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RESOURCE ARTICLE

Non-invasive age estimation based on faecal 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 relied on used biopsy samples. However, if DNA-based age estimation can be developed from faecal samples, age estimation can be performed entirely non-invasively. 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 faecal 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 collected from 30 individuals. 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 faecal samples to estimate the age of marine mammals.

KEYWORDS

ageing, demography, DNA methylation, ecology, epigenetics, Mikura Island

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1 | INTRODUCTION

Life history is a crucial factor that determines the ecology of animal species, and each species exhibits a specific life-history strategy. Understanding life history is also necessary for wildlife management because data on age at maturity, age-related changes in reproductive success, number of calves over a lifespan and longevity should be collected to predict population dynamic parameters. Growth stages can roughly be 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 & Lackey, 2011; killer whales (*Orcinus orca*): Olesiuk et al., 2005; Franks et al., 2016, belugas (*Delphinapterus leucas*): Ellis et al., 2018), and age-related changes in sociality (red deer (*Cervus elaphus*); Albery et al., 2022). 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 & 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 have been developed recently. One is the method using age-related external appearance changes on the body, including scars and body colouration (e.g. risso's dolphins (*Grampus griseus*): Hartman et al., 2015; Indo-Pacific humpback dolphins (*Sousa chinensis*): Guo et al., 2020). Krzyszczyk and 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 and 21 years due to the spots appearing age and the upper limit of the age-known individuals.

Ageing occurs in most organisms and it leads to various changes at the tissue and cellular levels (Petralia et al., 2014). Although ageing is thought to be caused by the combined effects of various factors (López-Otín et al., 2013), one of the factors that regulate ageing is epigenetic changes, which are inhibitory functional systems that accompany ageing at the gene level (Booth & Brunet, 2016). DNA methylation, such as CpG (cytosine-phosphate-guanine) methylation, is an example of an epigenetic change. Recently, the correlation between DNA methylation rate and ageing 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 the entire class of mammals and some non-mammalian species (e.g. all mammals: Lu et al., 2023; birds: De Paoli-Iseppi et al., 2019; fish: Weber et al., 2022; crustaceans: Fairfield et al., 2021). Following this pioneering study, similar approaches have been successfully applied to other cetacean species (common bottlenose dolphins (*Tursiops truncatus*): Beal et al., 2019; Antarctic minke whales (*Balaenoptera bonaerensis*): Tanabe et al., 2020; belugas: Bors et al., 2021; fin whales (*Balaenoptera physalus*): García-Vernet et al., 2021; Indo-Pacific bottlenose dolphins: Peters et al., 2023; bowhead whales (*Balaena mysticetus*) and killer whales: Parsons et al., 2023). These previous studies on cetaceans using epigenetic clock analyses relied on the samples obtained from invasive methods including commercial whaling (fin whales: 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., 2023, bowhead and killer whales: Parsons et al., 2023). 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 a single instance of death has occurred (Bearzi, 2000). By using faecal samples, DNA can be collected non-invasively without the need to touch individuals. However, there is a limited number of studies based on faecal-sampled epigenetic clocks. To our knowledge, the only studies to have developed epigenetic clocks using faecal 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, faecal 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 faecal samples from individuals, making the population well suited for faecal sample-based age estimation studies.

Here, we investigated the correlation between DNA methylation rate and age in faecal samples using a low-cost and convenient

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method called methylation-sensitive high-resolution melting (MS-HRM) analysis (Tse et al., 2011; Wojdacz et al., 2008; Wojdacz & Dobrovic, 2007). We focused on the genes *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, sex and 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 causing acceleration in epigenetic age (Ryan et al., 2018). This study aimed to develop a non-invasive age estimation model for Indo-Pacific bottlenose dolphins using DNA extracted from faecal samples. This methodology would be extensible to other mammals, and could contribute to ecological and conservation studies.

