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Telomere length provides a new technique for aging animals

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Abstract Field biologists often work with animals for which there is no prior history. A marker of an animal's age would offer insight into how age and experience affect reproductive success and other life history parameters. Telomere length shortens with age in cultured cells and mouse and human tissues. We found that lengths of telomere restriction fragments cleaved from blood cell DNA shorten predictably with age in the zebra finch (*Taeniopygia guttata*). If this relationship holds in other species, it should be possible, once the relationship between telomere length and age has been determined for a given species, to use blood samples to estimate ages of free-living animals. This will allow the incorporation of age into estimates of factors affecting life history parameters in cases where previous histories of animals are unknown.

Keywords Aging · Blood · DNA · *Taeniopygia guttata* · Telomere

Introduction

Knowledge of the age structure of a population is useful for understanding dynamics of population growth and estimating life history parameters (Stearns 1992). Likewise, knowledge of an individual's age allows insight into how aging differentially affects reproduction and behavior (Pianka 1983). Knowledge of age structure is important in the management of endangered species (Fiedler and Kareiva 1998). Unfortunately, determining the age of free-living individuals is often difficult, because historical data for individuals in most natural populations is lacking. Field biologists usually must spend considerable effort to determine the age structure of populations by tagging individuals as neonates (e.g., Brown

et al. 1997) or using age-specific characters like plumage (see Pyle 1997), dental wear (e.g., Roth and Shoshani 1998) and skeletochronology (e.g., Bjørndal 1998). For many studies these techniques either take too long to implement, provide information on relatively few age classes, or require destructive sampling. As a result, only a few ecological studies include age of individuals as a covariable in population or life history models, or in annual estimates of survival and fecundity (Clutton-Brock 1984; Coulson et al. 2001). An accurate estimate of an animal's age that could be obtained quickly and with minimal impact would therefore benefit biologists working on natural populations.

The aging process, or senescence, is the result of processes that are progressive and irreversible (Kohn 1971). As animals age, there is often a decline in fertility and expected survival (Harvey et al. 1989), supporting evolutionary theory that suggests that there are reproductive trade-offs with age (Bell 1984). One theory of senescence suggests aging is regulated by a molecular clock, in which each cellular division marks a tick of the clock. After a certain number of divisions, cells reach a replicative limit, as observed in cultured cells (Hayflick and Moorhead 1961). Olovnikov (1973) suggested that this cellular senescence was caused by the gradual decrease in the length of the telomere.

Telomeres are short, tandem repeated sequences of DNA found at the ends of linear eukaryotic chromosomes, whose sequence $(T_2AG_3)_n$ is highly conserved among vertebrates (Meyne et al. 1989). Telomeres function in stabilizing chromosomal end integrity (Prowse and Greider 1995), inhibiting aberrant fusions and rearrangements that occur on broken chromosomes (McClintock 1941) and aiding in the completion of duplication (Watson 1972). During each cell cycle telomeric repeats are lost because DNA polymerase is unable to completely replicate the 3' end of linear DNA (Watson 1972). In some tissues such as germ cells and carcinomas, telomeric repeats are maintained by telomerase, a ribonucleoprotein capable of elongating telomeres de novo (Greider and Blackburn 1985). In the absence of

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adequate telomerase activity, however, telomeres shorten with each cell division. This occurs in cultured fibroblasts (Harley et al. 1990) and in a variety of mouse and human tissues in vivo (Hastie et al. 1990; Allsopp et al. 1992; Vaziri et al. 1994; Slagboom et al. 1994; Coviello-McLaughlin and Prowse 1997; Frenck et al. 1998; Friedrich et al. 2000). This suggested to us that telomeres could serve as a marker of chronological age, and telomere length could then provide essential information for aging animals in free-living populations.

