Novel epigenetic age estimation in wild-caught Gulf of Mexico reef fishes

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Abstract: Cutting-edge DNA methylation-based epigenetic aging techniques were applied to Gulf of Mexico northern red snapper (*Lutjanus campechanus*; $n = 10$; 1–26 years old) and red grouper (*Epinephelus morio*; $n = 10$; 2–14 years old). Bisulfite-converted restriction site-associated DNA sequencing was used to identify CpG sites (cytosines followed by guanines) that exhibit age-correlated DNA methylation, and species-specific epigenetic clocks developed from 100s of CpG sites in each species showed strong agreements between predicted and otolith-derived ages ($r^2 > 0.99$ for both species). Results suggest epigenetic age estimation could provide an accurate and efficient approach to mass-aging fishes in a non-invasive manner.

Keywords: DNA methylation, epigenetic clock, age estimation, stock assessment, fisheries, red snapper, red grouper

Introduction

Age data are fundamental to determining life history parameters (e.g. age-at-length, age-at-maturity, age-related fecundity) and are thus critical for fisheries assessment and management, particularly when age-structured stock assessment models are used to estimate if a stock is overfished or undergoing overfishing. Fish age is traditionally determined by counting growth zones in a range of hard structures, including otoliths, vertebrae, scales, and fin rays (Campana 2001). Such techniques can be costly and time intensive (Helser et al. 2019), are of low accuracy for some species, are subject to reader bias, and are necessarily lethal in the case of otoliths and vertebrae (Campana 2001; Anastasiadi and Piferrer 2019). Moreover, protected species, brood stock for hatchery programs, and commercially valuable whole fish cannot have their otoliths or vertebrae extracted, and thus cannot be accurately aged using traditional techniques. As the demand for fish age composition data is increasing (Helser et al. 2019), there is a need to develop alternative age estimation methods.

Epigenetics refers to molecular-level mechanisms that affect gene expression without altering the underlying DNA sequence and that are heritable down cell lines or from parent to offspring (Kilvitis et al. 2014). DNA methylation is the most studied epigenetic mechanism and refers primarily to the addition of methyl groups (CH_3) to cytosines located within CpG dinucleotides (cytosines followed by guanines; Kilvitis et al. 2014). Recent studies have demonstrated that changes in DNA methylation levels at certain CpG sites exhibit strong correlations with chronological age, leading to the development of age-predictive models based on DNA methylation, referred to as epigenetic clocks (reviewed in Parrott and Bertucci 2019). Previously developed epigenetic clocks have involved methylation levels at 3 to 353 CpG sites (Horvath 2013; Polanowski et al. 2014) identified in a range of tissue types, including skin

(Polanowksi et al. 2014), blood (Thompson et al. 2018), muscle (Anastasiadi and Piferrer 2019), and fin clips (Mayne et al. 2020), and patterns have been shown to be tissue-specific (Horvath 2013; Thompson et al. 018). While epigenetic clocks have been derived predominantly for mammalian species, a handful of epigenetic clocks have been developed for laboratory-raised fishes, including for European sea bass (*Dicentrarchus labrax*; Anastasiadi and Piferrer 2019), zebrafish (*Danio rerio*; Mayne et al. 2020), and medaka (*Oryzias latipes*; Bertucci et al. 2021). In addition, Mayne et al. (2021) developed epigenetic clocks for three species of threatened fishes (Australian lungfish, *Neoceratodus forsteri*; Murray cod, *Maccullochella peelii*; and Mary River cod, *Maccullochella mariensis*), using a combination of wild and laboratory-raised individuals. While the development of epigenetic clocks in the aforementioned species suggests DNA methylation levels may generally be predictive of age in fishes, the majority of the aforementioned clocks were developed using samples from fishes reared in laboratories. Fish reared in controlled environments are not exposed to the same degree of environmental variation experienced by fishes in the wild and thus may not exhibit the same degree of environmentally induced epigenetic change. Moreover, Mayne et al. (2021) targeted CpG sites with methylation levels that are known to significantly correlate with age in laboratory-raised zebrafish and that were conserved between zebrafish and the target species. Therefore, the potential *de novo* identification of CpG sites exhibiting age-correlated methylation in wild-caught fishes of management concern, and the use of such sites to accurately estimate age, remains unknown.

