Development of DNA methylation-based epigenetic age predictors in loblolly pine (*Pinus taeda*)

Running Title: Epigenetic aging in *P. taeda*

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Abstract:

Biological aging is connected to life history variation across ecological scales, as well as informing a basic understanding of age-related declines to organismal function. Altered DNA methylation dynamics are a conserved aspect of biological aging and have recently been modeled to predict chronological age among vertebrate species. In addition to their utility in estimating individual age, differences between chronological and predicted ages arise due to acceleration or deceleration of epigenetic aging, and these discrepancies are linked to disease risk and multiple life history traits. Although evidence suggests that patterns of DNA methylation can describe

aging in plants, predictions with epigenetic clocks have yet to be performed. Here, we resolve the DNA methylome across CpG, CHG, and CHH-methylation contexts in the loblolly pine tree (*Pinus taeda*) and construct epigenetic clocks capable of predicting ages in this species within 8% of its lifespan. Although patterns of CHH methylation showed little association with age, both CpG and CHG methylation contexts were strongly associated with aging, largely becoming hypomethylated with age. Among age-associated loci were those in close proximity to malate dehydrogenase, NADH dehydrogenase, and 18S and 26S ribosomal RNA genes. This study reports one of the first epigenetic clocks in plants and demonstrates the universality of age-associated DNA methylation dynamics which can inform conservation and management practices, as well as our ecological and evolutionary understanding of biological aging in plants.

Introduction

Alterations to the epigenome are a conserved hallmark of biological aging, and recent findings have demonstrated that age-associated DNA methylation patterns can be modeled to generate epigenetic age predictors capable of estimating chronological and biological age with unprecedented accuracy (Berdyshev, Korotaev, Boiarskikh, & Vaniushin, 1967; Christensen et al., 2009; Hannum et al., 2013; Richardson, 2003). In one of the first DNA methylation-based age predictors or “epigenetic clocks” developed by Horvath (2013), the methylation status of 353 cytosines predicts human chronological age with an error of ± 3.6 years. Epigenetic clocks have subsequently been developed in a variety of other mammalian (Horvath, 2013; Weidner et al., 2014), avian (Raddatz et al., 2021), and fish species (Anastasiadi & Piferrer, 2020; Bertucci, Mason, Rhodes, & Parrott, 2021; Mayne et al., 2020), and are currently being applied to

biomedical and conservation problems, as well to questions regarding their relationship to the underlying biology of aging and senescence (Bertucci & Parrott, 2020; Kabacik, Horvath, Cohen, & Raj, 2018). However, whereas the phenomenon of epigenetic aging appears to be a conserved aspect of biological aging in vertebrates, age-associated changes to DNA methylation and their ability to predict chronological age in plants is relatively unexplored (Parrott & Bertucci, 2019).

DNA methylation refers to the covalent addition of a methyl group to the 5' carbon of cytosine nucleotides (Jung & Pfeifer, 2015; Ng & Adrian, 1999; Suzuki & Bird, 2008), and although the functional consequences of these modifications vary across genomic context and taxonomic groups, DNA methylation is broadly associated with repressed transcriptional activity of genes and transposable elements through direct silencing and promotion of repressive chromatin states (Ng & Adrian, 1999; Zilberman, 2008). Similar to vertebrates, changes in DNA methylation levels in plants are observed with exposure to stress, age, and development (Dubrovina & Kiselev, 2016; Jiang et al., 2014; Law & Jacobsen, 2010; Probst & Mittelsten Scheid, 2015). However, compared to the distribution of cytosine methylation within vertebrate genomes (which almost exclusively occurs in CpG dinucleotides) DNA methylation in plant genomes is frequently observed within CpG, CHG and CHH contexts (where H = A, T, or C). Methylated cytosines located within gene bodies in plants primarily occurs in CpG contexts (Takuno, Ran, & Gaut, 2016), whereas CpG, CHG, and CHH methylation is typically found within highly repetitive genomic regions, potentially functioning to silence transposable element activity (Ausin et al., 2016; Slotkin & Martienssen, 2007). How these different sequence contexts might relate to age-associated DNA methylation patterning is not resolved.

Despite the ability of current epigenetic clocks to predict chronological age, discrepancies between epigenetic age and chronological age are observed and reflect variation in biological age

(Horvath & Raj, 2018; Parrott & Bertucci, 2019; Xiao, Wang, & Kong, 2019). Accelerated epigenetic aging is associated with age related losses in organismal function and in humans, and predicts risk for age-associated disease and mortality (Levine et al., 2018; Perna et al., 2016; Zheng et al., 2016). In addition, epigenetic-to-chronological age discordance mirrors variation in life history traits (Anderson et al., 2021; Hamlat, Prather, Horvath, Belsky, & Epel, 2021). For example, birth weight, age and size at maturity, as well as the timing of reproductive senescence are all correlated to epigenetic age in humans, demonstrating intriguing links between aging processes and life history variation (Binder et al., 2018; Ryan et al., 2018; Simpkin et al., 2015). However, the links between the rate of epigenetic aging and variation in organismal function and life history traits (including those with potential commercial implications) are largely unexplored in plants.

Loblolly pine is a large tree with a broad geographic range spanning from southern New Jersey to eastern Texas, including parts of northern Florida (Baker & Langdon, 1990) and is capable of living for up to 275 years (Baker & Langdon, 1990). The loblolly pine is often commercially harvested for timber and is also used to diversify forest habitats, control erosion, and improve water quality (Baker & Langdon, 1990). Research and management practices aimed at improving growth and yield from loblolly pine stands often manipulate resource availability (Albaugh, Lee Allen, Dougherty, & Johnsen, 2004; Coyle, Aubrey, & Coleman, 2016; Fox, Lee Allen, Albaugh, Rubilar, & Carlson, 2007). However, the underlying biological mechanisms promoting optimal growth rates across variable environmental conditions remain unclear (Albaugh et al., 2004), and resolving age-associated methylation patterns in plants may aid in developing strategies for increasing traits such as leaf area and growth efficiency, which are linked to stand productivity (Dubrovina & Kiselev, 2016; Fox et al., 2007; Medlyn, Barrett,

Landsberg, Sands, & Clement, 2003; Samuelson, Stokes, Cooksey, & McLemore III, 2001). Furthermore, a recent study examining DNA methylation in *Populus trichocarpa* reported correlations between epimutation and age (Shahryary et al., 2020; Yao, Schmitz, & Johannes, 2021), raising the possibility that these patterns could be used to predict other physiological traits such as plant growth (Hu, Morota, Rosa, & Gianola, 2015).

