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Research article

Analysis of telomere length and telomerase activity in tree species of various life-spans, and with age in the bristlecone pine *Pinus longaeva*

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Abstract

Normal somatic cells have a finite replicative capacity. With each cell division, telomeres (the physical ends of linear chromosomes) progressively shorten until they reach a critical length, at which point the cells enter replicative senescence. Some cells maintain telomere length by the action of the telomerase enzyme. The bristlecone pine, *Pinus longaeva*, is the oldest known living eukaryotic organism, with the oldest on record turning 4770 years old in 2005. To determine what changes occur, if any, in telomere length and telomerase activity with age, and what roles, if any, telomere length and telomerase activity may play in contributing to the increased life-span and longevity of *P. longaeva* with age, as well as in other tree species of various life-spans, we undertook a detailed investigation of telomere length and telomerase activity in such trees. The results from this study support the hypothesis that both increased telomere length and telomerase activity may directly/indirectly contribute to the increased life-span and longevity evident in long-lived pine trees (2000–5000 year life-spans) compared to medium-lived (400–500 year life-span) and short-lived (100–200 year life-span) pine trees, as well as in *P. longaeva* with age.

Introduction

Telomeres, specialized structures at the physical ends of eukaryotic chromosomes consisting of highly conserved repeated DNA sequences (Kipling 1995; Shay 1999), shorten with each round of DNA replication (Harley et al. 1990) due to the inability of DNA polymerases to completely replicate linear DNA molecules (Olovnikov 1971, 1973, 1996; Watson 1972). Telomeres in nearly all plants and trees consist of the heptanucleotide repeat $(TTTAGGG)_n$ (Ganal et al. 1991; McKnight et al. 1997), although recent research has suggested that the plant order Asparagales have nearly all TTAGGG telomeric repeats (Sykorova et al. 2003; Rotkova et al. 2004). Telomere length can be used as a predictor of the future replicative capacity of cells (Allsopp et al. 1992), and depends on both the age of the cell and the number of times the cell has already divided (Harley et al. 1990). Telomere shortening can be used as both an *in vitro* (Harley et al. 1990; Allsopp et al. 1992; Harley et al. 1992; Flanary and Streit 2004) and *in vivo* (Lindsey et al. 1991; Kajstura et al. 2000; Wright and Shay 2002; Flanary and Streit 2003) marker of cell replication and cell aging. Normal somatic cells undergo a finite number of cell divisions *in vitro* before entering cellular senescence (Hayflick 1961). Senescence, which ultimately culminates in cell death, is characterized by an irreversible arrest of cell proliferation (Hayflick 1965), substantial alterations in patterns of gene expression (i.e., SAGE: Senescence-Associated Gene Expression)

(Bernd et al. 1982; Shelton et al. 1999; Funk et al. 2000), an increasing resistance to apoptosis (Spaulding et al. 1999), cell-type specific changes in cell function, and concomitant telomere shortening (Harley et al. 1990). Elongation of telomeres can occur by the ribonucleoprotein enzyme telomerase, which (in trees) adds tandem heptanucleotide repeats (i.e., TTTAGGG) *de novo* to 3′ ends of telomeres using its own RNA as a template (Greider and Blackburn 1985; Fajkus et al. 1996; Cech et al. 1997). Telomerase can compensate for the continual shortening of telomeres that would otherwise occur.

For eons, the bristlecone pine trees (*Pinus aristata* and *Pinus longaeva*), have flourished in the arid mountains of the Great Basin (which extend from California to Colorado), apparently thriving in rocky, dolomite (limestone substrate), at high altitudes [over 3000 m (10,000 feet) above sea level], in low precipitation [annual precipitation is less than 30 cm (12 inches), most of which falls as snow in winter], and in cold temperatures [annual temperature range is 2 °C to −32 °C (36 °F to −26 °F)]. Collectively, the average age of the bristlecone trees is about 1000 years, with several specimens still alive at well over 4000 years of age (as determined by counting annual growth rings following core boring). Indeed, the bristlecone pines are among the oldest known and longest-lived organisms on Earth (Schulman 1958). However, there may be older trees/eukaryotes existing elsewhere that have yet to be discovered. In 1958, the U.S. Forest Service established a 28,000 acre ancient bristlecone pine forest preserve, naming it the Schulman Memorial Grove, in honor of the founder of modern bristlecones, Edmund Schulman. One year earlier, in 1957, Schulman discovered the oldest living bristlecone pine on record (aptly named ''Methuselah''), which turns 4770 years old in 2005. Although the name is Biblical, Methuselah predates Christ by nearly 3000 years and was a little over 200 years old when construction on the Great Pyramid of Giza in Egypt began in approximately 2550 B.C.