Red snapper (*Lutjanus campechanus*) and red grouper (*Epinephelus morio*) are two of the most important species in commercial and recreational fisheries in the U.S. Atlantic and Gulf of Mexico. Both species are long-lived (>50 years for red snapper and >25 years for red grouper), and both are of management concern (SEDAR 52; SEDAR 61). Age estimates for both species

have been generated through traditional otolith increment analysis (Lombardi-Carlson et al. 2002; Lombardi 2017), and otolith-derived age estimation for red snapper has been validated with the bomb radiocarbon chronometer (Barnett et al. 2018). However, as with many managed species, the regulatory histories of red snapper and red grouper stocks have been complex, requiring large investments of time, effort, and financial resources into processing ever-increasing numbers of samples to generate age composition data that is input into stock assessment models (Passerotti et al. 2020). For example, over 49,000 otolith samples were processed and aged for the most recent stock assessment of Gulf of Mexico red snapper (Lombardi 2017).

Given the importance of age data for fisheries assessments and the lack of effective non-lethal methods for estimating age, the development of epigenetic clocks in species of management or conservation concern would be of great utility. In the present study, we sought to investigate the possibility of creating epigenetic clocks for wild-caught red snapper and red grouper, through the *de novo* identification of loci exhibiting age-correlated DNA methylation in two different tissue types (muscle and fin clips), both of which can be sampled non-lethally in the course of fisheries research.

Methods

Tissues were obtained from red snapper (muscle, $n = 10$ individuals) and red grouper (fin clips, $n = 10$ individuals) collected in the northern Gulf of Mexico and for which age was estimated via counts of opaque zones in otolith thin sections (range: 1–26 years for red snapper and 2–14 years for red grouper). In addition, all red snapper samples analyzed in this study had their ages directly validated via application of the bomb radiocarbon chronometer (Barnett et al.

2018; Patterson et al. 2021). Genomic DNA was extracted from tissue samples using a Mag–Bind Blood & Tissue DNA Kit (Omega Bio-tek, Inc., Norcross, USA), and DNA libraries were prepared for bisulfite-converted restriction site-associated DNA sequencing (bsRADseq), following a modified version of the Trucchi et al. (2016) protocol (described in Supplemental Material[1]). Libraries for both species were split into two portions, and one portion was bisulfite-treated using an EpiTect Plus Bisulfite Kit (Qiagen, Hilden, Germany). Bisulfite treatment converts unmethylated cytosines into uracils through chemical deamination, and uracils are subsequently replaced by thymines during PCR. This results in predictable base substitutions at all unmethylated cytosines, which can be identified by comparing sequences from the treated library to the untreated library (Trucchi et al. 2016). Libraries were sequenced across a single lane per species on an Illumina HiSeq4000 (Illumina, Inc., San Diego, USA). Red snapper reads were mapped to a draft of the red snapper genome (Portnoy, D.S. unpublished data), while the red grouper bisulfite-treated reads were mapped to a reference genome constructed from the untreated reads, using the *dDocent* pipeline (Puritz et al. 2014) for c = 0.88, $K_1 = 2$, and $K_2 = 1$. Mapped reads were then filtered to retain primary alignments, proper pairs, and those with a mapping quality ≥ 40.

CpG sites that could not be successfully genotyped in the untreated library or that were identified as potential single nucleotide polymorphisms (SNPs; defined as sites where >5% of total untreated reads across individuals displayed a cytosine to thymine substitution on the forward strand or guanine to adenine substitution on the reverse strand) were removed from the dataset. For each species, CpG sites were then filtered to retain only sites present in ≥90% of individuals. To identify CpG sites exhibiting age-correlated methylation, a Bayesian framework

[1]Supplementary data are available online.

was used to estimate the parameters of a generalized linear model (GLM) that included otolith-derived age as a fixed factor and individual as a random factor, using the *rstanarm* package (version 2.19.3; Goodrich et al. 2020; described in Supplemental Material[1]). The response variable was the binomial expression of the number of methylated reads (n) and the total number of reads for each sample (k) at a given CpG site $\binom{n}{k}$. GLMs were considered to have converged if the n_eff, a crude measure of the effective sample size, was greater than 2,000 or the Gelman-Rubin convergence diagnostic was less than 1.01 (Lunn et al. 2013; Muth et al. 2018). CpG sites with a 95% credible interval that did not include zero for the slope of otolith-derived age versus methylation were considered to exhibit significant age-correlated methylation.

