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Research Article

Telomeric Attrition with Increasing Age in Short- (Chihuahua Dog) and Long- (Asian Elephant) Life Span Animals

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Abstract

Here, we explored the rate of telomere attrition with increasing age by real-time quantitative PCR (qPCR) in a short- (Chihuahua dog) and long- (Asian elephant) lived species. A total of 122 Asian elephants (female = 106, male = 16) ranging from 24-840 months of age, and 89 Chihuahuas (female = 65, male = 24) 1-179 months of age were used in this study. We found that young (pre- and peri-pubertal) Asian elephants had a higher relative telomere length (RTL) compared to dogs. A low, but significant negative relationship between RTL and increasing age was observed in both Chihuahuas (R^2=0.0490, P=0.0017) and Asian elephants (R^2=0.0177, P=0.0210). The estimated rate of telomere loss for males and females of both species ranged from -0.0023 to -0.0065, with no clear differences between gender or species. Results suggest that Asian elephants may start with longer telomeres than Chihuahuas, as RTL was higher, but then the rate of telomere attrition proceeds at a similar rate in both species. Age accounted for only a small percentage of the variation in RTL in both Chihuahua dogs and Asian elephants, however. Thus, its use as a biological tool for age estimation would appear to be limited for these species.

Keywords: *Age, Asian elephant, Dog, Telomere*

Kısa (Chihuahua köpek) ve Uzun (Asya fili) Ömürlü Hayvanlarda Artan Yaş İle Birlikte Telomerik Yıpranma

Özet

Bu çalışmada, kısa (Chihuahua köpek) ve uzun (Asya fili) ömürlü hayvanlarda artan yaş ile birlikte telomerik yıpranma oranı gerçek zamanlı kantitatif PCR (qPCR) kullanılarak araştırıldı. Yaşları 24-840 hafta arasında değişen toplam 122 Asya fili (106 dişi ve 16 erkek) ile 1-179 aylık Chihuahua (65 dişi ve 24 erkek) çalışmada kullanıldı. Genç Asya filleri (pre- ve peri-puberte) köpekler ile karşılaştırıldığında daha fazla orantısal telomer uzunluğuna sahip olduğu tespit edildi. Hem Chihuahua (R^2=0.0490, P=0.0017) hem de Asya fillerinde (R^2=0.0177, P=0.0210) orantısal telomer uzunluğu ile artan yaş arasında düşük ama anlamlı negatif yönlü bir ilişki gözlemlendi. Her iki tür için erkek ve dişi hayvanlardaki tahmini telomer kayıp oranı 0.0023 ile 0.0065 arasında değişirken tür veya cinsiyet yönünden bir fark tespit edilmedi. Elde edilen sonuçlar daha fazla orantısal telomer uzunluğuna sahip olan Asya fillerinin Chihuahua köpeklerden daha uzun telomer ile başladıklarını ancak daha sonra telomer yıpranmanın her iki türde de benzer oranda şekillendiğini gösterdi. Hem Chihuahua köpeklerde hem de Asya fillerinde orantısal telomer uzunluğundaki varyasyonun sadece küçük bir yüzdesi yaşa bağlıdır. Bu sebeple, yaş tayininde bir biyolojik araç olarak kullanılması bu türlerde sınırlı gözükmektedir.

Anahtar sözcükler: *Yaş, Asya fili, Köpek, Telomer*

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INTRODUCTION

Field biologists and veterinarians often work with animals for which there are no data on birth dates. Yet, knowledge of the age structure of populations or individuals is important for understanding factors affecting survival and how to improve species management, both *ex situ* and *in situ*. Unfortunately, age determination is difficult for most species, especially on living animals. Some methods involve using age-specific characteristics like tooth eruption and dental wear [1,2], skeletal morphology [1,3], body morphometrics [4] and bone ossification [5], although most of these are applicable only after death. In addition, radiocarbon dating and aspartic acid racemization can serve as a tool for age estimation [6,7], but again, not for living animals. An accurate estimate of age that could be easily obtained with minimal impact would therefore benefit biologists working on natural populations.

