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Sperm of the Solitary Coral *Ctenactis echinata* Exhibit Longer Telomeres than that of Somatic Tissues

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Michiko C. Ojimi, Yossi Loya, and Michio Hidaka (2012) Sperm of the solitary coral *Ctenactis echinata* exhibit longer telomeres than that of somatic tissues. *Zoological Studies* **51**(8): 1475-1480. Because many corals reproduce asexually through regeneration from fragments, establishing a simple relationship between the age and size of a coral is problematic. Here, we attempted to test the coral telomere length as an indicator of age and senescence, because telomere shortening occurs during cell division. We examined the association between the size (weight) of a coral and the average length of its telomeres as determined by single telomere length analysis (STELA) products. We chose as our study species the solitary coral *Ctenactis echinata*, which is unlikely to reproduce via fragmentation and has never been observed to reproduce asexually via budding. We amplified DNA fragments containing part of a telomere with part of the subtelomeric region using a dual-suppression PCR, and determined DNA sequences of subtelomeric regions in order to design chromosome-specific primers for the STELA. The average length of STELA products was calculated from densitometric data, and it was plotted against the weight of the coral. Although sperm exhibited a longer telomere length than that of somatic tissues, there was no significant relationship between the average length of the STELA products and the weight of individuals. These results suggest that telomere shortening occurs during early development in this solitary coral. Moreover, our findings are expected to provide a genetic basis for coral aging. Future research on the range of telomere changes occurring in this coral would further our understanding of how age and size are related. http://zoolstud.sinica.edu.tw/Journals/51.8/1475.pdf

Key words: Ageing, Coral, Lifespan, Senescence.

Scleractinian corals display asexual reproduction in various ways, such as fragmentation (Highsmith 1982, Smith and Hughes 1999), transverse fission (Wells 1966), polyp bailout (Sammarco 1982), polyp expulsion (Karmarsky-Winter et al. 1997), and asexual planulae (Stoddart 1983), in addition to sexual reproduction. Corals also display a high capacity to regenerate from small pieces of tissue (Krupp et al. 1992, Vizel et al. 2011). Currently, it is not possible to determine whether both asexually produced corals and those regenerated from fragments are of the same age as their source colonies. Furthermore, the occasionally occurring fission and fusion of coral colonies make it difficult to estimate the age of corals from their size (Hughes and Jackson 1980, Hughes and Connell 1987, Babcock 1991).

Coral age (in years) can be estimated from growth bands, but this method is applicable only to corals with a massive growth form. It is hard to estimate the age of branching corals due to difficulties in obtaining a complete growth band, and to possible fragmentation. It is thus important to establish a method with which to estimate coral age based on soft tissues. Such a method would contribute much to our understanding of coral population biology, demographics, and various life history traits such as age at reproductive

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maturation, ageing, and senescence (Rinkevich and Loya 1986).

Telomeres are non-coding short tandem repeats of DNA at the ends of eukaryotic chromosomes (Blackburn 1984, LeBel 2005, Shawi and Autexier 2008). Telomeres protect chromosomes against degradation and fusion with other chromosome ends, and they shorten during cell division due to incomplete replication at the 5'-end of DNA strands (Urquidi et al. 2000, Nakagawa et al. 2004). Thus telomere length is expected to reflect the number of cell divisions and, therefore, the age of an organism. Indeed, in birds and mammals, telomere length generally reflects the age of an individual, and there are correlations between the telomere-shortening rate and the lifespan of a species, with some exceptions (Nakagawa et al. 2004). Baird et al. (2003) established the single telomere length analysis (STELA), which offers a method by which to measure the telomere length of specific chromosomes. The STELA is a sensitive and reliable method for estimating the length of individual telomeres in humans, where genome sequences are available (Baird et al. 2003, Kimura et al. 2007). This method is not readily applicable to non-model organisms, however, because the DNA sequences of subtelomeric regions are required. We therefore determined the DNA sequence of a subtelomeric region in tested corals using a dual-suppression polymerase chain reaction (PCR; Lian et al. 2006), and designed a chromosome-specific primer for the STELA.