Here, we investigate age-associated DNA methylation patterns in *P. taeda* and evaluate their genomic distributions among differing methylation contexts. We then test differing modeling strategies to develop a novel epigenetic clock for *P. taeda*, which can be used to investigate the factors most important to growth, development, and tree aging. This study demonstrates the utility of epigenetic clocks in non-vertebrate models and indicates that age-associated DNA methylation may be a universal aspect of organismal aging.

Materials and Methods

Sample Collection

Between December 31, 2019 and January 14, 2020, cambium samples from standard coring procedures were obtained from 24 *P. taeda* individuals ranging from 1 to 119 years of age. This study was conducted at the United States Department of Energy's Savannah River Site (Aiken, SC, USA), a National Environmental Research Park. The United States Department of Agriculture (USDA) Forest Service manages the natural resources of the Savannah River Site (Kilgo and Blake 2005). Using records from the Forest Service, we identified stands which were planted between 1 and 55 years prior and sampled three trees from each stand (1, 10, 19, 28, 37, 46, and 55 years old). Three additional trees of advanced age (82, 97, and 119 years old) were

identified using core samples as planting records for older stands were unavailable. Cambium samples from adult trees (>1-year) were taken at breast height (1.37 meters) using a metal hole punch. Saplings (1-year old) were sampled at the base of the primary stem with a metal blade. All samples were immediately stored in RNAlater at -20°C until DNA extraction. Diameter at breast height was measured using a diameter tape and height was measured using a TruPulse 200x Rangefinder (Laser Technology, Inc., Centennial, CO).

DNA extraction

DNA was extracted from cambium tissue using Qiagen's DNeasy Plant Pro Kit (catalog # 69204, Qiagen, Hilden, Germany) following the manufacturer's protocol with the addition of 100 µl of the Solution PS per sample due to high amounts of phenolic compounds in pine species. Briefly, samples were cut into small pieces using a sterile blade and then homogenized using a Mini-Beadbeater (BioSpec, Bartlesville, OK) for 4-8 min at 2000 oscillations/min. DNA was eluted in 50 µl of the supplied elution buffer, and the concentration and purity of DNA samples were assessed using a Qubit fluorometer 2.0 (Invitrogen, Carlsbad, CA) and Nanodrop spectrometer (Thermo-Scientific, Waltham, MA), respectively.

Reduced Representation Bisulfite Sequencing Library Preparation

Reduced Representation Bisulfite Sequencing (RRBS) libraries were prepared using Diagenode's Premium RRBS Kit (catalog #C02030032, Diagenode, Denville, NJ). Due to the occurrence of CHG methylation in plant genomes, we adapted the protocol by digesting genomic DNA with the BsaWI restriction enzyme (Catalog #R0567S, New England BioLabs, Ipswich, MA) instead of MspI, as BsaWI cuts the recognition site W[^]CCGGW. Digestion efficiency of

BsaWI in our samples was confirmed by visualizing digested genomic DNA from test samples using standard gel electrophoresis. For library preparations, 200 ng of genomic DNA from each sample was digested with 5 units/ng of BsaWI for 12 hr at 60°C, after which samples underwent a 20 min heat inactivation of the restriction enzyme at 80°C. To obtain sufficient library concentrations, two RRBS libraries were prepared for each sample. In the second library preparation, the protocol was further altered to add additional extension time in the final amplification (72°C for 1 min during cycling and 10 min during final extension.) Libraries were eluted in 22 µl of the supplied elution buffer and stored at -80°C until sequencing. Other than these alterations, the manufacturer's protocol was followed exactly.

RRBS Sequencing, Quality Control, and Alignment

RRBS libraries were assessed for concentration and fragment size distribution on a Fragment Analyzer (Advanced Analytical Technologies, Inc., Ames, IA) at the Georgia Genomics and Bioinformatics Core at the University of Georgia. Libraries were then pooled and sequenced single-end for 100 cycles on the Illumina NextSeq 2000 with 20% PhiX control added. Two library preparations for each sample were sequenced across five high-output flow cells and approximately 400 million reads from each flow cell were generated. The quality of the resulting reads was assessed using FastQC (v0.11.5) (Andrews, 2017). Reads were trimmed using TrimGalore! (v 0.4.5) to remove adapter sequences and low-quality reads (Phred score <25), using the --rrbs option for RRBS data. Trimmed reads were concatenated into a single file and aligned to a bisulfite converted index of the loblolly genome (Ptaeda2.0) using Bismark (v0.20.0) (Krueger & Andrews, 2011) allowing for one mismatch (option n -1). The subsequent alignments were sorted and indexed using SAMtools (v 0.1.19) (Li et al., 2009).

File processing

The resulting Bam files were analyzed using the methylKit package (Akalin et al., 2012) in R (version 4.0.5) (2021). An average of 18,177,435 (+/- 1,342,074) reads comprised each Bam file, and cytosines from each individual were divided into CpG, CHG, or CHH contexts using the read.context parameter in the processBismarkAIn function of methylKit. To broadly assess age-associated DNA-methylation, while also developing an epigenetic clock, we filtered our data using two approaches. In the first approach, we filtered out cytosines covered by < 5x reads and not represented in < 80% of samples. To ensure comparability across CpG, CHG, and CHH contexts, cytosines on opposite strands were not merged (destrand = FALSE). This data set is considered our “exploratory” data set, in which general age-associated patterns could be resolved. In the second approach, we increased coverage requirements ($\geq 10x$) to yield a dataset that could be used to construct epigenetic age predictors.