Materials and methods

Model organism

We used a captive population of zebra finches (*Taeniopygia guttata*) to determine the relationship between telomere length and age. Twenty-seven birds of known age between 1 and 24 months old were used in this experiment. An additional four individuals were older than 24 months. Zebra finches can live to approximately 5 years in the wild and 5–7 years in captivity (Zann 1996). Zebra finches were initially assigned to three age groups: juvenile birds (<4 months, $n=11$), young birds (11–15 months, $n=9$), and old birds (>18 months, $n=11$).

We collected blood (approximately 50 μ l) from the brachial vein into EDTA rinsed capillary tubes and then immediately transferred it into 100 μ l ice-cold 2% EDTA in phosphate-buffered saline (one part blood:two parts buffer). All procedures described were approved by the Iowa State University Committee on Animal Care (COAC, 10-0-4650-1-Q).

DNA isolation and restriction enzyme digestion

DNA was extracted from isolated erythrocyte nuclei within 24 h of blood collection using a salt extraction, alcohol precipitation method. Genomic DNA was digested for 16 h with the restriction enzymes *Hae*III, *Hin*I, and *Msp*I (50 U each; New England Biolabs, Beverly, Mass.) at 37°C, and DNA yield was determined using fluorometry. These restriction enzymes have no recognition sites within the telomeric repeat and yielded terminal restriction fragments comprised of telomeric repeats plus telomere-adjacent DNA sequences (Prowse and Greider 1995). Below we refer to these fragments as telomeres.

Ten micrograms of all digested DNA fragments was separated on a 0.5% non-denaturing agarose gel (3 V/cm) for 18 h, which was then soaked in 2 \times salt sodium citrate (SSC; Sambrook et al. 1989) for 30 min at room temperature and dried under vacuum for 30 min at room temperature.

We ran DNA from all zebra finches on one of two agarose gels (Fig. 1b). We also ran three ³²P-labeled lambda ladders on each gel to test for uniformity of DNA migration rate across the gel, and one sample was run 3 times on each gel to determine the intra- and inter-gel coefficients of variation (0.7% and 1.3%, respectively).

Gel hybridization and telomere length analysis

Non-denatured gels were hybridized for 16 h at 37°C with ³²P-labeled (C₃TA₂)₄ oligonucleotides in hybridization solution (Sambrook et al. 1989). Each chromosome has a single-stranded overhang at each end (G-strand overhang) to which the oligonucleotide binds. The gels were rinsed briefly with 0.25 \times SSC, and then washed in 0.25 \times SSC for 30 min at room temperature, followed by two washes for 1 hour each in 0.25 \times SSC at 37°C. We used a phosphor imager system (Molecular Dynamics) to visualize the telomeres and densitometry (ImageQuant V 1.2) to determine the position and strength of the radioactive signal in each of the

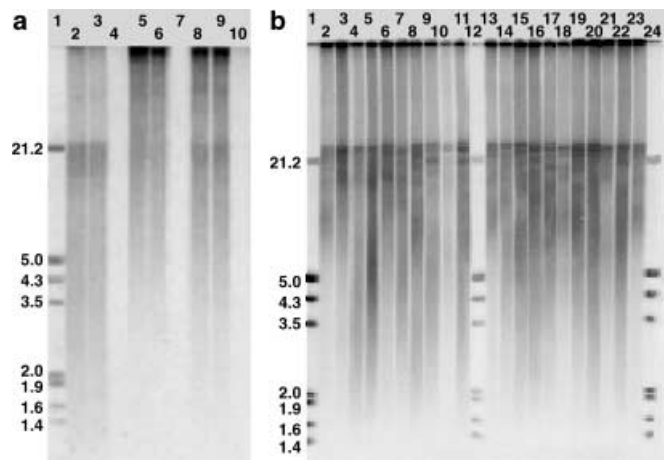


Fig. 1a, b Telomere lengths in zebra finch erythrocytes hybridized to the G-strand overhang. Lambda ladder size markers (in kilobases) are in lane 1 (a) or lanes 1, 12 and 24 (b). **a** Triplicate samples for three individuals (e.g., lanes 2, 3, 4, for one bird) with the last lane in each triplicate (lanes 4, 7, 10) treated with mung bean nuclease to remove the single-strand overhang. **b** Southern blot showing terminal restriction fragments. DNA from the same individual was run 3 times in each gel (lanes 2, 13 and 23) to determine intra- and inter-gel coefficients of variation

