Non-invasive age estimation based on fecal DNA using methylation-sensitive high-resolution melting for Indo-Pacific bottlenose dolphins

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Abstract

Age is necessary information for the study of life history of wild animals. A general method to estimate the age of odontocetes is counting dental growth layer groups (GLGs). However, this method is highly invasive as it requires the capture and handling of individuals to collect their teeth. Recently, the development of DNA-based age estimation methods has been actively studied as an alternative to such invasive methods, of which many have used biopsy samples. However, if DNA-based age estimation can be developed from fecal samples, age estimation can be performed without touching or disrupting individuals, thus establishing an entirely non-invasive method. We developed an age estimation model using the methylation rate of two gene regions, *GRIA2* and *CDKN2A*, measured through methylation-sensitive high-resolution melting (MS-HRM) from fecal samples of wild Indo-Pacific bottlenose dolphins (*Tursiops aduncus*). The age of individuals was known through conducting longitudinal individual identification surveys underwater. Methylation rates were quantified from 36 samples. Both gene regions showed a significant correlation between age and methylation rate. The age estimation model was constructed based on the methylation rates of both genes which achieved sufficient accuracy (after LOOCV: MAE = 5.08, $R2 = 0.34$) for the ecological studies of the Indo-Pacific bottlenose dolphins, with a lifespan of 40-50 years. This is the first study to report the use of non-invasive fecal samples to estimate the age of marine mammals.

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Abstract

Age is necessary information for the study of life history of wild animals. A general method to estimate the age of odontocetes is counting dental growth layer groups (GLGs). However, this method is highly invasive as it requires the capture and handling of individuals to collect their teeth. Recently, the development of DNA-based age estimation methods has been actively studied as an alternative to such invasive methods, of which many have used biopsy samples. However, if DNA-based age estimation can be developed from fecal samples, age estimation can be performed without touching or disrupting individuals, thus establishing an entirely non-invasive method. We developed an age estimation model using the methylation rate of two gene regions,*GRIA2* and *CDKN2A,* measured through methylation-sensitive high-resolution melting (MS-HRM) from fecal samples of wild Indo-Pacific bottlenose dolphins (*Tursiops aduncus*). The age of individuals was known through conducting longitudinal individual identification surveys underwater. Methylation rates were quantified from 36 samples. Both gene regions showed a significant correlation between age and methylation rate. The age estimation model was constructed based on the methylation rates of both genes which achieved sufficient accuracy (after LOOCV: MAE = 5.08, R^2 = 0.33) for the ecological studies of the Indo-Pacific bottlenose dolphins, with a lifespan of 40-50 years. This is the first study to report the use of non-invasive fecal samples to estimate the age of marine mammals.

1 | Introduction

Life history is a crucial factor that determines the ecology of animal species, and each species exhibits a specific life history strategy. Growth stages can be roughly classified based on life history traits such as the timing of weaning, maturation, and lifespan. However, the allocation of resources towards growth, survival, and future reproduction varies among species (Williams, 1966), and each species has a different duration for each life stage (e.g. in female Indo-Pacific bottlenose dolphins (*Tursiops aduncus*): calf (pre-weaning): 0–3.5 years, subadult (immature): 3.5–10.3 years, adult (after first parturition): 10.3–50 years, Kogi *et al* ., 2004; Kogi, 2013; Wang, 2018). There have been reports on age-related cessation of reproductive ability (e.g. post reproductive lifespan in humans (*Homo sapiens*): Levitis & Bingham, 2011; killer whales (*Orcinus orca*): Croft *et al* ., 2015; Franks *et al* ., 2016, belugas (*Delphinapterus leucas*): Ellis *et al* ., 2018), and age-related changes in sociality (red deers (*Cervus elaphus*): Albery *et al* ., 2020). These reports provide evidence of the changes in resource allocation occurring after maturity, indicating that information on age is necessary to clarify the life history of a species, rather than growth stage.

However, longitudinal observation is expensive and time-consuming, especially for long-lived animals like the Indo-Pacific bottlenose dolphins which can live about 50 years (Wang, 2018). Therefore, age estimation methods are essential to efficiently investigate the age structure of a specific population.

A commonly used method for age estimation in toothed whales (odontocetes) is counting dental growth layer groups (GLGs) (see Perrin and Myrick, 1980). This method requires capturing of individuals to collect dental samples. The invasive nature of measuring dental GLGs, makes it unsuitable for small or threatened populations. It is also difficult to estimate age using dental GLGs for populations living in offshore areas where capture can be difficult and for populations that are targeted for tourism. Thus, methods of non-invasive age estimation for toothed whales has developed recently. One is the method using age-related external appearance changes on the body, including scars and body cololations (e.g. risso's dolphins (*Grampus griseus*): Hartman *et al.,* 2015; Indo-Pacific humpback dolphins (*Sousa chinensis*): Guo *et al* ., 2020). Krzyszczyk & Mann (2012) and Yagi *et al* . (2022) described the age-related changes to the speckle appearance patterns on the Indo-Pacific bottlenose dolphins in Shark Bay, Australia, and Mikura Island, Japan respectively. Yagi*et al.* (2023) developed a speckle-based age estimation model that showed high accuracy (R^2 = 0.77, standard deviation (SD) = 2.58). However, the model is only limited to estimating the ages between 7.68–21 years due to the spots appearing age and the upper limit of the age-known individuals.