2 | MATERIALS AND 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 faecal 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 (33°90′ N, 139°60′ 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 to 45 m. Boat-based surveys generally use only dorsal fins for individual identification (e.g. Würsig & Würsig, 1977). In contrast, the underwater survey utilized natural marks on the whole body, including scars (mainly cookie-cutter shark rake marks), fin notches and other characteristic marks (e.g. the absence of a dorsal fin) for individual identification. We considered two dolphins to be the same if three or more natural marks matched in each image (see Kogi et al., 2004). 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 to 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

Faecal 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 faecal samples along with the environmental water. The recordings were then used to identify which faecal samples were collected from the individuals that defecated. The collected faecal 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°C after being transferred into containers with 70% ethanol or higher. DNA was extracted from weighing 0.6 g of ethanol-preserved faecal 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 faecal samples obtained from 45 individuals between 2014 and 2021. Due to the nature of faecal 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 in different years, we treated each data point as an independent data point. Under these conditions, 25 samples were excluded and 36 samples from 30 individuals were analysed. Among them, 9, 15 and 12 individuals were males, nursing females and non-nursing females respectively. Figure 1 shows the age distribution of the dataset, which covers 1–27 years.

2.4 | Standard DNA

Zero percentage and hundred percentage methylated standard DNA was created to calculate the methylation rate of the samples. High concentration 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

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piece of skin and muscle sample was cut into smaller pieces, DNA was extracted using the DNeasy Blood and 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 the *TET2* (ten 11 translocation 2), *GRIA2* and *CDKN2A* methylation rate and age in the common bottlenose dolphins (a species closely related to the Indo-Pacific bottlenose dolphins) using DNA extracted from skin samples. Similar correlations for the same genes were seen using skin DNA of 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 Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/) database using the Standard Nucleotide Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). PCR primers to amplify the three target genes (reference genomes: *TET2* and *GRIA2*: NC_047038; *CDKN2A*: NC_04739) were designed. 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. The target gene regions included 11 and 10 CpGs in *GRIA2* and *CDKN2A* respectively. Primers were designed using Methyl Primer Express v1.0 (Thermo Fisher Scientific, San Jose, CA, USA, Table 1).

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).

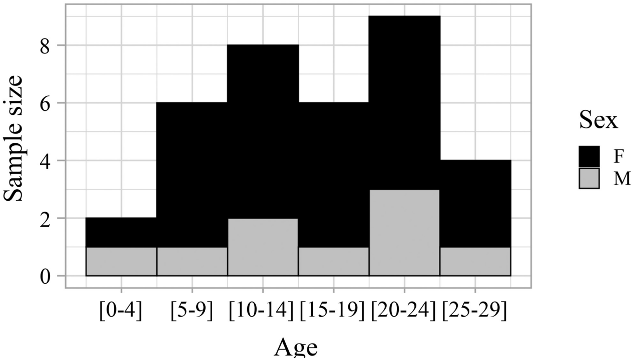

FIGURE 1 Age distribution of male and female individuals used for the analyses.

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 bisulphite conversion using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA). This converts unmethylated cytosines to uracil. The differences between the bisulphite-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 (bisulphite-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 5 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 s 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 genes.

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 at least 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.

TABLE 1 Details of target gene, primer and PCR information and accession no.

Gene	Primer	n (CpGs)	Length (bp.)	PCR condition	Accession no
GRIA2	F: GTAGTTTGTTGGAAGTTGTATTTAG R: CCTCCTCTTCCTTCTTTAAC	11	204	95°C (5 min), [95°C (10 s); 55°C (30 s); 72°C (10 s)]*40 cycles	NC_047038
CDKN2A	F: TTTAGAAGGATGAGGTTGG R: CCTCTAAATCTTCATACCAATC	10	198	95°C (5 min), [95°C (10 s); 55°C (30 s); 72°C (10 s)]* 37 cycles	NC_047039