We have found no previously published research that has examined telomere length or telomerase activity in tree species of any kind. Telomere dynamics with age has never been examined as a contributing factor in the expected life-span and/or longevity of tree species. Therefore, we examined telomere length and telomerase activity in: (1) age-matched controlled pine tree species of various life-spans (i.e., 100–200, 400–500, and 2000–5000 years), and (2) various aged (i.e., 20–3500 years old) long-lived bristlecone pine trees (*Pinus longaeva*). These experiments were performed to determine what changes, if any, occur in telomere length and telomerase activity with age, and what roles, if any, telomere length and telomerase activity may play in contributing to the increased life-span and longevity of these tree species.

Materials and methods

Collection of tree samples

Details of the actual sample collection were identical for all trees. To collect core samples, an area on the tree trunk having a relatively flat surface was located, and a standard tree coring device (i.e., consisting of a steel pipe with a pointed end) was introduced into this area by hammering. As the pipe drove into the wood, it separated a portion of the wood into the center of the pipe. After the pipe was advanced to a sufficient depth within the tree (approximately 3–5 cm), it was repeatedly twisted to cause the core sample inside (approximately 5 mm in diameter) to break off. After removing the pipe from the tree, the sample was then removed from the interior of the pipe. To collect root samples, the soil near the base of the tree trunk was removed to excavate the living roots of the tree. A small quantity (approximately 3–5 g) of the root tissue was collected. Needle samples were collected from the end of an accessible branch containing approximately 10–50 individual needles. Following collection, all samples were placed in plastic containers, which were immediately stored on dry ice until being permanently stored at −80 °C prior to analysis.

Samples (core, needle, and root) were collected by permission from the United States National Arboretum in Washington, DC as per Sue Martin on July 25, 2003 from the following age-matched tree species: the coastal redwood, *Sequoia sempervirens*, and the bristlecone pine,

Pinus aristata, (2000–5000 year life-span: ''long-lived trees''); the Western white pine, *Pinus monticola*, and the red pine, *Pinus resinosa*, (400–500 year life-span: ''medium-lived trees''); the loblolly pine, *Pinus taeda*, and the longleaf pine, *Pinus palustris*, (100–200 year life-span: ''short-lived trees''). Samples were collected when each tree was 18 years of age (except for *S. sempervirens*, which was 16 years old at the time of collection). Samples (core, needle, and root) were collected by permission from the Inyo National Forest in Bishop, CA as per John Louth on December 11, 2003 from the bristlecone pine *Pinus longaeva*. Samples were collected from a total of nine *P. longaeva* trees of various ages (in years): 20 ± 5, 37, 54, 63, 180, 334, 1200 ± 100, 2000 ± 200, 3500 ± 100.

Determination of telomere length

Tree samples (i.e., core, needle, root) were placed in a mortar and ground into a fine powder using a pestil and liquid nitrogen. Genomic DNA was isolated from approximately 20.0 mg freshly ground powder of each sample (dry weight) using the DNeasy DNA isolation kit (Qiagen, Valencia, CA). Only needle and root samples were used for Southern blot analysis, since core samples did not provide adequate DNA quantity for analysis. To measure telomere length (i.e., telomere restriction fragment (TRF) length), Southern blot analysis using chemiluminescent detection and the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche, Indianapolis, IN) was employed as previously described (Flanary and Streit 2003, 2004), with the following modifications. Following electrophoresis of 3.0 g genomic DNA, hybridization of telomeric repeats was accomplished by using a digoxigenin-labeled tree telomere-specific oligonucleotide probe (TTTA GGG)$_3$.

Determination of telomerase activity

Tree samples (i.e., core, needle, root) were placed in a mortar and ground into a fine powder using a pestil and liquid nitrogen. Total protein was harvested from approximately 5.0 mg freshly ground powder of each sample (dry weight) and telomerase activity was measured as previously described (Flanary and Streit 2003, 2004) using the telomere repeat amplification protocol (TRAP).