Aging occurs in many organisms and it leads to various changes at the tissue and cellular levels (Petralia *et al* ., 2014). Although aging is thought to be caused by the combined effects of various factors (López-Otin *et al.* , 2013), one of the factors that regulates aging are epigenetic changes which are dysfunctional systems that accompany aging at the gene level (Booth & Brunet, 2016). DNA methylation is an example of an epigenetic changes, in which DNA methylation rates at CpG sites (cytosine-phosphate-guanine) in specific gene regions changes with age. Recently, the correlation between DNA methylation rate and aging has been used to develop an age estimation method, known as the epigenetic clock. This method was initially developed for humans (Horvath, 2013) and has since been applied to several species (e.g. Bechstein's bats (*Myotis bechsteinii*): Wright *et al.,* 2018; domestic cats (*Felis catus*) & snow leopards (*Panthera uncia*): Qi *et al* ., 2021; brown bears (*Ursus arctos*): Nakamura *et al* ., 2023). In cetaceans, Polanowski *et al* . (2014) was the first to report the application of DNA extracted from skin samples in humpback whales (*Megaptera novaeangliae*). Following this pioneering study, similar approaches have been successfully applied to other cetacean species (belugas: Bors*et al.* , 2020; Antarctic minke whales (*Balaenoptera bonaerensis*): Tanabe *et al* ., 2020; common bottlenose dolphins (*T. truncatus*): Beal *et al* ., 2019; Indo-Pacific bottlenose dolphins: Peters *et al* ., 2022). Capture methods from research whaling, commercial whaling and hunting are extremely invasive. These previous studies on cetaceans using epigenetic clock analyses relied on the samples obtained from invasive methods including commercial whaling (fin whales (*B. physalus*): García-Vernet *et al* ., 2021), whale research program (Antarctic minke whales: Tanabe *et al* ., 2020), capture and release of wild individuals (common bottlenose dolphins: Beal *et al.,* 2019), stranded carcasses (belugas: Bors*et al* ., 2021), and the use of rifles and crossbows for biopsy (humpback whales: Polanowski *et al* ., 2014; belugas: Bors *et al* ., 2021; Indo-Pacific bottlenose dolphins: Peters *et al* ., 2022). Although biopsy procedures are less invasive compared to capture methods including whaling and hunting, it remains at a certain level of invasiveness, particularly for small cetacean species where instances of mortality have been reported (Weller *et al* ., 1997; Bearzi, 2000; Noren & Mocklin, 2012). By using fecal samples, DNA can be collected non-invasively without the need to touch individuals. However, there is a limited number of studies based on fecal-sampled epigenetic clocks. To our knowledge, the only studies to have developed epigenetic clocks using fecal samples are from Nakano *et al* . (2019, 2020) which reported a significant correlation between the methylation rate of*ELOVL2* (Elongation of very long chain fatty acids protein 2) and age in chimpanzees (*Pan troglodytes*) and Japanese macaques (*Macaca fuscata*).

To examine the correlation between methylation rate and age, fecal samples from age-known individuals are required. At our research field, coastal water off Mikura Island located approximately 200 km south of Tokyo, Japan, around 160 Indo-Pacific bottlenose dolphins were living year-round (Kakuda *et al* ., 2002; Kogi *et al* ., 2004). Since 1994, longitudinal individual identification surveys using underwater video data have been conducted around this island (Kogi *et al* ., 2004). These underwater surveys allow tracking the actual ages of individuals born after 1994 and collection of fecal samples from individuals, making the population well-suited for fecal sample-based age estimation studies.

Here, we investigated the correlation between DNA methylation rate and age in fecal samples using a low-cost and convenient method called methylation-sensitive high-resolution melting (MS-HRM) analysis (Wojdacz & Dobrovic, 2007; Wojdacz *et al* ., 2008; Tse *et al* ., 2011). We focused on the genes *TET2* (ten eleven translocation 2),*GRIA2* (glutamate receptor Ia2/AMPA2), and *CDKN2A* (cyclin dependent kinase inhibitor 2A), which reported a correlation between age and methylation rate in skin samples of a closely related species, the common bottlenose dolphins (Beal *et al.,* 2019). Furthermore, we developed an age estimation model using the methylation rates of these genes. We also assessed the effects of biological factors (sex differences and female nursing states) on methylation rate because in humans, various stressors are known to affect the epigenetic clock (e.g. Lawn *et al* ., 2018, Marini *et al* ., 2020) such as increased frequency of pregnancy causes acceleration in epigenetic age (Ryan*et al.,* 2018). This study aimed to develop a non-invasive age estimation model using DNA extracted from fecal samples ahead of other mammals and to contribute to ecological and conservation studies.

2 | Material & Methods

2.1 | Ethics statement

This study was conducted in accordance with the 'Agreement of the Dolphin Watching Operation around Mikura Island'. The research protocol was approved by Mikurashima Village, permitting us access to the protected areas of the sea around Mikura Island under the 'Agreement of the Rational Use of Nature Conservation Promotion Area at Mikura Island'. We obtained approval from the Mikurashima Tourism Association to use individual identification data and fecal samples for this study (Approved number: No. 20220726). We followed the 'Guidelines to Study Wild Animals' of the Wildlife Research Center of Kyoto University to minimize disturbance.