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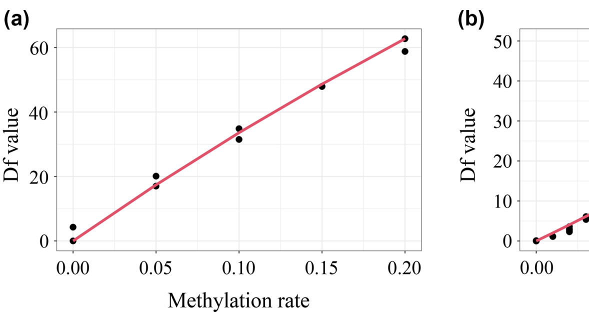

FIGURE 2 Standard curve of methylation rate and Df value. (a: *GRIA2*, b: *CDKN2A*).

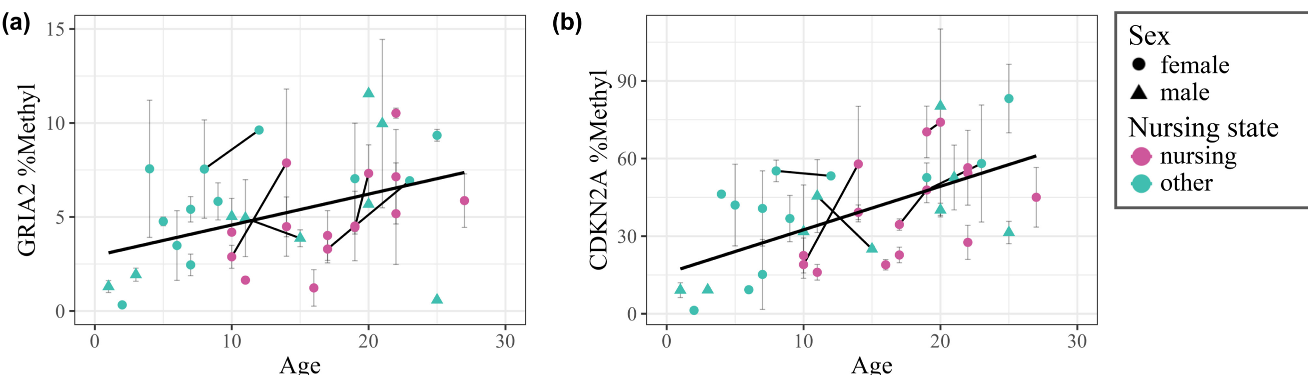

FIGURE 3 Correlation between methylation rate and age. (a) shows the result of *GRIA2*, and (b) shows the result of *CDKN2A*. Plots connected together are from the same individuals analysed across different years. The error bar indicates the standard deviation of multiple measurements for each sample.

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 Warnecke et al. (1997) modified by Hamano et al. (2017) as follows:

$$\frac{a \times M}{b - M} = \frac{Df}{Df_{max} - Df}$$

where *a* is the coefficient, *b* is the maximum methylation rate used for the standard curve construction, *M* is the methylation rate, Df_{max} is the 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) and leave-one-individual-out cross-validation (LOIOCV) was performed to validate the overfitting of the models. This is because in contrast to LOIOCV, LOOCV treats samples obtained from a single individual but differing years as independent data which may result in an overfitted model. We compared the LOOCV and after LOIOCV errors to evaluate the individual differences. The precision and accuracy were calculated both before and after LOOCV and LOIOCV. 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, 2020) and "car" (Fox & Weisberg, 2019) were used for the

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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 faecal samples. The error in the estimated ages was considered based on the year of first identification, growth stage during faeces collection, and life event (weaning and first giving birth) year. The growth stages were confirmed as described by Kogi et al. (2004). Five individuals were recorded in the life event years such as weaning and first giving birth (Table S2).