The aging process, or senescence, is related to progressive and irreversible cellular changes, like a molecular clock, and that after a certain number of divisions, cells reach a replicative limit [8,9]. One theory is that this cellular senescence is caused by the gradual decrease in the length of the telomere [10,11]. Telomeres are comprised of several canonical repeated nucleotides. Located at each end of a chromosome, they protect the chromosome from deterioration or damage [12]. Telomere length shortening occurs normally in somatic cells during DNA replication [13], throughout the life of an individual [14-20]. Thus, measures of telomere length have been used to estimate age in several mammalian species, including mice [19], dogs [15,21,22], sea lions [20] and humans [18,23]. However, results have been inconsistent; correlations between telomere length and age have been found in some [14,16], but not all studies [15]. Thus, additional studies across a broader array of species is needed to determine if measures of telomere length could be used as a means of age estimation. Today, there are three methods used to assess telomere length: southern blotting or terminal restriction fragments (TRFs) [14,16,24], flow fluorescence *in situ* hybridization (FISH) [25] and quantitative PCR (qPCR) [17-20]. TRF requires a large amount of DNA (0.5-5 µg/individual) and is time-consuming (3-5 days), whereas flow FISH limits the type of tissues for analysis [14,16,24]. The qPCR technique may be more useful because it can be applied to a variety of tissues, is easier to use and relies on a high throughput system [17,24].

In this study, we used a qPCR-based technique to examine telomere lengths in two species with vastly different lifespans: Chihuahua dogs represent short- (~10 years) and Asian elephants represent long- (~70 years) lived species. Elephants were of interest because of their endangered status and limited information on aging biology, plus they have a long lifespan similar to human. Dogs are the most popular companion animal, but have a limited lifespan. Our hypothesis is that relative telomere length is related to age in both long- and short-lived animals.

MATERIAL and METHODS

Animal and Samples

Animals in this study included 89 Chihuahuas *(Canis familiaris)* (female=65, male=24) ranging in age from 1-179 months) and 122 captive Asian elephants *(Elephas maximus)* (female=106, male=16) aged 24-840 months *(Table 1)*. Animal age was known for each individual based on interviews with owners and existing record. Two to three blood samples were collected between May and August 2015 from each individual for hematology and serum chemistry analyses, and for DNA extraction. All animals were deemed healthy based on a normal physical examination and unremarkable serum chemistry results,

Table 1. *Age distribution of Chihuahuas and Asian elephants*

Chihuahuas				Asian Elephant			
Age (year)	**Male**	**Female**	**Total**	**Age (year)**	**Male**	**Female**	**Total**
<1	8	9	17	<10	4	14	18
1-2	2	10	12	10-20	5	15	20
2-3	3	5	8	20-30	2	21	23
3-4	3	15	18	30-40	2	29	31
4-5	2	7	9	40-50	3	20	23
5-6	3	6	9	50-60	0	3	3
6-7	2	4	6	60-70	0	4	4
7-8	0	1	1	total	16	106	122
8-9	1	4	5	-	-	-	-
>9	0	4	4	-	-	-	-
Total	24	65	89	-	-	-	-

including liver function (alkaline phosphatase and alanine aminotransferase) and kidney function (blood urea nitrogen and creatinine), and normal complete blood counts (hematocrit and hemoglobin levels, red blood cell count, white blood cell count and platelet count). Animals had no history of infectious disease or injury in the 3 months prior to the sample collection. This study was approved by the Animal Use Committee of the Faculty of Veterinary Medicine, Chiang Mai University, Thailand, in 2015 (S17/2558 and S25/2558).

DNA Extraction and Real Time Quantitative PCR

Blood was extracted according to manufacturer instructions of the genomic DNA Extraction kits (catalog number: 1RBC-RT011, RBC Bioscience, Taiwan). DNA was measured qualitatively and quantitatively using agarose gel electrophoresis and absorbance at A260 to be given 50 ng/μL of stock DNA. Subsequently, DNA (50 ng) was used for estimation of telomere length of individuals by qPCR as described by Cawthon [17]. Briefly, the final concentrations of reagents in the PCR were 1x real-time master mix (catalog number: MBL-BIO-98005, Bioline), containing telomere primer concentrations of 270 nM of tel1:5'-GGTTTTTGAGG GTGAGGGTGAGGGTGAGGGTGAGGGT-3', and 900 nM of tel 2: 5'-TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA-3' in a total volume of 10 μL [17]. The acidic ribosomal phosphoprotein PO or 36B4 (single copy gene) primer concentrations were 400 nM of forward: 5'-CAGAGTGAYGTGCAG CTGAT-3' and 400 nM of reverse: 5'-AGCACTTCAGGG TTGTAGATGCTGCC-3'; the primer pair was designed based on the multiple alignment of the 36B4 gene from various species, including mouse, African elephant, dog, human and cat, and confirmed by Sanger sequencing. The cycling profile for the telomere (T) PCR was as follows: 40 cycles of 95°C for 15 s, 65°C for 2 min. For 36B4 as single-copy gene (S) there followed 30 cycles of 95°C for 15 s, 68°C for 1 min. After that, cycle threshold (C_t) values were acquired from qPCR to be used for calculating the relative telomere length (RTL). The RTL was derived from the following formula: $2^{-\text{delta(ct(telomere)-ct(36B4 gene))}}$ and was expressed as T/S [17-20]. The rate of telomere loss was obtained from 1/slope of linear regression model (RTL/year) [26].

