Full paper

A PRELIMINARY STUDY OF EPIGENETIC ESTIMATION OF AGE OF THE ANTARCTIC MINKE WHALE *BALAENOPTERA BONAERENSIS*

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Abstract

Age is one of the most important life history parameters for assessment and management of marine living resources. Counting of the growth layers deposited in the earplugs is the most accepted technique for determining chronological age of baleen whales. However unreadable growth layers form in the earplugs of some individual whales. In such cases, alternative methods of age estimation are required. The objective of the present study was to examine the utility of the DNA methylation technique as a proxy to estimate chronological age in the Antarctic minke whale. For this purpose, skin tissues of a total of 100 Antarctic minke whales sampled in the Pacific region of the Antarctic by JARPAII surveys were used. Earplug-based age data from the same whales were used for calibration purposes. Seven CpG sites in three genes (TET2, CDKN2A and GRIA2) were selected for the analysis. In a previous study, these sites showed significant correspondence between methylation levels and age in humpback whales. Methylation levels of the seven CpG sites were scored successfully. Four CpG sites showed significant regressions with age, which contrasted with the case of the humpback whale where all seven sites showed significant regressions with age. The assay predicted age from skin samples with a standard deviation of 8.865 years. This low precision makes the age estimated by the CpG methylation technique unsuitable for use in population dynamics models such as the statistical catch-at-age (SCAA). Furthermore CpGs methylation levels fluctuated among body positions of the whale, particularly between dorsal (exposure to sunlight) and ventral sides. The precision of the CpG methylation technique for age estimation could be improved by increasing the number of CpG sites showing a good correlation with age, and this work is ongoing. In addition, other factors including variation of CpG methylation levels between different tissues should be examined to further evaluate the utility of the DNA methylation techniques as a proxy of age estimation in baleen whales.

Keywords Antarctic minke whale, age estimation, epigenetics, DNA methylation, earplugs

Introduction

Age is one of the most important life history parameters for assessment and management of marine living resources. In baleen whales, age has been determined using a variety of methods such as examination of baleen plates (Nishiwaki, 1951; Zenitani and Kato, 2010), earplugs (Lockyer, 1984) and tympanic bulla (Christensen, 1995). Counting of the growth layers deposited in the earplugs is the most accepted technique for determining chronological age of baleen whales (Lockyer, 1984). Earplug-based age determination has the advantage that it is time- and cost-efficient, and the technique can be used on available historical samples.

The Antarctic minke whale (*Balaenoptera bonaerensis*) is one of the smallest balaenopterid species, which is widely distributed in the Southern Hemisphere. This species is considered the most abundant baleen whale species, with a total abundance estimated at 515,000 (IWC, 2012). During the assessment of this species, the International Whaling Commission Scientific Committee (IWC SC) successfully applied statistical catch-at-age (SCAA) analyses. A summary history of the application of SCAA to this species was presented by Punt (2014), and an assessment of Antarctic minke whales using SCAA was reported by Punt *et al*. (2014). Among the most important outputs from the SCAA analyses was the information on the age-specific natural mortality and the historical trends of the stocks. The key input data for the SCAA analyses consisted of catches, abundance estimates, length frequency data and age-at-length data. Age data was obtained from reading earplugs of Antarctic minke whales caught during past commercial whaling operations and by scientific surveys by the Japanese Whale Research Program under Special Permit in the Antarctic (JARPA/JARPAII).

While earplug's growth layers reading is considered the most acceptable technique for age determination in whales, unreadable growth layers form in the earplugs of some individual baleen whales (Maeda *et al.*, 2013; George *et al.*, 1999). In the case of the Antarctic minke whale, determining the age by reading earplugs of immature whales is particularly difficult. In such cases, alternative methods of age determination are required.

Indices of chronological age of whales have been developed using molecular approaches. These include length of telomeres (Olsen *et al.*, 2012; 2014), and epigenetic technique based on DNA methylation (Polanowski *et al.*, 2014). Another technique is based on enantiomers of aspartic acid in eye lens, the aspartic acid racemization (AAR) technique, which was successfully applied to Antarctic minke whales (Yasunaga *et al*., 2017).