Exploratory data set

The resulting exploratory data set was analyzed using the files generated for CpG, CHG, and CHH cytosine-methylation contexts. We filtered out cytosines displaying zero or near-zero variance in methylation status across samples using the nearZeroVar function from the Classification And Regression Training (caret) package (Kuhn, 2015) in R. Following filtering of invariant cytosines, we performed Spearman correlations between the methylation status of each cytosine and chronological age using the corr.test function from the psych package (Revelle, 2019) in R. Given the exploratory nature of the downstream analyses, we considered cytosines with an absolute correlation coefficient greater than 0.5 ($|R| > 0.5$) as being “age-associated”, and

those with p-values less than 0.05 ($P < 0.05$) following an FDR correction for multiple comparisons as “significantly correlated” with age.

To assess genomic characteristics of age-associated and significantly correlated CpG, CHG, and CHH cytosines, we classified the genomic locations of all covered cytosines according to genomic context within the *P. taeda* genome using the GenomicRanges package (Lawrence et al., 2013). We first used CpG plot (Madeira et al., 2019) to identify CpG islands (CGIs; parameters: widow = 100, min len = 200, minoe = 0.6, minpc = 50). We then used CGI coordinates to generate coordinates for shore regions (± 2000 bp from CGIs), and shelf regions (± 2000 -4000 bp from CGIs) using Bedtools and Samtools (H. Li et al., 2009; Quinlan & Hall, 2010). All remaining sites not falling in island, shore, or shelf regions were characterized as open sea regions. Enrichment above background within each methylation context was performed with binomial tests using all cytosines following invariant filtering within each context (21,566, 25,501, and 33,151 CpG, CHG, and CHH cytosines, respectively) as the background levels. To identify genes in close proximity to cytosines according to age-associated methylation patterns, we performed BLAST searches on 400 bp regions (200bp upstream and 200 bp downstream) centered around each of the 35 cytosines from CpG and CHG contexts with the greatest correlations with age.

Clock data set

Our exploratory analyses indicated that the methylation status of CHH cytosines was not associated with age; thus, we only generated epigenetic clocks using CpG and CHG cytosines. We first removed any invariant sites using the nearZeroVar function from the caret package (Kuhn, 2015) and performed imputation on missing data using a K-nearest neighbor (KNN)

approach in the impute package (Hastie et al. 2021) in R. We set $k=2$ as each age group among our samples was only represented by a maximum of three samples. We then split our data into a training data set ($n = 19$; distributed among ages 1, 10, 19, 28, 37, 46, 55, and 97 years old), and a test data set ($n = 5$; ages: 1, 28, 55, 82, and 119 years old). Individuals comprising the test set were selected by dividing the age range (1 – 119 years) into 5, then choosing 5 individuals with ages separated by approximately 24 years.

Elastic net clocks

We trained elastic net models for CpG and CHG methylated cytosines using the glmnet package (Friedman, Hastie, & Tibshirani, 2010) in R. We used an elastic net penalized regression model ($\alpha = 0.5$, family = gaussian) to select CpG- and CHG-methylated cytosines and assign penalties to individual model coefficients using the subset of CpG-loci and CHG-loci. We used a leave-one-out cross validation approach ($nfold = 19$) to select the optimal lambda value (value resulting in minimum mean error) for the training set model ($n = 19$ samples). We then used our training set model to predict ages of the individuals in our test set ($n = 5$). Following the generation of our elastic net epigenetic clock, we performed BLAST searches of 400 bp regions centered around each clock cytosine and used GenomicRanges to determine the genomic contexts for these sites (genes and CpG islands, shores, shelves, or open seas).

Pearson clocks

To construct linear models capable of predicting age, we identified the top 5 and 10 cytosines with the greatest Pearson correlation with age ($|R| > 0.5$) from our training set using the corr.test function (using FDR correction, $\alpha = 0.05$) from the psych package. We generated our linear models using the lm function in R, and then used them to predict the ages of the

individuals in our test data set. Following the generation of our linear model epigenetic clocks, we performed BLAST searches of 400 bp regions centered around clock cytosines and used GenomicRanges to determine genomic context (genes and CpG islands, shores, shelves, or open seas).

To compare performance of our elastic net and Pearson clocks, we used multiple regression to compare the mean absolute errors (MAE) generated when using each clock to predict ages of trees from our test set. We used clock type as a factor and chronological age as a covariate and corrected resulting P values using Bonferroni correction for multiple comparisons. Additionally, overfit of each clock to our training set was evaluated by comparing the MAE for training and test sets from each clock using t-tests.

Results

Characterizing age-related cytosine methylation

Among the CpGs analyzed, 533 (2.47%) displayed age-associated methylation, with 207 CpGs having positive correlations (max $R = 0.84$) and 326 having negative correlations (min $R = -0.79$) (Figure 1). Following FDR correction for multiple comparisons, 10 CpGs (9 negative and 1 positive) showed significant correlations with age. When examining locations of our age-associated cytosines with respect to genomic contexts, CpG-methylated cytosines were enriched in island ($P = 4.85e-10$) and shore regions ($P = 0.038$), while being depleted in shelf ($P = 9e-04$) and open-sea regions ($P = 2.20e-06$) (Figure 2). Among the age-associated CpG-methylated sites found in islands, many became hypomethylated with age (Table 1). Whereas this pattern was also observed for CpGs in shore and open-seas, CpGs in shelf regions were more evenly split between hypo- and hypermethylation with increasing age. Malate dehydrogenase (*mdh*), as well as 18S

and 26S ribosomal genes of related pine species, were among the genes that a few of our methylated CpG-containing 400bp regions mapped to (Supplemental Table 1).