2.2 | Study area and individual identification survey

We observed Indo-Pacific bottlenose dolphins around Mikura Island, Japan (33o90'N, 139o60'E) which is a small (approximately 20.54 km^2) oceanic island. Ongoing individual identification surveys have been conducted since 1994 using underwater video recordings taken within 300 m of the coastline, at depths ranging from 2-45 m. The sex of each individual was determined by observing the genital and mammary slits in both males and females and/or an erection in males (see Kogi *et al* ., 2004). The nursing females were determined if the mother and calf pair was frequently observed swimming in echelon, infant, or lactating positions (see Kogi *et al* ., 2004). Kakuda*et al.* (2002) identified the *Tursiops* sp. around Mikura Island as *Tursiops aduncus* . From 1994–2022, a total of 382 individuals have been identified. The birth year is known for 243 individuals. In this study, 45 age-known individuals were used. The actual age of each individual was calculated by subtracting their birth year from the year of sample collection.

2.3 | Sample collection and DNA extraction

Fecal samples were collected by skin diving. We dived underwater with small action cameras (Hero3, Hero 7 white, Hero 7 black, GoPro Inc, San Mateo, Canada) and polyethylene small container (polyethylene petit tube, Ryohin Keikaku, Tokyo, Japan). We recorded individuals that were close to the camera. When the filmed individuals defecated, polyethylene small containers were used to suction the fecal samples along with the environmental water. The recordings were then used to identify which fecal samples were collected from the individuals that defecated. The collected fecal samples were refrigerated in a cooler box on the boat and upon return to land, the samples were processed by discarding the supernatant of the environmental water. The remaining material was preserved at 4 degC after being transferred into containers with 70% ethanol or higher. DNA was extracted from weighing 0.6 g of ethanol-preserved fecal samples using QIAmp DNA Stool Kit or QIAmp Fast DNA Stool Mini Kit (QIAGEN, GmbH, Hilden, Germany) with 200 μl of water. The concentration of extracted DNA was measured with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

We used a total of 61 fecal samples obtained from 45 individuals between 2014–2021. Due to the nature of fecal samples, some samples failed to quantify DNA methylation rates due to the low concentration of target DNA. Samples in which methylation rates could not be quantified more than twice for each gene were excluded from the analysis. If multiple samples were collected from the same individual but in different years, we treated each sample as an independent dataset. Under this condition, 36 datasets from 30 individuals were used in the analysis. Fig.1 shows the age distribution of the dataset which covers 1–27 years of age.

2.4 | Standard DNA

0% and 100% methylated standard DNA was created to calculate the methylation rate of the samples. High concertation of DNA is needed for the preparation of standard DNA. We used skin and muscle samples from four individuals that were bycatch in gill nets off Mikura Island. The individual number and bycatch years are as follows: #165 and #259 in 2008, #406 in 2013, and an unknown individual in 2005 (Table S1). After, a small piece of skin and muscle sample was cut into smaller pieces, DNA was extracted using the DNeasy Blood & Tissue kit (QIAGEN GmbH, Hilden, Germany). The 0% methylated standard DNA was obtained by performing whole genome amplification treatment using the REPLI-g Mini Kit (QIAGEN GmbH, Hilden, Germany). The 100% methylated standard DNA was obtained by fully methylating with

CpG methyltransferase (M.SssI; New England Biolabs, Beverly, MA, USA). Each standard DNA was purified using High Pure PCR Product Purification Kit (Roche Molecular Systems, Pleasanton, CA, USA).

2.5 | Primer design

Beal *et al* . (2019) reported a correlation between methylation rate and age in the common bottlenose dolphin (a closely related species to the Indo-Pacific bottlenose dolphin) using three gene regions: *TET2* , *GRIA2* , and *CDKN2A* from DNA extracted from skin samples. Similar correlations using the same genes were seen in humpback whales (Polanowski *et al* ., 2014) and Antarctic minke whales (Tanabe *et al.,* 2020). These findings suggest that these genes can be commonly used for epigenetic clock analyses in cetaceans. However, we were unable to find the genomic information on Indo-Pacific bottlenose dolphins. Therefore, primer designs were referred to the genomic information on common bottlenose dolphins, available at the National Centerfor Biotechnology Information (NCBI,*https://www.ncbi.nlm.nih.gov/*) database using the Standard Nucleotide Basic Local Alignment Search Tool (BLAST) (Altschul *et al* ., 1990). We attempted to design PCR primers to amplify three target genes (reference genomes: *TET2* &*GRIA2* : NC_047038; *CDKN2A* : NC_04739). Primers were designed using Methyl Primer Express v1.0 (Thermo Fisher Scientific, San Jose, CA, USA, Table 1). However, we were unable to design a primer for the *TET2* gene that would successfully amplify the target region. As a result, we proceeded with the analysis using only *GRIA2* and*CDKN2A* genes.

There was a need for additional verification to ensure that the designed primers specifically amplified the DNA of the target species and not the prey species. To confirm this, we used the NCBI database using the BLASTN tool (*https://blast.ncbi.nlm.nih.gov/Blast.cgi*) and homogenous gene regions were searched against reference genomes to ensure that the same sequences were not found in the prey species for this population, as reported by Takahashi *et al* . (2020) and Kita*et al.* (2018).