The objectives of this study were to establish a method by which to measure telomere length in single chromosomes of corals using the STELA, test whether the telomere length indeed reflects the coral weight (age), and compare telomere lengths between somatic tissues and sperm. We used a solitary coral, *Ctenactis echinata*, as our study organism. This coral has a rigid skeleton and is unlikely to reproduce asexually via fragmentation. We therefore assumed that the weight of the coral reflects its age. We compared telomere lengths of specific chromosomes among individuals of various sizes (255-5000 g). We also compared the telomere length of sperm with that of somatic tissues. This is the 1st study to use the modified STELA to measure telomere lengths in a coral and investigate the relationship between telomere length and weight of a coral.

MATERIALS AND METHODS

Collection of corals and sperm

Individuals of the solitary coral *Ctenactis echinata* were collected from a patch reef near Sesoko I., Okinawa, Japan (26°39'50"N, 127°52'24"E), several days before the expected spawning period in 2008 and 2009.

Individuals were tagged (Loya and Sakai 2008), placed in separate containers, and kept in running-seawater tanks supplied with unfiltered seawater. The containers, each with one individual, were removed from the tanks during the night of the expected spawning period. Male individuals released sperm on the nights of 22 Aug. 2008 and 13 July 2009. Seawater containing sperm was collected using a beaker and filtered through a 40-μm mesh. It was then centrifuged at 5000 rpm for 10 min, and the resulting pellet was placed in a 2-ml microtube and stored at -80°C until use.

Somatic tissue samples were removed from six individuals of *C. echinata* of different sizes (255-5000 g in weight). Unfortunately, no individuals smaller than 255 g were available. A few septa were taken from the oral surface of each individual, 1-2 cm from the periphery, in order to sample somatic tissues without gonads. The sampled tissues were put in a CHAOS solution (4 M guanidine thiocyanate solution containing 0.1% *N*-lauroylsarcosine sodium, 25 mM Tris-HCl at pH 8.0, and 0.1 M β-mercaptoethanol), to preserve the DNA.

DNA extraction

Genomic DNA of *C. echinata* was extracted either from tissue samples or sperm using the sodium dodecylsulfate (SDS)-proteinase K method. Sperm samples were incubated in DNA extraction buffer (50 mM EDTA, 50 mM Tris pH 8.0, and 0.4 M NaCl) containing 1% SDS and 2% β-mercaptoethanol at 65°C for 1 h, followed by the addition of 0.5 mg/ml proteinase K and incubation at 37°C overnight. Tissue samples in the CHAOS solution were incubated at 55°C for 1 h. DNA was then extracted from the samples according to the protocol described by Ojimi and Hidaka (2010).

Sequencing of the subtelomeric region and design of chromosome-specific primers

The flanking regions of telomeres were

amplified using the dual-suppression PCR method (Lian et al. 2006). *Eco*RV-digested DNA was ligated to an adaptor, and the PCR amplified using the adaptor primer, AP2, and either the telorette 42 or tel32 primer (Table 1). The presence of an amine residue at the 3'-end of the shorter adaptor strand blocks polymerase-catalyzed extension of the shorter strand. Flanking regions of telomeres were amplified using a previously described protocol (Ojimi and Hidaka 2010).

Based on three cloned sequences containing TTAGGG repeats at one end, a chromosome-specific primer (Ce1) located in the subtelomeric region was designed with PRIMER 3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). The primer sequence is shown in table 1.

Modified STELA

To estimate the length of a telomere of a specific chromosome, we used a modified version of the STELA described by Ojimi and Hidaka (2010). An oligonucleotide called telorette1 (Table 1), which comprises seven bases (5'-CCCTAAC-3') complementary to the telomere motif, TTAGGG, and a non-complementary tag region (teltail), was annealed to a single-stranded region (G-tail or 3'-overhang) of the telomere. The telorette linker was then ligated to the 5'-end of the complementary C-rich strand of telomeric DNA at 16°C overnight in a 40 μl volume containing 50 ng of genomic DNA, 5 μM telorette linker, and 350 U of T4 DNA ligase (TaKaRa Bio, Ohtsu, Japan) in 1 × manufacturer's ligation buffer. We carried out triplicate PCRs for each DNA sample in a 10 μl volume containing 125 pg of telorette-ligated DNA, 0.5 μM telomere-adjacent primer (Ce1) and a teltail primer (Table 1), 2.5 mM $MgCl_2$, 0.4 mM dNTPs, 10 × *LA Taq*™ reaction buffer (TaKaRa Bio), and 0.5 U *LA Taq*™ polymerase (TaKaRa Bio). The PCRs were performed under the following conditions: 94°C for 1 min, followed by 27 cycles of 10 s at 98°C and 10 min at 68°C, and then kept for 10 min at 72°C. DNA fragments were resolved by 0.8% Tris-acetate-EDTA (TAE) agarose gel electrophoresis. Three negative controls were performed: one containing the telorette but without ligase, one containing ligase but without the telorette, and one lacking both constituents.