Statistical Analysis

Differences in RTL between young (pre- and peri-pubertal) Asian elephants (0-15 years) [27] and Chihuahua dogs (0-24 months) and between males and females of each species were determined using Student's t-tests**.** Correlations between relative telomere length and age were determined as the coefficients of determination (R^2) by a linear regression model in R program [17-20]. *P*-values were based on slopes being zero or not using analysis of variance *(ANOVA)*. The rate of telomere loss was obtained from the slope of the linear regression model and expressed as RTL/year. Differences were considered significant if $P<0.05$.

RESULTS

In this study, multiple alignment of acidic ribosomal phosphoprotein PO or the 36B4 gene (used as a single-copy gene for calculating RTL) from different species, including mouse, African elephant, dog, human and cat *(Fig. 1)*, was performed based on a 129 bp-sized amplicon. The RTL determined by qPCR was greater in Asian elephants than Chihuahua dogs, with no difference between sexes within species for the youngest animals (pre- and peri-pubertal) *(Fig. 2)*. When considering in total for both male and female, a general decline in RTL with increasing age was then observed *(Fig. 3)*. The overall coefficients of determination (R^2), which indicate the relationship between age and RTL, were significant ($P<0.05$) for both species, with a very low correlation of 0.0490 and 0.0117 in Chihuahua dogs and Asian elephants, respectively *(Table 2)*; although when gender was considered separately, the only significant correlation between RTL and age was in female Chihuahuas *(Table 2, Fig. 3A)* in contrast to female Asian elephants *(Table 2, Fig. 3B)*. However, our study found no correlation between age and RTL in males in both species *(Table 2, Fig. 3A,B)*. The estimated rate of telomere loss for males and females of both species was variable and ranged between -0.0023 and -0.0065 *(Table 2)*.

DISCUSSION

Based on our knowledge, this is the first study to measure telomere length in Asian elephants by qPCR and compare age-related telomere loss between a long- and short-lived species. Our results demonstrated that RTL was greater in Asian elephants compared Chihuahua dogs, and that telomere length attrition was linked to increasing age in both species, although correlations were low and accounted for only about 5 and 2% of the variation, respectively.

There are few studies of telomere shortening with age in dogs, although numbers of subjects were generally limited [15]. Investigations of telomere length in three dog breeds, Labrador retriever (n=22), miniature schnauzer (n=17) and beagle (n=8), by telomeric restriction fragment analysis found a negative relationship between age and telomere length across but not within breeds [15]. By contrast, we did find a significant negative correlation between age and telomere length in Chihuahua dogs (n=89). A shortening of telomeres with cell replications of *in vitro* canine fibroblasts has been detected [22], and Fick et al.[21] showed a connection between telomere loss with age in 15 dog breeds (n=175), similar to our results. Furthermore, Fick et al.[21] reported that different dog breeds varied in telomere length, which contributed to average lifespan. Moreover, dog breeds with shorter telomeres exhibited higher susceptibility to cardiovascular, gastrointestinal, musculoskeletal and respiratory disorders.

