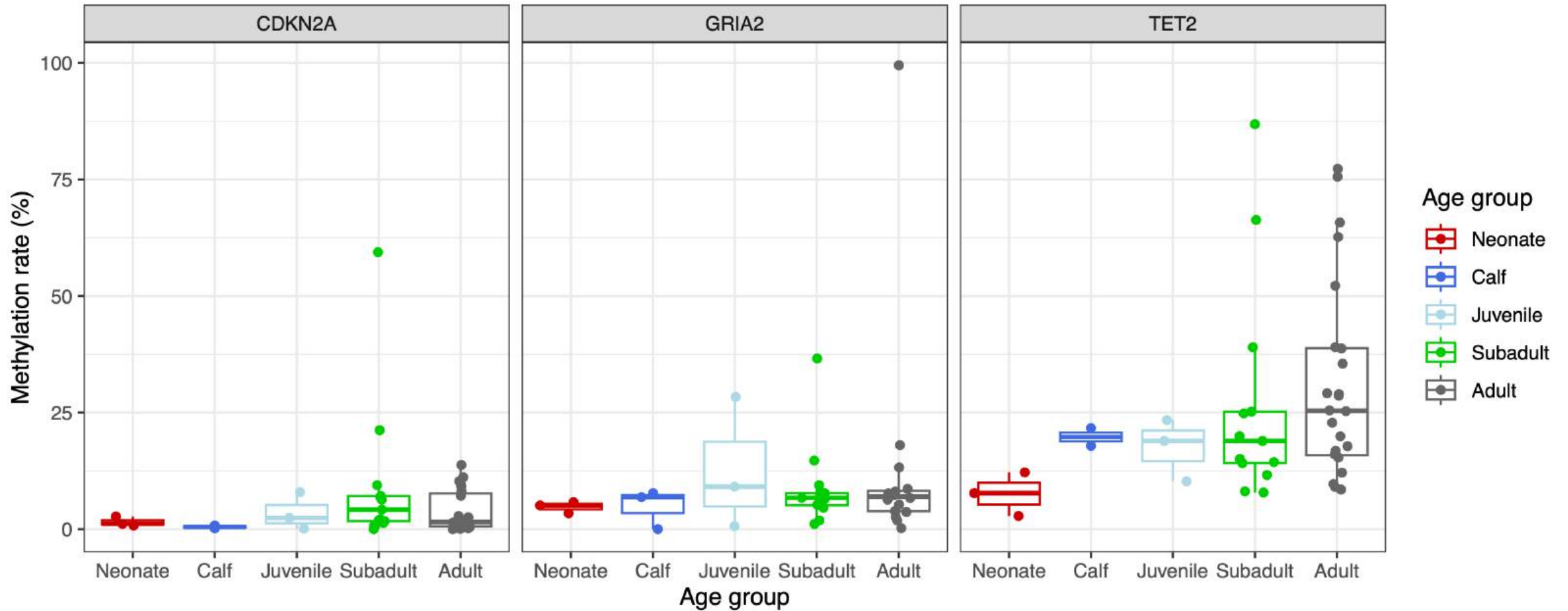
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Stranded finless porpoise individuals collected in Hong Kong coastal regions
(In total, $n = 64$; but some metadata is lacking from several of them)



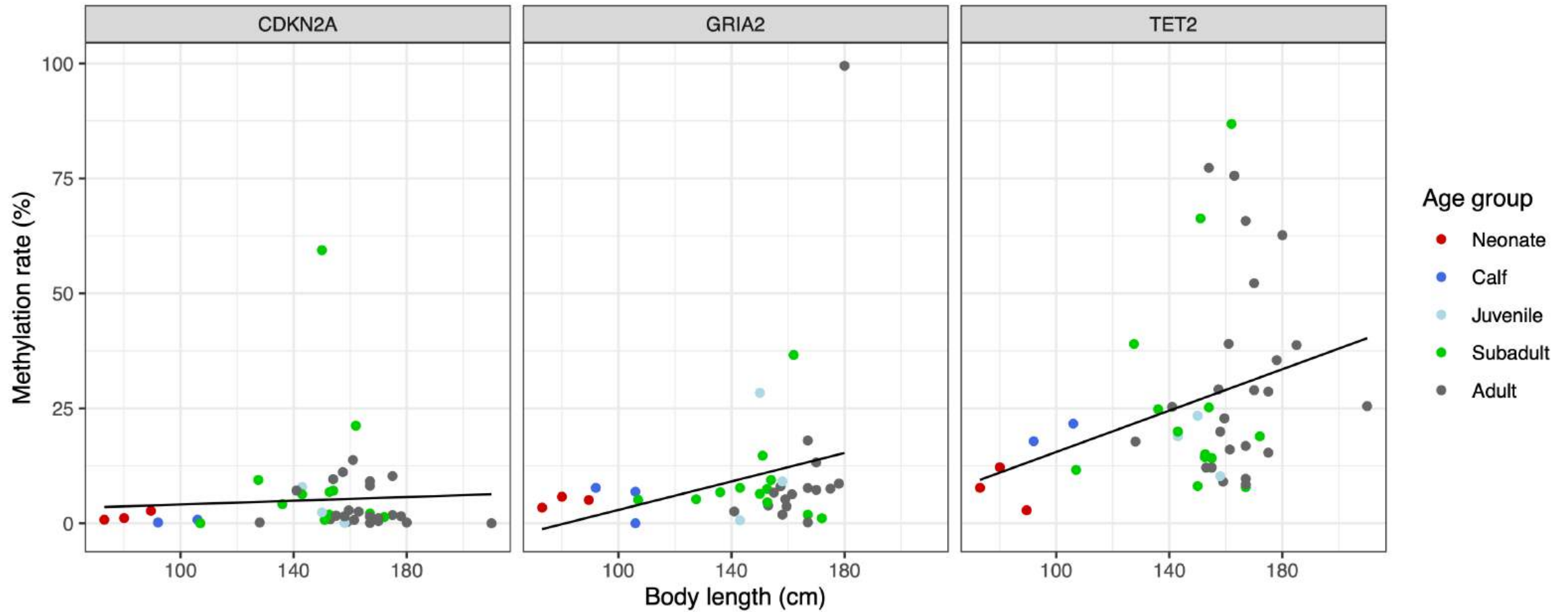
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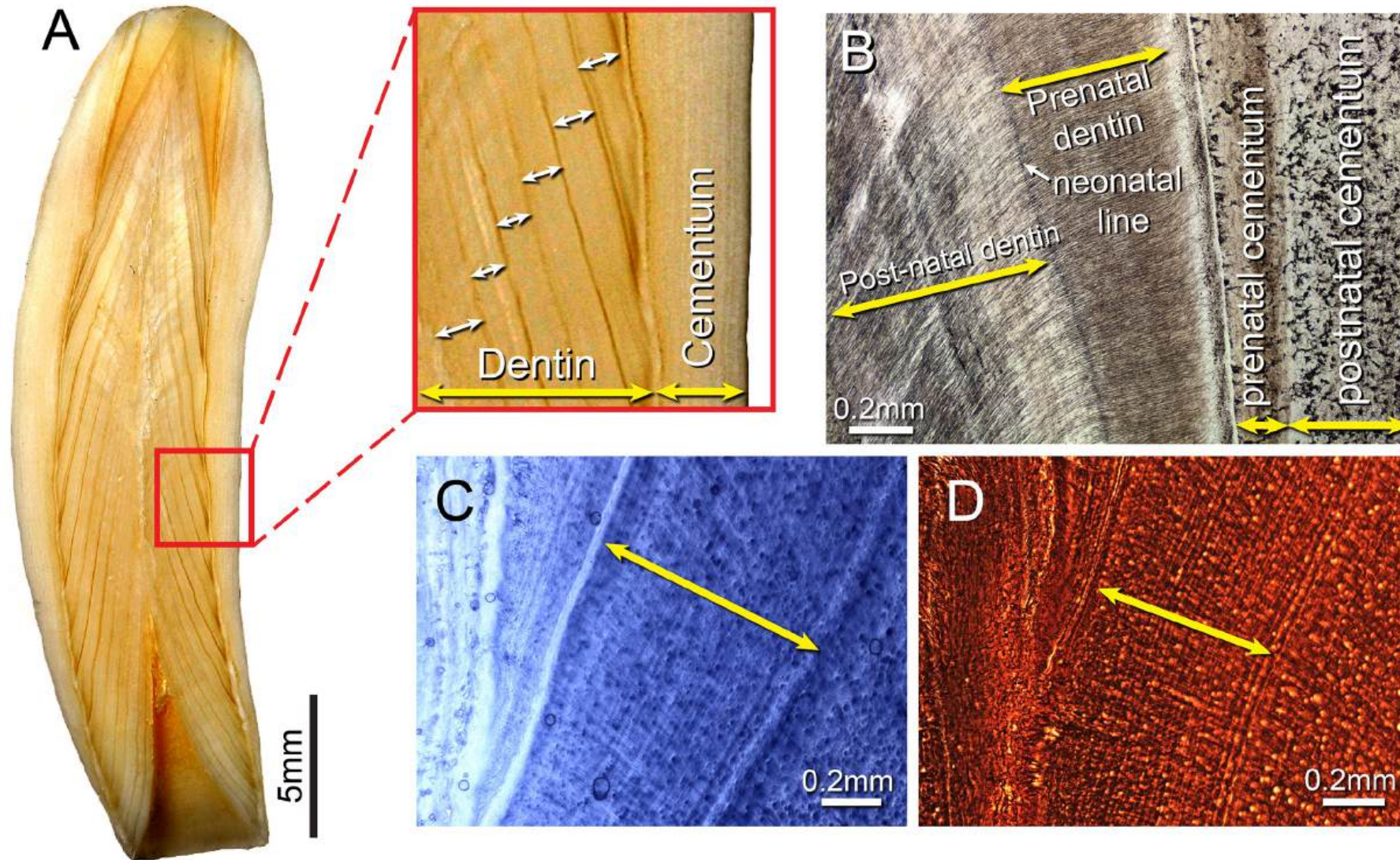


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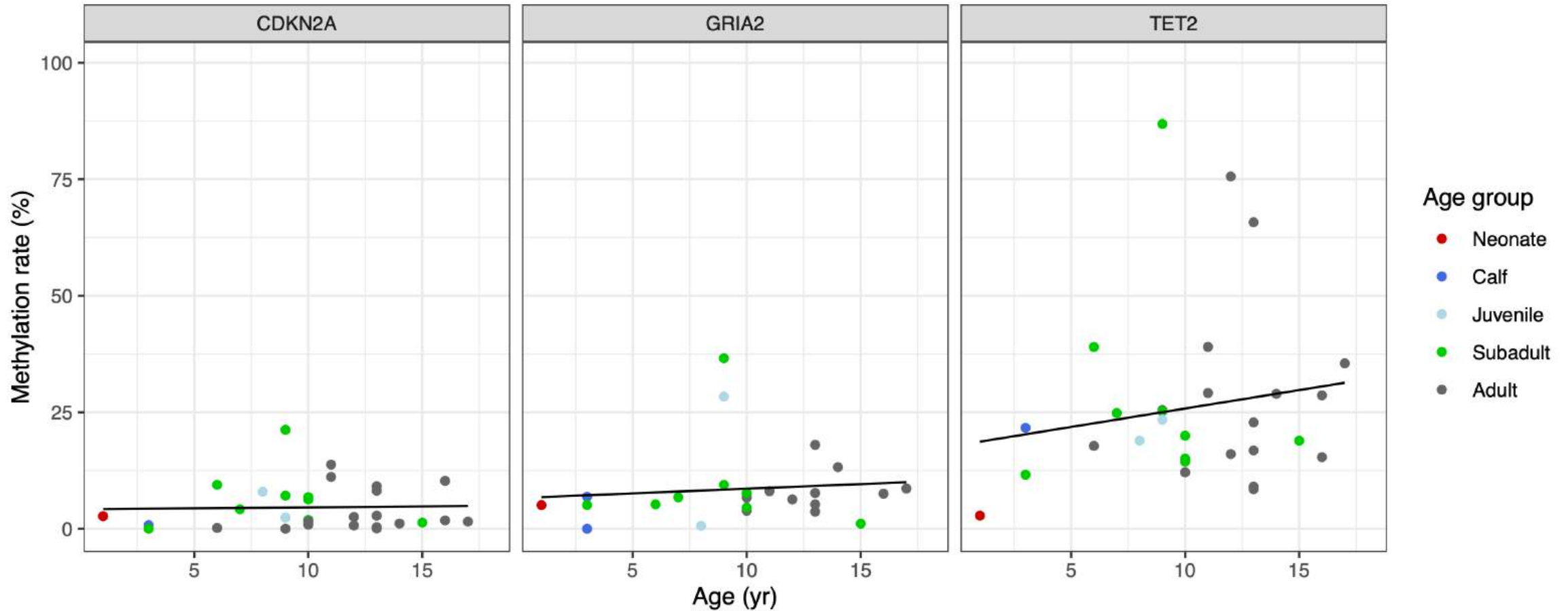
Growth Layer Groups (GLGs)



Beluga (*Delphinapterus leucas*)

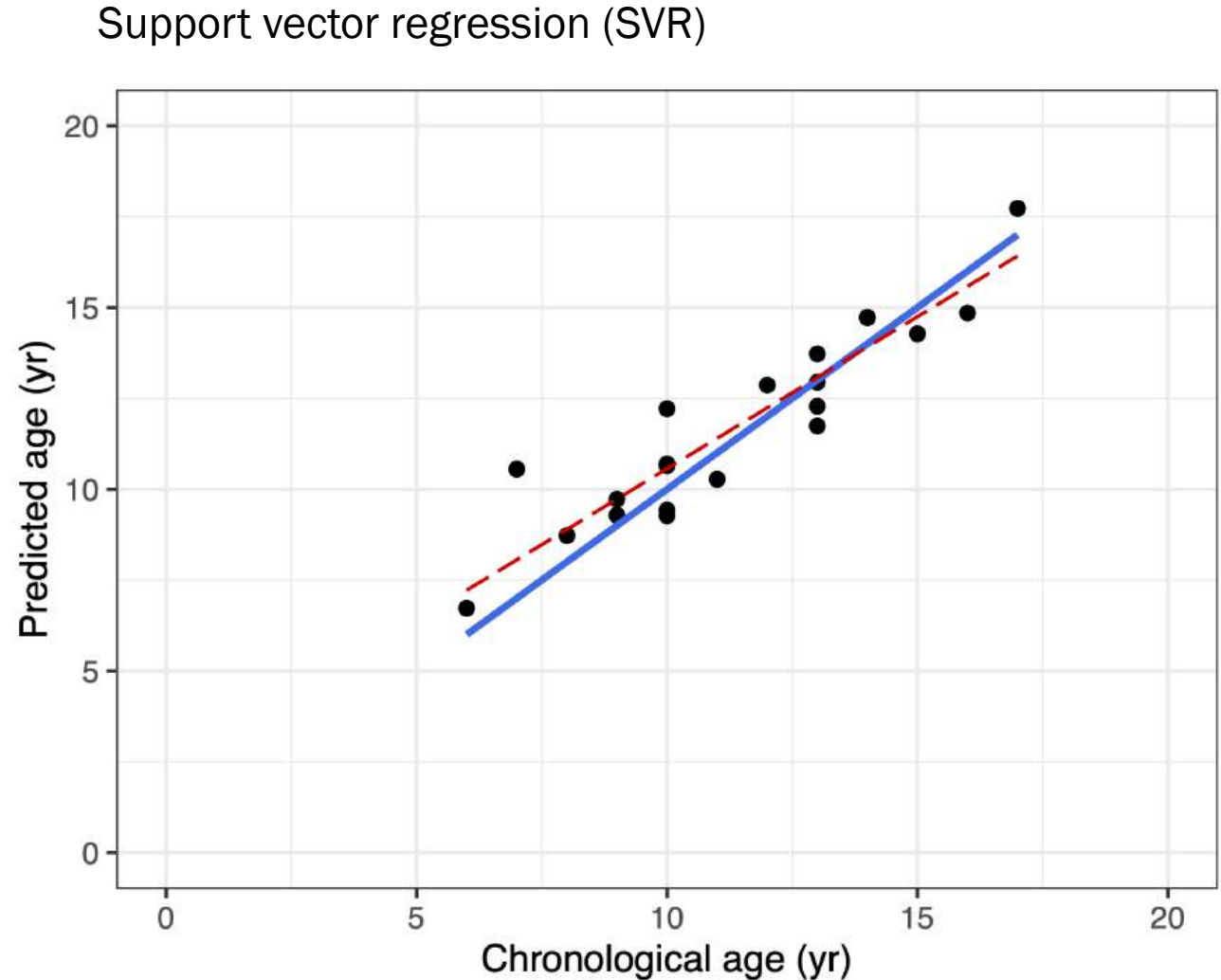
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- Support Vector Regression (SVR) was applied to capture nonlinear relationship between ages and DNA methylation rates.
- Model: Age $\sim$ Three genes
- Chronological ages can be accurately predicted from DNA methylation rates