For each CpG site that exhibited age-correlated methylation, percent methylation was estimated as the number of methylated reads divided by the total number of reads, and per-site 95% confidence intervals were calculated around the estimate in each individual (Clopper and Pearson 1934). Only those sites with confidence intervals <0.60 in at least 8 individuals per species were retained (Thompson et al. 2017). For retained sites, individuals with over-dispersed confidence intervals (>0.60) were entered as missing data. Because downstream analysis does not allow for missing data, methylation frequencies at missing individuals/sites were imputed using the *MICE* package (version 3.13.0; van Buuren and Groothuis-Oudshoorn 2011) in *R* (version 3.6.0).

The relationship between otolith-derived age and percent methylation across CpG sites was characterized using elastic net penalized regression modeling, a method that reduces the magnitude of coefficients and the number of predictor variables to reduce model complexity. Modeling was implemented in *glmnet* (version 4.0.2; Friedman et al. 2010) with an alpha parameter of 0.5 (considered the optimal merging of a ridge and lasso model; Thompson et al.

2017; Mayne et al. 2020; Bertucci et al. 2021). The internally cross-validated version of *glmnet* (*cv.glmnet*) was utilized to automatically select the optimal penalty parameter (λ), with 10-fold cross validation. Linear regressions were then computed to visualize the relationship between predicted age and otolith-derived age.

Results

Red snapper

For red snapper, 1,674,121 CpG sites were recovered across all samples. A total of 408,745 sites either could not be successfully genotyped in the untreated library or were identified as potential SNPs and were removed from the dataset. Of the remaining 1,265,376 sites, 49,189 sites were present in at least 90% of samples. Mean global CpG methylation across the retained sites was 85.72% (± 0.31%; SD). Bayesian GLMs identified 3,224 sites that exhibited significant age-correlated methylation, and a total of 1,829 sites had sufficiently tight confidence intervals in at least 80% of samples. Penalized regression analysis retained 199 CpG sites in the age-predictive model ($\lambda = 0.1104$). The slope and y intercept (± SE) for the regression of predicted age versus otolith-derived age were 0.98 (± 0.01) and 0.25 (± 0.09; $r^2 = 0.9995$; Fig. 1A). Of the 199 sites included in the final model, 80.40% of sites ($n = 160$) exhibited hypomethylation (i.e., a decrease in methylation with increasing age; Fig. 1C).

Red grouper

For red grouper, 1,238,719 CpG sites were recovered across all samples. A total of 163,925 sites either could not be successfully genotyped in the untreated library or were identified as potential SNPs and were removed from the dataset. Of the remaining 1,074,794 sites, 9,834 sites were present in at least 90% of samples. Mean global CpG methylation across

the retained sites was 85.45% (± 1.08%; SD). Bayesian GLMs identified 690 sites that exhibited significant age-correlated methylation, and a total of 307 sites had sufficiently tight confidence intervals in at least 80% of samples. Penalized regression analysis retained 49 CpG sites in the age-predictive model ($\lambda = 0.0501$). The slope and y intercept (± SE) for the regression of predicted age versus otolith-derived age were 0.98 (± 0.01) and 0.17 (± 0.05; $r^2 = 0.9996$; Fig. 1B). Of the 49 sites included in the final model, 75.51% of sites ($n = 37$) exhibited hypomethylation (Fig. 1D).

Discussion

The present study demonstrates the *de novo* identification of loci exhibiting age-correlated DNA methylation in wild-caught fishes and the utility of using such loci to estimate age. The epigenetic clocks developed show strong agreement between predicted and otolith-derived ages ($r^2 > 0.99$ for both species), supporting the notion that this may be an important yet currently untapped tool for fisheries science.