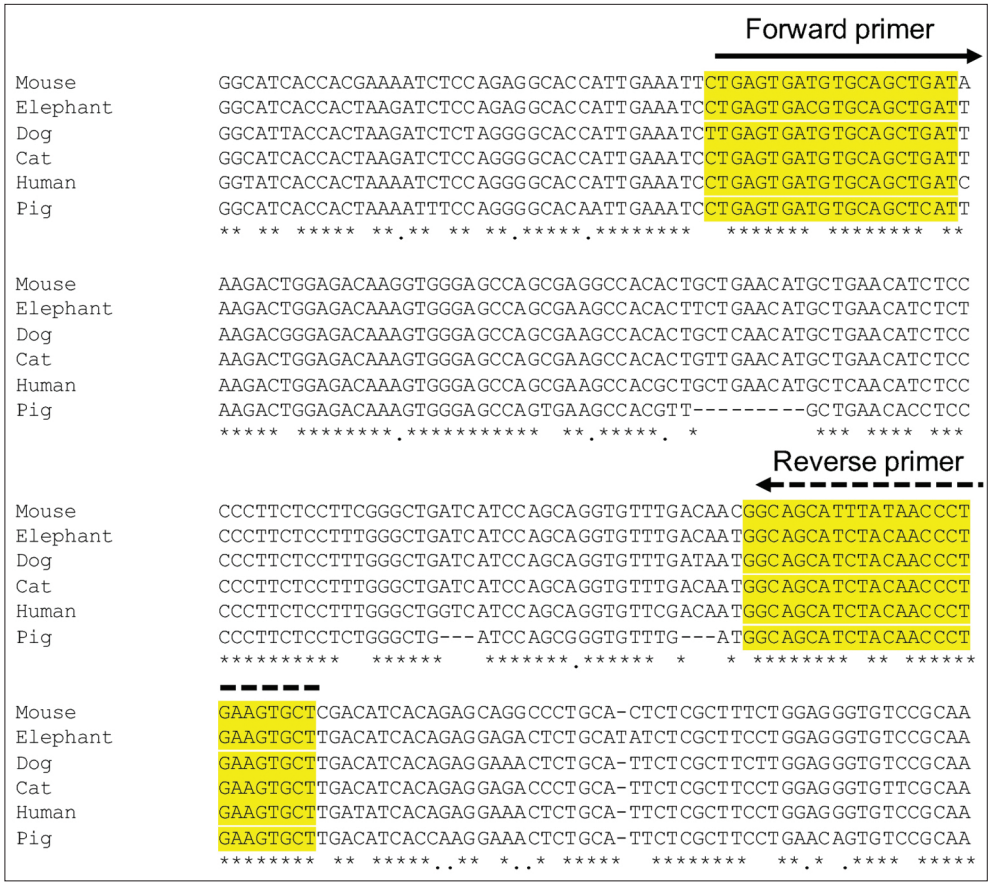

Fig 1. Multiple alignment of acidic ribosomal phosphoprotein PO or the 36B4 gene from various species. Below the nucleotide sequence is a key denoting conserved nucleotide (*) and semi-conservative mutation (.)

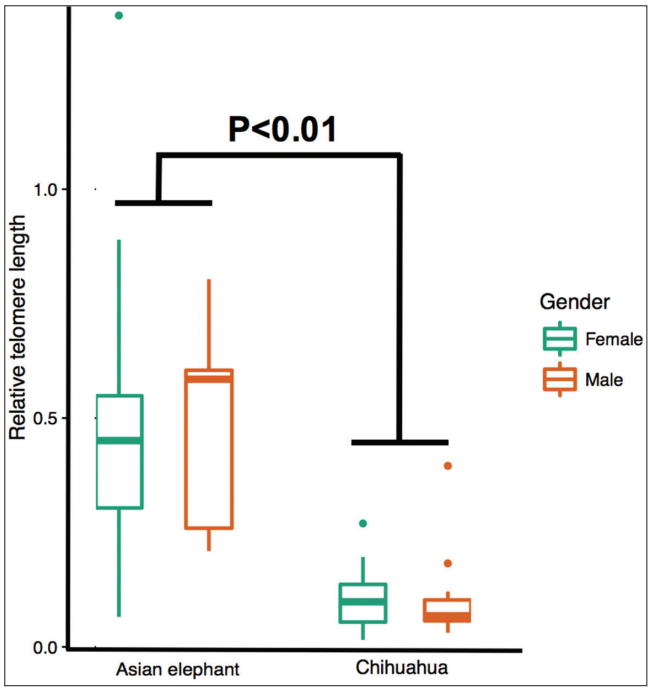

Fig 2. Comparison of relative telomere length before maturity in male and female Asian elephants (<15 years of age) and Chihuahua dogs (<2 years of age). Box plots present the median, 25% and 75% confidence intervals, and non-outlier minimum and maximum whiskers