Conclusions

1. We have developed qPCR and eDNA metabarcoding methods to detect cetaceans in Hong Kong waters.
2. The performance of all the primers and probes was carefully evaluated and applied to natural seawater samples.
3. Resident cetaceans were successfully detected from seawater samples.
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Acknowledgements

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- HKUST and OCES
- Ocean Park and AFCD in HK



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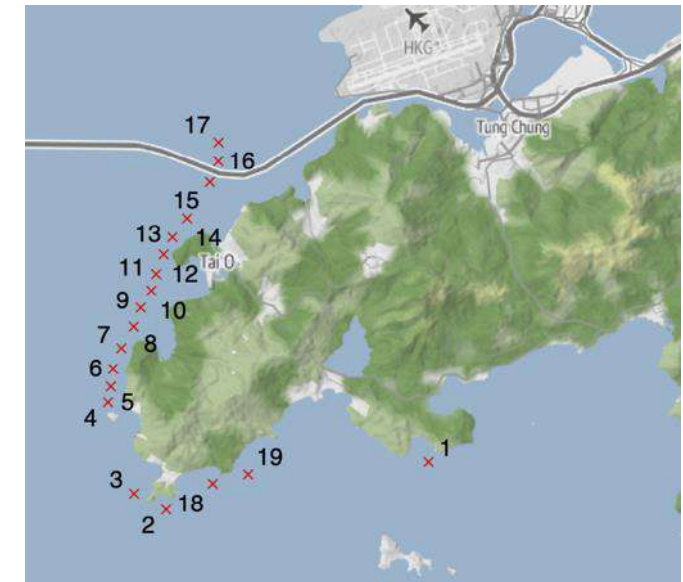
Lindsay Portor

Metabarcoding project
Masaki Miya, Tetsuya Sado
Sachie Ozawa, Oka Shin-Ichiro

qPCR project
Satsuki Tsuji

DNA methylation project
Genfu Yagi, Xia Fei
Itsuki Hirayama, Yuen Wa Ho (Derek)

Understanding **cetacean** ecology from water samples: **Cetacean environmental DNA** studies in Hong Kong waters



Masayuki USHIO ushio@ust.hk

OCES, The Hong Kong University of Science and Technology (HKUST)



Cetacean eDNA studies in Hong Kong

Cetaceans and their roles in ecosystems

- **Cetaceans** are large aquatic mammals, which are often iconic and flagship species in marine ecosystems.
- **Cetaceans** play essential roles in maintaining the marine ecosystem functions and services, such as **nutrient cycling**, **trophic cascades**, and **eco-tourism** (Roman & McCarthy 2010; Estes et al. 2011; Kiszka et al. 2022).



Cetaceans are highly threatened

- Megafauna in coastal ecosystems are highly threatened.
- However, conservation of these cetaceans and other megafauna is often neglected as a key component in the conservation scheme, which is referred to as the “**megafauna gap**” (Li et al. 2023 *Science Advances*).
- Thus, efficient and effective cetacean monitoring methods are needed.

SCIENCE ADVANCES | RESEARCH ARTICLE

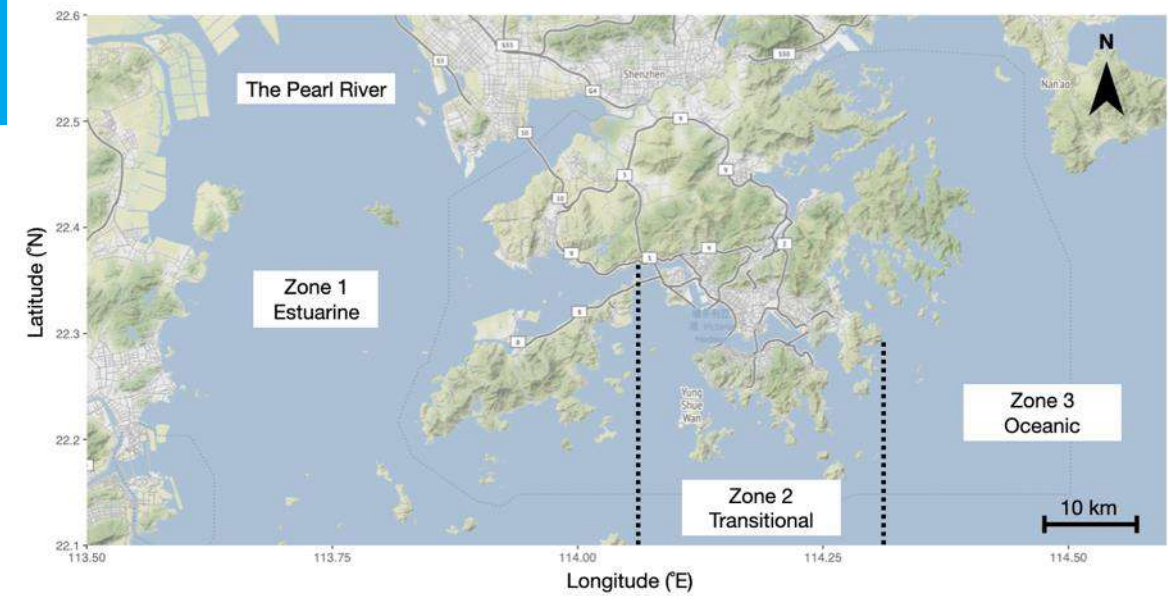
APPLIED ECOLOGY

A wide megafauna gap undermines China's expanding coastal ecosystem conservation

Xincheng Li¹, Hanchen Wang¹, Douglas J. McCauley², Andrew H. Altieri³, Brian R. Silliman⁴, Jonathan S. Lefcheck^{5,6}, Jihua Wu⁷, Bo Li⁸, Qiang He^{1*}

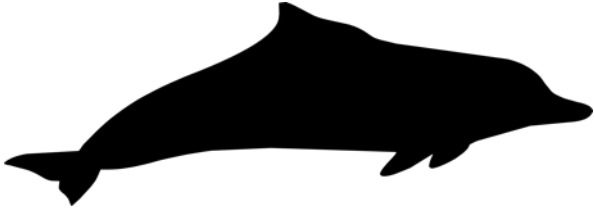
HK resident cetaceans

Two resident cetaceans in HK waters
(they are in HK waters all year around)



HK resident cetaceans: Visual monitoring survey

Chris huh (CC BY-SA 3.0)

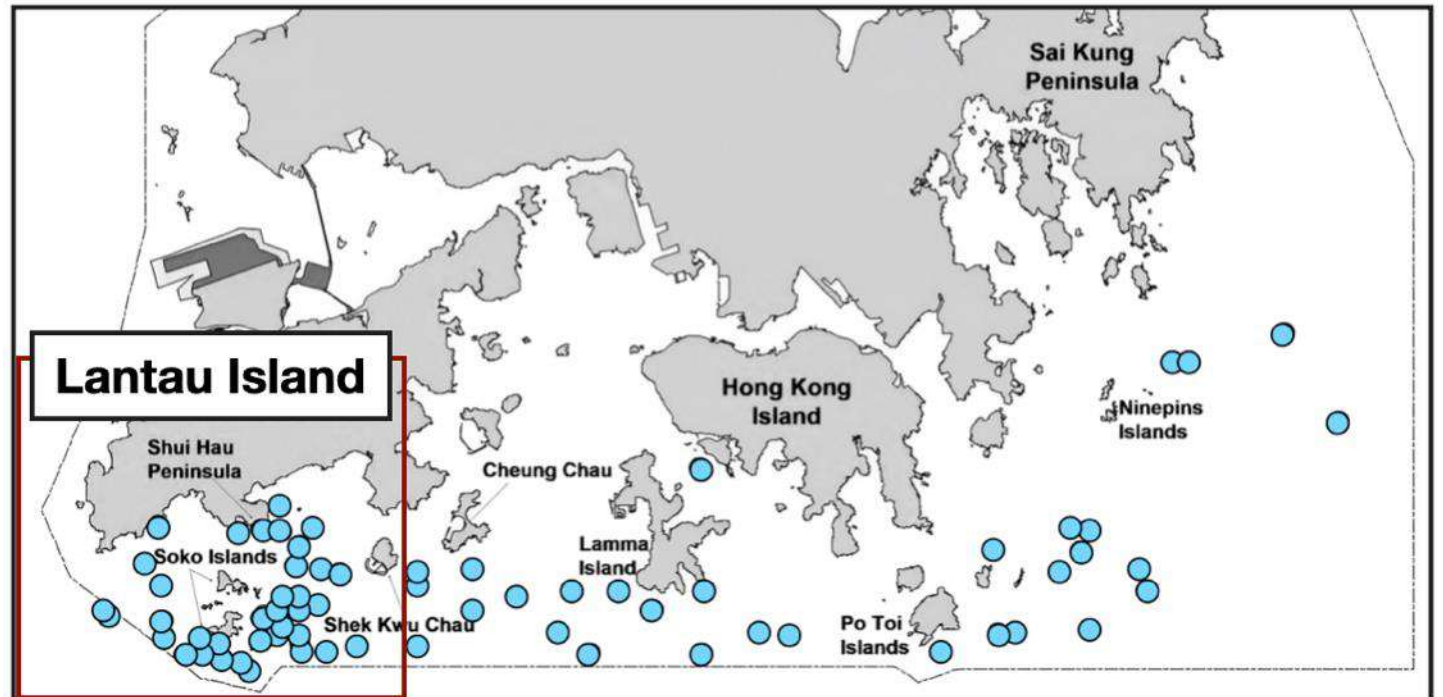
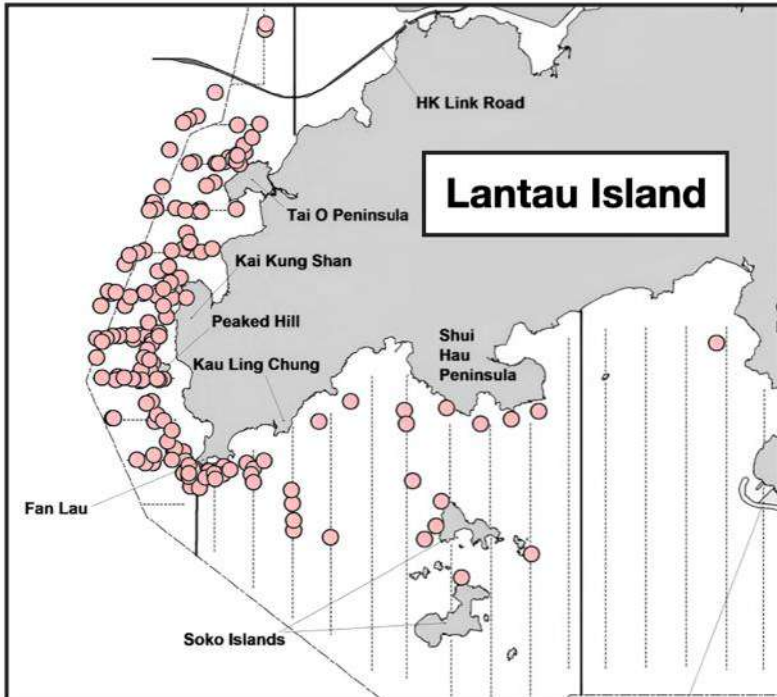


Chinese White Dolphin
(*Sousa chinensis*)

Chris huh (CC BY-SA 3.0)



Finless porpoise
(*Neophocaena phocaenoides*)



HK residential cetaceans

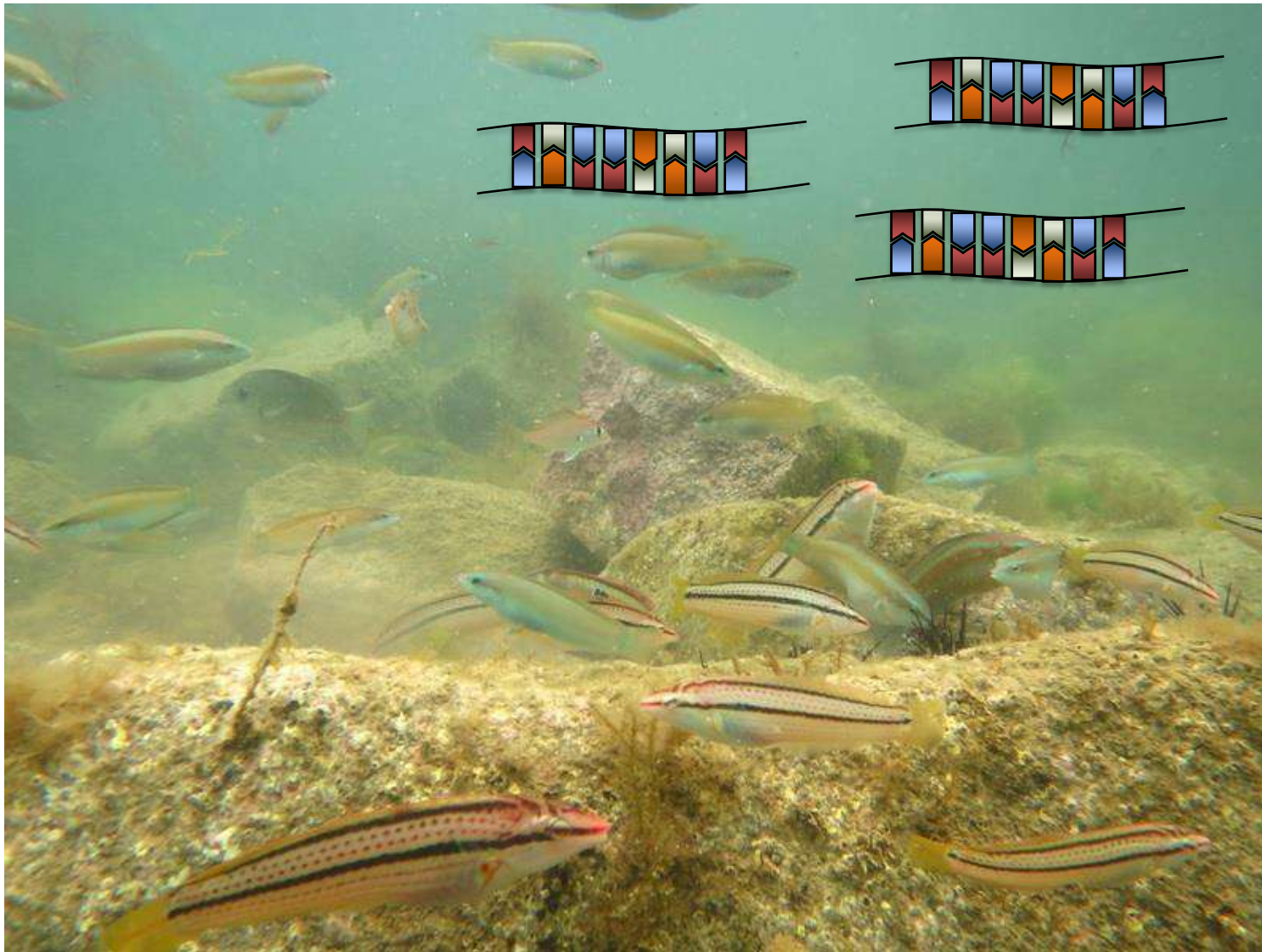


- In Tai O village, there are dolphin-watching boat (40HKD/each for 20 min).
- Chinese White Dolphin is important for eco-tourism, too.