For CHG methylation, 869 cytosines (3.41%) were age associated, with 350 displaying positive correlations (max $R = 0.87$) and 519 negatively correlated with age (min $R = -0.89$; Figure 1). Following FDR corrections, 70 of these 869 age-associated sites (60 hypomethylated and 10 hypermethylated) showed significant ($P < 0.05$) correlations with age. Compared to background levels, age-associated CHG cytosines were enriched in island regions ($P = 3.62e-05$) and were depleted in shores ($P = 1.3e-03$) (Figure 2). The majority of CHG-methylated cytosines were hypomethylated with age, regardless of genomic context (Table 1). One of the top 35 CHG age-associated cytosine sites mapped to an apparent homolog of *mdh* and became hypermethylated with age.

Within CHH-methylation contexts, 308 (0.093%) cytosines were found to be age-associated, with 187 displaying a positive correlation with age (max $R = 0.76$) and 121 negatively correlated to age (min $R = -0.71$; Figure 1). However, following FDR corrections, no cytosines were significantly ($P < 0.05$) correlated with aging, and compared to background levels, there were no differences in the distributions of age-associated CHH-methylated cytosines based on genomic context ($P > 0.05$). CHH-methylated cytosines found in island regions showed a trend of being mostly hypermethylated (67%) with age (Table 1), while CHHs found in shore regions were mostly hypomethylated (59%) with age. Shelf-region CHHs were more evenly split between hypo and hypermethylation with age (48% vs 52%), while most of the open-sea region CHH cytosines showed hypermethylation with age (63%) (Figure 2).

Construction of epigenetic clocks capable of predicting age

Individual age predictors were initially constructed using elastic net regularized regression approaches using either CpG methylation, CHG methylation, or a combination of CpG and CHG methylation. All three elastic net clocks predicted ages of trees from our training set within 0.62 years, and predicted ages were highly correlated with chronological ages for these trees ($R^2 = 0.99$) (Table 2). However, the ability of these models to predict chronological ages of trees in the test set were slightly lower and R^2 values ranged from 0.79 to 0.90, with the CHG model outperforming the CpG model, and the CpG and CHG combined model performing the best.

In addition to our elastic net models, we manually constructed epigenetic clocks for each methylation context (CpG, CHG, and CpG + CHG) using cytosines with the greatest Pearson correlation coefficients. When the top 5 Pearson age-correlated cytosines were selected, ages were predicted within 3.91 years and were highly correlated to chronological age ($R^2 = 0.89$ to 0.95) (Table 4). Predictions from the combined model again showed the highest correlations with chronological age. Correlations (R^2) between predicted and chronological ages ranged from 0.83 when using only CpG-methylated cytosines to 0.9 for our model using a combination of CpG and CHG cytosines. We then constructed models using the top 10 Pearson age-correlated cytosines. Relative to the models using the top 5 correlated cytosines, these models performed better on the training set ($R^2 = 0.94$ to 0.98); however, performance suffered on the test set, R^2 values ranging from 0.03 when using only CpG-methylated cytosines to 0.87 when using both CpG and CHG-methylated cytosines.

When comparing predictive performance among our elastic net and Pearson clocks, MAE of predicted ages for trees from our test set ranged from 20.86 to 38.14 years (7.58-13.9% of the

total lifespan for *P. taeda*). Although MAE for our test set did not differ based on clock type ($P > 0.05$), MAE of predicted ages increased by 0.43 (± 0.06) years with each chronological year increase in age for trees in our test set ($t_{35} = 7.04$, $P = 1.02\text{e-}06$) (Figures 3 and 4). To assess the potential overfit of our models, we compared the MAE of the training sets to those of test sets. None of our models were overfit to our training set ($P > 0.05$) (Tables 2 and 4).

We next used BLAST to assess if clock cytosines were proximal to specific genes. Among the loci that mapped to known genes were *mdh*, NADH dehydrogenase, and rRNA (18S and 26S). These loci were found in both methylation contexts (CpG and CHG) among our clocks, and were distributed among all genomic contexts (islands, shores, shelves, and open seas). Both *mdh* and NADH dehydrogenase sites became hypermethylated with age, and sites mapping to rRNA became hypomethylated with age (similar to patterns we observed in our exploratory data set). Within our combined CpG+CHG elastic net clock 10 of 47 cytosines returned hits based on homology to these genes (Table 5). Among the 4 cytosines that mapped to *mdh*, the 2 CpG loci were located next to each other on the same scaffold, and both were in shore regions. The remaining 2 CHG loci that mapped to *mdh* were in shore and open sea regions on different scaffolds. Similar to *mdh*, the 2 cytosines that mapped to NADH dehydrogenase were also located on different scaffolds, 1 being a CpG and located in an open sea region and the other a CHG found in an open sea region. Cytosines in rRNA genes (18S and 26S) were CpG-methylated, with the 18S cytosine being found in an open sea region and the 26S cytosines (3) found in island, shore, and open sea regions on differing scaffolds of the *P.taeda* genome, respectively. For annotations of cytosines from CpG and CHG clocks, see Supplemental Tables 3-6.

Discussion

We demonstrate the first use of methylation patterns to generate epigenetic clocks for *P. taeda*, which are capable of predicting chronological ages within ~8% of the total lifespan (275 years) of this species. Although there were no differences in test set MAE among our clocks, predicted ages from clocks combining methylation in both contexts showed the highest correlations with age, indicating that methylation in both contexts is useful when predicting ages for *P. taeda* and potentially other plant species. Although age-associated CpG and CHG methylation was distributed across genomic regions, we observed an enrichment of age-associated CpG methylation within island and shore regions, and CHG methylation in island regions. Within both methylation contexts, age-associated methylation mostly resulted in hypomethylation, regardless of genomic (island, shore, shelf, or open sea) context. In regard to potential functional implications, only CpG-methylation has been previously associated with gene expression in *P. taeda* (Takuno et al., 2016). As increased CpG methylation at promoter sequences (commonly associated with CpG island regions) is associated with reduced expression of downstream genes (Gehring & Henikoff, 2007), hypomethylation at many island-region sites may indicate that expression of specific genes increases with age in *P. taeda*. In contrast to CpG-methylation, CHG-methylation is associated with gene splicing (Chaudhary, Jabre, & Syed, 2021; Zhang, Lang, & Zhu, 2018). Alterations in CHG-methylation status can affect the proper splicing of genes (Chaudhary et al., 2021; Zhang et al., 2018) which can have deleterious consequences (Ong-Abdullah et al., 2015). When comparing age-related changes in methylation status among cytosines from both CpG and CHG contexts mapping to known genes in our BLAST searches, gains and losses were consistent for sites mapping to similar genes for both contexts. Thus, our findings taken together with current functional understanding indicates a