lanes over the range of 3–17 kb (Harley et al. 1990). Telomere restriction fragments come from cells of potentially different ages and from all chromosomes within the cell. Different chromosomes have different restriction sites relative to the telomeric end, so the resulting fragments vary in size producing a smear on the gel rather than a sharp band. However, the mean telomere length can be determined by averaging across the smear (Harley et al. 1990). Average labeled fragment length in each lane was calculated as the mean of the optical density using the formula: $L = \frac{\sum(OD_i \times L_i)}{\sum(OD_i)}$, where OD_i is the densitometry output at position i (arbitrary units), and L_i is the length of the DNA (bp) at position i . We estimated the precision with which we could determine L by running one sample 6 times. This yielded a mean (\pm SD) fragment length of $9,427 \pm 123$ bp.

Bird DNA is known to have substantial interstitial “telomeric” sequences within the centromere region that could bind the probe if the DNA was single-stranded (Venkatesan and Price 1998). To ensure that our probe was binding only to the G-strand overhang of the telomere and not to any interstitial telomeric sequences, some samples were digested with a single-strand specific endonuclease (mung bean nuclease; 40 U) to remove the overhang before restriction enzyme digestion and hybridization to the probe.

Results

Figure 1a shows the hybridization of the telomeric probe (C₃TA₂)₄ to digested DNA from three different individuals run in triplicate. The DNA from one replicate for each individual was digested with mung bean nuclease to cleave off the telomere G-strand overhang before restriction digestion (Venkatesan and Price 1998). Removal of the G-strand overhang, which produced blunt ends, abolished hybridization to the probe, indicating that our probe bound only to telomeres and not to any interstitial telomeric sequences.

Densitometry indicated that mean telomere length decreased as age increased in zebra finches (Fig. 1b,

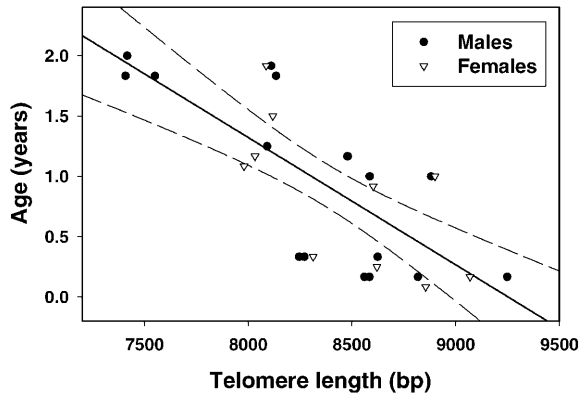


Fig. 2 Change in age as a function of telomere lengths in zebra finches. The line is the best-fit regression through the data described by the equation $\text{age} = 9.77 - 0.00106 \text{ telomere length}$. The 95% confidence interval for the slope is 0.001 ± 0.0002 . Dashed lines represent the 95% confidence interval for the mean age predicted from a given telomere length

Table 1 Telomere length (bp) of juvenile (<4 months), young (11–15 months) and old (>18 months) zebra finches. Means followed by different letters differ from each other (Tukey's honestly significant difference test, $P < 0.05$)

Age group	Number	Mean telomere length (bp) ^a	SEM
Juvenile	11	8,656 a	104
Young	9	8,448 a	115
Old	11	7,979 b	104

^a $F_{2,28} = 11.04$, $P = 0.0003$

Table 1). Males and females did not differ ($F_{1,29} = 0.24$, $P = 0.63$), although gender differences have been reported in mice (Coviello-McLaughlin and Prowse 1990). For the 27 birds of known age, mean telomere length decreased significantly with increasing age ($F_{1,25} = 29.89$, $P < 0.0001$, $r^2 = 0.54$). There was an approximate reduction in telomere length of 516 ± 94 bp/year (\pm SE) in zebra finch erythrocytes (Fig. 2). Thus, knowing telomere length provides a means to estimate age in zebra finches.