Results

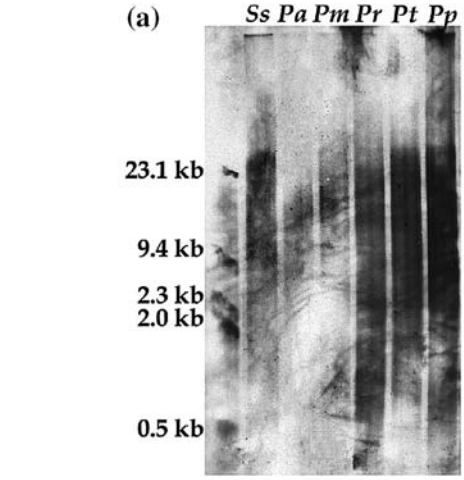
Telomere length dynamics in trees of various life-spans

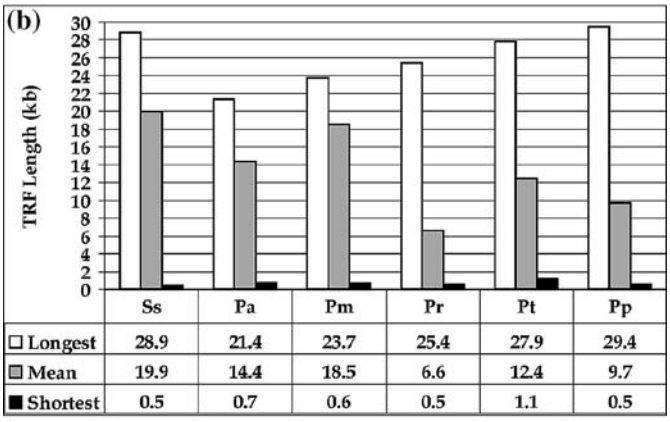
A representative Southern blot image used for measurement and quantitation of TRF length is shown in Figure 1a. In needle samples (Figure 1b), TRF length was correlated with the expected life-span of each species. The average of the longest TRF lengths for long-lived trees (25.12 kb) was higher compared to medium-lived trees (24.58 kb), but shorter compared to short-lived trees (28.66 kb). The average of the mean TRF lengths for long-lived trees (17.14 kb) was higher compared to both medium-lived (12.56 kb) and short-lived (11.06 kb) trees. The average of the shortest TRF lengths for long-lived trees (0.60 kb) was higher compared to medium-lived trees (0.58 kb), but shorter compared to short-lived (0.81 kb) trees.

In root samples (Figure 1c), TRF length was also consistent with the expected life-span of each species. The average of the longest TRF lengths for long-lived trees (52.51 kb) was higher compared to both medium-lived (38.57 kb) and short-lived (28.48 kb) trees. The average of the mean TRF lengths for long-lived trees (16.66 kb) was higher compared to both medium-lived (11.13 kb) and short-lived (5.72 kb) trees. The average of the shortest TRF lengths for long-lived trees (2.67 kb) was higher compared to both medium-lived (1.83 kb) and short-lived (2.51 kb) trees.

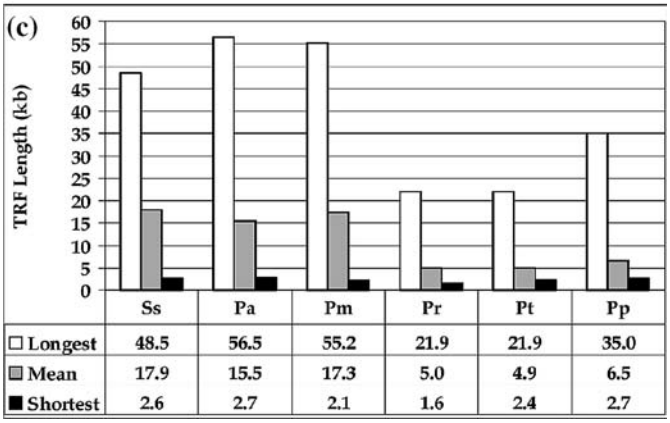
Telomerase activity in trees of various life-spans