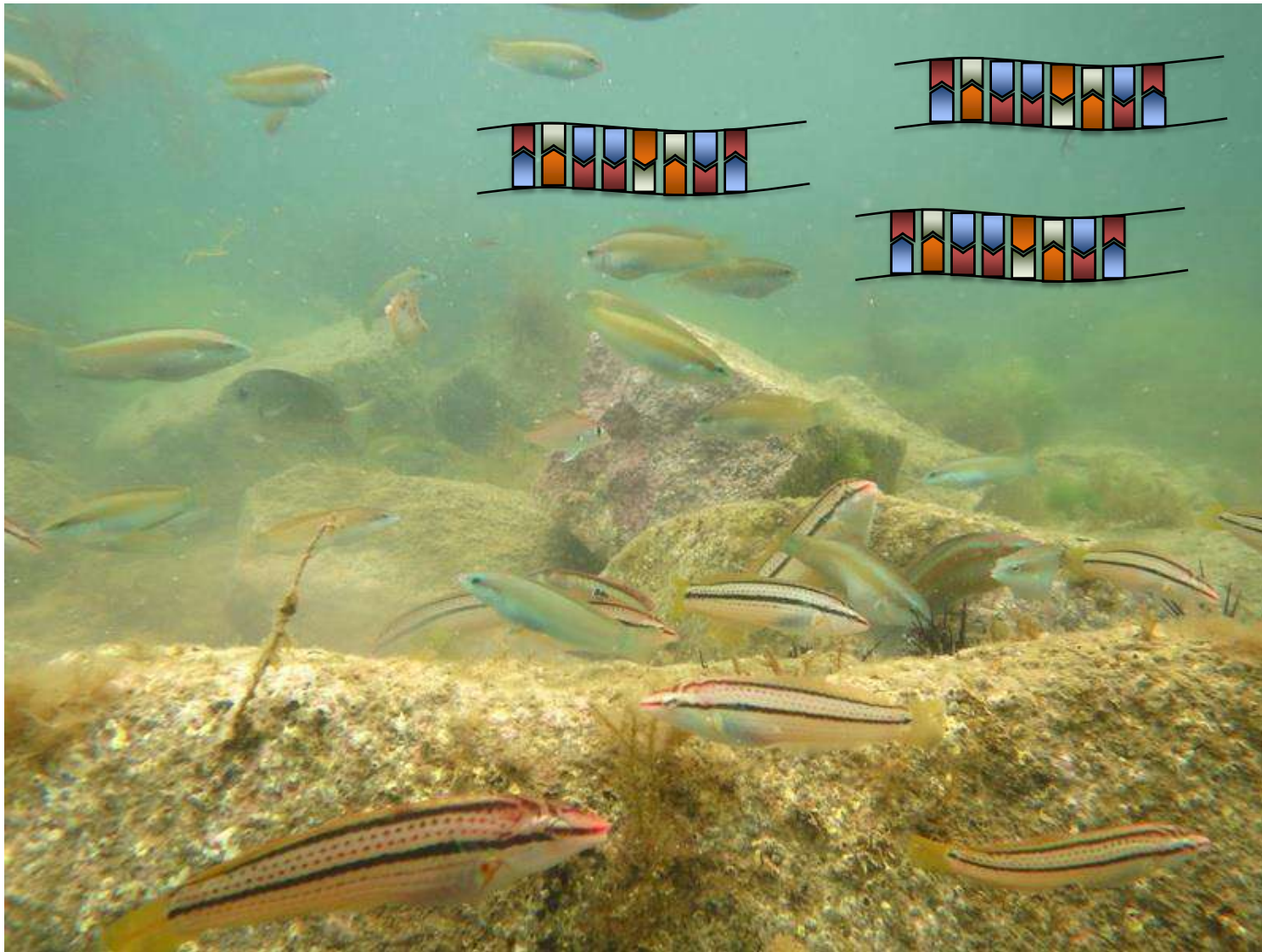
Environmental DNA analysis to complement traditional survey methods

Environmental DNA analysis



- **Definition:** “Environmental DNA (eDNA) is a complex mixture of genetic DNA from many different organisms found in an environmental sample” (Taberlet et al. 2012, 2018)
- eDNA can be extracted from soil, sediment, water, air, feces, and so on.
- Some studies include intracellular DNA (e.g., microbes) (= broad definition) and others do not (narrow definition)

Environmental DNA analysis



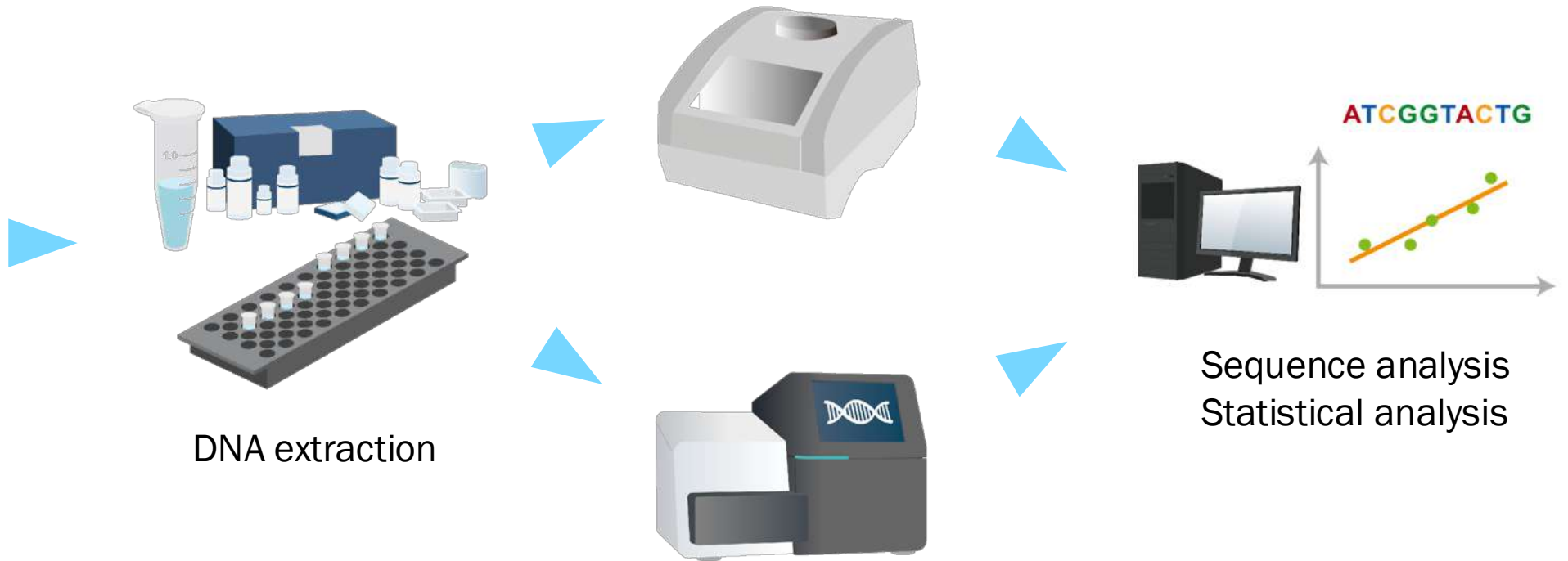
- **eDNA analysis:**
- Highly sensitive
- Low time- and labor-cost in field (e.g., we just need to collect water samples)
- We do not need taxonomic identification skill

Environmental DNA analysis

Two major methods



Collect samples
(e.g., water filtration)



Quantitative PCR

Analyze **single or a few species**, but can quantify DNA concentrations

eDNA metabarcoding

Analyze **many species**, but lower quantitative capacity

Sequence analysis
Statistical analysis

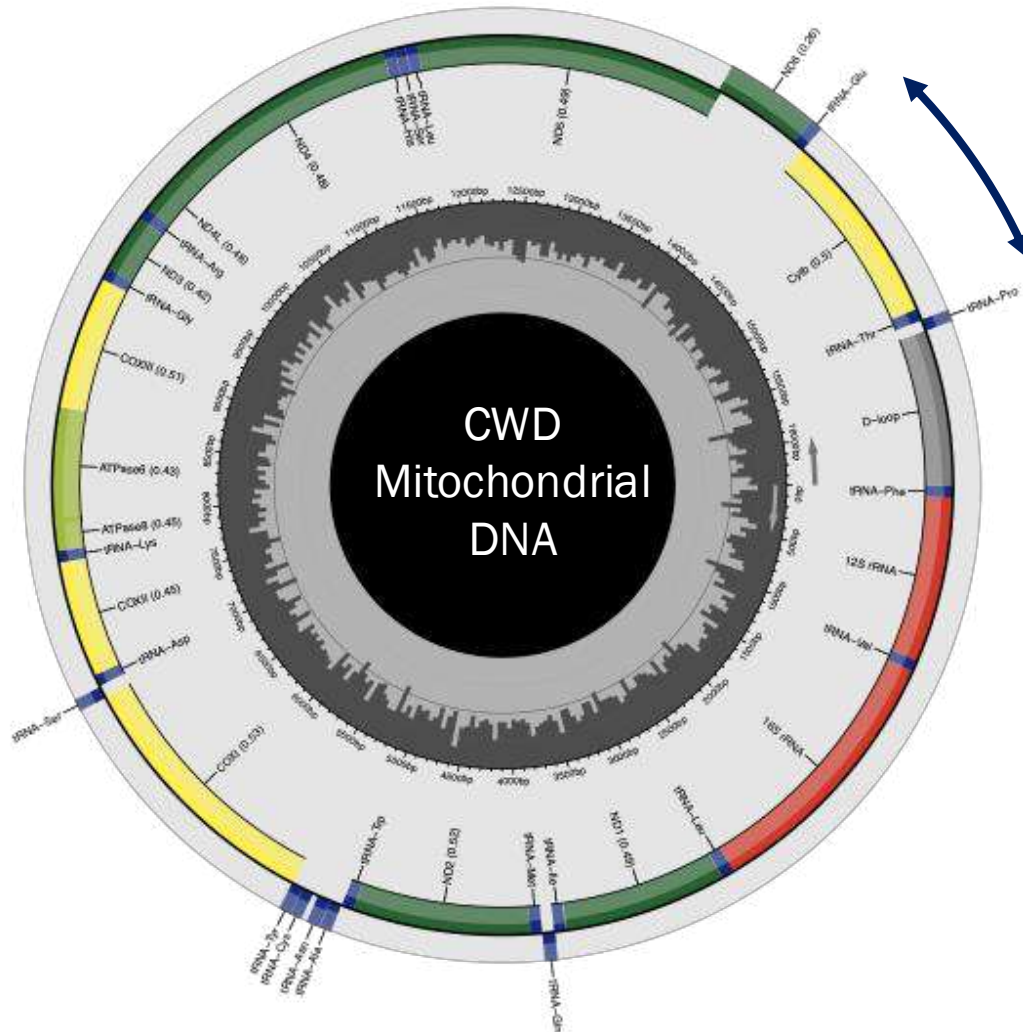
Quantitative PCR

Analyze **single or a few species**, but can quantify DNA concentrations



qPCR-based detection of HK cetaceans

qPCR primers: Chinese white dolphins and Finless porpoises



qCWD-F: TCGCTTTCCACTTTATCCTTCCC

qCWD-R: GATGTCTTTGATTGTATAATAGGGGTGA

qCWD-P: AGCAGCCGTTACCTGCTATTCCTACAT

LOD = 3.64 copies/reaction (95% detection prob.)

LOQ = 69 copies/reaction (< 35% C.V.)



Kiser et al. (2025) *bioRxiv*

<https://doi.org/10.1101/2025.10.30.685690>

Nas-Cytb-F: TACGTTCTACCCTGAGGGCAA Hashimoto et al.

Nas-Cytb-R: GCTTTGTCTACAGAGAACCCGC (2024)

Nas-Cytb-P: TGTCAGCAATCCCTTATATTGGCAGCACAC

LOD = 3.0 copies/reaction (95% detection prob.)

LOQ = 34 copies/reaction (< 35% C.V.)