possible link between epigenetic aging and increases in gene expression and splicing variation with age. Compared to CpG and CHG-methylation, CHH-methylation in plants has largely been associated with transposable element silencing (Dubin et al., 2015), and rates of epimutations in transposable element regions are generally low (van der Graaf et al., 2015). Thus, the stability of CHH methylation through development could explain the poor associations between CHH methylation and aging observed in the current study.

Although the majority of cytosines in our analyses did not map to annotated genes, the expression of critical genes involved in conserved molecular pathways that regulate life-history traits has been observed to influence multiple physiological processes (Partridge & Gems, 2002; Perls et al., 2002; Flatt & Partridge, 2018). Among the genes proximal to several of our age-associated CpG and CHG-methylated sites in the exploratory and clock data sets were malate dehydrogenase (mdh), NADH dehydrogenase, and 18S and 26S rRNA. The expression of mdh, a multi-subunit metabolic enzyme in many organisms including plants (Longo & Scandalios, 1969; Yudina, 2012), has been linked to respiration and CO₂ assimilation rates, and normal plant growth and development (Tomaz et al., 2010). Expression has been shown to increase with age, which is postulated to maintain aerobic metabolism (Sharma & Patnaik, 1982). NADH dehydrogenase, another important metabolic enzyme involved in the electron transport chain (Weiss, Friedrich, Hofhaus, & Preis, 1992), is also associated with plant growth and development (Sweetman et al., 2019). Proper subunit splicing of this enzyme is essential for its function (Bonen, 2008; Malek & Knoop, 1998), with defective splicing leading to decreased activity of the electron transport chain, slower growth, and delayed germination and phenotypic development (Hsieh et al., 2015). Expression and activity of rRNA have been shown to increase with increased

metabolic demands (Russell & Zomerdijk, 2005), also affecting growth, cell adaptation, stress responses, and cell proliferation (Russell & Zomerdijk, 2005). Increased methylation near rRNA promoter regions greatly reduces expression (Ghoshal et al., 2004; Zatsepina et al., 1993); therefore, the loss of methylation we observed for cytosines proximal to 18S and 26S rRNA genes in island and shore regions coupled with the methylation gains we observed for CpG-methylated cytosines near *mdh* and NADH dehydrogenase in shore and open sea regions may indicate expression of these genes increases to match increasing metabolic demands as *P. taeda* trees age. If so, an increased requirement for proper splicing of NADH and *mdh* would be needed (Gendrel, Lippman, Yordan, Colot, & Martienssen, 2002; Jeddeloh, Stokes, & Richards, 1999), which might explain the increased CHG-methylation near these genes with age; although these predictions require further evaluation.

Beyond their ability to predict chronological age, the epigenetic clocks developed here are likely to find utility in forest management applications and addressing questions surrounding the basic biology of aging. The directionality and magnitude of epigenetic-to-chronological age discordance in humans and other vertebrates correlates to variation in life history traits (Anderson et al., 2020; Hamlat et al., 2021; Ryan, 2021). Variation in life history traits can have consequences for stand productivity (Kellner & Swihart, 2016; Schulze, 2003). If epigenetic-to-chronological age discordance, especially in early life, is connected to variation in tree growth, productivity, wood quality, and/or responses to disturbances, epigenetic clocks might inform breeding programs and could be used in evaluating efficiencies of experimental manipulations to increase early-life growth and subsequent wood yield from managed *P. taeda* stands (Baker & Langdon, 1990; Clason, 1989). Age-associated variation in DNA methylation patterns is also

associated with species-specific lifespans in vertebrates (Mayne, Berry, Davies, Farley, & Jarman, 2019). The findings presented here raise the possibility that age-related DNA methylation patterns might be used to estimate lifespan and yield insights into the underlying genetic and epigenetic determinants across different plant species.

A limitation of this study was the relatively small number of individual trees analyzed. The *P. taeda* genome, like that of many tree species, is quite large (20 billion base pairs) (Neale et al., 2014). As a result, resolving patterns of genomic methylation are sequencing intensive, and more cost-efficient technical approaches (e.g. amplicon sequencing, bait capture techniques) are likely needed if measures of epigenetic age are to be scaled to stand and population applications (Meek & Larson, 2019). Due to the age distributions of our sampled trees, age predictions for our individuals older than 55 years of age were poor, which may have contributed to the greater MAE from our clocks compared to those predicting ages for many vertebrate species (Bertucci et al., 2021; Bors et al., 2021; Lemaître et al., 2020; Meer, Podolskiy, Tyshkovskiy, & Gladyshev, 2018; Stubbs et al., 2017). As we initially removed sites not present in at least 80% of our individuals and many of our trees were within stand ages (Cunningham, n.d.; Li et al., 1999; Stiff & Stansfield, 2003), additional sampling of older trees could allow for retention of higher numbers of cytosine sites whose methylation status remains dynamic in later life, allowing for more accurate age predictions of older trees in further clock studies. Despite these limitations, we clearly demonstrate the relationship between DNA methylation and chronological age in a long-lived tree species and indicate that alterations in DNA methylation may be a universal aspect of aging across the tree of life.

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Data accessibility: Data used to support the claims in this manuscript will be made publicly available in the NCBI Sequence Read Archive (SRA) upon acceptance for publication. Scripts used to analyze data will be publicly available at <https://github.com/stg0015/Loblolly-scripts>.

