

Uncovering Age-Associated Genomic DNA Methylation Signatures in Asian Elephants

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Kana Arai

1 | Introduction

Ageing is a fundamental biological process shared across all living organisms. Accurate age estimation is needed for understanding life-history strategies, population dynamics, and individual health trajectories, particularly in long-lived species. In Asian elephants (*Elephas maximus*), age structure is closely linked to reproductive potential, survival patterns, social functions, and population resilience. However, traditional morphological approaches can be subjective and lose precision once physical growth stabilises, making it difficult to distinguish between individuals, especially in mature and older individuals. These limitations highlight the need for objective biomarkers that can estimate age across the entire lifespan and provide insight into biological ageing beyond chronological time.

DNA methylation has emerged as one of the most promising molecular biomarkers of ageing, exhibiting reproducible age-associated changes across a wide range of taxa. It is an epigenetic modification that involves the addition of methyl groups at cytosine-guanine dinucleotide (CpG) sites. These changes form the basis of epigenetic clocks, which can predict age from methylation patterns at specific CpG sites. Although such tools have been extensively developed in humans and model organisms, their application to Asian elephants has remained limited despite the species' exceptional longevity, complex social systems, prolonged reproductive lifespan, and endangered status. Asian elephants, therefore, represent both a conservation priority and a uniquely informative system for investigating molecular ageing in long-lived mammals.

This thesis aimed to establish a comprehensive molecular framework for DNA methylation-based age estimation in Asian elephants, integrating multiple methodological scales and extending beyond chronological age prediction to explore signatures of biological ageing. Rather than developing a singular model, this work adopts a progressive, integrative strategy, combining targeted marker validation, genome-wide discovery, non-invasive translation, and exploratory physiological interpretation. Together, these studies advance both the methodological foundations and conceptual understanding of epigenetic ageing research in Asian elephants.

2 | Materials and Methods

Two molecular techniques were employed. First, a targeted methylation-sensitive high-resolution melting (MS-HRM) approach was used to validate conserved age-associated markers in a cost-effective and accessible format. Second, a genome-wide reduced

representation bisulphite sequencing (RRBS) strategy was implemented to identify novel, species-specific age-associated CpG sites and construct a high-resolution epigenetic clock. To explore field applicability, faecal-derived DNA was assessed as a non-invasive approach for methylation-based age estimation. Finally, genome-wide methylation patterns were integrated with longitudinal hair cortisol measurements to examine potential molecular signatures of biological ageing.

The MS-HRM model was constructed using 53 blood samples using two candidate genes (*RALYL* and *TET2*) previously identified as age-associated in other mammals. Bisulphite-converted DNA was amplified, and methylation levels were inferred from melting profiles using standard curves. Support vector regression with leave-one-individual-out cross-validation (LOIOCV) was used to evaluate predictive performance.

For the genome-wide analysis, RRBS libraries were prepared from 91 blood samples and sequenced on a high-throughput next-generation platform. After quality filtering, 144,611 CpG sites were retained for downstream analyses. CpG sites showing strong age correlations were pre-selected before elastic net regression with LOIOCV to construct the epigenetic clock. Functional enrichment analyses were conducted to examine biological pathways associated with age-related regions.

For non-invasive applications, 63 faecal samples were evaluated using MS-HRM. 18 candidate regions derived from the RRBS dataset were screened; however, due to DNA degradation and primer design constraints in CpG-dense regions, only 35 samples and three regions (*MKGPRF*, *CACNB2*, *MACROD1*) were suitable for model construction.

Beyond chronological age estimation, this framework was also used to explore molecular signatures of biological ageing, which reflect functional and physiological divergence from chronological time. To examine whether cortisol levels were associated with biological ageing, genome-wide methylation patterns were compared between seven individuals with high versus low baseline cortisol levels. Differentially methylated sites were identified and functionally annotated, and epigenetic age acceleration was calculated as residuals from DNA methylation age regressed on chronological age.