The present study focuses on the estimation of age in Antarctic minke whale based on the epigenetic approach. The best studied class of epigenetic change in vertebrates is the methyl group presence or absence at the C5 position of Cytosine residues that are adjacent to Guanidine residues ('CpG sites'). CpG methylation levels play an

important role in the control of gene expression, where higher methylation levels ('hypermethylation') generally reduce gene transcription rate. Methylation changes at specific CpGs have been linked to age in mice (Maegawa *et al*., 2010) and humans (Christensen *et al*., 2009; Gronniger *et al*., 2010; Bocklandt *et al*., 2011; Koch and Wagner, 2011; Hannum *et al*., 2013). It should be noted that the CpG methylation technique does not provide the chronological age of the individuals but rather a physiological age that can be used as a proxy for chronological age.

The CpG methylation approach was recently applied to known age (from photo-identification studies) humpback whales (Polanowski *et al*., 2014). These authors assayed 37 cytosines for methylation level in humpback whale skin of which seven had significant age-related profiles. They selected the three most age-information cytosine markers for a humpback whale epigenetic age assay. The assay had a coefficient of estimation (R^2) of 0.787, and predicted age from skin samples with a standard deviation of 2.991 years.

The objective of the present study was to examine the utility of the CpG methylation technique as a proxy to determine chronological age in the Antarctic minke whale, by using the same three informative cytosine markers of the humpback whale (Polanowski *et al*., 2014). The ages of the Antarctic minke whales examined for this epigenetic study were available from earplug's readings, and these data were used for calibration purposes.

Apart from the investigation of informative CpG sites, several other factors should be considered to further investigate the utility of the CpG methylation technique for determining age of whales for population-level analyses (see summary in IWC, 2017). For example, it was suggested there is a need to better understand the 'stressors' (e.g. sunlight) that may affect the calibration of the methylation approach. A secondary objective of the present study was therefore to investigate changes of the CpG methylation levels with regard the body positions where the skin samples were obtained, *i.e.* parts with more or less exposure to sunlight.

Materials and methods

Whale sampling

Antarctic minke whales used in the present study were caught in the austral summer seasons 2010/11 and 2011/12 in the Pacific sector of the Antarctic comprised of the area between 130°E-145°W by surveys of the Second Phase of the Japanese Whale Research Program under Special Permit in the Antarctic (JARPAII). For the CpG methylation study a total of 100 whales were selected from the total number of whales caught in the two austral summer seasons. The selection was based on the quality of their age information from earplugs, information used for calibration purposes. The age determination based on earplugs was conducted by a researcher from the Institute of Cetacean Research (ICR) following Lockyer (1984). Each individual age was determined by counting growth layers appearing on the bisected surface of the earplug using a stereoscope microscope, assuming an annual deposition of growth layers (i.e. one pair of dark and pale laminae accumulated per year). For the 100 whales selected, age information from the earplugs was considered as 'Excellent' (very clear Growth Layer Groups (GLGs), and low likelihood of aging reading error) or 'Good' (most of the GLGs are clear, and moderate likelihood of aging reading error). Table 1 shows the number of individuals used in this study by sampling season, sex and age classes.

Tissue sampling and DNA extraction

Skin tissue samples were obtained from each of the 100 whales, from the left lateral part of their body (sampling position '6' in Fig. 1). Tissue samples were stored in 95% ethanol until DNA extraction. Genomic DNA was extracted from 0.05 g of skin tissues using Gentra Puregene kits (QIAGEN). Extracted DNA was stored in the TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The IWC guidelines for DNA data quality (IWC, 2009) were followed as much as possible (see Kanda *et al*., 2014).

Identification of age-related CpG sites in the Antarctic minke whale

The procedure for identification of age-related CpG sites and measurement of methylation levels in Antarctic minke whales followed Polanowski *et al.* (2014), and their explanation of procedures is repeated below. The following three genes (seven CpG sites) were selected because they showed significant correspondence between CpG methylation levels and age in humpback whales: TET2 (CpG+16, CpG+21 and CpG+31 sites); CDKN2A (CpG+297, CpG+303 and CpG+309 sites), and GRIA2 (CpG+202 site).

Three candidate regulatory region sequences of Antarctic minke whale (Kishida *et al*., 2015) were taken from GenBank and BLAT searches of the humpback whale genome. Where candidate genes had a clearly orthologous regulatory region in the humpback whale genome, primers for amplification of Antarctic minke whale sequences were designed by eye based on homologous humpback whale sequences (Table 2).