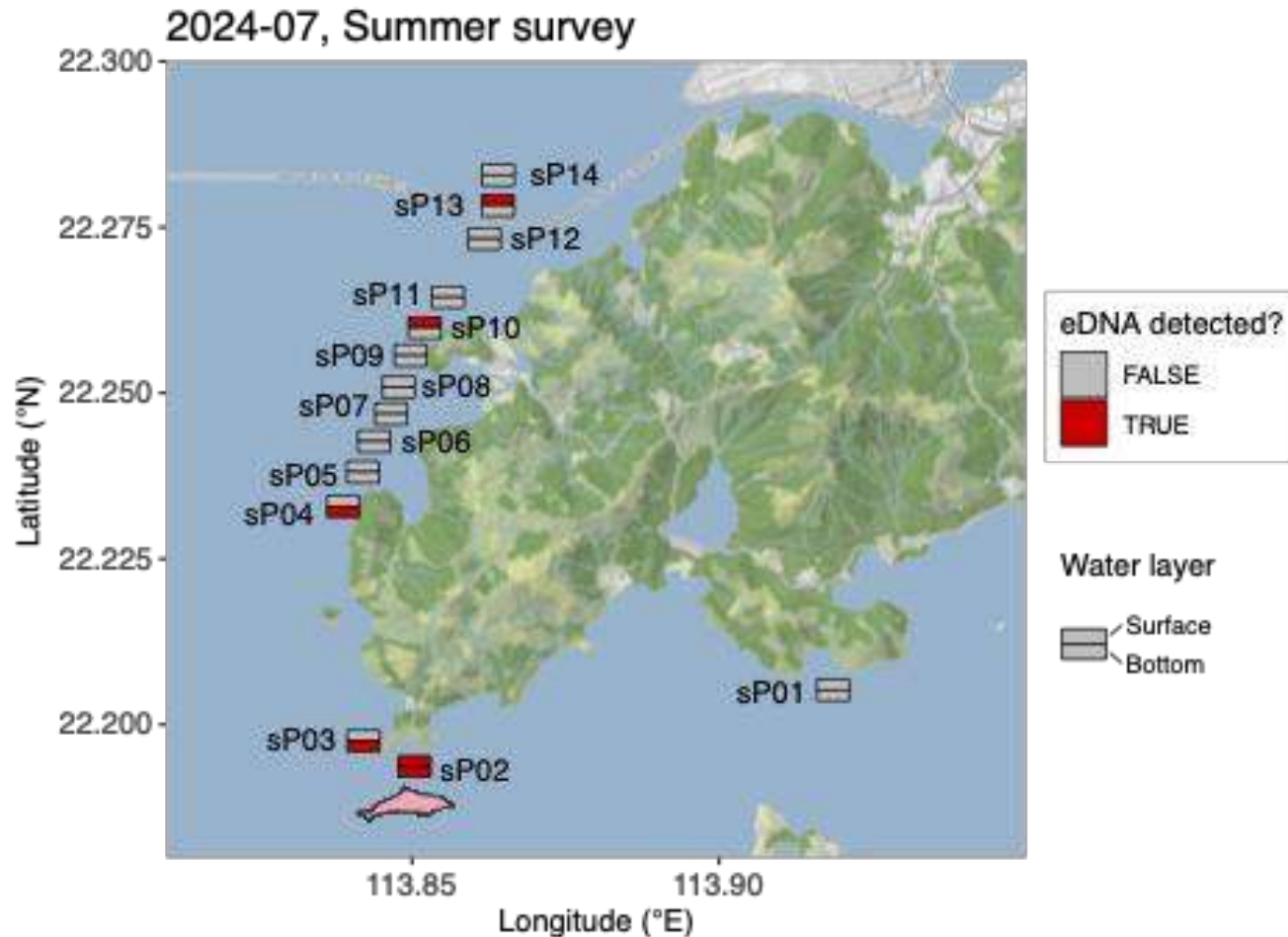
Lin & Ushio (2025) *bioRxiv*

<https://doi.org/10.64898/2025.12.21.695310>

qPCR: Results of Chinese white dolphins

Lantau Island survey: Summer (July 2024) and winter (Jan 2025)

1 L of seawater, gravity filtration, bottom and surface water, eDNA extracted using B&T kit



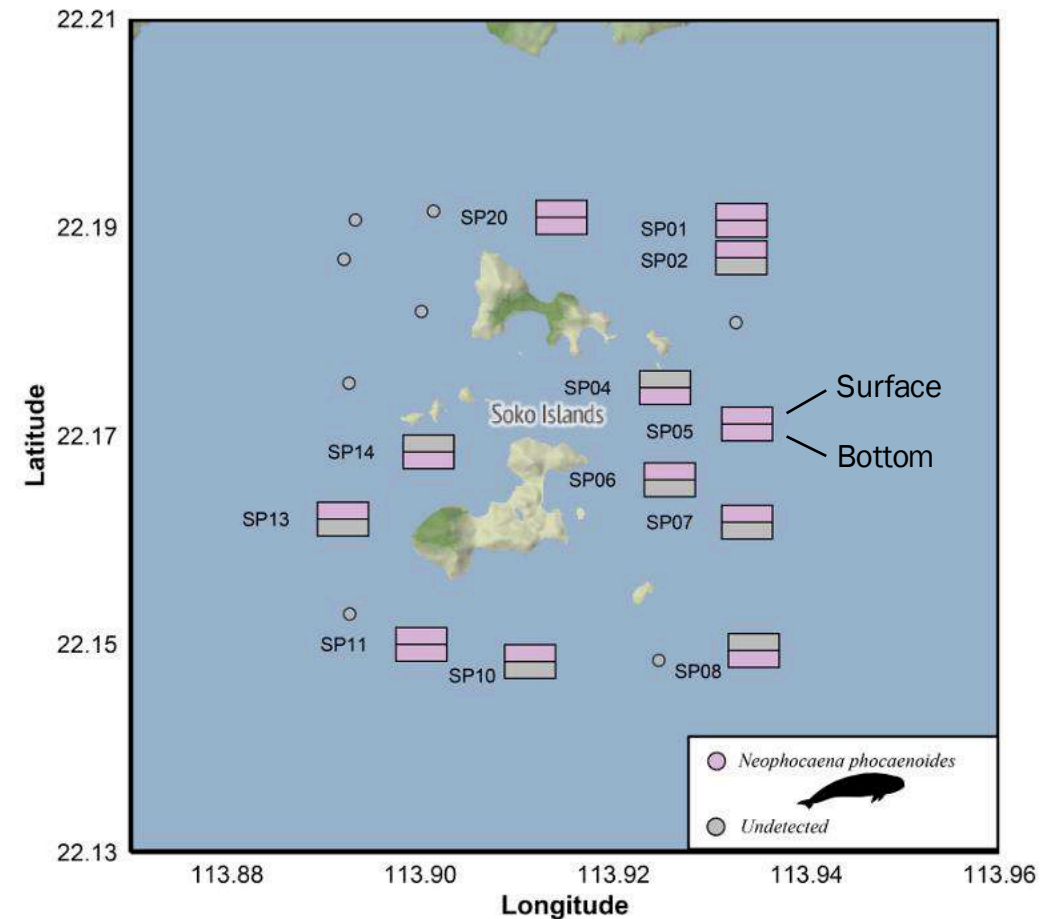
qPCR: Results of Indo-Pacific finless porpoises

Soko Islands survey: Spring survey (Mar 2025)

1 L of seawater, gravity filtration, bottom and surface water, eDNA extracted using B&T kit



Xiaoqi Lin



qPCR surveys

- The primers' performance was evaluated very carefully (specificity, LOD, and LOQ).
- The primer sets successfully detected eDNA of Chinese white dolphins and finless porpoises.
- All detected values were below LOQ (Limit of Quantification), suggesting that cetacean eDNA concentrations in natural environments can be very low (but still detectable).
- We need further refinements to the methods, e.g., water sampling volume, water layer, etc.



eDNA metabarcoding

Analyze **many species**, but lower quantitative capacity

eDNA metabarcoding-based detection

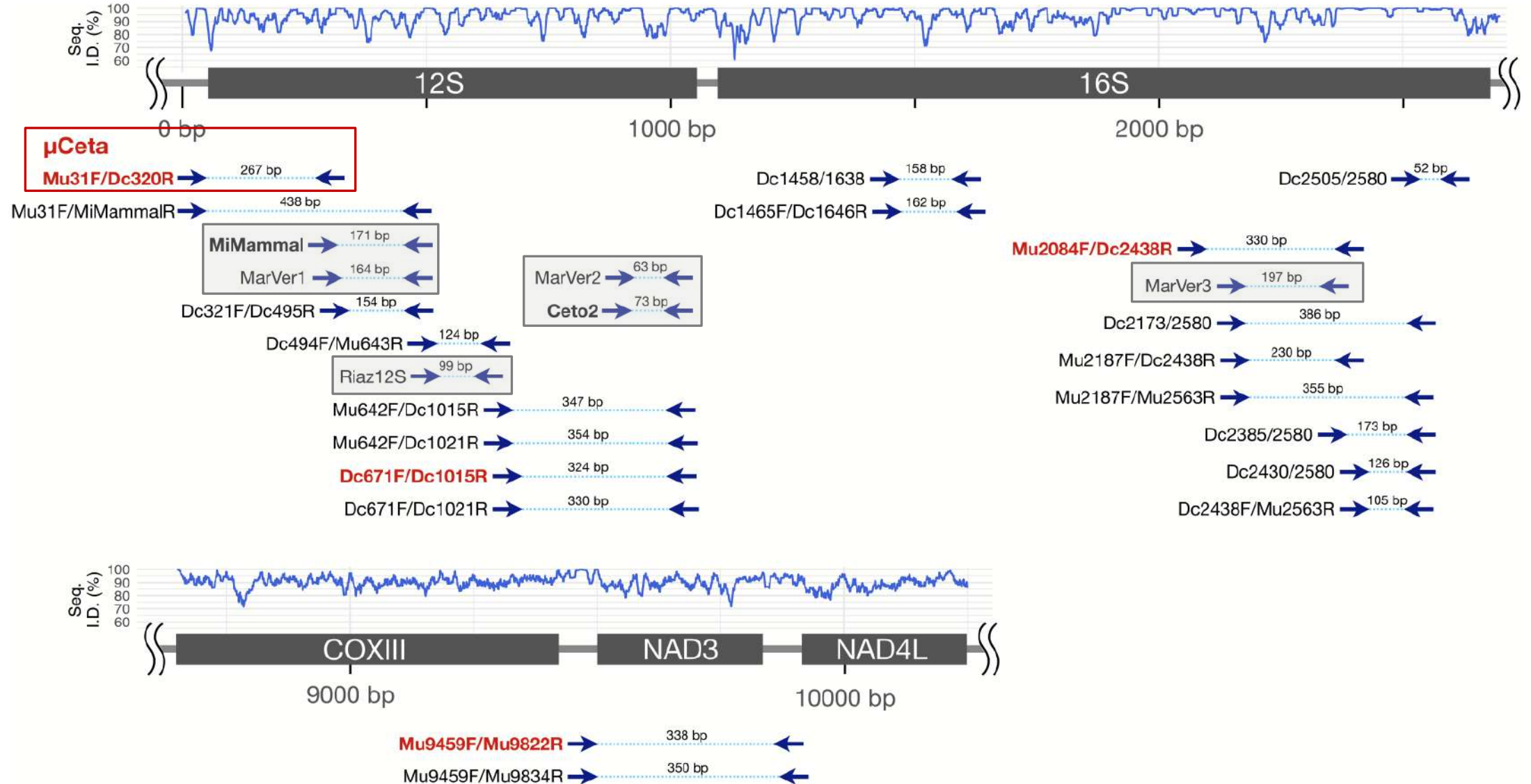


μCeta: A Set of Cetacean-Specific Primers for Environmental DNA Metabarcoding With Minimal Amplification of Non-Target Vertebrates

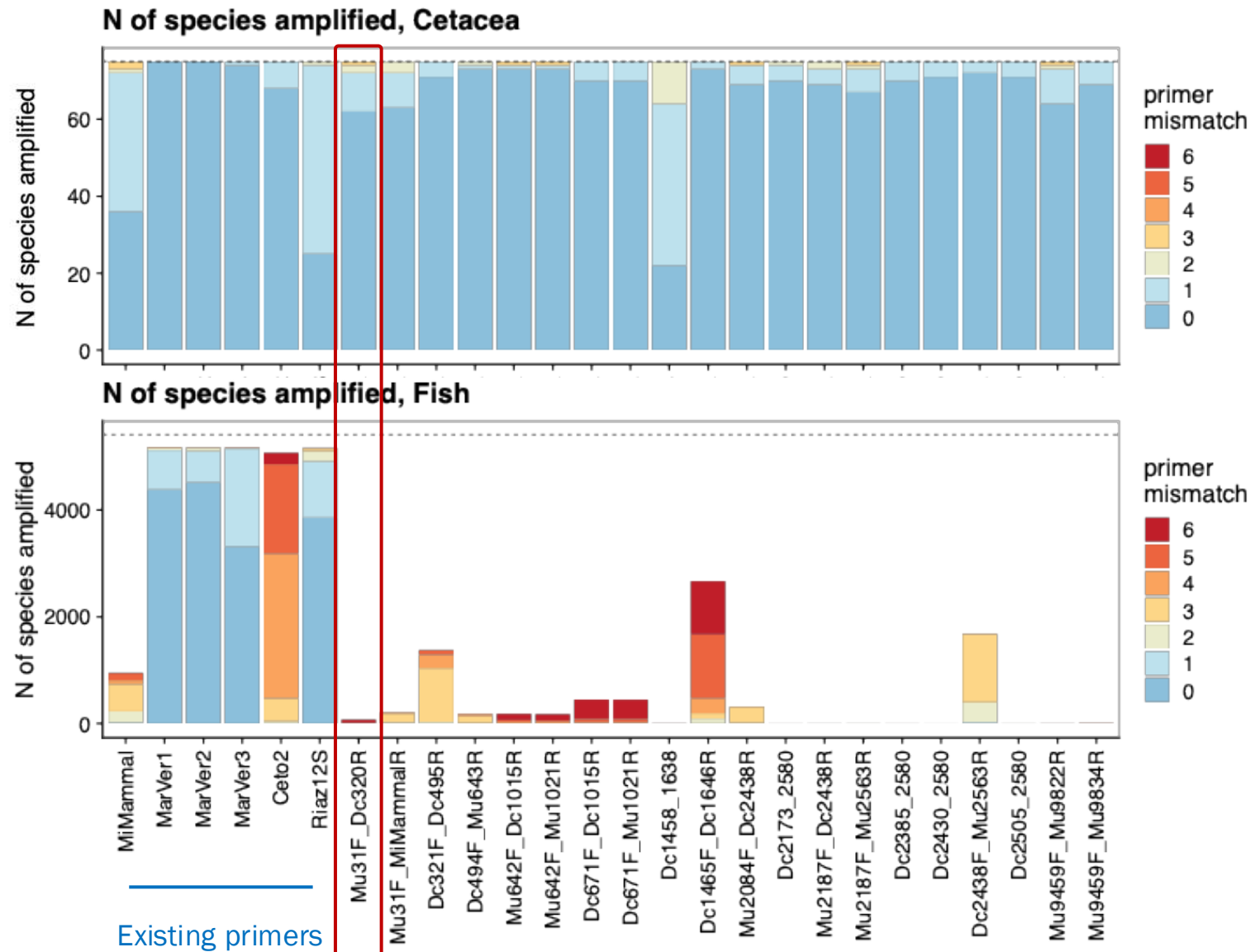
Masayuki Ushio¹  | Sachie Ozawa² | Shin-ichiro Oka² | Tetsuya Sado³ | Robinson O. Kiseru¹ | Lindsay Porter⁴ | Eszter Matrai⁵ | Masaki Miya³