A representative TRAP analysis image used for measurement and quantitation of telomerase activity is shown in Figure 2a. In needle samples (Figure 2b), telomerase activity correlated with the expected life-span of each species. The average telomerase activity for long-lived trees (15.28) was higher compared to both medium-





	Ss	Pa	Pm	Pr	Pt	Pp
Longest	28.9	21.4	23.7	25.4	27.9	29.4
Mean	19.9	14.4	18.5	6.6	12.4	9.7
Shortest	0.5	0.7	0.6	0.5	1.1	0.5



	Ss	Pa	Pm	Pr	Pt	Pp
Longest	48.5	56.5	55.2	21.9	21.9	35.0
Mean	17.9	15.5	17.3	5.0	4.9	6.5
Shortest	2.6	2.7	2.1	1.6	2.4	2.7

Figure 1. Representative Southern blot image (a) used for measurement and quantitation of telomere restriction fragment (TRF) length. Shown here is the Southern blot image for needle samples of tree species of various life-spans: *Ss* = *Sequoia sempervirens* and *Pa* = *Pinus aristata* (2000–5000 year life-span: ''long-lived trees''); *Pm* = *Pinus monticola* and *Pr* = *Pinus resinosa* (400–500 year life-span: ''medium-lived trees''); *Pt* = *Pinus taeda* and *Pp* = *Pinus palustris* (100–200 year life-span: ''short-lived trees''). Genomic DNA was isolated from needle and root samples of tree species of various life-spans and probed with a digoxigenin-labelled tree telomere-specific oligonucleotide ($TTTAGGG_3$). Densitometric quantitation of averaged TRF length [in kilobases (kb)] in each life-span group revealed longer telomeres (i.e., longest, mean, shortest) in nearly all needle samples (b), and in all root samples (c), of long-lived trees compared to both medium- and short-lived trees.

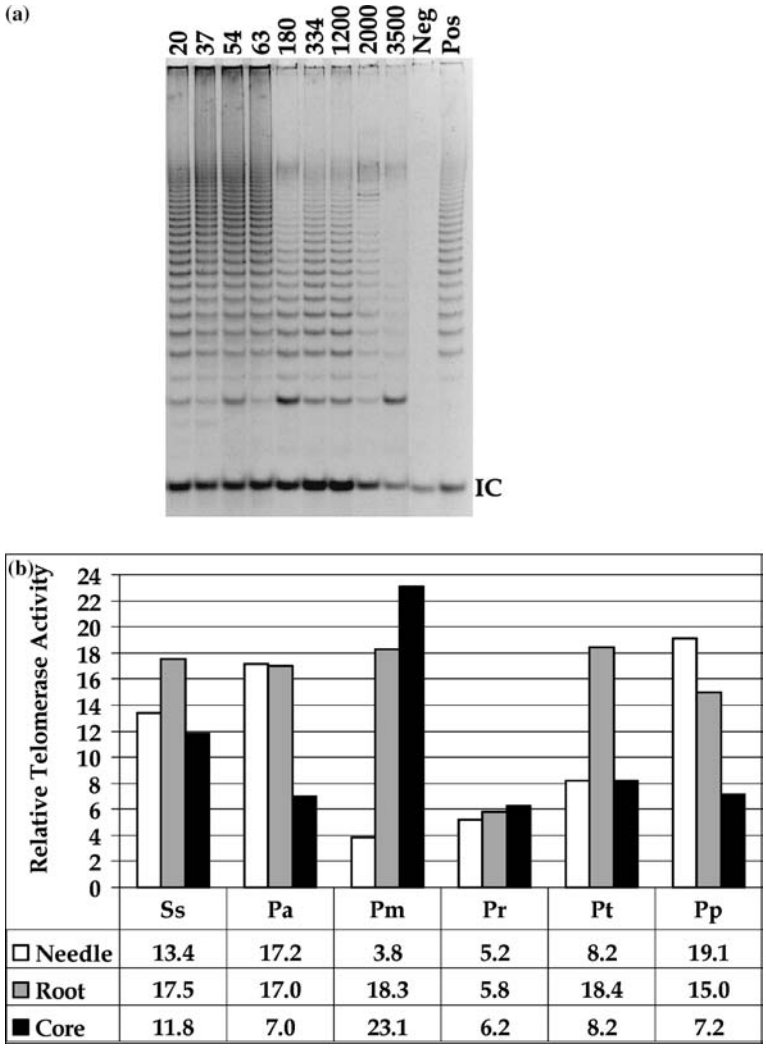
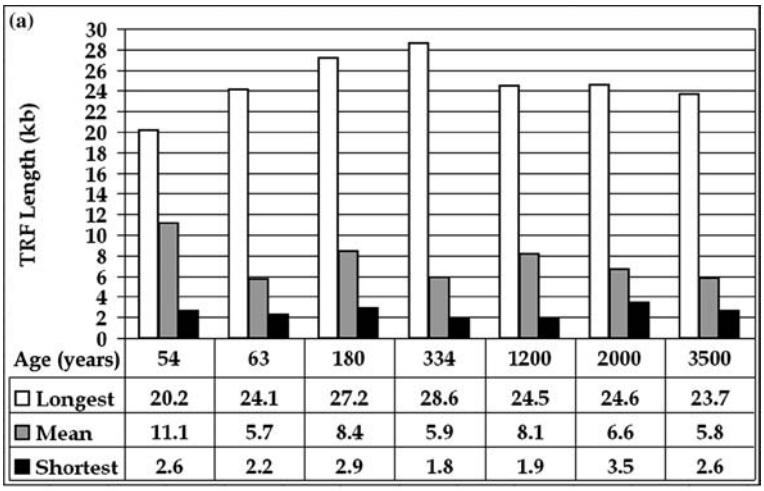