697 **Author contributions:**

698 E.M.B., B.B.P, D.P.A., and A.H. developed the concept for the paper. E.M.B. and R.S. collected
699 the samples. E.M.B. prepared the libraries and assisted S.G. with data analysis. S.G. analyzed the
700 data and wrote the paper while being assisted by E.M.B. and B.B.P.

Figure 1. Distributions of Spearman correlation coefficients of cytosine methylation status with age following filtering of invariant sites across CpG, CHG, and CHH methylation contexts from 24 Loblolly pine trees of differing ages. (A) Of 21,567 CpGs analyzed, 533 (2.5%) showed correlations between methylation status and age of $R > |0.5|$. (B) Of 25,501 CHGs analyzed, 869 (3.4%) displayed correlations between methylation status and age of $R > |0.5|$. (C) Of 33,151 CHHs analyzed, 308 (0.93%) showed correlations between age and methylation status of $R > |0.5|$.

Figure 2. Enrichment and depletion of age-related DNA methylation within genomic regions with varying CpG densities. Cytosines for which methylation status was associated with age ($|R| > 0.5$) were compared against all RBSS-captured cytosines (background). (A) Age-associated CpG methylation was enriched in CpG island and shore regions and were depleted in CpG shelf and open-sea regions. (B) Age-associated CHG methylation was enriched in CpG islands and depleted in shore regions. (C) There were no differences in the distributions of age-associated CHH-methylated cytosines compared to background CHH methylated cytosine levels (Asterisks indicate $P < 0.05$).

Figure 3. Elastic net models used to predict chronological age in *P. taeda* for both training and test subsets. Clock accuracy is measured by the Pearson's correlation coefficient and precision by the mean absolute error (MAE) (+/- standard error). (A) 35 CpGs out of 18,844 CpGs following invariant filtering were selected in our elastic net model. (B) 50 CHGs out of 22,263 CHGs were selected and incorporated into the clock. (C) Combining CpG and CHG datasets, there were 41,107 cytosines following invariant filtering, with 47 incorporated into the clock. Values of individual datapoints represent the error between predicted ages from our models compared to chronological ages among test individuals, with positive values indicating overestimation and negative values indicating underestimation of chronological age.

Figure 4. Pearson models incorporating the five cytosines with the strongest age-associated methylation patterns predict chronological ages in *P. taeda* for both training and test subsets for CpG, CHG, and combined (CpG+CHG) models. Clock accuracy is measured by the Pearson’s correlation coefficient and precision by the mean absolute error (MAE) (+/- standard error). Values of individual datapoints represent the error between predicted ages from our models compared to chronological ages among our test individuals, with positive values indicating overestimation and negative values indicating underestimation of chronological age.

Table 1. Distributions of hypo- (-) and hypermethylated (+) cytosines with age among genomic regions

Context	Cytosine subset	Island Cytosines		Shore Cytosines		Shelf Cytosines		Open sea cytosines	
		-	+	-	+	-	+	-	+
CpG	Age-associated	111	14	56	35	7	9	152	149
	Significantly correlated	2	0	2	1	0	0	4	1
CHG	Age-associated	105	24	57	30	33	27	325	269
	Significantly correlated	21	0	5	1	1	0	33	9
CHH	Age-associated	7	14	20	14	11	12	84	147

Table 2. Performance of elastic net models when predicting ages of trees from training and test

Context	Elastic Net							
	# Initial sites	# Filtered sites	# Clock sites	Train R2	Train MAE (years)	Test R2	Test MAE (years)	Overfit? (t-test p-value)
CpG	26520	18844	35	0.9998	0.62 (+/- 0.08)	0.79	22.68 (+/- 12.02)	No (0.14)
CHG	30728	22263	50	0.9999	0.56 (+/- 0.10)	0.843	25.00 (+/- 11.52)	No (0.10)
CpG+CHG	57248	41107	47	0.9999	0.53 (+/- 0.10)	0.899	22.53 (+/- 9.99)	No (0.09)