Measurement of cytosine methylation levels

Methylation levels in the CpG sites were measured with Qiagen PyroMark assays. The pyrosequencing assays were designed using PYROMARK Assay Design Software (Version 2.0.1, Qiagen). Antarctic minke whale DNA was converted using the Epitect Bisulphite Conversion Kit (Qiagen). The assay regions were PCR amplified using a biotinlabelled, HPLC-purified primer and standard sequencing grade primer (Table 2). Amplification reactions consisted of 12.5 µl PYROMARK mastermix, 2.5 µl Coral Load, 1 µl each of 5 µM forward and 5 µM reverse primers, 2 µl of bisulphite converted template DNA and 6 µl of water. Thermocycling conditions were 15 min at 95°C followed by 45 cycles of 30 s at 95°C, 30 s at 56°C and 30 s at 72°C and a final extension step of 10 min at 72°C. Pyrosequencing was performed on a PYROMARK 24 Pyrosequencing System (Qiagen). The PYROMARK Q24 software gave percentage methylation values for each CpG site.

Comparison between methylation level of CpG sites and age determined by earplug readings

The methylation levels for each of the seven CpG sites were compared to the ages determined by earplug readings, for n=100 individuals. Linear regression was used to investigate how much of the variation in CpG site methylation was explained by age differences. The accuracy and precision of the Antarctic minke whale epigenetic age assays were assessed with a Leave One Out Cross Validation (LOOCV) (Picard and Cook, 1984) analysis for the seven methylation sites combined.

Variation of the methylation levels of CpG sites with body position

Six Antarctic minke whales sampled during the 2016/2017 New Scientific Whale Research Program in the Antarctic Ocean (NEWREP-A) survey in the sector comprised of the area between 45°-150°E were used for this experiment. Skin samples were collected from four positions of the dorsal side (positions 1-4), two positions of the lateral side (positions 5-6), and one position of the ventral side (position 7) (Fig. 1, Table 3). Also, in the case of five whales (all except whale No. 6 in Table 3), additional sampling was carried out at three positions (8-10) that corresponded to parts of the body injured or with Cookie cutter shark (*Isistius brasiliensis*) marks (Table 4). Cookie cutter sharks live in the depths of all the oceans near the equator. Their top teethes are small, pointy and sharp to grasp hold of the whale or other prey's skin. Turning in a circle, this shark carves a round chunk of flesh

out with its larger razor-sharp, serrated or saw-like bottom teeth. In a flash, the cookie cutter scoops out the meal. Its preys are left with an almost perfectly round mark that looks like someone used a round cookie cutter on its body (Compagno, 1984). In the case of whale No. 6, only one additional position was investigated (position 8; Table 3). In total, 58 skin samples were collected for this experiment.

Tissue preservation, DNA extraction and DNA-M procedures were the same as explained earlier. Methylation levels of the seven CpG sites in each of the six whales were compared among the seven body positions shown in Fig. 1.

Results

Identification of age-related epigenetic markers in Antarctic minke whale

The PCR amplifications using a biotinlabelled primer set for the three genes were checked for quality by the agarose gel. The three genes for all individuals were amplified successfully. After pyrosequencing assay, the quality checks by PYROMARK Q24 software were evaluated as high (passed) for the three genes of all individuals and methylation levels for each CpG site were assessed.

Comparison between cytosine methylation level and age determined by earplug readings

The regressions of age and methylation levels in each of the seven CpG sites are shown in Fig. 2. The highest R^2 was observed in site TET2_CpG+31 (0.1874) and the lowest in site CDKN2A_CpG+297 (< 0.0001). Four CpG sites had a significant regression relationship with age (TET2_CpG+16, P=0.014; TET2_CpG+21, P=0.009; TET2_CpG+31, P=6.88e-06; and CDKN2A_CpG+309, P=0.005). The other three sites (GRIA2_CpG+202, CDKN2A_CpG+297 and CDKN2A_CpG+303) had a non-significant regression relationship with age (P > 0.05).

The precision of the Antarctic minke whale epigenetic age assay as assessed by the LOOCV is shown in Fig. 3. The overall precision was estimated as the standard deviation of the mean difference between known and estimated ages, which was 8.865 years.