3 | RESULTS

Figure 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 inferred methylation rates of *GRIA2* and *CDKN2A* ranged from 0.32–11.56% and 1.32–83.20% respectively. 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*: $R^2 = 0.34$, $p < 0.01$; Figure 3). We examined the changes in methylation rate over the years for five individuals for which samples were collected across multiple times (Figure 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*: $p > 0.05$; Tables 2 and 3). On the other hand, nursing females showed significantly lower methylation rates (ANCOVA, *GRIA2*: $p < 0.05$, *CDKN2A*: $p < 0.05$; Tables 2 and 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 R^2, mean absolute error (MAE) and error range of the model before LOOCV were 0.74, 2.63 years and −11.6–8.24 respectively (Figure 4a). However, the R^2, MAE and error range of the model after LOOCV were 0.33, 5.08 years and −14.5–19.0 of the error range respectively (Figure 4b). The model exhibited an R^2 of 0.31 and an MAE of 5.55 after LOIOCV (Figure S1). Thus, the difference between the results after LOOCV and LOIOCV was not conclusive, and the effect of individual differences on the model was low.

In comparison with models 2 and 3, which included sex and nursing status as an explanatory variable, respectively, model 1, which did not include sex and nursing status, 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). The estimated age of four individuals (individual numbers: #014MA, #068FA, #077FA and #182MA) was more than 10 years younger than the continuous observation period. When comparing the results of life-event years with the estimated ages, no additional individuals exhibited differences exceeding 10 years when considering the typical events reported by Kogi et al. (2004) and Kogi (2013).

4 | DISCUSSION

In this study, we found a significant correlation between age and methylation rate in the gene regions, *GRIA2* and *CDKN2A* using DNA extracted from a non-invasive faecal sample. Although sex did not affect the correlation between age and methylation rate, the methylation rate of nursing females was lower than expected and thus, their age tends to be underestimated. 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 two genes and DNA extracted from faecal samples to develop an age estimation model. We estimated the ages of 19 age-unknown individuals using our model (Table S2). The estimated results were inaccurate for four individuals from long-term individual identification records. These errors were reasonable given the error range in our study. Moreover, the accuracy of our model was lower than that for other models using cetacean skin samples (e.g. humpback whales: MAE = 3.575, Polanowski et al., 2014; common bottlenose dolphins: RMSE = 5.14, Beal et al., 2019; fin whales: MAE = 4.264,

TABLE 2 Results of ANCOVA for *GRIA2*.

Explanatory variable	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

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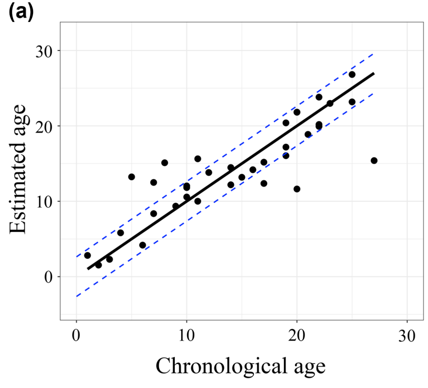
TABLE 3 Results of ANCOVA for *CDKN2A*.

Explanatory variable	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



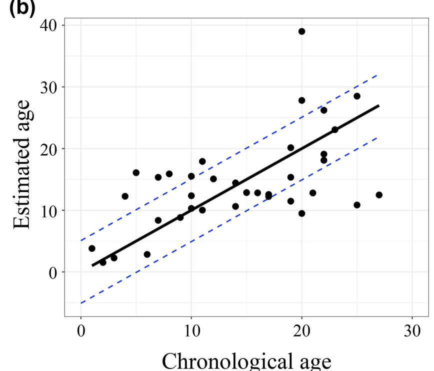


FIGURE 4 Relationship between chronological age and estimated age in model 1. (a) shows the result before LOOCV and (b) shows the result after LOOCV where the blue dotted line represents the MAE range and the black line represents y=x.

TABLE 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

García-Vernet et al., 2021, MAE=2.1, Peters et al., 2023). Thus, our model may need further enhancement.