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te	Context	Scaffold	Position	Pearson Correlation	P value	Cytosine region	Blast (400 bp window)	Score	E value	Model Coefficient
33	CpG	APFE030056171.1	85519	0.654965751	0.1790736	Shore	gb AC241314.1 Pinus taeda clone PT_7Ba3152O20, complete sequence	420	0	1.92E-02
70	CpG	APFE030124122.1	12062	0.766388533	0.03356732	Open Sea	gb BT124256.1 Picea sitchensis clone WS0472_E12 unknown mRNA	70	7.00E-08	6.13E-02
95	CpG	APFE030296703.1	22823	0.635707517	0.2388488	Shore	gb CP023113.2 Lupinus angustifolius cultivar Tanjil chromosome ...	46	1.00E+00	4.04E-02
92	CpG	APFE030429279.1	23186	0.426113678	0.7692602	Open Sea	ref XM_002178996.1 Phaeodactylum tricornutum CCAP 1055/1 predic...	50	4.80E-02	2.15E-02
87	CpG	APFE030610080.1	1827	-0.618603432	0.2685608	Island	gb KY860920.1 Pinus resinosa 26S ribosomal RNA gene, partial se...	559	0.00E+00	-1.43E-02
155	CpG	APFE030649645.1	41591	-0.595468448	0.2685608	Open Sea	gb DQ987890.1 Picea smithiana 18S ribosomal RNA gene, partial s...	236	5.00E-58	-1.19E-02
374	CpG	APFE030665456.1	32141	-0.454116977	0.676632	Island	gb AC241277.1 Pinus taeda clone PT_7Ba0032C08, complete sequence	72	2.00E-08	-2.48E-02
146	CpG	APFE030813233.1	186110	0.628514942	0.24590978	Shore	gb HM853297.1 Pinus pinaster malate dehydrogenase (mdhB) gene, ...	244	2.00E-60	1.62E-02
147	CpG	APFE030813233.1	186111	0.810819503	0.01143114	Shore	gb HM853297.1 Pinus pinaster malate dehydrogenase (mdhB) gene, ...	244	2.00E-60	1.55E-02
365	CpG	APFE030896286.1	7992	0.520639615	0.4273731	Open Sea	gb KM244285.1 Pinus ayacahuite isolate E4095 NADH dehydrogenase...	72	2.00E-08	8.38E-02
243	CpG	APFE030921614.1	53436	0.638958619	0.2314296	Open Sea	gb AC241337.1 Pinus taeda clone PT_7Ba3875B09, complete sequence	82	2.00E-11	1.68E-02
746	CpG	APFE031018063.1	67260	0.809419608	0.01143114	Open Sea	gb BT124256.1 Picea sitchensis clone WS0472_E12 unknown mRNA	62	2.00E-05	5.21E-02
760	CpG	APFE031293786.1	340	-0.657638981	0.175754	Open Sea	gb KY860920.1 Pinus resinosa 26S ribosomal RNA gene, partial se...	591	0.00E+00	-1.70E-02
399	CpG	APFE031355998.1	7890	-0.656292592	0.1774374	Shore	gb KY860920.1 Pinus resinosa 26S ribosomal RNA gene, partial se...	747	0.00E+00	-2.91E-02
133	CpG	APFE031387609.1	58957	0.697040128	0.1108949	Open Sea	emb AM460409.1 Vitis vinifera, whole genome shotgun sequence, c...	50	6.40E-02	1.28E-02
787	CpG	APFE031573243.1	563	-0.39575534	0.873483	Open Sea	gb AC241294.1 Pinus taeda clone PT_7Ba2900I02, complete sequence	456	0.00E+00	-1.52E-02
33	CHG	APFE030022174.1	103673	-0.631271133	0.24523123	Shelf	emb CR378014.1 Pinus pinaster SSR, clone PPB1C11	168	1.00E-37	-4.70E-02
55	CHG	APFE030116531.1	169841	0.437955931	0.736395	Open Sea	ref NC_039746.1 Pinus Taeda mitochondrion, complete genome	696	0	7.32E-02
95	CHG	APFE030198195.1	116	0.456408321	0.6683698	Open Sea	ref NG_052992.1 Homo sapiens zinc finger and BTB domain contain...	52	0.016	2.93E+00
02	CHG	APFE030266102.1	32477	0.764090945	0.03356732	Open Sea	gb JQ519677.1 Rhopalosoma sp. SK-2012 voucher DM400 elongation ...	56	1.00E-03	1.60E-02
03	CHG	APFE030266102.1	32482	0.746860297	0.04826154	Open Sea	gb JQ519677.1 Rhopalosoma sp. SK-2012 voucher DM400 elongation ...	56	1.00E-03	1.94E-02
93	CHG	APFE030339785.1	101999	0.707142325	0.09913354	Open Sea	gb AC241312.1 Pinus taeda clone PT_7Ba3106J15, complete sequence	64	4.00E-06	6.97E-02
32	CHG	APFE030384252.1	33429	0.475514945	0.5982055	Open Sea	gb CP020630.1 Oryzias latipes strain HSOK chromosome 10	44	3.90E+00	4.92E-02
59	CHG	APFE030474771.1	62256	-0.663102568	0.1617216	Open Sea	gb AC241319.2 Pinus taeda clone PT_7Ba3200G14, complete sequence	129	9.00E-26	3.93E-02

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91	CHG	APFE030543629.1	29207	0.671762877	0.1433964	Open Sea	gb KM244267.1 Pinus merkusii isolate 919/1 NADH dehydrogenase s...	248	1.00E-61	6.18E-02
141	CHG	APFE030641925.1	181993	0.731095016	0.06161909	Shore	gb HM853297.1 Pinus pinaster malate dehydrogenase (mdhB) gene, ...	194	2.00E-45	8.83E-03
114	CHG	APFE030706012.1	190247	0.306476627	0.9928919	Open Sea	emb LK065319.1 Apteryx australis mantelli genome assembly AptMa...	50	6.40E-02	3.11E-03
196	CHG	APFE030845544.1	125944	-0.605217158	0.2685608	Open Sea	gb AC241347.1 Pinus taeda clone PT_7Ba4144L02, complete sequence	111	2.00E-20	-8.22E-03
161	CHG	APFE030912860.1	23919	-0.786726873	0.01950128	Open Sea	gb AC241341.1 Pinus taeda clone PT_7Ba4050C22, complete sequence	101	2.00E-17	-2.30E-02
164	CHG	APFE030912860.1	23950	-0.762353973	0.034064	Open Sea	gb AC241341.1 Pinus taeda clone PT_7Ba4050C22, complete sequence	101	2.00E-17	-3.43E-04
165	CHG	APFE030912860.1	23958	-0.768712593	0.03356732	Open Sea	gb AC241341.1 Pinus taeda clone PT_7Ba4050C22, complete sequence	101	2.00E-17	-1.93E-02
197	CHG	APFE030944291.1	28610	-0.474078374	0.60426607	Open Sea	gb JQ262265.1 Pinus radiata isolate 5586 anonymous locus O_1789...	117	3.00E-22	-7.98E-02
118	CHG	APFE030960074.1	37277	-0.584417322	0.2685608	Shore	gb AC241357.1 Pinus taeda clone PT_7Ba4350E08, complete sequence	466	0.00E+00	-1.16E-02
119	CHG	APFE030960074.1	37304	-0.520006454	0.4296008	Shore	gb AC241357.1 Pinus taeda clone PT_7Ba4350E08, complete sequence	466	0.00E+00	-2.13E-02
122	CHG	APFE030995986.1	235721	-0.66335366	0.16172156	Island	gb AC241265.2 Pinus taeda clone PT_7Ba0002H13, complete sequence	74	4.00E-09	-3.77E-02
123	CHG	APFE030995986.1	235730	-0.84349085	0.00465065	Island	gb AC241265.2 Pinus taeda clone PT_7Ba0002H13, complete sequence	74	4.00E-09	-5.42E-02
124	CHG	APFE030995986.1	235737	-0.7415851	0.05307257	Island	gb AC241265.2 Pinus taeda clone PT_7Ba0002H13, complete sequence	74	4.00E-09	-1.90E-02
194	CHG	APFE031021371.1	131664	0.634258251	0.2395202	Open Sea	gb AC241265.2 Pinus taeda clone PT_7Ba0002H13, complete sequence	272	9.00E-69	4.77E-02
179	CHG	APFE031170467.1	52691	-0.80960342	0.01143114	Island	gb AC241357.1 Pinus taeda clone PT_7Ba4350E08, complete sequence	196	4.00E-46	-2.09E-02
161	CHG	APFE031246774.1	57161	0.16958204	0.9928919	Island	gb AC241265.2 Pinus taeda clone PT_7Ba0002H13, complete sequence	90	7.00E-14	4.27E-02
157	CHG	APFE031412923.1	164330	0.570400422	0.2794292	Open Sea	gb HM853297.1 Pinus pinaster malate dehydrogenase (mdhB) gene, Ö	170	3.00E-38	2.86E-03
168	CHG	APFE031420108.1	79663	-0.482785312	0.5711723	Open Sea	emb LR131964.1 Parambassis ranga genome assembly, chromosome: 14	44	3.90E+00	-2.87E-02
143	CHG	APFE031468650.1	339818	-0.638966355	0.2314296	Shore	gb AC241351.1 Pinus taeda clone PT_7Ba4290D18, complete sequence	444	0.00E+00	-4.08E-03
171	CHG	APFE031470839.1	87114	0.560775989	0.30052775	Open Sea	gb AC241310.1 Pinus taeda clone PT_7Ba3100G06, complete sequence	68	3.00E-07	3.71E-02
104	CHG	APFE031487846.1	62255	-0.670249113	0.1433964	Island	gb CP009524.1 Methanosarcina sp. Kolksee, complete genome	62	2.00E-05	-2.09E-02
162	CHG	APFE031619745.1	77285	-0.634856423	0.2395202	Shore	gb AC241270.1 Pinus taeda clone PT_7Ba0014H13, complete sequence	240	3.00E-59	-3.40E-03
168	CHG	APFE031626435.1	55430	-0.749901258	0.04805051	Open Sea	gb JQ262265.1 Pinus radiata isolate 5586 anonymous locus O_1789...	101	2.00E-17	-3.29E-02
									Intercept	38.04695