¹Department of Ocean Science, The Hong Kong University of Science and Technology, Kowloon, Hong Kong SAR, China | ²Okinawa Churashima Foundation, Motobu-cho, Okinawa, Japan | ³Natural History Museum and Institute, Chiba, Chiba, Japan | ⁴Southeast Asia Marine Mammal Research, Lippo Centre, Queensway, Hong Kong SAR, China | ⁵Ocean Park Hong Kong, Aberdeen, Hong Kong SAR, China

New cetacean-specific primers for eDNA metabarcoding



New primer development | eDNA metabarcoding



Collection of water samples from aquarium tanks



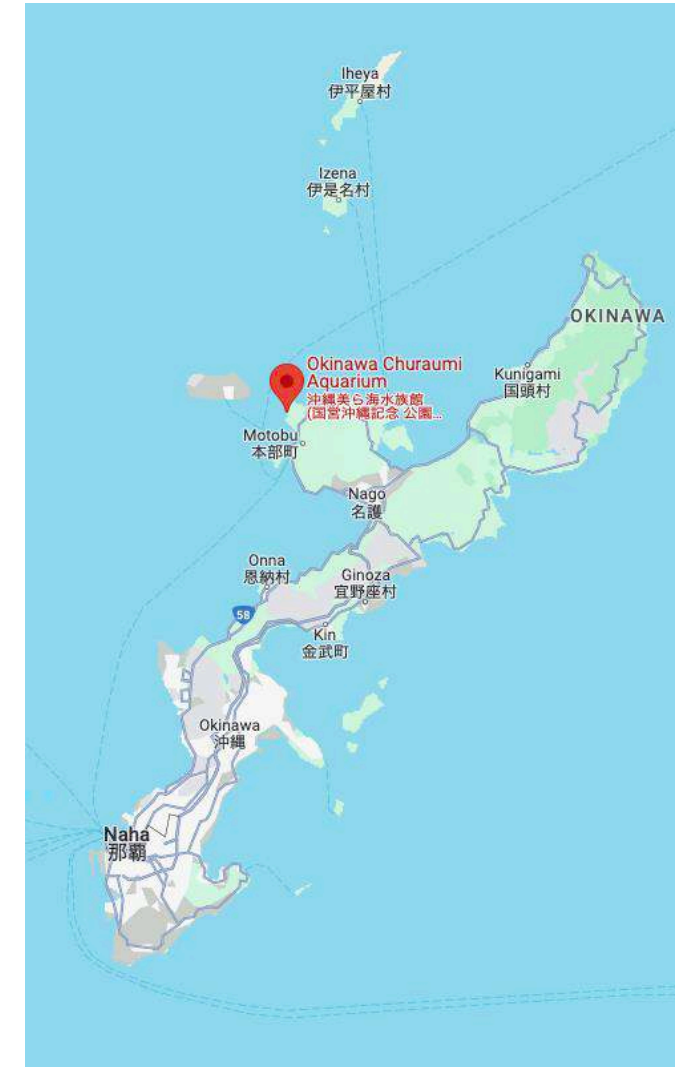
@Okinawa Churaumi Aquarium, Japan

Collection of water samples from aquarium tanks



@Okinawa Churaumi Aquarium, Japan

Aquarium pools with a “known” species composition



Churaumi pool samples



Common bottlenose dolphin
Tursiops truncatus



Indo-Pacific bottlenose dolphin
Tursiops aduncus



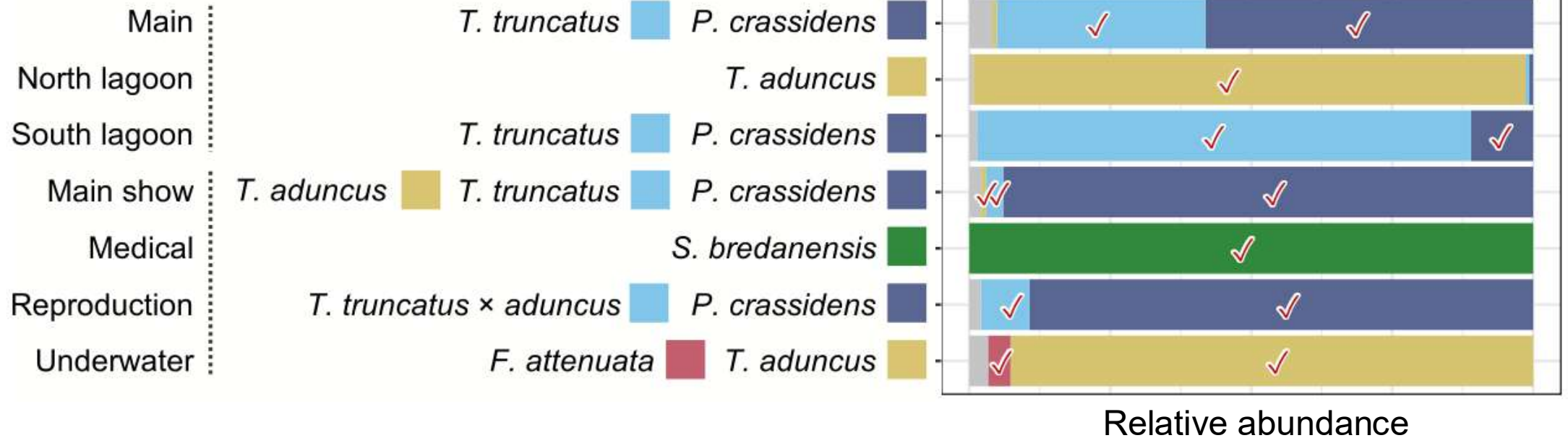
False Killer Whale
Pseudorca crassidens



Pygmy Killer Whale
Feresa attenuata



Rough-toothed dolphin
Steno bredanensis



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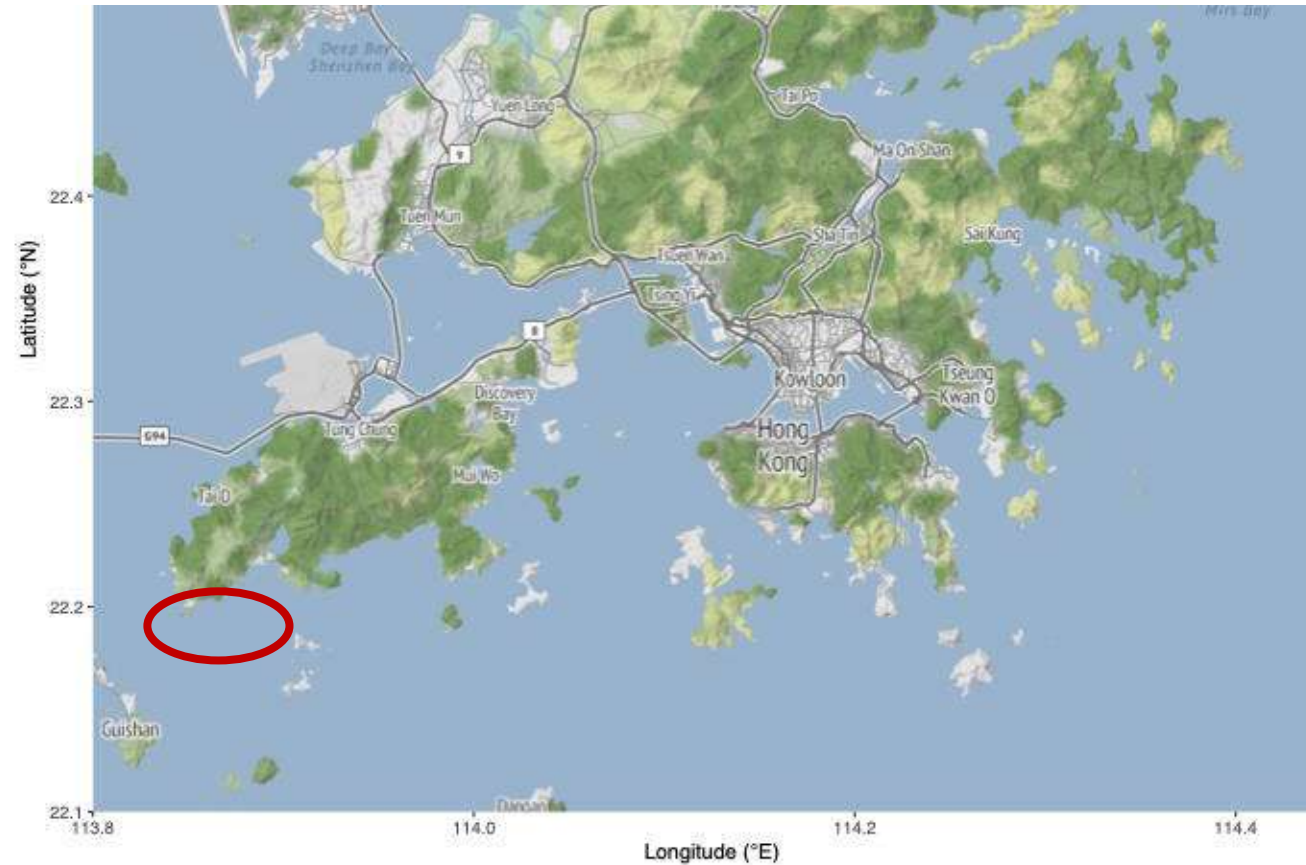
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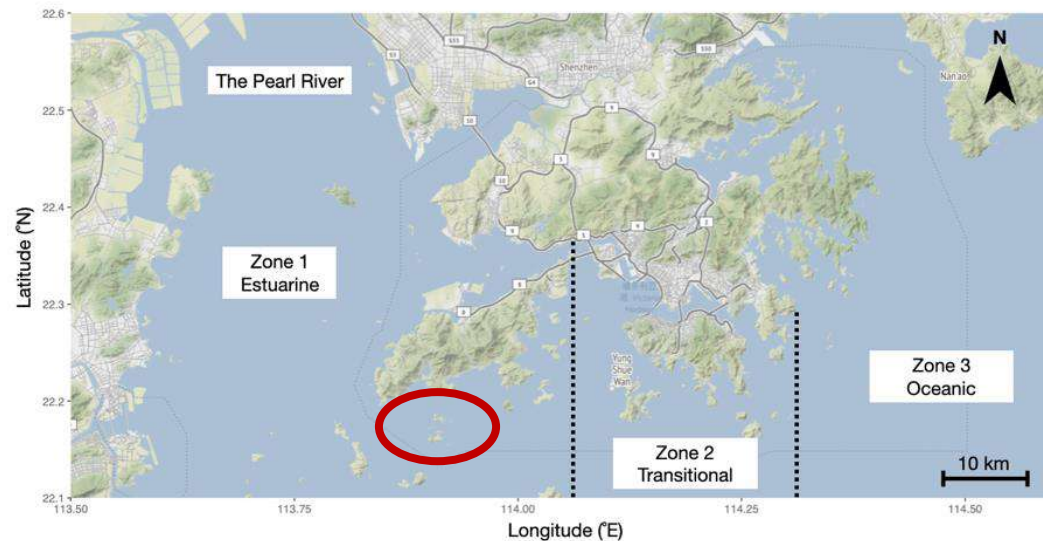
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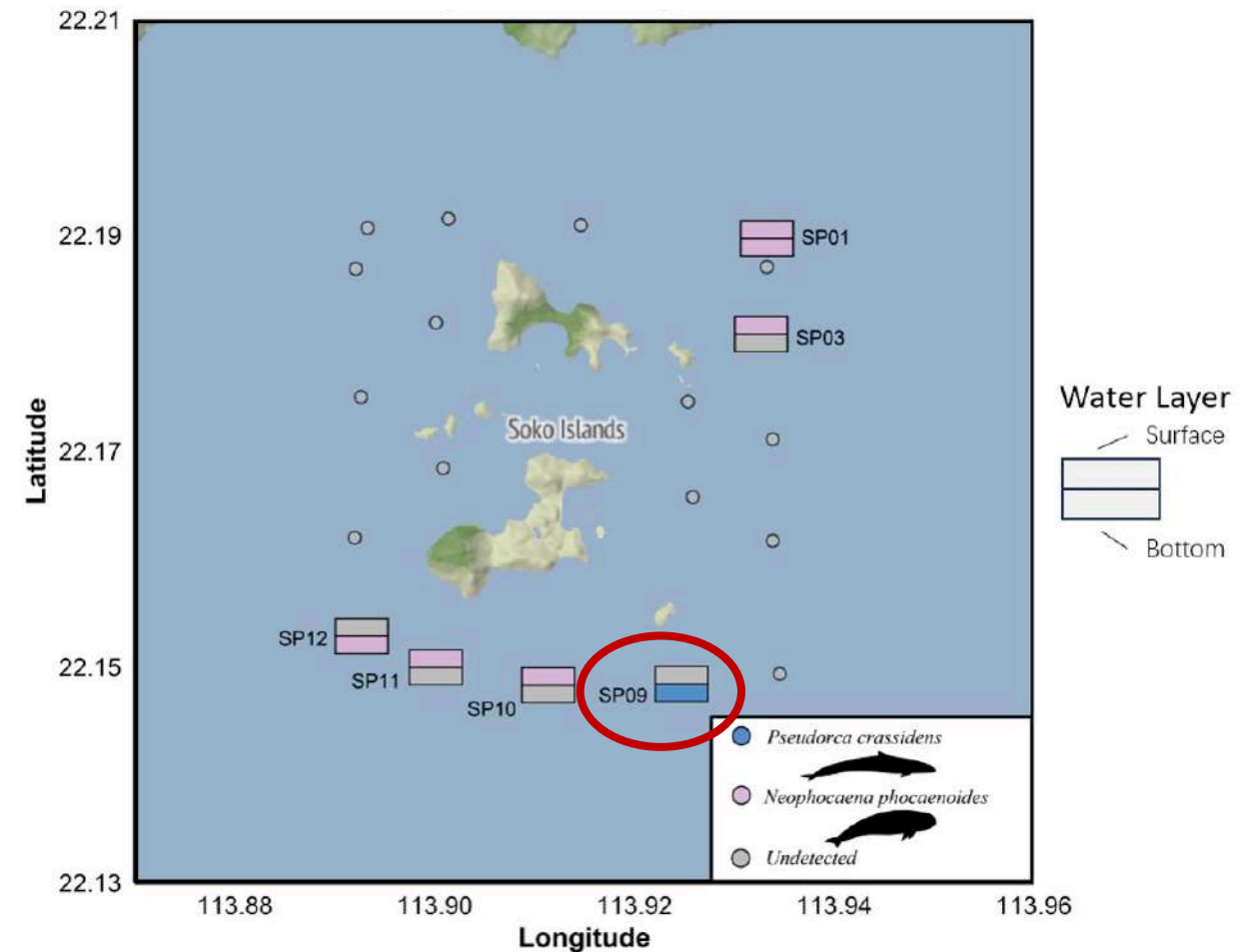
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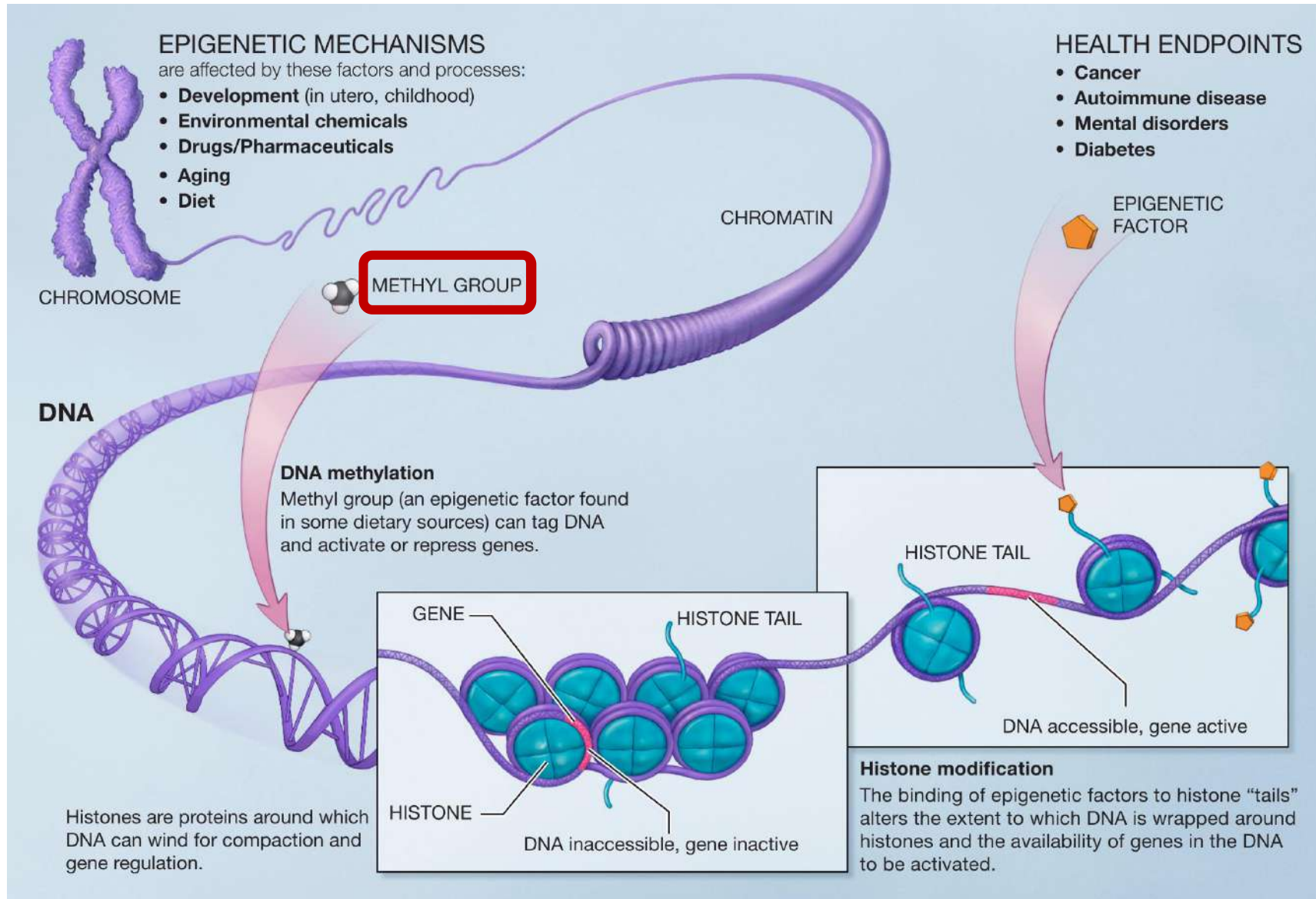