Similar patterns of telomere length shortening with increasing age have been shown in other mammalian species, like human, mouse and sea lion [15,17-20]. Karlsson et al.[23] reported high variability in the degree of telomere repeats in humans, however, even within the same age group (~20 years). Other studies in birds, such as terns *(Stena hirundo)*, wandering albatrosses *(Diomedea exulans)* and Leach's storm-petrel *(Hydrobates pelagicus)*, also found high variability in telomere length with age at hatching [14,16]. In addition to the variation in telomere length at birth, environmental and lifestyle factors can affect the acceleration of telomere loss in cells. For example, in rats *(Rattus norvegicus)*, poor nutrition during the growth phase resulted in accelerated telomere shortening [28]. In shag *(Phalacrocorax aristotelis)*, a long-lived bird species, a faster growth rate was linked to a higher rate of telomere loss [29]. Thus, variation in telomere length across individuals may be a consequence of (i) growth rates of somatic, especially in early age [30], (ii) variation in telomere length at birth [14,16,31] and (iii) differences in telomere attrition rate [31,32]. The animals in this study had vastly differing backgrounds, which could explain the inhomogeneous nature of telomere lengths observed among individual Chihuahuas and Asian elephants, although in both species RTL did decline with age.

Table 2. *Summary of the relationship between telomere length and age in Chihuahua dogs and Asian elephants*

Parameter	Chihuahua Dog			Asian Elephant		
	All (n=89)	Female (n=65)	Male (n=24)	All (n=122)	Female (n=106)	Male (n=16)
[1]Adjusted R^2	0.0490	0.0537	0.0167	0.0177	0.0109	0.1427
RTL/year	-0.0048	-0.0048	-0.0065	-0.0023	-0.0023	-0.0065
P-value	0.0017*	0.0352*	0.4389	0.0210*	0.1442	0.0825

RTL = relative telomere length; [1]Adjustment of R^2 based on the number of variables in the model