2.6 | Measuring methylation rate

The 20 μl of 300 ng extracted DNA samples (section 2.3) and purified standard DNA (section 2.4) were subjected to bisulfite conversion using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA). This converts unmethylated cytosines to uracil. The differences between the bisulfite-converted DNA samples were measured using MS-HRM using a LightCycler 480 Instrument II (Roche Molecular Systems, Branchburg, NJ, USA) equipped with the Gene Scanning Software in a 25 μl total volume containing 1x EpiTect HRM PCR Master Mix (EpiTect HRM PCR kit: QIAGEN GmbH, Hilden, Germany), 750 nM of each primer, and 2–4 μl of template DNA (bisulfite-converted DNA). The chosen detection format was set to SYBR Green I/HRM Dye. First, the initial PCR activation step was performed at 95 @C for five mins to activate the polymerase, then the three-step PCR was performed (PCR conditions are presented in Table 1). Finally, MS-HRM analysis was initiated by cooling the samples to 65 @C for 1 sec and then heated to 95 @C at a ramp rate of 0.02 @C/s. Gene Scanning Software was used to normalize the raw melt curves, allowing the comparison of samples. After MS-HRM, the normalizing process was performed, the pre-melt temperature region was set to 72–74 @C and post-melt temperature region to 82-84 @C for both gene.

We defined the minimum absolute value of the relative signal difference from the difference plots as the Df value. For the analysis, we used the average Df value, which was calculated by measuring each sample twice. To construct a standard curve that correlates the Df value with the methylation rate, we used the 100% and 0% methylated standard DNA samples described in section 2.2. These standard DNA samples were mixed in various ratios, resulting in a set of standard DNA samples with different methylation percentages (0, 5, 10, 15, 20, 50, and 100%). The Df values of the standard curve were obtained from the MS-HRM using the standard DNA samples for both genetic regions. The plotted standard curve followed a non-linear regression model designed by Wamecke*et al.* (1997ed by Hamano *et al* . (2016) as follows:

$$\frac{a * M}{b - M} = \frac{\mathrm{Df}}{\mathrm{Df}_{\max} - \ Df}$$

Where a: coefficient, b: The maximum methylation rate used for the standard curve construction, M: methylation rate,Df_{max}: Df value of the maximum methylated standard samples (*GRIA2*: 0.2, *CDKN2A*: 1.0).

Due to the low range of methylation percentages observed in *GRIA2*during subsequent analysis, the standard curve was redrawn using the standard DNA samples with the methylation percentages of 0, 5, 10, 15, and 20%. All subsequent analyses of *GRIA2* utilized this recalibrated calibration curve to quantify the methylation percentage of the samples.

2.7 | Regression analysis

The correlation between methylation rate and age for both gene regions was examined using a single linear regression analysis. The effect of sex and female nursing state on methylation rate in both gene regions was assessed using analysis of covariance (ANCOVA).

To develop the age estimation model, we used the support vector regression (SVR). We constructed three models and assessed their precision and accuracy, as listed below:

Model 1: *GRIA2* methylation rate + *CDKN2A* methylation rate

Model 2: *GRIA2* methylation rate + *CDKN2A* methylation rate + sex

Model 3: *GRIA2* methylation rate + *CDKN2A* methylation rate + female nursing state

Leave-one-out cross-validation (LOOCV) was performed to validate the overfitting of these models. Precision and accuracy were calculated both before and after LOOCV. All computations were performed using R (version 4.0.2) statistical software (R Core Team, 2020). The R package "Pamesures" (Wang & Li, 2018), "e1071" (Meyer *et al* ., 2022), "MuMIn" (Bartoń, 2022), and "car" (Fox & Waisberg, 2019) were used for the analysis. The output of coefficient of "a " was carried out using the "nls" command. The two parameters, "cost" and "epsilon" for the SVR models were optimized using the "tune" command with the fixed set of "type = eps-regression, kernel = radial, gamma = 0.5". The coefficient of determination (R^2) and mean absolute error (MAE) was used to indicate how well an estimated age fitted the model. Differences were considered significant at$p < 0.05$ for all analyses.

2.8 | Application of the age estimation model towards individuals of unknown age

Using the best-constructed age estimation model, we estimated the age of 19 age-unknown individuals (7 males and 12 females) around Mikura Island, using DNA extracted from fecal samples. In this population, the age of weaning (average: 3.5 years, SD = 0.74, Kogi *et al* ., 2004) and the age of first birth (average: 10.31 years, SD = 1.75, Kogi, 2013) are reported. Of the individuals whose actual age was unknown, we selected only those individuals for which we could observe weaning and/or first birth. We then assigned their age in the year when the feces were collected, with an age of 3.5 years for weaning and 10.31 years for the year of first birth. We compared the estimated age derived from the genes developed in this study with the ages estimated from weaning or first birth.

3 | Results

Fig 2 shows the standard curve of *GRIA2* and *CDKN2A* and the value of coefficient "a " was calculated as 1.151 and 0.7838, respectively.

The methylation rate of *GRIA2* and *CDKN2A* ranged from 0.32–11.56% and1.32–83.20%, respectivly. The simple linear analysis showed a significant correlation between age and methylation rate in both genes (*GRIA2* : $R^2 = 0.17$, $p = 0.01$; *CDKN2A* : $R^2 = 0.34$, $p< 0.01$; Fig 3). We examined the changes in methylation rate over the years for five individuals for which samples were collected across multiple times (Fig.3). Although overall, the methylation rate of both genes increased with age, amongst these individuals, the methylation rate of *GRIA2* decreased with age for one individual (individual number: #592MS) and that of *CDKN2A* for two individuals (individual number: #592MS and #604FA).