3 | Results

3.1 | Building an age estimation model using a cost-effective targeted candidate gene approach

The resulting blood-based model achieved a mean absolute error (MAE) of 7.36 years after LOIOCV, corresponding to 9.2 % of the maximum reported lifespan. Both regions showed significant correlations with chronological age, with *RALYL* becoming hypermethylated and *TET2* hypomethylated with increasing age. No significant sex differences were detected, and longitudinally sampled individuals were consistently predicted to be older at later time points,

demonstrating within-individual temporal consistency. This chapter establishes a proof-of-concept that conserved age-associated methylation patterns can be leveraged for molecular age estimation in elephants using a low-cost, scalable approach.

3.2 | *Building a genome-wide epigenetic clock: Identifying age-associated CpG sites*

Building on this foundation, a genome-wide RRBS analysis identified 389 CpG sites strongly associated with age. These were used to construct a more accurate epigenetic clock with an MAE of 4.82 years (6 % relative error). Age-associated CpGs exhibited characteristic genomic distributions, with hypermethylation predominantly occurring in CpG islands and gene bodies, and hypomethylation in shores and intergenic regions, which were patterns consistent with epigenetic ageing observed in humans. Functional enrichment analyses revealed associations with developmental processes, transcriptional regulation, nervous system development, and pathways related to social behaviour and reproduction. These findings suggest that elephant epigenetic ageing reflects both conserved mammalian mechanisms and life-history adaptations.

3.3 | *Towards non-invasive age estimation: Validating methylation analysis using faecal samples*

To extend this framework toward non-invasive application, faecal-derived DNA was evaluated using MS-HRM. Despite substantial technical challenges related to DNA degradation and primer design in CpG-dense regions, three genomic regions (*MRGPRF*, *CACNB2*, and *MACROD1*) were successfully validated, yielding a model with an MAE of 9.31 years (11.64 % relative error). Although less precise than blood-based models, this approach demonstrates the feasibility of non-invasive molecular age estimation and should be interpreted as a methodological proof-of-concept rather than a fully optimised field-ready tool.

3.4 | *Exploring links between epigenetics and cortisol levels in Asian elephants*

Beyond chronological age estimation, this thesis explored molecular signatures of biological ageing by integrating genome-wide methylation profiles with longitudinal hair cortisol data. Individuals with high versus low baseline cortisol levels exhibited distinct methylation signatures, with 5,592 differentially methylated CpG sites. Cortisol-associated CpGs showed minimal overlap with age-associated CpGs, and pathway enrichment analyses highlighted inflammation, metabolic signalling, and cellular senescence, distinct from those associated with chronological ageing. A positive association was observed between cortisol levels and epigenetic age acceleration ($R^2 = 0.64$, $r = 0.89$, $p < 0.05$), suggesting that physiological stress may be linked to accelerated biological ageing. These findings are exploratory and do not imply causality but provide some of the first molecular evidence that

intrinsic ageing processes and stress-related pathways may operate through partially distinct mechanisms in Asian elephants.

4 | Discussion

Taken together, this thesis establishes one of the first comprehensive molecular frameworks for studying ageing in Asian elephants. By integrating targeted validation, genome-wide discovery, non-invasive translation, and physiological exploration, this work advances both methodological development and biological understanding. It demonstrates that DNA methylation can serve as an objective biomarker of both chronological and biological ageing, with potential relevance to health assessment, longevity research, and conservation management.

More broadly, this work positions Asian elephants as a valuable model for investigating ageing in long-lived mammals. By distinguishing between chronological and biological age, this framework offers new opportunities to study why individuals of the same chronological age may differ in resilience, reproductive potential, and survival. While several components of this thesis, particularly the faecal and cortisol-based analyses, should be viewed as proof-of-concept rather than fully validated tools, they provide an essential foundation for future longitudinal, field-based, and cross-population studies. Ultimately, this thesis contributes to the growing field of comparative epigenetic ageing and provides a conceptual and methodological basis for integrating molecular biomarkers into demographic inference, welfare assessment, and conservation strategies for long-lived endangered species.