Figure 2. Representative TRAP analysis image (a) used for measurement and quantitation of telomerase activity in tree species of various life-spans (listed in Figure 1). Shown here is the TRAP image for core samples of the indicated age (years) from the bristlecone pine *Pinus longaeva*. Neg = telomerase-negative control; Pos = telomerase-positive control; IC = internal control. Total protein was isolated from core, needle, and root samples. Densitometric quantitation (b) revealed higher relative telomerase activity (arbitrary units) in all needle and root samples, and in nearly all core samples, of long-lived trees compared to both medium- and short-lived trees.

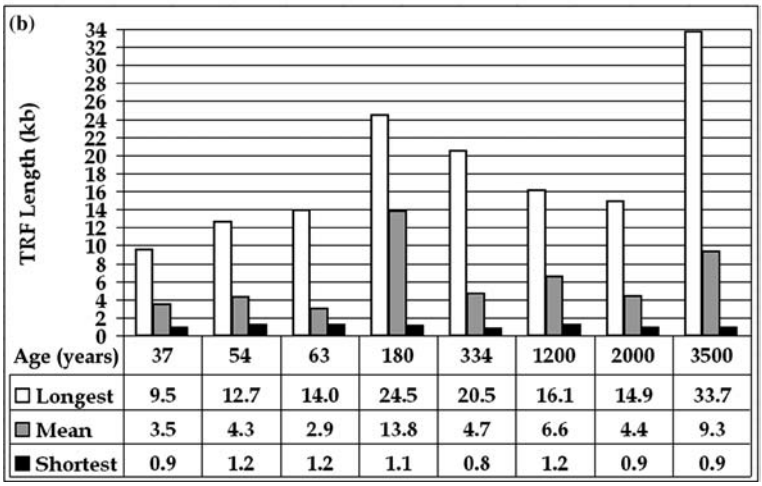
lived (4.50) and short-lived (13.65) trees. In root samples, the average telomerase activity for long-lived trees (17.26) was higher compared to both medium-lived (12.03) and short-lived (16.70) trees. In core samples, the average telomerase activity for long-lived trees (9.42) was higher compared to short-lived trees (7.70), but lower compared to medium-lived trees (14.65).