No sex differences were observed in the correlation between methylation rate and age in both genes (ANCOVA, *GRIA2:* $p > 0.05$,*CDKN2A:* $p > 0.05$; Table 2–3). On the other hand, nursing females showed significantly lower methylation rates (ANCOVA,*GRIA2* : $p < 0.05$, *CDKN2A* : $p < 0.05$; Table 2–3).

An age estimation model was developed using the methylation rates of both genes, *GRIA2* and *CDKN2A* as explanatory variables in SVR (model 1). The model before LOOCV was performed, exhibited aR^2 of 0.74 and a mean absolute error (MAE) of 2.63 years (Fig 4a). After LOOCV, the model exhibited aR^2 of 0.33 and an MAE of 5.08 years (Fig 4b).

In comparison to model 2 which included sex as an explanatory variable, and model 3 with female nursing states as an explanatory variable, model 1 showed the best precision and accuracy of estimation (Table 4).

The resulting model was used to estimate the ages of 19 individuals of unknown age (Table S2). Among these individuals, we compared the absolute difference between the age estimated from our model and the age of first births or weaning for five individuals. The analysis revealed an MAE of 9.41 years (Range = 21.56–4.58, Table S2).

4 | Discussion

In this study, we found a significant correlation between age and methylation rate in the gene regions, *GRIA2* and *CDKN2A*using a DNA extracted from a non-invasive fecal sample. Although sex was not affected by the correlation between age and methylation rate, the methylation rate decreased for the females in nursing states. We also succeeded in constructing an age estimation model using the methylation rates of both genes. This study is the first to report the use of multiple genes and DNA extracted from fecal samples to develop an age estimation model. We estimated the ages of 19 unknown-age individuals using our model (Table. S2). The error between the estimated age from our model and the assigned age based on the year of first birth or weaning exceeded the MAE after LOOCV was performed. On the other hand, only one of the five individuals (individual number: #068FA), had a high residual error, while the average error among the other four individuals was 6.37 years (range: 4.58–8.53), which is closer to the MAE after LOOCV. The reasons for the high estimation errors in the individual is unknown. However, considering that fecal sample collection was conducted underwater, it is plausible that feces from a different individual, outside the camera's field of view might have been mistakenly collected. Indo-Pacific bottlenose dolphins are known to swim in groups of 20-50 individuals (Wang, 2018), which increases the likelihood of accidentally collecting fecal samples from a different individual. To enhance the accuracy of age estimation using this method, it is preferable to collect fecal samples multiple times from the same individual and estimate the age based on those replicated samples.

The age estimation model which exhibited the highest accuracy and precision were from the methylation rates of *GRIA2* and*CDKN2A* (model 1), with an MAE of 5.08 years. This is 10–13% (percentage error) of the life span of a Indo-Pacific bottlenose dolphin which is 40–50 years (Wang, 2018) and it provides a sufficient level of accuracy for ecological and conservation studies. In a study focusing on Bechstein's bats, a similar approach using multiple genes, including*GRIA2* from wing tissues, achieved an age estimation with a standard deviation of 1.52 years (Wright *et al* ., 2018). Assuming a lifespan of 20 years for this species, the error corresponds to 7.6% of the lifespan. In the case of blood samples of domestic cats, an age estimation error of 3.83 years has been reported (Qi *et al* ., 2021). Assuming a lifespan of 12 years for domestic cats, the error accounts for approximately 31.9% of its total lifespan. Notably, despite the use of fecal samples, which are associated with lower precision, the range of error to lifespan was comparable to studies conducted on other species. However, our model shows lower accuracy compared to other cetaceans using skin samples (humpback whale: MAE = 3.575, Polanowski *et al* ., 2014; common bottlenose dolphin: RMSE = 5.14, Beal *et al* ., 2019; fin whale: MAE = 4.264, García-Vernet*et al* ., 2021). In contrast to previous studies that used pyrosequencing to measure methylation rates with DNA extracted from skin samples, we used MS-HRM with DNA extracted from fecal samples. The accuracy of MS-HRM was reported to be similar to that of pyrosequencing (Migheli *et al* ., 2013), thus, the differences in detection limits between the methods is not considered to be the cause of lower accuracy. However, pyrosequencing allows for the estimation of

methylation rate at individual CpG sites, and only the most strongly correlated sites can be selected for the estimation model. MS-HRM quantifies the methylation rate within a certain range that encompasses multiple CpG sites. Depending on the target regions, it may include several CpG sites with varying degrees of correlation to age which can potentially lead to a decrease in accuracy. Previous studies have also reported variations in the correlation between methylation rates at individual CpG sites with age (e.g. *GRIA2* : 0.48–0.75, *CDKN2A* : 0–0.44, Beal*et al* ., 2019). In addition, the fecal samples used in this study may also include not only the target dolphin's DNA (Kita *et al.*,2017), but could also include DNA of intestinal bacteria (Suzuki*et al.,* 2021), and prey species (Kita *et al.,* 2018). The low concentration of the target DNA may have caused low estimation accuracy. As mentioned above, as samples were collected underwater, there is a risk of mixing feces from other individuals that have defecated nearby. In the future, when similar analyses are conducted using DNA extracted from fecal samples, it may be possible to solve this problem by using multiple replicated samples obtained from the same individual to then detect and discard samples with relatively high concentrations of target DNA or contamination from other individuals' feces.