STELA with Southern hybridization

STELA products were detected using Southern blot hybridization with a telomere probe, $(TTAGGG)_{10}$. DNA extracted from somatic tissues of 6 individuals (255-5000 g) and sperm from three other individuals (541-2115 g) were used. The PCR products were separated on a 0.8% TAE agarose gel, and then transferred to a Hybond-N+ membrane (GE Healthcare, Backinghamshire, UK). Labeling of the probe, and hybridization and detection of the signal were done using the AlkPhos Direct Labeling and Detection System (GE Healthcare) following the protocols provided. Signals were detected under a luminescence imager (Light-Capture AE-6981FC, ATTO, Tokyo, Japan). Sizes of the STELA products were calculated from the positions of band peaks using CS Analyzer vers. 3.0 (ATTO). In this study, sizes of the STELA products were averaged, and variations in the intensities of the PCR bands were not considered.

RESULTS

We designed a chromosome-specific primer, Ce1, for the STELA based on the subtelomeric sequence of a chromosome (Table 1). PCRs using the primer and a 'teltail' primer produced DNA fragments, which showed a smear with occasional bands on the membrane following Southern hybridization with the telomere probe

Table 1. List of primers

Primer name	Sequence (5' - 3')	Reference	Accessions No.
Telorette42	TGCTCCGTGCATCTGGCATCCCCTAACCCTAACCCTAACCCT	Ojimi and Hidaka (2010)	
Tel32	CCCTAACCCTAACCCTAACCCTAACCCTAACC	Ojimi and Hidaka (2010)	
Ce1	ACAAACGTGCATTTGTTCA	This study	AB561863
Telorette1	TGCTCCGTCGATCTGGCATCCCCTAAC	Baird et al. (2003)	
Teltail	TGCTCCGTGCATCTGGCATC	Baird et al. (2003)	
AP2	CTATAGGGCACGCGTGGT	Lain et al. (2006)	

(Fig. 1). STELA products were not generated in the negative controls, in which either ligase, the telorette linker, or both were omitted.

After transforming distance data to fragment size (bp) data using the λ*Hind*III-digested fragments (molecular markers) as a standard, the mean size of the STELA products, which indicates the average length of the telomere, was calculated. Mean sizes of STELA products were averaged for the replicated PCRs, and all fragments and average values were used to examine the relationship between telomere lengths and weights of *C. echinata* individuals. There was no significant correlation between the average telomere length of somatic tissues and the weight of individuals (Steel Dwass, $p > 0.05$, Fig. 2).

The telomere length of sperm was estimated using sperm DNA from 3 individuals. The average telomere length of sperm was significantly longer than that of somatic tissues (Welch *t*-test, $p < 0.01$, Fig. 2).

DISCUSSION

By designing a primer based on the nucleotide sequence of the flanking region of a telomere, we were able to apply the STELA to a non-model organism, the solitary coral *Ctenactis echinata*. For the STELA, we amplified a double-stranded region of the telomere together with a part of the subtelomeric region, using a chromosome-specific forward primer and a 'teltail' primer. Thus, sizes of the STELA products offered a good estimate of the length of the specific telomere (Baird et al. 2003). Electrophoretic patterns of STELA products varied among replicated PCRs with the same DNA sample, as reported by Baird et al. (2003) and Kimura et al. (2007). In this study, telomere lengths obtained from triplicate PCRs were averaged.

Two species of dinoflagellates and *Symbiodinium* sp. were reported to have the *Arabidopsis* consensus telomeric sequence $(TTTAGGG)_n$ (Alverca et al. 2007, Zielke and Bodnar 2010). Thus, the 3'-end nucleotide of the telorette cannot be annealed to the G-rich strand of *Symbiodinium* telomeres, making the ligation of the telorette to the 5'-end of the C-rich strand difficult. This strongly suggests that STELA products were derived from the coral and not from its algal symbionts.