Xiaoqi Lin



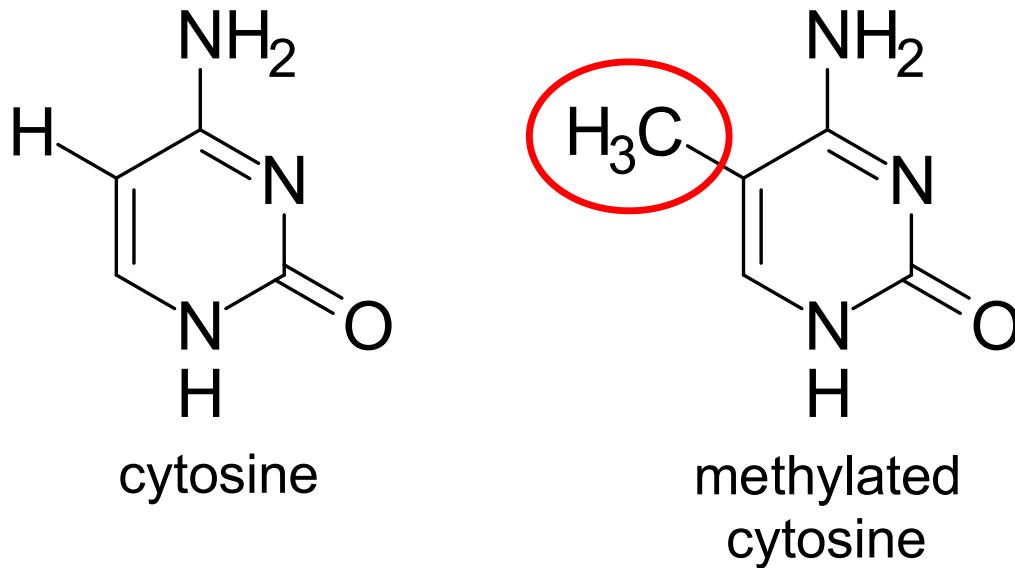
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nature aging



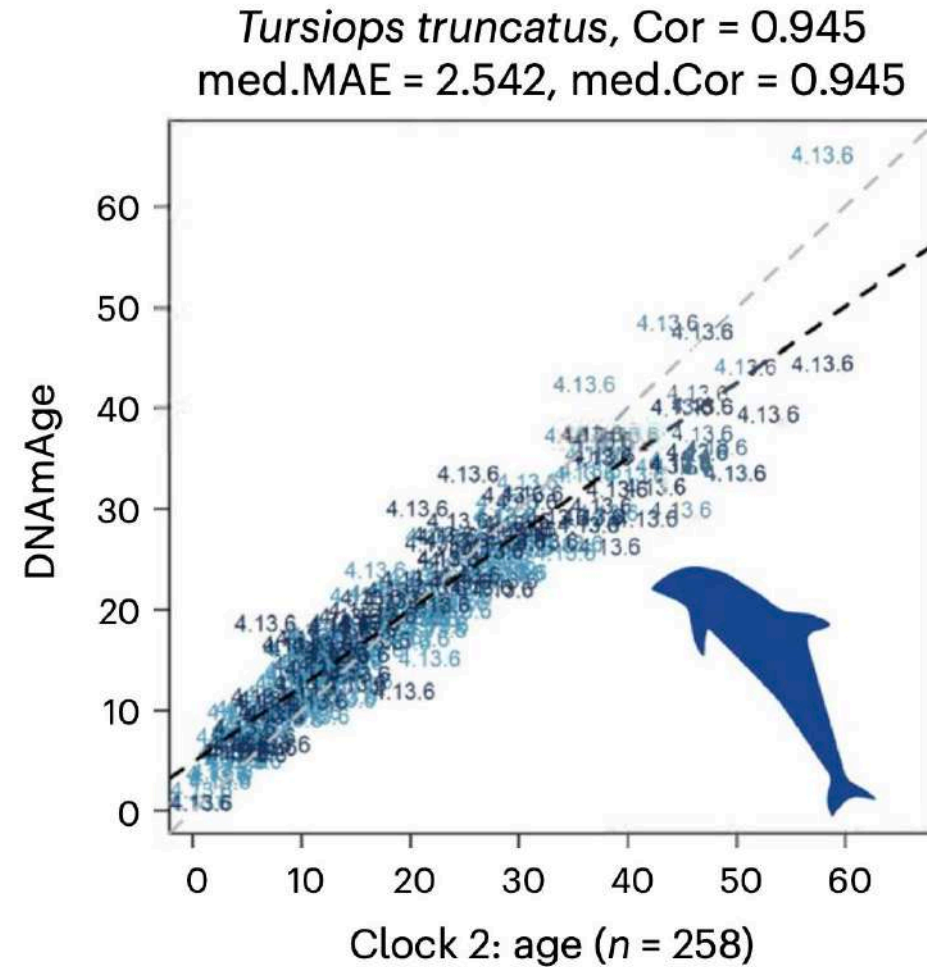
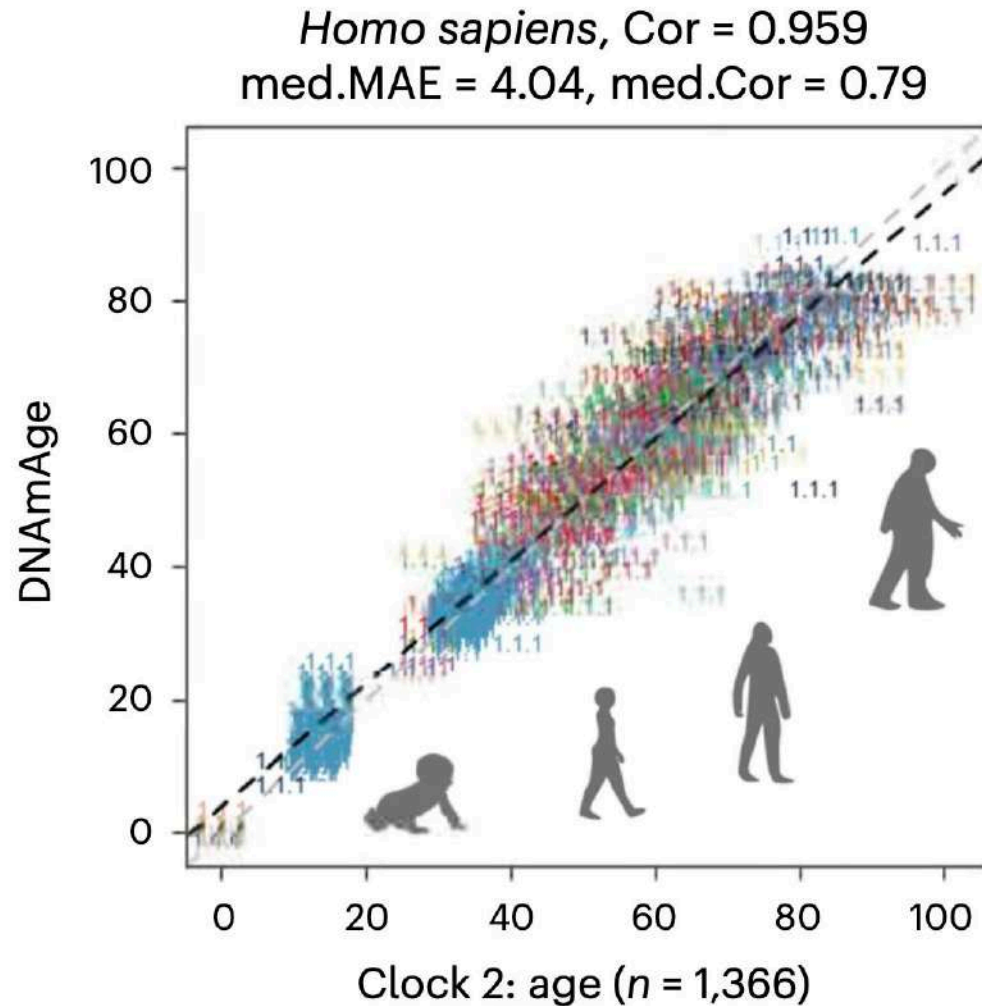
Resource