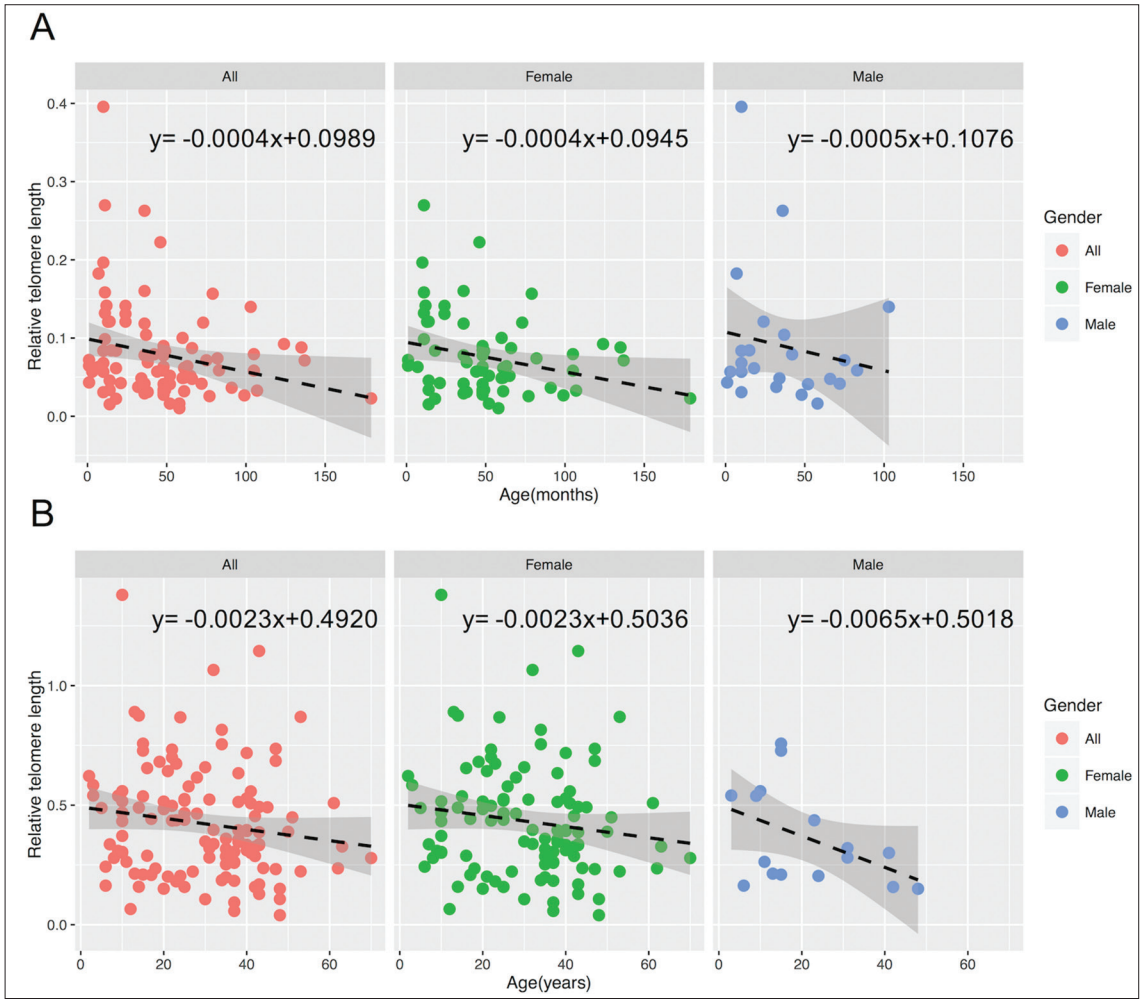

Fig 3. Correlation of relative telomere length and age from blood samples of Chihuahua dogs **(A)** and Asian elephants **(B)** using real-time quantitative PCR. Data are relative telomere length in which the C_t of telomere region **(T)** was normalized by that of 36B4 gene as single copy gene. Linear regression and 95% confidence intervals are shown as dashed lines with grey shading

The negative relationship between RTL and age was higher in Chihuahuas (R^2=0.049) than Asian elephants (R^2=0.0177), although there did not appear to be a difference in the estimated rate of telomere loss between these two species despite markedly differing lifespans. Within a species, expected life span may be related to the number of telomeric DNA repeats, something that has been found in dogs [21]. Canine telomeres range from 11 to 29 kbp [21]; however, the number of telomere repeats in Asian elephants is unknown. Presumably, the telomere length of Asian elephants is longer than that of Chihuahua dogs because they had a higher RTL. However, these relationships will not be completely understood until the actual telomere length in Asian elephants is determined. A slower loss of telomere DNA repeats has been observed in long-lived birds and mammal species [15,16,19,31]. On the other hand, telomere length and average lifespan has shown no connection across other vertebrate groups [16,31]. Hence, although the initial telomere length important appears to be important, the rates of telomere erosion and telomere restoration may also be related to life expectancy [16,31].

Sex differences in telomere length in mammalian species may be due to heterogametic influences, which account for some of the observed sex-bias in mortality [33]. In our study, however, no gender differences between age and RTL were observed in Asian elephants. By contrast, in Chihuahua dogs, we found a significant correlation between RTL and age in females. This result contrasts with that of Fick et al.[21] though, who found a relationship in male, but not female dogs. That study did not consider breed in data analyses, so differential results might be due to a species difference. In rats, shorter telomere lengths in males than females have been reported, but in both sexes, telomere attrition increased with age in most tissues [34]. However, these observations have not been consistently observed in other species; for example, Australia sea lion *(Neophoca cinerea)* [20]. In women, average telomere length in females was higher, and telomere length attrition was less than that of males [35]. However, there was no correlation of RTL and age in males of both species, which might be due to a smaller number of males. Additionally, in both species, the oldest individuals were female.

In conclusion, identifying and understanding the connection between telomere length and senescence or aging could potentially help in age estimation in some species. However, in our study, age accounted for only a small percentage of the variation in RTL in both Chihuahua dogs and Asian elephants (<5%). Thus, its use as a forensic tool for age discrimination would appear to be limited in these species. Rather, more research is needed to determine how numbers of telomere repeats at birth, rate of telomere loss and environmental factors affect overall length on an individual basis in these and other species, and if any are related to aging and/or mortality.

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Authors' Contributions

K.N. designed and conducted all the experiments. K.B. assisted in the experiments, performed the statistical analysis and support of information for discussion. W.K. and I.K. performed all the experiments. K.N. and S.C. collected dog blood sample C.S and C.T. collected elephant blood sample. S.P. gave a statistical advice. K.N., K.B., J.B. and C.T. assisted in discussions and writing of the manuscript. All authors read and approved the final manuscript.

Competing Interest

We have no competing interests.

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