Telomere length dynamics in the bristlecone pine, Pinus longaeva

The longest, mean, and shortest TRF lengths in needle samples of *Pinus longaeva* exhibited a cyclical pattern of lengthening and shortening in size with age (Figure 3a). Overall, TRF length in needle samples remained relatively unchanged with age. In root samples, all TRF lengths (i.e.,



Age (years)	54	63	180	334	1200	2000	3500
Longest	20.2	24.1	27.2	28.6	24.5	24.6	23.7
Mean	11.1	5.7	8.4	5.9	8.1	6.6	5.8
Shortest	2.6	2.2	2.9	1.8	1.9	3.5	2.6



Age (years)	37	54	63	180	334	1200	2000	3500
Longest	9.5	12.7	14.0	24.5	20.5	16.1	14.9	33.7
Mean	3.5	4.3	2.9	13.8	4.7	6.6	4.4	9.3
Shortest	0.9	1.2	1.2	1.1	0.8	1.2	0.9	0.9

Figure 3. Densitometric quantitation of averaged TRF length from the bristlecone pine *Pinus longaeva* at the indicated age (54–3500 years) revealed that all telomeres (i.e., longest, mean, shortest) underwent a cyclical pattern of lengthening and shortening in size with age in both needle (a) and root (b) samples. Overall, TRF length remained unchanged with age in needle samples, and increased with age in root samples. Insufficient DNA was isolated to enable measurement of needle samples at 37 years of age.

longest, mean, and shortest) also underwent a similar cyclical pattern of lengthening and shortening in size with age (Figure 3b). Overall, TRF length in root samples was found to increase with age, with a slight increase in TRF length evident during the early growth stages (e.g., the first few hundred years).

Telomerase activity in the bristlecone pine, Pinus longaeva

Telomerase exhibited a cyclical pattern of increasing and decreasing activity in all samples analyzed (core, needle, and root) from 20 to 3500 years of age in the bristlecone pine, *Pinus longaeva* (Figure 4). Overall, telomerase activity was found to increase with age in root samples, slightly decrease with age in needle samples, and substantially decline with age in core samples. From age 1200 to 3500, both the pattern and relative level of telomerase activity in needle and root samples are nearly the same, with activity in both samples ending in a moderate decline at age 3500. In core samples, telomerase activity underwent minor fluctuation early in life (i.e., from age 20 to 54), and subsequently steadily declined with age. In all

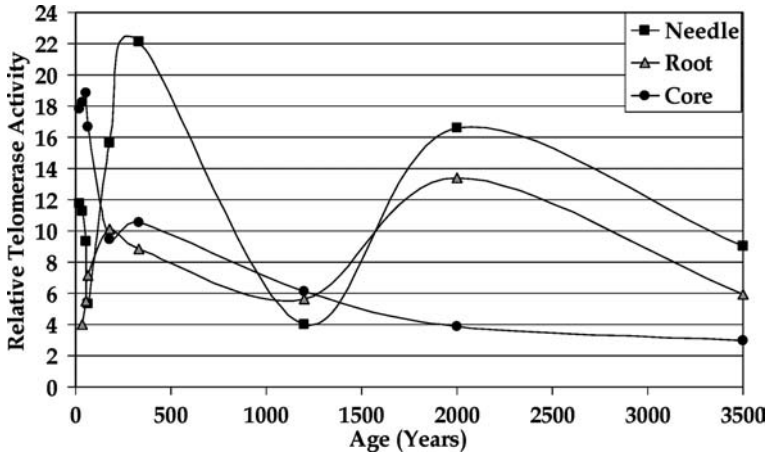


Figure 4. Densitometric quantitation of telomerase activity (arbitrary units) in samples (needle, root, core) from the bristlecone pine *Pinus longaeva* at the indicated age revealed a cyclical pattern of increasing and decreasing activity with age, especially in needle and root samples. Overall, telomerase activity slightly decreased with age in needle samples, and slightly increased with age in root samples. In all samples (i.e., core, needle, root), telomerase activity remained present and active at all ages analyzed (even up to 3500 years of age).

samples (i.e., core, needle, root), telomerase activity remained present and active at all ages analyzed (even up to 3500 years of age).

Discussion

What enables some species of pine tree to live for several thousands of years, while other species are capable of living only a few hundred years? The answer to this has continued to elude researchers for decades. The results from this study, which is the first to report on telomere length and telomerase activity in any tree species, suggests that telomere length and telomerase activity may play essential roles that directly/indirectly regulate life-span in certain tree species.