<https://doi.org/10.1038/s43587-023-00462-6>

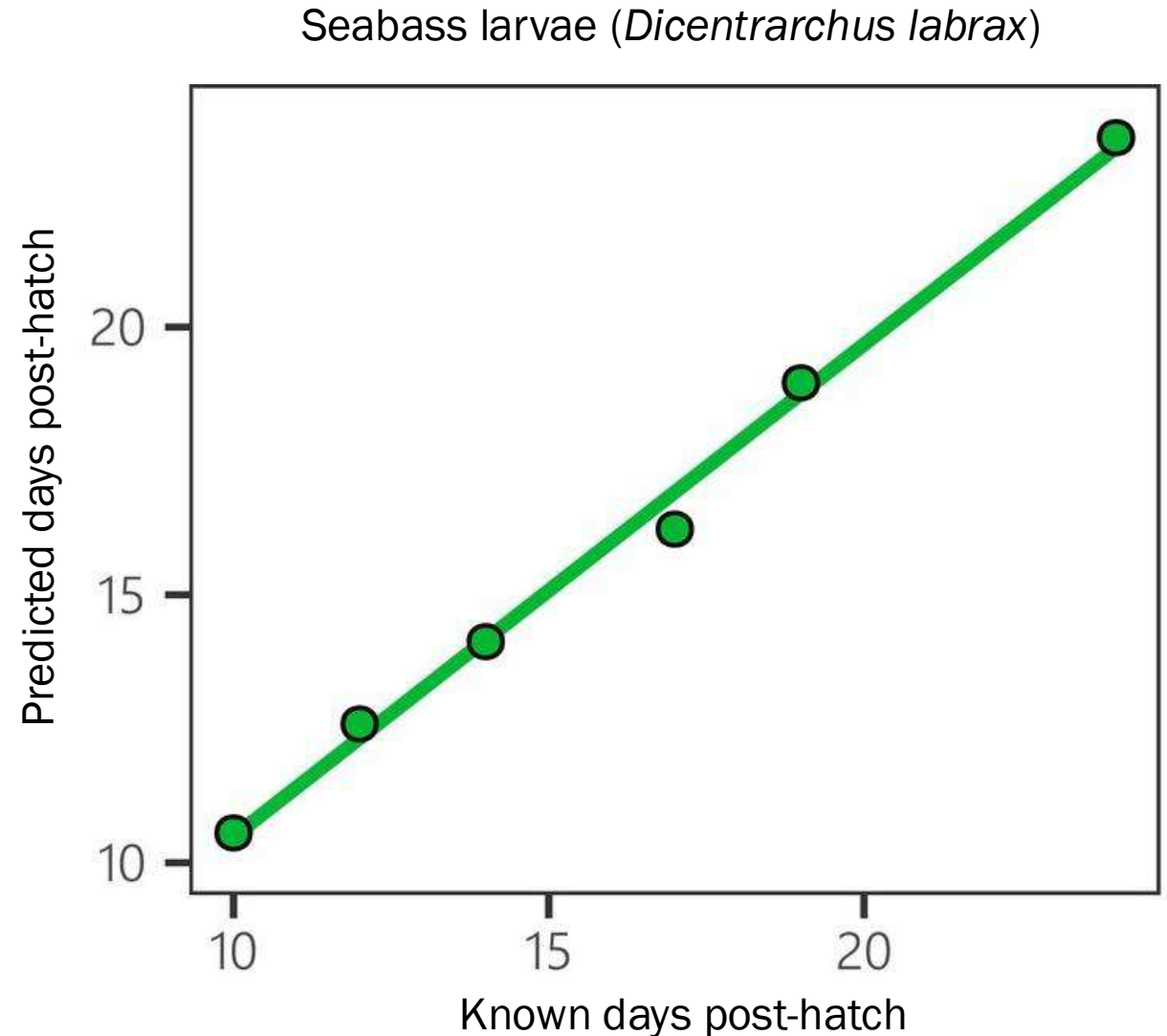
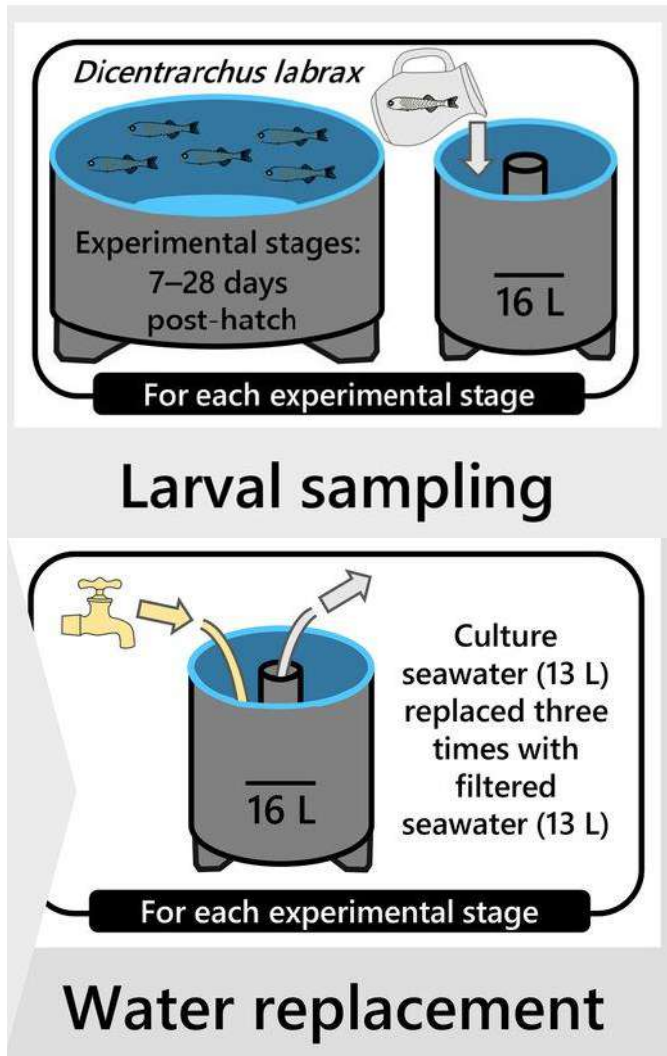
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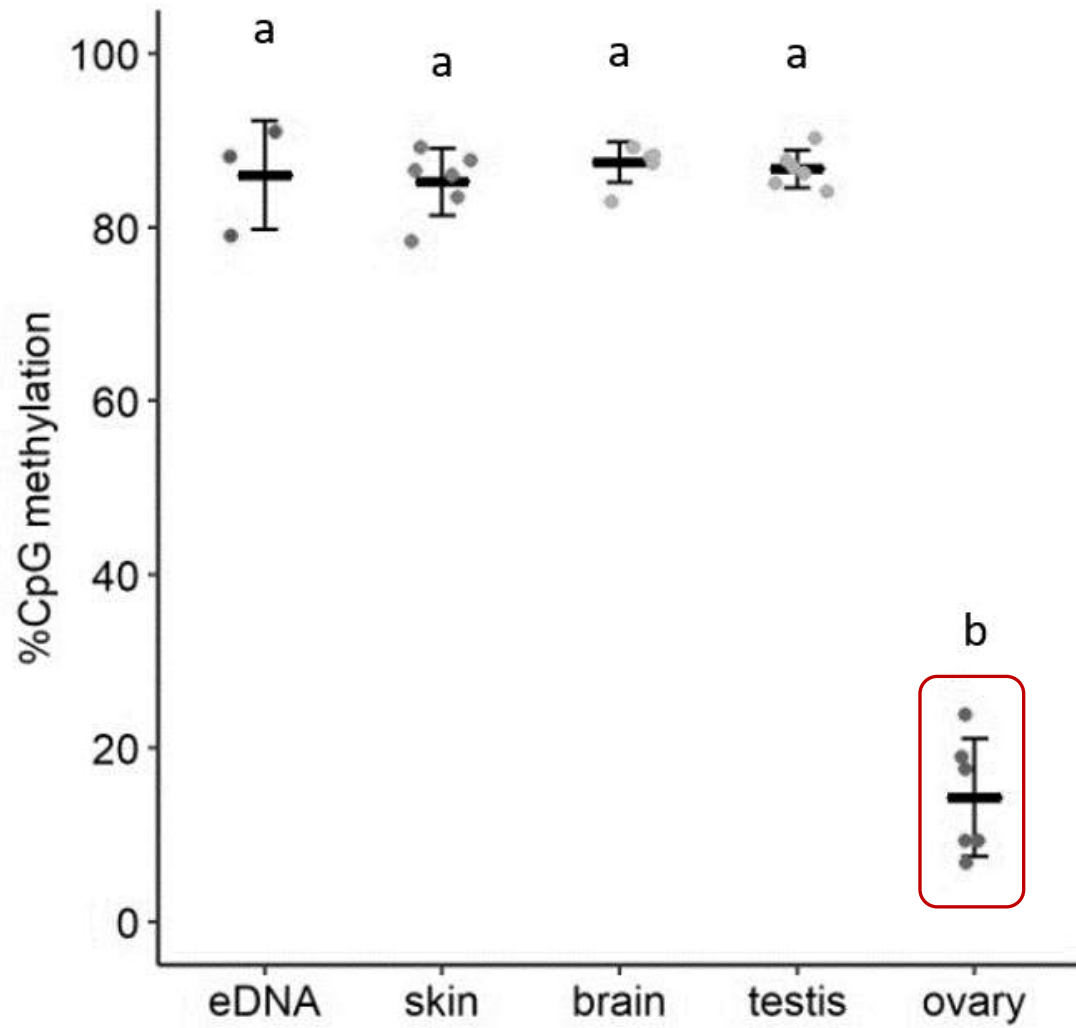
DNA and age estimation



eDNA and age estimation



eDNA and age estimation



Zebrafish (*Danio rerio*)

D. Terver (CC BY-NC 4.0)

Young tissue has lower DNA methylation rate!



<https://doi.org/10.1038/s42003-026-10496-2>

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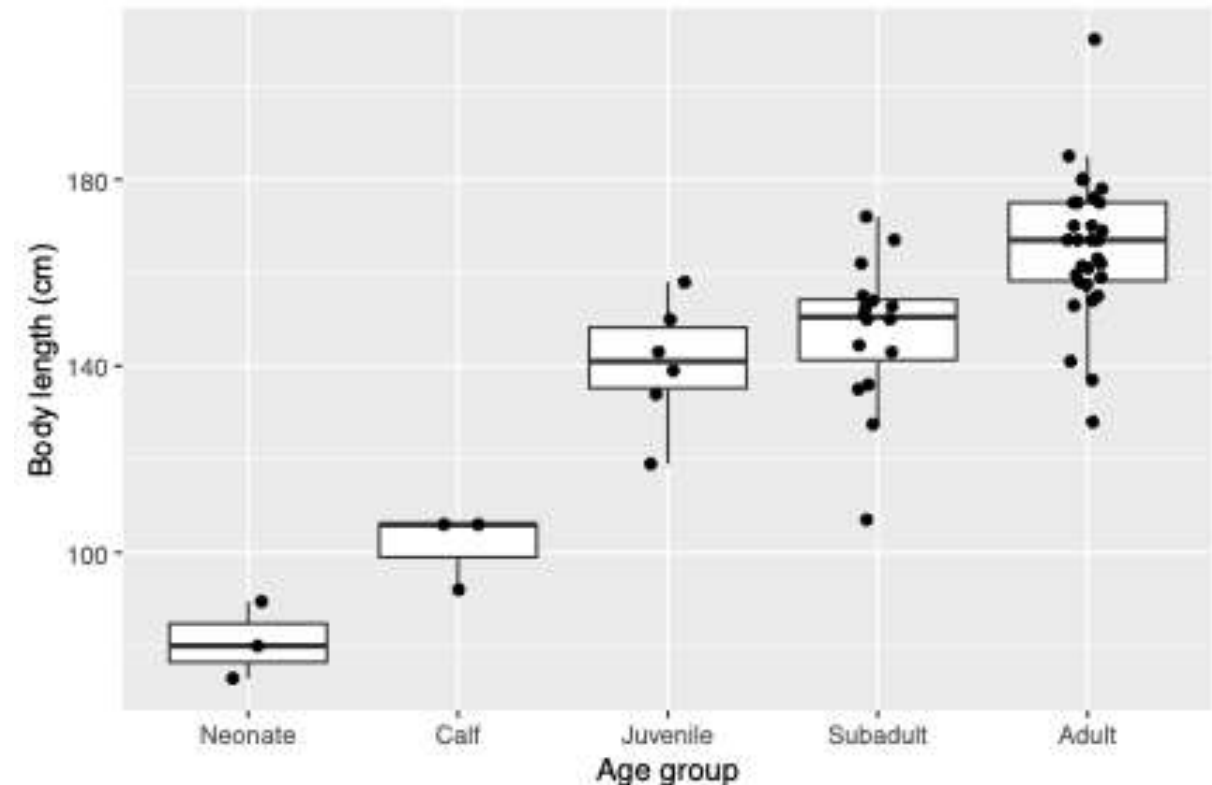


Check for updates

Chengbin Liu ¹ , Xia Fei², Itsuki T. Hirayama³, Genfu Yagi⁴ & Masayuki Ushio ¹ 

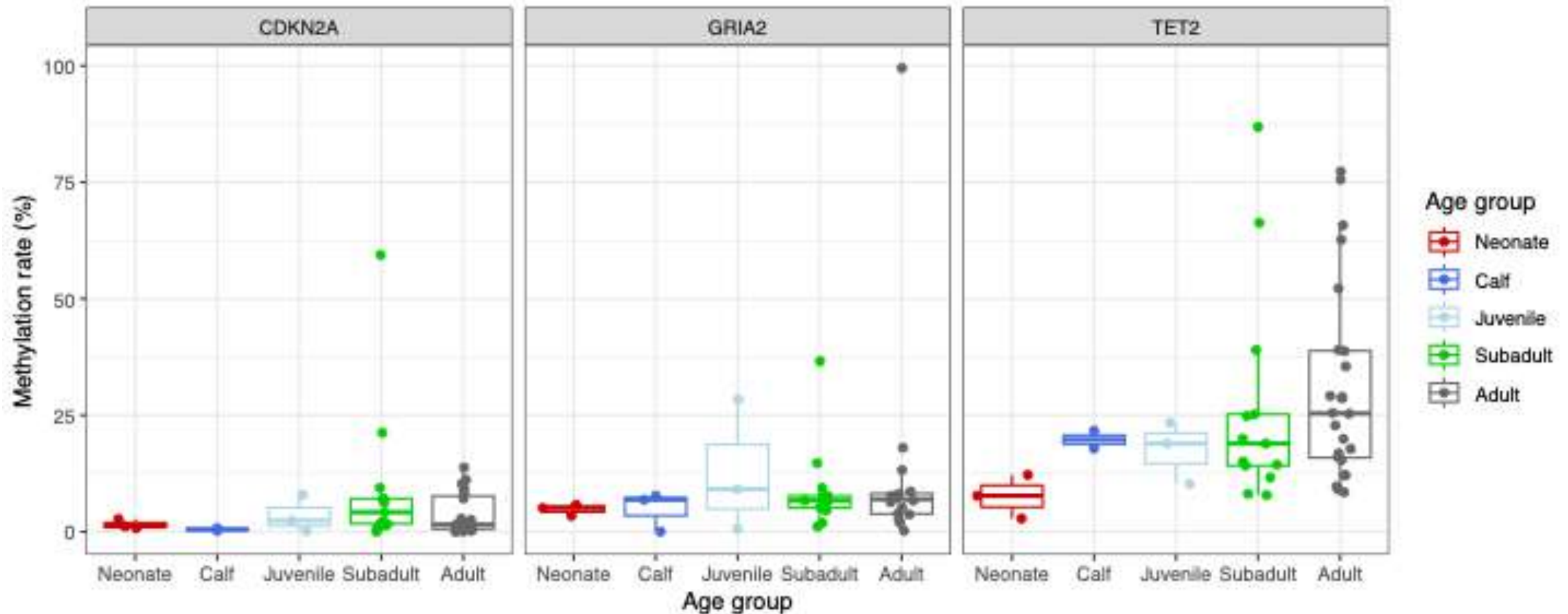
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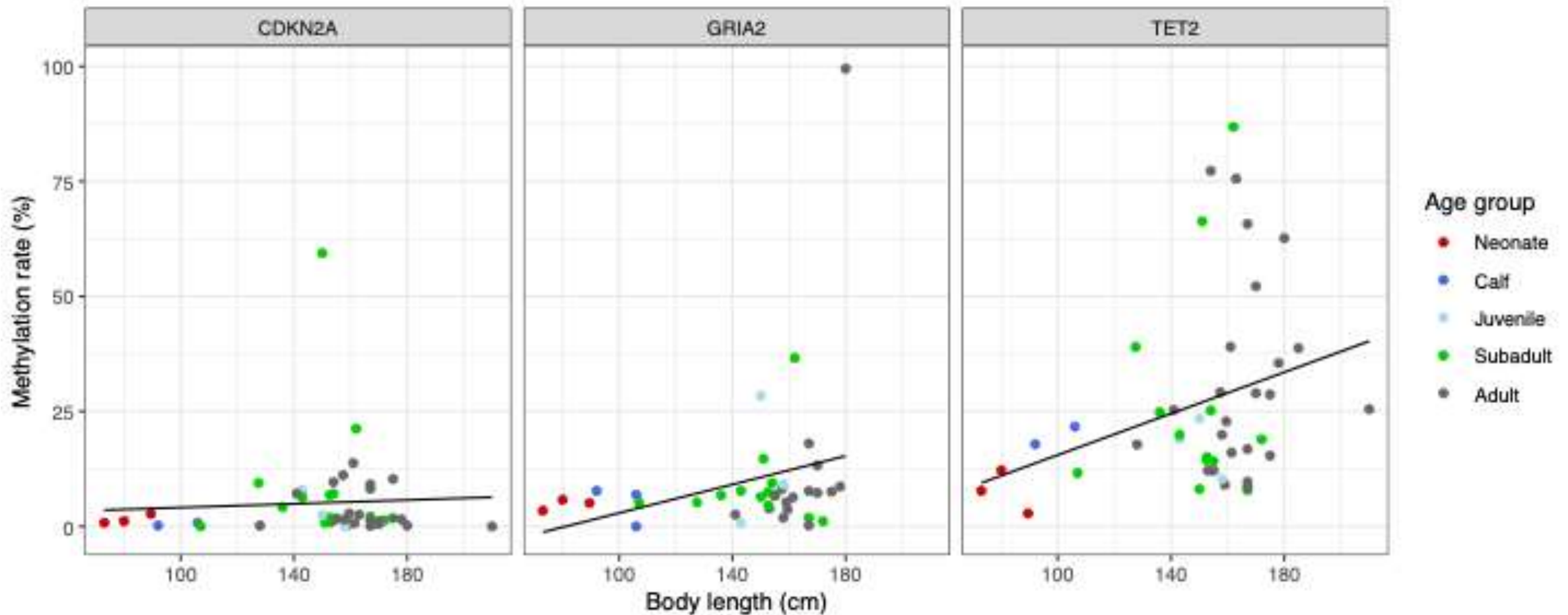
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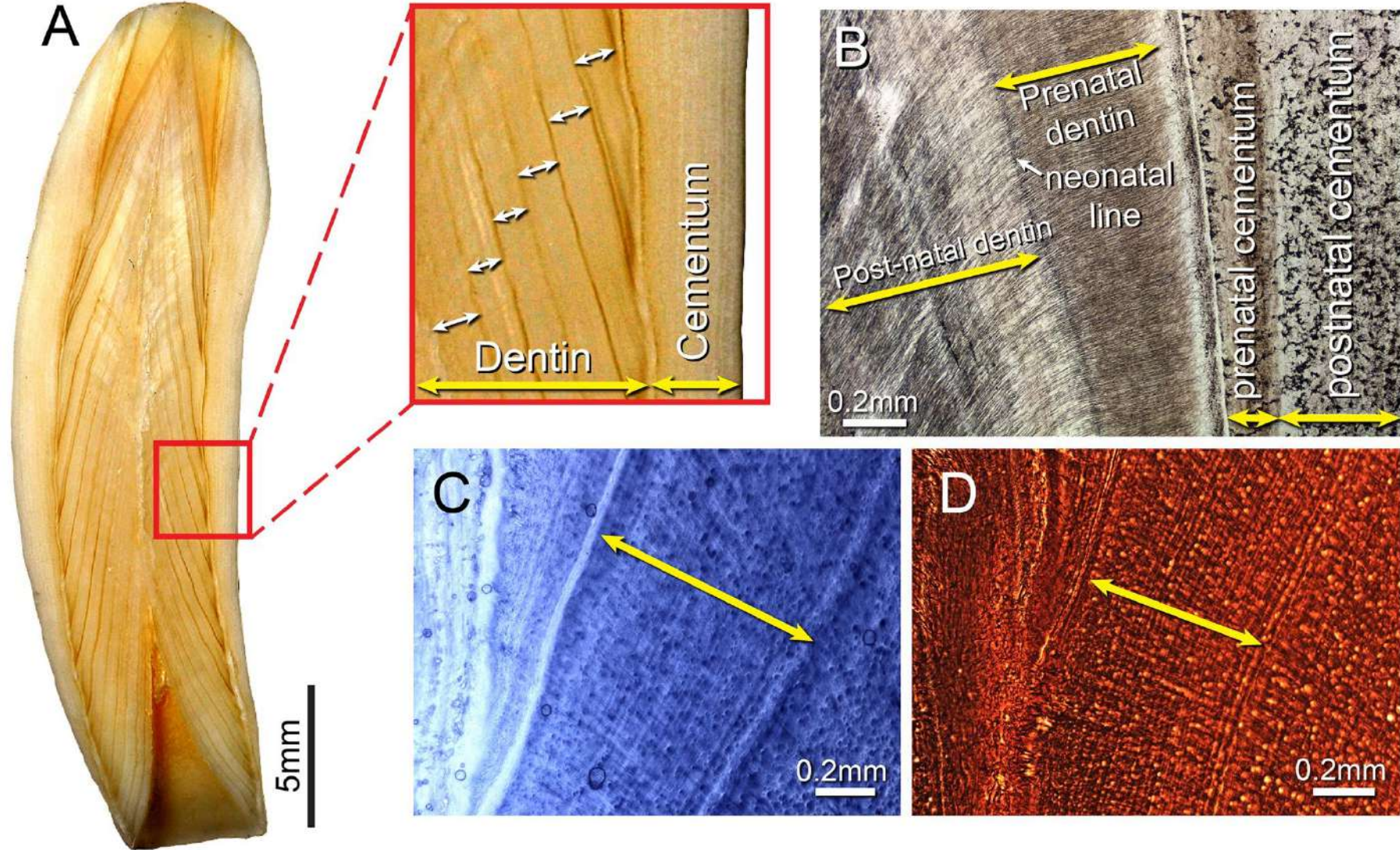