The epigenetic clocks developed for red snapper and red grouper performed well relative to those previously developed (Anastasiadi and Piferrer 2019; Mayne et al. 2020, 2021; Bertucci et al. 2021). The strong agreement between predicted and otolith-derived ages reported here is promising and may be the result of characterizing a large number of independent CpG sites exhibiting age-correlated methylation in both species. However, the low sample sizes ($n = 10$ for both species) precluded the application of more rigorous predictive approaches, involving training and testing datasets (Mayne et al. 2021) to minimize model overfitting. To more thoroughly validate the predictive capability of these epigenetic clocks, larger numbers of samples spread evenly across ages, with replicates at ages, will be necessary. In addition, while

the results reported here indicate that the epigenetic clocks developed are potentially highly accurate, a larger number of samples is needed to assess precision.

While previously developed epigenetic clocks involved fishes reared in the laboratory (Anastasiadi and Piferrer 2019; Mayne et al. 2020; Bertucci et al. 2021), the results of the present study indicate accurate epigenetic clocks can also be developed for wild-caught fisheries species. Changes in patterns of DNA methylation can be induced by aspects of the environment, such as water temperature, salinity, and toxin levels (reviewed in Beal et al. 2018). Thus, fish reared under controlled conditions will not experience the same degree of environmental fluctuation (process error), and therefore environmentally induced epigenetic change, as fishes in the wild. Having said that, the predictive capabilities of epigenetic clocks previously developed for laboratory-raised European sea bass (Anastasiadi and Piferrer 2019) and medaka (Bertucci et al. 2021) were not significantly affected by increases in water temperature or exposure to ionizing radiation, respectively. Nonetheless, the results presented here not only indicate that accurate epigenetic clocks can be developed for wild-caught fishes despite potential noise introduced by environmental heterogeneity, but also that different tissue types (muscle and fin clips), both of which can be sampled non-lethally, are appropriate for such work. Because studies in humans (Horvath 2013) and mice (Thompson et al. 2018) have shown that epigenetic clocks can be tissue-specific, comparisons across tissue types within species will be important to consider when creating epigenetic clocks for fisheries applications. Finally, hypomethylation was observed in a greater number of sites in the final models for both species and among all sites identified as exhibiting significant age-correlated methylation (58.86% and 74.44% for red snapper and red grouper, respectively). This finding is consistent with other studies that have

observed higher frequencies of age-correlated hypomethylation than age-correlated hypermethylation (reviewed in Johnson et al. 2012).

The ability to identify loci exhibiting age-correlated DNA methylation in wild-caught fishes through reduced representation sequencing approaches, demonstrated herein, suggests epigenetic clocks could be a widely applied tool for fisheries research. The development of epigenetic clocks would allow for the collection and incorporation of age-specific biological data into stock assessments without the need for destructive sampling, increasing the diversity of data sources that could be accessed. Moreover, age data are currently considered to be one of the more expensive sources of data utilized in stock assessments, due to the time required for otolith processing and analysis (Helser et al. 2019). The development of epigenetic clocks could allow for the construction of high-throughput multiplex PCR assays (Mayne et al. 2021) to assess specific age-correlated CpG sites (50-500 sites; Campbell et al. 2015), thus enabling accurate, rapid, cost-effective (<$10 per sample; Mayne et al. 2021), and concurrent age estimation in thousands of individuals.

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Contributors' statement

DNW: data collection, methodology, formal analysis, writing (original draft); **ATF:** conceptualization, methodology, formal analysis, writing (review and editing); **BKB:** data collection, writing (review and editing); **WFP:** conceptualization, data collection, writing (review and editing); **CH**: conceptualization, resources, writing (review and editing); **DSP:** conceptualization, methodology, resources, writing (review and editing).

Data availability statement

Datasets (raw and filtered) and scripts (including those used to create a *de novo* reference genome for red grouper, to conduct the Bayesian GLMs, to calculate 95% confidence intervals, and to conduct the elastic net regressions) are available at https://github.com/marinegenomicslab/Epi-Age-Est. Raw bsRADseq sequences will be made publicly available at the conclusion of a separate ongoing study.