No significant sex differences were reported in the results of the age estimation model that included *GRIA2* for the common bottlenose dolphins, which is closely related to our target species (Beal *et al* ., 2019). Similarly, we did not find significant sex differences between methylation rate and age in *GRIA2* and *CDKN2A*(Table 3). Comparisons between the models also showed that the model which excluded sex as an explanatory variable, demonstrated the highest precision and accuracy. The results suggest that sex may not be necessary for age estimation. It has been reported that on average, females tended to have lower epigenetic age than males, and this association strengthened over the course of human life (Simpkin *et al* ., 2016). The sex differences in the rate of aging are consistent with higher mortality rates and shorter average life expectancy in males (Crimmins *et al* . 2019). The tendency for males to have a shorter lifespan is a common phenomenon observed in mammals (Lemaitre *et al.,* 2020). In non-human mammals, a small number of species have reported sex differences between DNA methylation rate and age in the epigenetic clock (common bottlenose dolphin: Beal *et al* ., 2019; domestic cat: Qi *et al* ., 2021). While many other mammalian species have reported that there were no significant sex differences found (humpback whale: Polanowski *et al* ., 2014; fin whale: Garcia-Varnet *et al.,* 2020; beluga: Bors *et al.,* 2021; brown bear: Nakamura *et al* ., 2023). Most of these studies often do not investigate sex differences in DNA methylation rates for each gene but rather examine the significance of sex as one of the explanatory variables in age estimation formulas that utilize multiple genes. As a result of formulating estimation models with multiple genes, the impact of sex may be concealed and overlooked. To address this issue, further research focusing on the relationship between sex differences and methylation rate for each gene is needed.

This is the first study to suggest the effect of female nursing states on methylation rate and age in *GRIA2* , and *CDKN2A* .*GRIA2* are family of receptors (Henley & Wilkinson, 2013), while*CDKN2A* encodes for several tumor suppressor proteins (Foulkes*et al* ., 1997; Zhao *et al* ., 2016). From a functional perspective, it is difficult to consider the exact factors that could lead to the decrease in methylation rates during the calving period. However, a decrease in methylation could be associated with specific physiological changes that occur during suckling and other parenting- or calf-nursing-related activities. Although age-related changes in methylation rate decreased in females specifically, no significant sex differences were found overall. Therefore, it is possible that female methylation rates are adjusted after calving by unknown factors, and further studies examining the longitudinal variation in methylation rates of individuals are needed in the future. The female nursing states did not contribute to the precision and accuracy of the age estimation model. This may be due to the different regression methods employed. The ANCOVA was used to examine the sex differences in methylation rate changes with age for each gene based on the least squares method. This calculates the regression line by minimizing the distance from all data plots on the scatterplot. On the other hand, the SVR used in building the age estimation model in this study, is based on the maximum-margin principle, where the regression line is determined by maximizing the distance from the outer plots on the scatterplot. These conceptual differences between the regression methods suggest that an effect observed in one method may not be detectable in the other. It is recommended that future analyses of similar studies should take this effect into account.

Conventional age estimation has been based on counting the growth layers formed on the tooth cross section in odontocetes (see Perrin & Myrick, 1980). This method requires capturing of individuals to collect their teeth. In recent years, there have been attempts to develop an age estimation method using epigenetic clock analysis in various taxa (e.g. humans: Horvath, 2013; Bechstein's bats: Wright *et al* ., 2018; Asian elephants (*Elephas maximus*) & African elephants (*Loxodonta africana*): Prado *et al.,* 2020). All previous studies of epigenetic clock analysis on cetaceans have used skin samples, which require biopsy surveys or capture procedures to be conducted in the wild (Polanowski *et al* ., 2014; Beal *et al* ., 2019; Tanabe *et al* ., 2020; Bors *et al* ., 2021; Peters*et al* ., 2023). Biopsy surveys are generally considered less invasive compared to capture methods; however, it is worth noting that there have been instances of mortality, especially in small cetaceans (Bearzi, 2000; Noren & Mocklin, 2012). Our method allows for non-invasive age estimation as fecal samples were collected underwater, without touching and disrupting the dolphins. As stated by Qi *et al.,* (2021), pyrosequencing is the gold standard method for quantifying DNA methylation rate. However, each analysis requires 3–4 hours for completion and costs $14, while MS-HRM offers a more cost-effective alternative, with each analysis requiring only two hours and costing $7. This cost-effectiveness, combined with the shorter turnaround time, makes the MS-HRM method highly suitable for implementation in various research sites.

The framework of this study can be extended to other cetacean species and populations where fecal samples can be collected. For instance, the Atlantic spotted dolphins (*Stenella frontalis*) around the Bahamas and the dwarf minke whales (*Balaenoptera acutorostrata*subsp.) in Australia have conducted underwater identification surveys (see Herzing, 1997; Birtles *et al.,* 2002). These populations have favorable conditions for collecting fecal samples. In addition, fecal samples of large cetaceans can be collected whilst being on board (see Smith & Whitehead, 2000; Reidy *et al.,* 2022). Identification surveys on gray whales (*Eschrichius robustus*), North Atlantic right whales (*Eubalaena glacialis*), Southern right whales (*E. australis*), sperm whales (*Physeter macrocephalus*), blue whales (*B. musculus*), and humpback whales have been conducted using natural marks such as color patterns and the shape of flukes (Hammond *et al.,* 1990). Thus, it may be possible to introduce age estimation using fecal samples as well. Even in areas where long-term individual identification surveys have not been conducted by researchers, activities such as swim-with-dolphin programs, scuba diving, and wading in close proximity to observation targets have been developed in more than 11 genera of cetaceans, over 54 areas in 32 counties (Carzon *et al.,* 2023). Therefore, there is potential for collaboration with the tourism industry in these areas, where fecal samples could be collected in conjunction with tourism activities. This synergy between research and tourism allows for the collection of fecal samples while visitors engage in educational and conservation efforts. Terrestrial mammals may get better results because hydrolysis is less likely to occur on land. If the fecal samples-based age estimation can be applicated to terrestrial animals, it may lead to benefits for both study species and researchers because fecal samples may be collected non-invasively even in species that are difficult and/or dangerous to encounter.