The biggest limitations of this study include the number of target genes and sample size. A high number of explanatory variables generally improves model accuracy. Indeed, when comparing studies focused on *Tursiops* sp., those targeting tens of genes (MAE=4.84, Robeck et al., 2021; MAE=2.1, Peters et al., 2023) showed better accuracy than studies using only three genes or less (MAE=5.14, Beal et al., 2019). Mayne et al. (2021) suggested that a sample size of >70 is necessary to calibrate robust epigenetic clocks. However, some studies targeting wild animals have calibrated an epigenetic clock using <70 samples (e.g. $n=45$, Polanowski et al., 2014; $n=60$, Wright et al., 2018; $n=39$, Beal et al., 2019; $n=34$, García-Vernet et al., 2021; $n=49$, Nakamura et al., 2023). Nevertheless, the sample size in this study ($n=36$) was relatively small compared to those in previous studies. Despite using SVR to improve results with a limited sample size, and considering the errors in our model after LOOCV, it is apparent that the sample size was insufficient. In this study, we successfully estimated the age from faecal samples using a small sample size. However, future improvements in accuracy can be realized by increasing both the sample size and number of target genes.

GRIA2 and *CDKN2A* methylation rate quantification showed high errors in some samples (Figure 3). In contrast to previous studies that used pyrosequencing to measure methylation rates with DNA extracted from skin samples (humpback whales: MAE=3.575, Polanowski et al., 2014; common bottlenose dolphins: RMSE=5.14, Beal et al., 2019; fin whales: MAE=4.264, García-Vernet et al., 2021), we used MS-HRM with DNA extracted from faecal 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 are 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, faecal 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). The low target DNA concentration may have caused the high variance in each methylation rate measurement (Figure 3) and high estimation error. Although the variation was considerable, the overall correlation was statistically significant, suggesting that the error may have been caused by the accuracy of individual measurements rather than their precision. Future analyses conducted using DNA extracted from faecal samples may get closer to the true methylation rate by using more multiple replicated samples and taking the average. Considering that the faecal

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samples were collected underwater, faeces from different individuals outside the camera's field of view might have been mistakenly collected. We also recommend collecting faecal samples from an individual multiple times and estimating age based on replicated samples to enhance the accuracy of this method. In this study, we always monitor the defecation of dolphins to collect the faeces of known individuals and avoid collecting faeces from different individuals if two or more individuals defecated simultaneously. Therefore, the probability of contamination was low. Moreover, a method for faecal DNA quality assessment is needed before measuring the methylation rate to improve precision. Low-quality samples can be detected by checking whether the real-time PCR amplification curves are abnormal.

The age estimation model with the highest accuracy and precision was based on *GRIA2* and *CDKN2A* methylation rates (model 1), with an MAE of 5.08 years. This error was calculated for the age range used in this study up to 27 years of age. Assuming that the error is comparable over 27 years of age, this is 10–13% (percentage error) of the life span of an Indo-Pacific bottlenose dolphin, which is 40–50 years (Wang, 2018) and provides a sufficient level of accuracy for ecological and conservation studies. A study focusing on Bechstein's bats with 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 21 years for this species (see Wilkinson & South, 2002), the error corresponds to 7.2% of its lifespan. The age estimation error of blood samples from domestic cats was 3.83 years (Qi et al., 2021). Assuming a lifespan of 30 years for domestic cats, this error accounts for approximately 12.7% of the total lifespan. Notably, despite the use of faecal samples, which are associated with low precision, the range of error in lifespan was comparable to those studies conducted on mammalian species.