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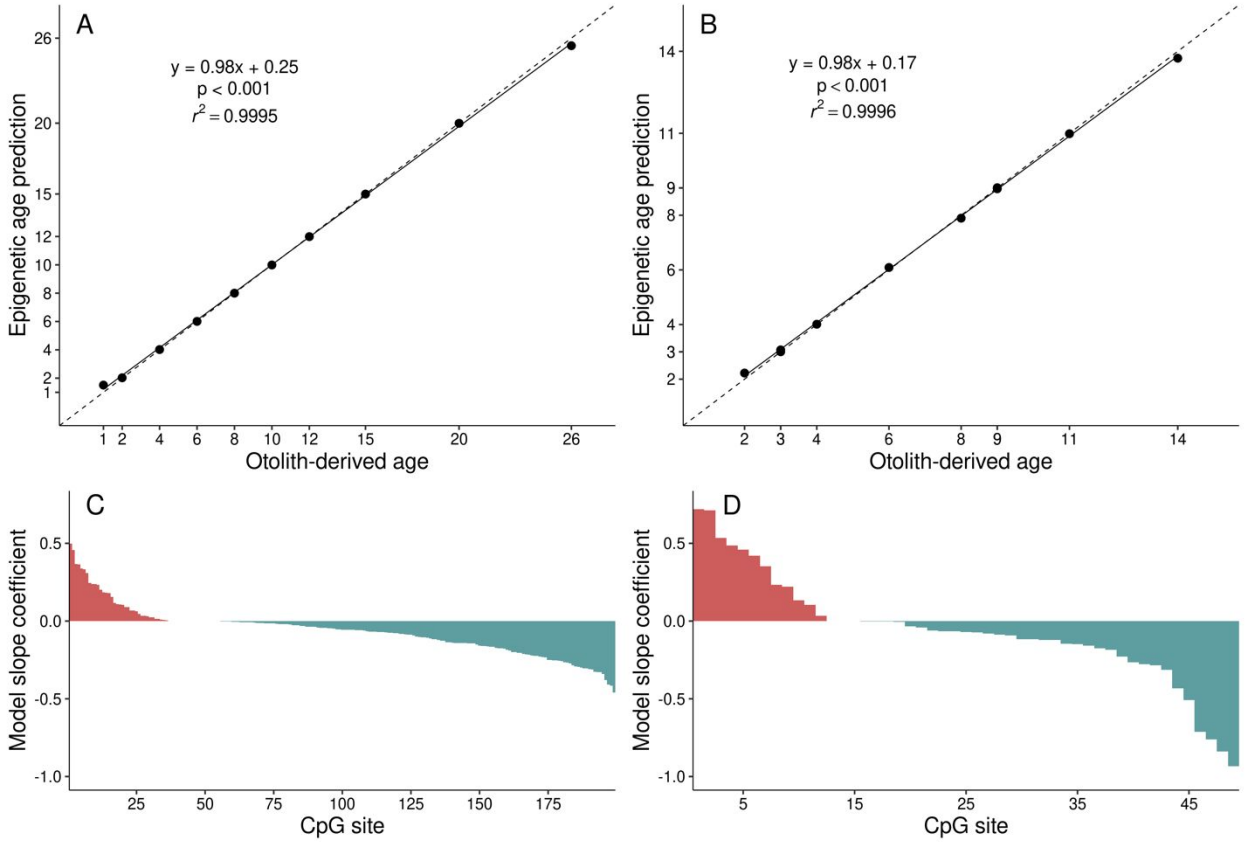

Fig. 1. Epigenetic age predictions versus otolith-derived ages for (**A**) red snapper (n = 10; 1–26 years old) and (**B**) red grouper (n = 10; 2–14 years old). Dashed lines indicate lines of 1:1 agreement between predicted and otolith-derived ages. Solid lines represent linear regression fits to the data, with equations indicated on panels. Panels **C** and **D** depict the final model slope coefficients for each CpG site included in the final model for red snapper and red grouper, respectively. Red bars indicate CpG sites exhibiting hypermethylation, while blue bars indicate CpG sites exhibiting hypomethylation.