The successful quantification of methylation rates using fecal samples of Indo-Pacific bottlenose dolphins in this study, suggests the potential applications of age estimation using the same genes in other cetacean species. This study serves as a steppingstone towards the widespread application of non-invasive age estimation methods in various mammal species, offering valuable contributions towards the understanding of their ecology through age-related information.

Conclusion

In this study, we were able to develop an age estimation model using an epigenetic clock analysis with DNA extracted from fecal samples of wild Indo-Pacific bottlenose dolphins. The accuracy of the age estimation model showed an MAE of 5.08 years, and the error ranges from 10–13% of its lifespan. The results provide a sufficient level of accuracy and could be used in ecological studies such as life history and demography. The accuracy of our model achieved in this study is comparable to studies which used similar gene regions in other species. To the best of our knowledge, this is the first study to have successfully quantified the methylation rates of multiple genes from fecal-derived DNA to estimate age in mammals.

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Author Contributions

Genfu Yagi : Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Software, Validation, Visualization, Writing; **Huiyuan Qi** : Investigation, Methodology; Software**Kana Arai** : Investigation, Methodology, Writing-review & editing; **Yuki F. Kita** : Resources, Supervision;**Tadamichi Morisaka** : Conceptualization, Supervision, Project administration, Writing-review & editing; **Kazunobu Kogi** : Data acquisition, Resources, **Motoi Yoshioka** : Supervision;**Miho Inoue-Murayama** : Funding acquisition, Methodology, Resources, Supervision, Writing-review & editing.

Data Accessibility

The data obtained from HRM analyses and R scripts used in this study have been made available in Dryad at DOI:*https://doi.org/10.5061/dryad.9zw3r22kr*

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Table 1 Details of target gene, primer, and PCR information, and accession number.

Gene	Primer	n (CpGs)	Length (bp.)	PCR condition	Accession number
GRIA2	F: GTAGTTTGTTG-GAAGTTGTA TTTAG R: CCTCCTCTTCCTTCTTTAAC	11	204	95 °C (5 min), [95 °C (10 s); 55 °C (30 s); 72 °C (10 s)]*40 cycles	NC_047038

Gene	Primer	n (CpGs)	Length (bp.)	PCR condition	Accession number
CDKN2A	F: TTTAGAAG-GATGAG-GTTGG R: CCTCTAAATCTTCATACCAATC	10	198	95 °C (5 min), [95 °C (10 s); 55 °C (30 s); 72 °C (10 s)]* 37 cycles	NC_047039

Table 2 Results of ANCOVA for *GRIA2* .

Explanatory varianble	Sum Sq	Df	F value	Pr(>F)
*GRIA2*_methyl_rate	417.21	1	11.415	0.002035
Sex	104.05	1	2.8468	0.101929
Nursing state	410.34	1	11.227	0.002189
*GRIA2*_methyl_rate:Sex	23.64	1	0.6468	0.427581
*GRIA2*_methyl_rate:Nursing_state	16.42	1	0.4493	0.507808

Table 3 Results of ANCOVA for *CDKN2A* .

Explanatory varianble	Sum Sq	Df	F value	Pr(>F)
*CDKN2A*_methyl_rate	650.63	1	23.0574	4.08E-05
Sex	110.57	1	3.9184	0.057001
Nursing state	316.71	1	11.2237	0.002192
*CDKN2A*_methyl_rate:Sex	0	1	0.0001	0.991066
*CDKN2A*_methyl_rate: Nursing_state	34.61	1	1.2264	0.276902

Table 4 Accuracy comparison among LOOCV models.

Model	R-square	MAE	p value
1. *GRIA2*_methyl_rate + *CDKN2A*_methyl_rate	0.334	5.079	0.0002234
2. *GRIA2*_methyl_rate + *CDKN2A*_methyl_rate + Sex	0.122	6.35	0.03648
3. *GRIA2*_methyl_rate + *CDKN2A*_methyl_rate + Nursing_state	0.260	5.41	0.001502

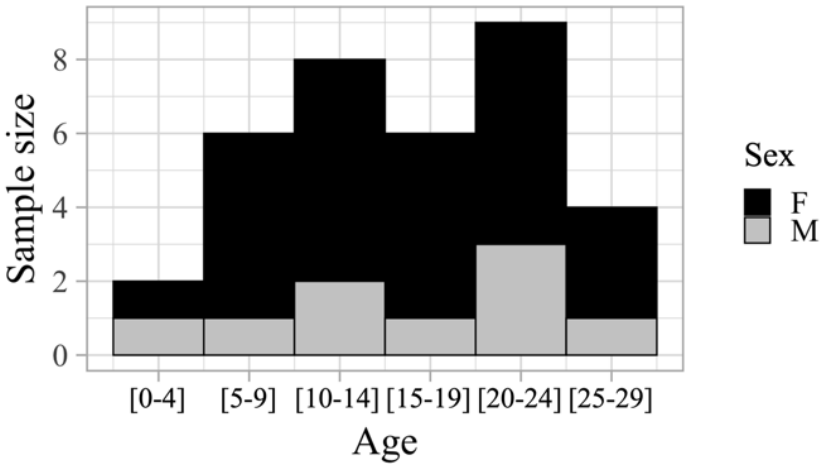

Figure 1. Age distribution of male and female individuals used for the analyses.