Most tree species die by one or more of the following causes: endogenous cell senescence, microorganism/insect attacks, and exogenous damage from their physical environment (e.g., fires). It has been noted that long-lived trees are (apparently) unable to succumb to cell senescence (Larson 2001). In fact, observations of the seemingly unlimited rejuvenation and division of cells within meristems (self-renewing populations of cells that can divide indefinitely and provide cells for future growth) in long-lived trees (e.g., giant sequoias), and the observation that ''foresters and other botanists who have studied the big trees have stated repeatedly that none is known to have died of ''natural'' causes'' (Westing 1964), support the hypothesis that lack of cell senescence and unlimited replicative capacity (especially within meristems) may be contributing factors to their longevity. We found only three previously published articles that studied biomarkers of aging in *Pinus longaeva*. One study (Connor and Lanner 1990) found no significant difference between number of xylem rays, longevity of both phloem and xylem rays, number of phloem cells, and thickness of phloem and tracheids in young compared to old trees. The same authors also examined needles from 1-year old and 35-year old bristlecone pines and found that both cuticle thickness and chlorophyll content remained unchanged throughout the life of the needle (Connor and Lanner 1991). Another study (Lanner and Connor 2001) examined *P. longaeva* trees ranging in age from 23 to 4713 years, and found no significant age-related differences in meristem function in embryos, seedlings, or mature trees in regards to tracheid diameter, shoot characteristics (shoot length and number of stem units), and seed/seedling characteristics (seed weight, seed germination, and biomass of seed-

lings). Overall, the results from our study support these three previous articles. We found no age-related decline in telomere length in needle samples, although root samples were found to slightly increase with age. Similarly, no age-related decline in telomerase activity was found in needle and root samples, although core samples did exhibit a overall decrease in telomerase activity.

In comparing trees of various life-spans, both needle and root samples in long-lived trees (2000–5000 year life-span) were found to possess higher average TRF lengths of the longest, mean, and shortest telomeres compared to both short-lived and medium-lived trees in all but two instances (i.e., except in longest and shortest telomeres of short-lived trees in needle samples). Additionally, in needle, root, and core samples, long-lived trees were found to possess higher average telomerase activity compared to both short-lived and medium-lived trees in all but one instance (i.e., except in core samples of medium-lived trees). Thus, both telomere length and telomerase activity overall correlated well with the expected life-span of each tree species, such that long-lived trees possessed longer average TRF lengths and exhibited higher telomerase activity compared to both medium-lived and short-lived trees. A correlation between TRF length and telomerase activity only existed for root samples, suggesting that telomerase activity may be more closely regulating telomere length in the roots (compared to needles) of the examined trees. The replicative potential (especially within meristems) and life-span of individual cells in long-lived trees may be higher than that in medium-lived and short-lived trees, as supported by (1) the presence of higher average telomere length in all root samples, and in nearly all needle samples in age-matched trees, (2) the presence of higher average telomerase activity in all needle and root samples, and in nearly all core samples in age-matched trees, and (3) the increased life-span of long-lived trees. Although only one age was examined [i.e., 18 years of age (except for *S. sempervirens*, which was 16 years of age)], the results obtained provide a glimpse into the realm of tree telomere biology and suggest an active role for telomere length regulation and telomerase activity in contributing to the longevity of trees.

All sample types (i.e., core, needle, root) possessed a very heterogenous population of different cell types, and thus the measurements of telomere length and telomerase activity were based on mixed cell populations present within each sample. The presence of non-dividing cells within samples would likely exhibit an absence of both telomere shortening (due to lack of division) and telomerase activity (unless telomerase has functions other than telomere maintenance). It is unknown how these non-dividing cells would affect telomere length, since it would depend on the length found within these cells in order to determine if they increased, did not affect, or decreased the overall measured telomere length of the mixed cell population. As for telomerase activity, the presence of non-dividing cells (which should not exhibit any activity) may contribute to an overall decrease in measured activity of the cell sample population as a whole. However, it remained difficult to accurately gauge the percentage of dead cells within particular tree samples. Dead cells present within (particularly core) samples may have contained fragmented DNA (if some cells were in the process of apoptosis during sample collection) or no DNA at all (which may explain why very little DNA was isolated from core samples). The presence of small quantities of fragmented DNA (if present) may have caused a higher quantity of "short" telomeres to be identified within that sample type, although this is not of concern in the present study since core samples were not analyzed for telomere length, and the other sample types (i.e., needle and root) would likely contain minuscule quantities of dead cells (and hence fragmented DNA), if present at all. Although the data collected did reveal a difference in telomere length and telomerase activity between trees of various life-spans and with age, it is impossible to accurately determine what roles were played by each individual cell type present within the arboreal milieu.