Variation of the methylation levels of CpG sites with body position

Fig. 4 shows the variation of the methylation levels of CpG sites at different positions in the whale body of six individual Antarctic minke whales. This analysis was made for the three genes and seven CpG sites (excepting some few for which amplification failed). Methylation level of the seven CpG sites fluctuated with body positions regardless of the body length of the whales. In particular, the three TET2's CpG sites showed that the difference between the maximum and the minimum values of methylation levels was 50% or more among body positions. The ratio of maximum and the minimum methylation level of the TET2's sites were 2.1-3.2 fold in the whale No. 2; 1.3-1.7 fold in the whale No. 5; and 1.6-2.4 fold in the whale No. 6 (Table 3; Fig. 4). The average methylation level per individual, per TET2's site was 12.5% in the four dorsal positions, 11.5% in the two lateral positions and 7.5% in the ventral position. The three TET2's sites in three individuals (whales No. 2, 5 and 6) showed the lowest methylation level in body position seven (ventral side), compared with the other positions (Table 3; Fig. 4).

Regarding additional sampling positions (8-10) that corresponded to parts of the body with Cookie cutter shark marks, the average methylation level per individual, per TET2's site in the whale No. 2 and 5 was 10.2%. This value was lower than the average methylation level in the four dorsal positions or in the two lateral positions, and higher than in the single ventral position.

Discussion

This study provided a good opportunity to compare the utility of the same CpG sites to determining age in two baleen whale species. The present study took advantages of a substantial number of Antarctic minke whales for which age had been determined independently by earplug readings. Such information was of great utility for calibration purposes.

As noted earlier chronological age is important for assessment purposes related to the Antarctic minke whale such as that conducted using SCAA (Punt *et al*., 2014). Such models require precise information on the age of individual whales. In this regard the precision of the age estimates by the CpG methylation approach was low in both humpback (standard deviation of 2.991 years, Polanowski *et al*., 2014) and Antarctic minke (standard deviation of 8.865 years) whales. It should be noted that the LOOCV estimates of standard deviation in both species are not comparable as the analysis in humpback whales was based on three sites while the Antarctic minke whale analysis was based on seven sites. In any case, the low precision in both cases makes the age estimated by the CpG methylation approach unsuitable for use in population dynamics models such as the SCAA. Kitakado (2016) showed that the precision of methylation-based recruitment in the Antarctic minke whale is much worse than that for earplug-based readings, with the methylation-based results hardly better than those without any age information at all.

On the other hand the CpG methylation technique does not provide the chronological age of the individuals but rather a physiological age that can be used as a proxy for chronological age. Thus physiological and metabolic differences between species, means that the technique will need to be calibrated for each species (IWC, 2016). This study made a contribution in this regard as the performance of the same genetic markers was compared between two different species of baleen whales. In fact, some differences were observed between the two species in the pattern of methylation levels with respect the age. For example in the case of the humpback whale all seven loci showed significant regression with age while in the case of the Antarctic minke whale only four of those loci showed a significant regression relationship. Tanabe *et al*. (2020) concluded that age-related CpGs can differ even between closely related species, and that it is necessary to find species-specific age-related CpGs for estimating the age of animals using this technique.

This present study found that CpG methylation levels vary substantially among different parts of the body from which skin samples were obtained. In particular differences were observed between dorsal (body parts exposed to

sunlight) and ventral (body parts not exposed to sunlight). Gronniger *et al*. (2010) showed that aging and sun exposure are associated with comparably small, but significant changes in the DNA methylation patterns of human epidermis and dermis samples. There is a need to better understand the 'stressors' (e.g. sunlight) that may affect the calibration of the methylation approach and further studies should be conducted in the future. On the other hand, the effects on methylation levels of acquired external injures including Cookie cutter shark marks might be minimal for the Antarctic minke whales.

The screening of a substantial number of CpG sites based on Human BeadChip microarray has already started for the Antarctic minke whale, and results will be presented in the near future. Precision of the CpG methylation technique for age estimation could be improved by increasing the number of CpG sites showing a good correlation with age.

Apart from increasing the number of CpG sites to increase the precision of the age estimates, other aspects should be considered to further evaluate the utility of the CpG methylation approach as a proxy of chronological age in baleen whales. For example, correlation between chronological age and methylation profile varies a great deal among different tissues (Horvath, 2013). In addition to skin, biopsy samples typically include connective tissue and the lipid filled fat cells and these tissues should also be investigated (Arner *et al.*, 2015). These studies should continue in the future using a large number of CpG sites and whales.

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