Table 4. Performance of Pearson models when predicting ages of trees from training and test sets

Pearson Model (5 cytosines)							
Context	# Initial sites	# Filtered sites	Train R2	Train MAE (years)	Test R2	Test MAE (years)	Overfit? (t-test p-value)
CpG	26520	18844	0.898	5.48 (+/- 1.13)	0.829	24.02 (+/- 9.9)	No (0.13)
CHG	30728	22263	0.911	5.84 (+/- 0.81)	0.873	20.86 (+/- 9.10)	No (0.17)
CpG+CHG	57248	41107	0.952	3.91 (+/- 0.72)	0.9	22.18 (+/- 9.41)	No (0.12)
Pearson Model (10 cytosines)							
Context	# Initial sites	# Filtered sites	Train R2	Train MAE (years)	Test R2	Test MAE (years)	
CpG	26520	18844	0.943	4.07 (+/-0.84)	0.03	38.14 (+/- 19.03)	No (0.15)
CHG	30728	22263	0.972	3.28 (+/- 0.45)	0.58	22.08 (+/- 13.41)	No (0.23)
CpG+CHG	57248	41107	0.979	2.59 (+/- 0.5)	0.87	21.12 (+/- 9.01)	No (0.11)

Table 5. Combined CpG and Pearson models (5 and 10 cytosines)

Site	Context	Scaffold	Position	Pearson Correlation	P value	Cytosine region	Blast (400 bp window)	Score	E value	Top 5 Model Coefficient	Top 10 Model Coefficient	Intercept
21133	CpG	APFE031387609.1	58957	0.697040128	0.111	Open Sea	emb AM460409.1 Vitis vinifera, whole genome shotgun sequence	50	6.40E-02	0.242	0.32154	
17723	CHG	APFE030995986.1	235730	-0.84349085	0.004	Island	gb AC241265.2 Pinus taeda clone PT_7Ba0002H13, complete sequence	88	3.00E-13	-0.231	-0.21276	
2070	CpG	APFE030124122.1	12062	0.766388533	0.033	Open Sea	gb BT124256.1 Picea sitchensis clone WS0472_E12 unknown mRNA	70	7.00E-08	0.223	0.20857	
15746	CPG	APFE031018063.1	67260	0.809419608	0.011	Open Sea	gb BT124256.1 Picea sitchensis clone WS0472_E12 unknown mRNA	62	2.00E-05	0.009	-0.15923	
6393	CHG	APFE030339785.1	101999	0.707142325	0.099	Open Sea	gb AC241312.1 Pinus taeda clone PT_7Ba3106J15, complete sequence	64	4.00E-06	0.281	0.37791	Top 30.7
12447	CpG	APFE030813233.1_	186111_	0.810819503	0.011	Shore	PREDICTED: Oryzias melastigma trace amine-associated receptor_	44_	3.9_		0.06751	
7132	CHG	APFE030384252.1	33429	0.807122	0.113	Open Sea	gb CP020630.1 Oryzias latipes strain HSOK chromosome 10	44	3.90E+00		-0.44427	
16261	CHG	APFE030912860.1_	23919_	0.786726873	0.020	Open Sea	gb AC241341.1 Pinus taeda clone PT_7Ba4050C22, complete sequence_	101_	2.00E-17_		-0.07754	
20779	CHG	APFE031170467.1_	52691_	-0.80960342	0.011	Island	gb AC241357.1 Pinus taeda clone PT_7Ba4350E08, complete sequence_	196_	4.00E-46_		0.05243	
5002	CHG	APFE030266102.1	32477	0.805784	0.113	Open Sea	gb JQ519677.1 Rhopalosoma sp. SK-2012 voucher DM400 elongation ...	56	1.00E-03		0.05348	Top 10 29.7