We did not find significant sex differences between *GRIA2* and *CDKN2A* methylation rate and age (Table 3). Comparisons between the models also showed that the model which excluded sex as an explanatory variable had the highest precision and accuracy. These results suggest that including sex may not be necessary for age estimation and are consistent with the results of many previous studies analysing the epigenetic clock in other mammals (humpback whales: Polanowski et al., 2014; fin whales: García-Vernet et al., 2021; belugas: Bors et al., 2021; brown bears: Nakamura et al., 2023). However, most of these studies did not investigate sex differences in DNA methylation rates for each gene but rather examine the significance of sex as an explanatory variable in age estimation formulas using multiple genes. The effect of sex may be concealed and overlooked because of the formulation of estimation models with multiple genes. In this study, nursing females showed a lower methylation rate than others, suggesting differences in the pattern of age-related methylation rates between sexes. This is surprising since this explanatory variable was included based on the assumption that the methylation rate would accelerate due to the increased stress from calf nursing. However, concluding the significance of this effect is difficult given the small sample size and low R^2, as well as the lack of knowledge about the relationship between nursing and methylation rate. Owing to the limited sample size of non-nursing females, we may have detected changes in methylation rates after maturity. Beal et al. (2019) reported no significant sex-related differences in the epigenetic clock analysis of common bottlenose dolphins. However, age-related methylation changes showed different sex-specific trends, suggesting that a small number of mature individuals in the dataset may hinder such contributions. Further studies on the relationship between sex and each gene methylation rate, particularly in mature individuals, are needed to address these issues.

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. Biopsy surveys are generally less invasive than capture methods (Noren & Mocklin, 2012); however, a single instance of death has occurred in small cetaceans (Bearzi, 2000). 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 (Beal et al., 2019; Bors et al., 2021; Peters et al., 2023; Polanowski et al., 2014; Tanabe et al., 2020). Our method allows for non-invasive age estimation as faecal samples were collected underwater, without touching and disrupting the dolphins. As stated by Qi et al. (2021), MS-HRM is quick and cost-effective. For each pyrosequencing analysis, the gold standard method for quantifying DNA methylation rate requires 3–4 h for completion and costs $14 and DNA microarray chip-based analysis requires approximately 1 h per chip and costs $150 per sample, while MS-HRM offers a more cost-effective alternative, with each analysis requiring only 2 h and costing $7. This cost-effectiveness, combined with the quick turnaround time, is an advantage for implementing MS-HRM in various research sites to estimate the age of many individuals, such as clarifying the age structure of a population.

The framework of this study can be extended to other cetacean species and populations where faecal samples can be collected. Both genes hypermethylated with age. Although common bottlenose dolphin skin DNA also hypermethylates with age (Beal et al., 2019), faecal *CDKN2A* methylation rates (range: 1.32%–83.20%, age: 1–27 years) were higher than that reported in previous studies (range: 0%–6.7%, age: 2–36 years; Beal et al., 2019). This is similar to the reported age-related hypermethylation of human *ELOVL2*, which hypermethylates with age in various tissues, although its slope is tissue specific (Bacalini et al., 2017). The correlation between age and methylation rates of *GRIA2* and *CDKN2A* has been reported in some cetaceans (Beal et al., 2019; García-Vernet et al., 2021; Polanowski et al., 2014), suggesting that methylation rates of these genes may be useful for age estimation in other cetacean species. 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 Birtles et al., 2002; Herzing, 1997). These populations have favourable conditions for

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collecting faecal samples. In addition, faecal samples of large cetaceans can be collected whilst being on board (see Reidy et al., 2022; Smith & Whitehead, 2000). Identification surveys on grey 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 colour patterns and the shape of flukes (Hammond et al., 1990). Thus, it may be possible to introduce age estimation using faecal 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 faecal samples could be collected in conjunction with tourism activities. This synergy between research and tourism allows for the collection of faecal 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 faecal sample-based age estimation can be applied to terrestrial animals, it may lead to benefits for both study species and researchers because faecal samples may be collected non-invasively even in species that are difficult and/or dangerous to encounter.

The successful quantification of methylation rates using faecal 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 stepping stone 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.

5 | CONCLUSION

In this study, we were able to develop an age estimation model using an epigenetic clock analysis with DNA extracted from faecal 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 faecal-derived DNA to estimate age in mammals.

AUTHOR CONTRIBUTIONS

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

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

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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