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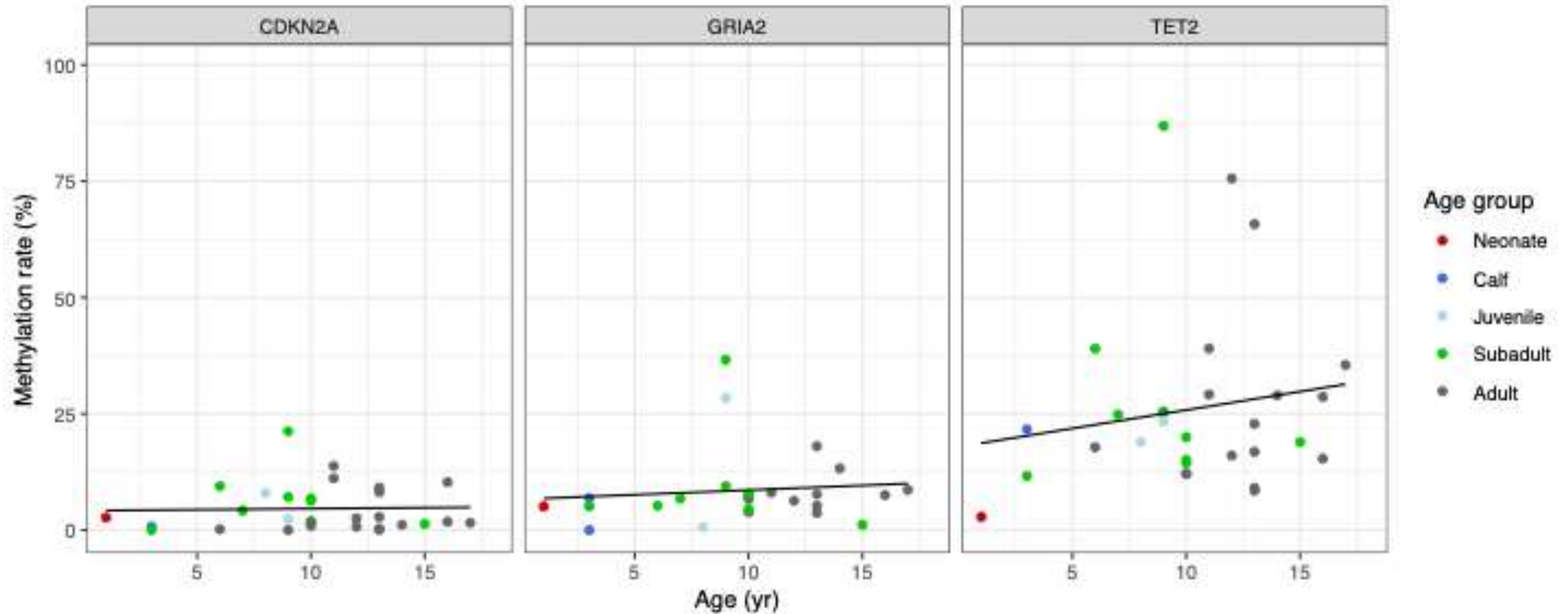
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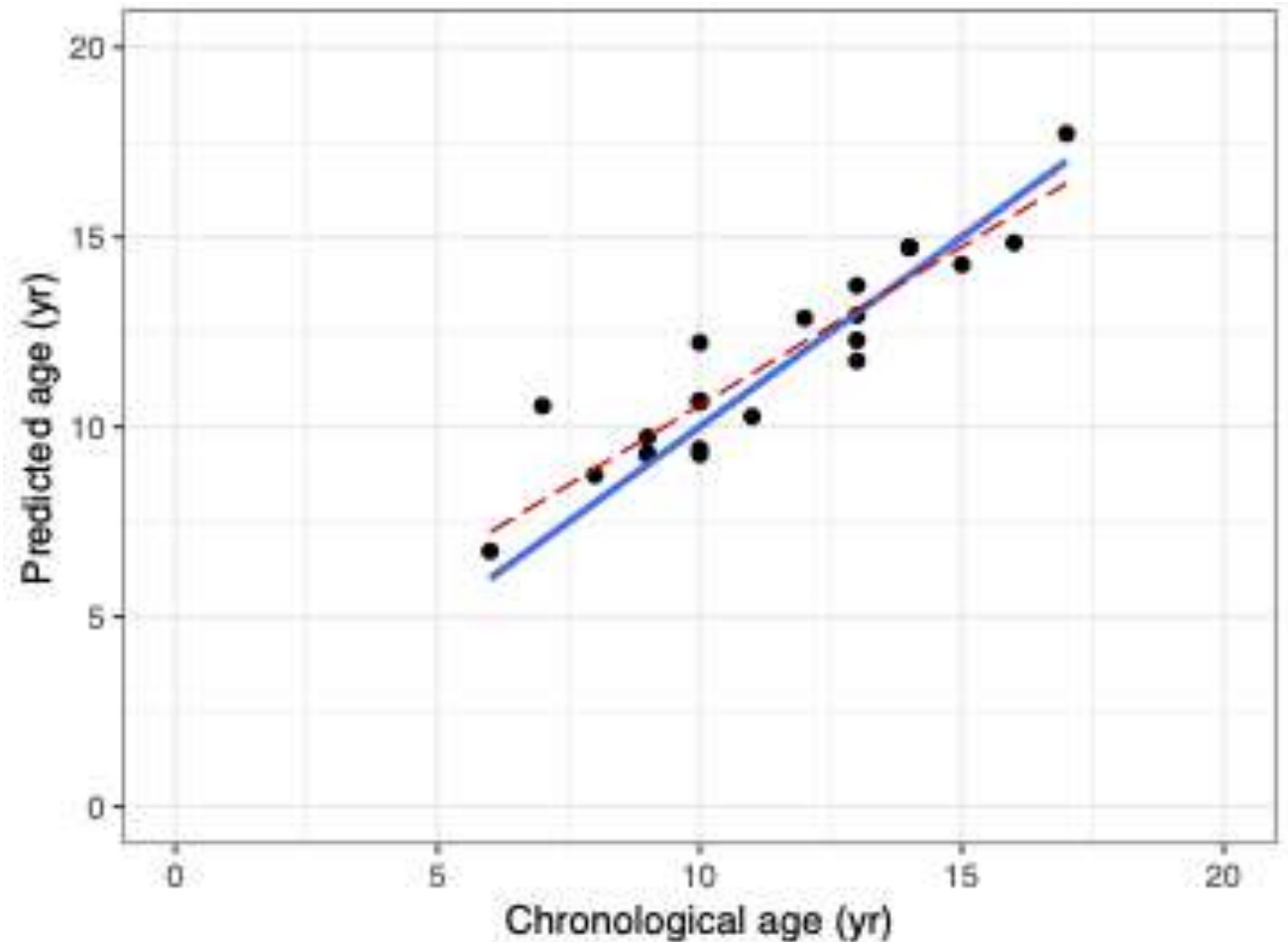
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- Model: Age $\sim$ Three genes
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Support vector regression (SVR)



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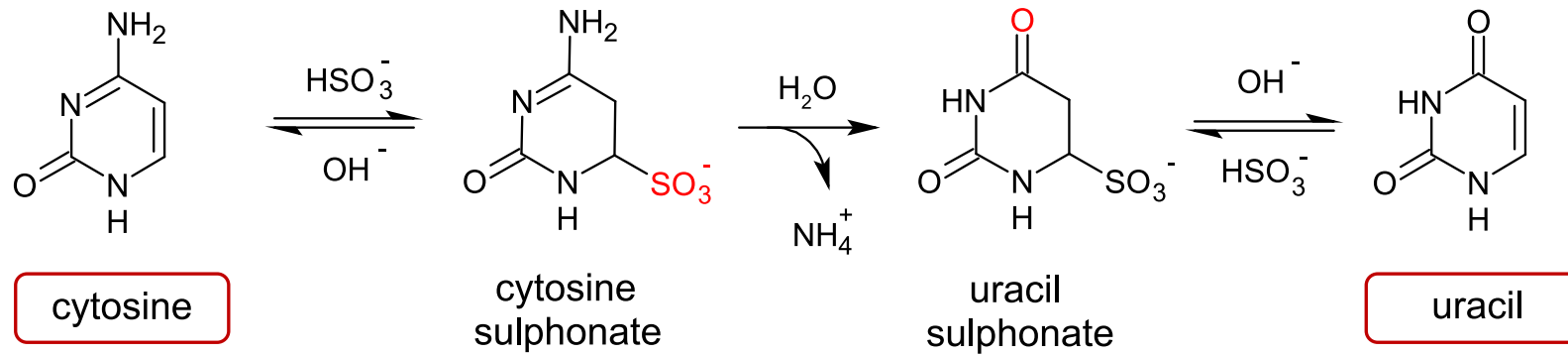
DNA methylation project
Genfu Yagi, Xia Fei
Itsuki Hirayama, Yuen Wa Ho (Derek)

Appendix

Methods to measure DNA methylation

Methods to measure DNA methylation

- Application of DNA methylation-based age estimation to eDNA studies
 - Can we estimate age from environmental DNA (eDNA)?
- How can we measure DNA methylation rate?
 - Bisulfate treatment



Treated

AT**U**GCA**C**G...

▲ Unmethylated C

Not treated

AT**C**GCA**C**G...

5-methylcytosine

Methods to measure DNA methylation

- After the bisulfate treatment, several methods can be used...
 - Bisulfate sequencing
 - Methylation-Sensitive High-Resolution Melting (MS-HRM) approach

Bisulfate sequencing

Treated

AT**U**GCA**C**G...

Not treated

AT**C**GCA**C**G...

MS-HRM

Treated

ATUGCACG...
|| || || || || || || ||
TAACGTGC...

▲ Unmethylated C

Not treated

AT**C**GCA**C**G...
TAG**G**CGTGC...

▼ methylated C

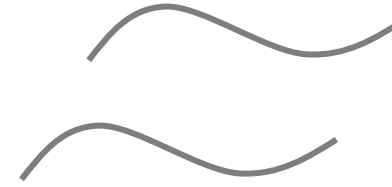
Melting temperature changes.

The more methylated, the stronger the bonds between two strands (after the bisulfate conversion), and the higher the melting temperature.

Methods to measure DNA methylation



Low temperature



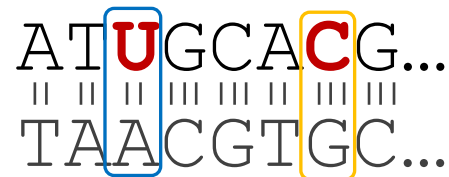
High temperature

Melting temperature

- Base compositions
- Sequence length
- Buffer composition
- ...

MSHRM

Treated



▲ Unmethylated C

Not treated



Melting temperature changes.

The more methylated, the stronger the bonds between two strands (after the bisulfate conversion), and the higher the melting temperature.

MS-HRM approach