Our current findings support the hypothesis that both increased telomere length and telomerase activity in long-lived trees, compared to medium-lived and short-lived trees, may play essential roles that directly/indirectly regulate life-span in longer-lived tree species. Their

increased expected life-span may be due to higher levels of telomerase activity, which could act to slow/prevent the attrition of telomeres (at least in needle and root samples, and to a possible lesser extent in core samples), thereby accounting for the presence of longer telomeres in long-lived tree species. Longer telomeres may, in turn, slow/prevent the subsequent entry of the associated cells into senescence (if occurring at all) that may otherwise occur in the absence of such telomerase activity (and possibly in the presence of critically short telomeres). As a result, if the individual cells within certain areas of a tree (e.g., needle, root, meristem) possess a long life-span [conferred by increased telomerase activity and telomere length (as observed in long-lived trees)], this may, in turn, result in the increased expected longevity of the organism as a whole.

In comparing samples collected from the bristlecone pine, *Pinus longaeva*, both TRF length and telomerase activity were found to exhibit a cyclical pattern with age. For both TRF length and telomerase activity in both needle and root samples in *P. longaeva*, there was no evidence of overall telomere shortening, or decrease in telomerase activity, with age (up to 3500 years of age). In addition, telomerase activity was detected in all samples (needle, root, and core) and ages (up to 3500 years) analyzed, suggesting that active telomerase may be present to slow/prevent telomere attrition in the samples analyzed. In core samples, telomerase activity started at a high level and declined gradually with age, yet there is still detectable activity up to 3500 years of age. In both needle and root samples of *P. longaeva*, the pattern of telomere shortening and lengthening that occurred with age closely correlated to the concomitant decrease and increase in telomerase activity over the same time period. This suggests that telomerase activity is capable of regulating telomere length with age in *P. longaeva*. A correlation between TRF length and telomerase activity only existed for root samples (when comparing *P. longaeva* with age), suggesting that telomerase activity may be more closely regulating telomere length in the roots (compared to needles) of *P. longaeva*.

Our current findings support the hypothesis that telomere length and telomerase activity may play essential roles that directly/indirectly regulate life-span in certain tree species, such as *P. longaeva*. Their long life-span may be due to the stable presence of telomerase activity (detectable in all samples analyzed up to approximately 3500 years of age), which could act to slow/prevent the attrition of telomeres (at least in needle and root samples up to 3500 years of age, and possibly to a lesser extent in core samples due to the declining levels of telomerase activity evident within core samples with age), and thereby preserve telomere length as well as limit the quantity of critically short telomeres present with age. The absence/low quantity of such short telomeres may, in turn, slow/prevent the subsequent entry of the associated cells into senescence (if occurring at all) that may otherwise occur in the absence of such telomerase activity (and in the presence of critically short telomeres). As a result, if the individual cells within certain areas of a tree (e.g., needle and root) possess a long life-span [conferred by stable telomerase activity and preservation of telomere length throughout the centuries (as observed in *P. longaeva* with age)], this may, in turn, result in the increased longevity of the organism as a whole (Harley et al. 1992; Fossel, 2000).

What secrets do the long-lived bristlecone pines possess that confer such great longevity and what are their limits, if any, in regards to maximum life-span? It has been hypothesized that the numerous structural and physiological characteristics possessed by long-lived trees (e.g., the continual retention of stem cell-like meristematic cells, specialized vascular system, and a hormonal control system) play critical roles in contributing to their longevity (Lanner 2002). These secrets remain hidden for now; however, finding the source of the great longevity evident in bristlecone pines (perhaps through the presence of stable telomerase activity and preservation of telomere length within its cells, especially within meristems, with age) may enable the development of a therapy to afford humans similar longevity (perhaps through preservation of telomere length in somatic cells via exogenous/endogenous telomerase activity). The implications of studying longevity in long-lived bristlecones is best summarized by Schulman

(1958) himself: ''The capacity of these trees to live so fantastically long may, when we come to understand it fully, perhaps serve as a guidepost on the road to the understanding of longevity in general.''

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