Discussion

A number of studies in rodents and humans have shown a gradual decrease with time in the length of telomeres from a variety of tissues (Hastie et al. 1990; Allsopp et al. 1992; Vaziri et al. 1994; Slagboom et al. 1994; Coviello-McLaughlin and Prowse 1997; Frenck et al. 1998; Friedrich et al. 2000). We have now shown this also occurs in avian erythrocytes. Measuring telomere length should offer a quick, relatively non-invasive method to estimate the age of individuals within a population once the rate of telomere shortening is known for that population.

The rate of telomere shortening in zebra finch erythrocytes is an order of magnitude greater than the annual base pair loss of leukocyte telomeres in humans

(31–33 bp/year; Hastie et al. 1990; Slagboom et al. 1994). Annual telomere loss from spleen cells in mice, another short-lived species, is 600 bp/year (Coviello-McLaughlin and Prowse 1997). The rate of telomere shortening may vary systematically with life span or body size, but such an analysis awaits further comparative data.

Nucleated erythrocytes should provide DNA for telomere determination in fish, amphibians, reptiles and birds. Mammalian erythrocytes usually lack nuclei, but leukocytes can be used to measure telomere length from blood samples (Hastie et al. 1990). Telomere lengths in peripheral blood cells probably reflect telomere lengths of the hematopoietic stem cells (Vaziri et al. 1994), because blood cells do not divide while in circulation. Although hematopoietic stem cells in humans do have some telomerase activity, they still lose telomeric repeats and thus proliferative potential over time (Vaziri et al. 1994; Englehardt et al. 1997).

Blood is an excellent candidate tissue for aging studies, although other tissues, such as skin, have also been used to measure telomere length (Allsopp et al. 1992; Friedrich et al. 2000). Blood can be obtained easily from most animals and is often sampled in the field for other reasons. In addition, blood cell telomere lengths may shorten at a greater rate than telomere lengths in other tissues (Hastie et al. 1990; Friedrich et al. 2000), because of the high turnover rate of blood cells (Chang and Harley 1995).

The ages of the zebra finches we used in this study ranged over about half of this species' natural life span and thus we do not know whether telomere length declines in a linear fashion across all ages. Most human studies investigating the loss of telomeric repeats over the entire life span have shown a linear decline (Hastie et al. 1990; Allsopp et al. 1992; Slagboom et al. 1994). One recent study in humans has suggested that telomeres shortened more rapidly immediately after birth than through the rest of life (Frenck 1998). In addition, whether or not the rate of shortening varies between populations is unknown. Differences among individuals or populations in blood turnover rate or intracellular stress (e.g., oxidative stress, von Zglinicki et al. 1995) could alter shortening rates, but this remains to be demonstrated.

We conclude that the gradual loss of telomeric sequences can be used as a marker of chronological age for species in which the clock can be calibrated using DNA from either known-age individuals from captive populations or marked individuals in long-term field studies. This will allow rapid determination of the age class of individuals and the age structure of a population in which DNA from a reasonable number of individuals can be sampled. Species-specific calibration curves for this technique should include individuals from the widest available range of known-age animals. This method will permit the inclusion of the estimate of age with other factors affecting life history parameters, even if previous histories of animals in the population are unknown. In addition, if individuals can be sampled repeatedly over

their life span, we should be able to determine how rate of telomere shortening affects longevity of individuals and if the rate of shortening is constant over time.

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