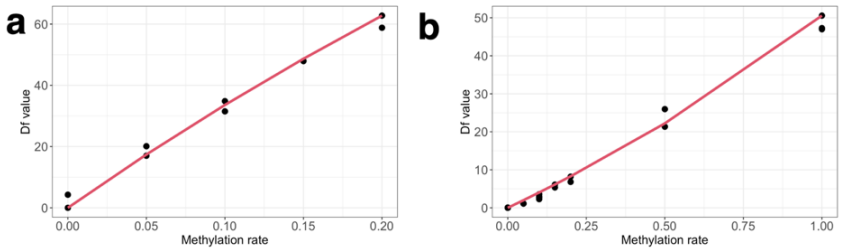

Figure 2. Standard curve of methylation rate and Df value. (a:*GRIA2* , b: *CDKN2A*)

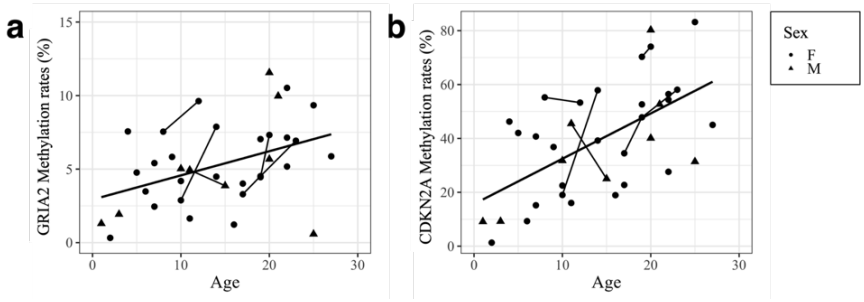

Figure 3. Correlation between methylation rate and age. (a) shows the result of *GRIA2* , and (b) shows the result of *CDKN2A* . Plots connected together are from the same individuals analyzed across different years.

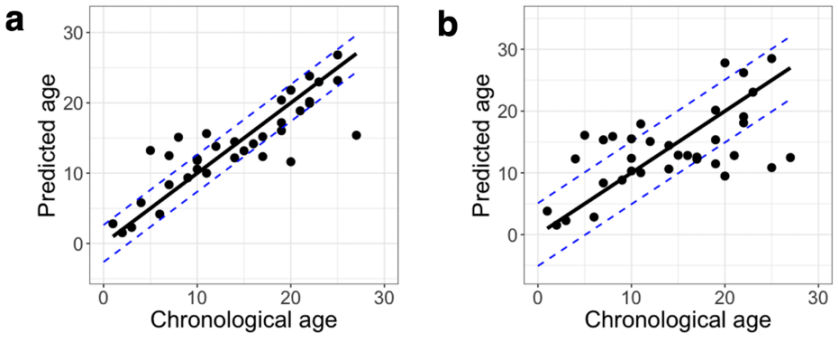

Figure 4. Relationship between chronological age and estimated age in model 1. (a) shows the result before LOOCV and (b) shows the result after LOOCV where the blue dotted line represents the MAE range and the black line represents y=x.

Table S1. List of tissue samples for standard DNA preparation.

Sample_No.	ID	Sex	Tissue
#1	#259	male	muscle
#2	#259	male	skin
#3	#165	male	skin
#4	#406	male	muscle
#5	#406	male	skin
#6	Unknown	female	muscle

Table S2. Results of age estimation for the age-unknown individuals. The 'Year' column indicates the year when the feces was collected, the 'First Identified' column indicates the year the individual was first identified, and the numbers in parentheses indicate the growth stage at that time (A: Adult, S: Subadult, J: Juvenile). The estimated age of the individual based on the year of weaning, or first birth is shown in the 'E A by life event' column. The parentheses indicate the event (W: Weaning, P: First birth).

ID no.	Year	Estimated age	First Identified	E A by life event
#004MA	2016	27.00	1994 (S)	NA
#014MA	2020	14.17	1994 (S)	NA
#025FA	2015	37.03	1994 (A)	NA
#033FA	2015	21.25	1994 (A)	NA
#036MA	2021	18.48	1994(S)	NA
#039FA	2015	15.48	1994(A)	NA
#055MA	2018	20.18	1994(S)	NA
#056FA	2015	31.09	1994(A)	NA
#068FA	2020	11.75	1994(S)	33.31 (P)
#072FA	2016	49.27	1994(A)	NA
#077FA	2016	9.78	1994(S)	18.31 (P)
#080FA	2016	35.05	1994(A)	NA
#086FA	2020	34.19	1994(A)	NA
#182MA	2016	4.67	1994(A)	NA
#551FA	2021	24.30	2002(J)	18.5 (W)
#558FA	2021	12.74	2003(S)	19.31 (P)

ID no.	Year	Estimated age	First Identified	E A by life event
#620FA	2021	16.89	2012(S)	12.31 (W)
#639MS	2021	14.96	2016(S)	NA
#652MS	2021	26.26	2018(S)	NA