No STELA product was generated in the negative controls that omitted ligase, the telorette, or both. Thus, ligation of the telorette to the 5'-end of the C-rich strand of the telomere is necessary in order to obtain STELA products. This demonstrates that the STELA products obtained represented the double-stranded region of the telomere rather than telomere-like sequences interspersed within the chromosomes (Nomoto et al. 2001, Nanda et al. 2002). At present, the possibility cannot be excluded that the STELA products in this study contained telomeres of more than one chromosome, since we do not know

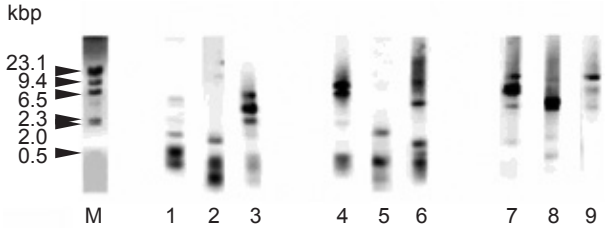

Fig. 1. Southern hybridization images of STELA products obtained from somatic tissues and sperm of *C. echinata*. Results of triplicate PCRs for somatic tissues from 2 individuals (lanes 1-3, 255 g skeletal weight; lane 4-6, 5000 g), and sperm from another individual (lanes 7-9, 2115 g) are shown. M, λ*Hind*III molecular marker.

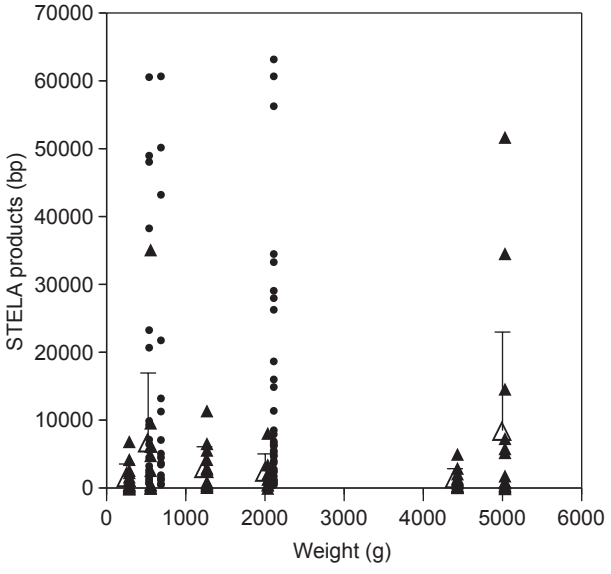

Fig. 2. STELA products of the solitary coral *C. echinata* of various sizes using Southern hybridization with somatic tissues and sperm. Closed triangles represent band patterns detected by Southern hybridization with tissue, and open triangles represent average fragment size (mean ± S.E.). Closed circles represent band patterns detected by Southern hybridization with sperm.

whether every subtelomeric region of the coral possesses a unique DNA sequence.

Our objective was to compare telomere lengths of specific chromosomes among individuals of *C. echinata* of various weights. Since the coral is solitary and has a rigid skeleton, the weight of the individual is expected to reflect its age. If the telomere length indeed shortens as corals age, we expected to find a negative correlation between the telomere length and the weight of individuals. However, there was no significant correlation between the average length of the STELA products and the weight of *C. echinata* individuals. This strongly suggests that telomere length is maintained throughout the polyp stage (255-5000 g). An alternative possibility is that the rate of shortening of telomeres with age is low, and the size range of very young *C. echinata* individuals used in this study was not large enough (i.e., no individuals of < 255 g were tested) to detect potential significant shortening in the telomeres. Another possibility that could mask the results is the possible occurrence of asexual reproduction, as reported for some other fungiid species (Wells 1966, Yamashiro and Nishihira 1998, Colley et al. 2000). However, there is no report of budding *C. echinata*, nor have we observed any cases of budding in this species during our field studies. By combining the modified STELA with another telomere length estimation (e.g., telomere restriction fragment (TRF)), the reliability and sensitivity of the coral age estimation might be improved.

The coral sperm revealed a longer telomere length than that of somatic tissues. This strongly suggests that telomere shortening occurs during early development, and that the telomere length of germ-line cells is maintained or restored during germ-cell maturation. The maximum observed telomere length in human sperm is also longer than that of other cell types such as leucocytes and fibroblasts (Nakagawa et al. 2004).

Telomere length rates of change (TROCs) differ among species and tissues (Nakagawa et al. 2004). Different TROC values were reported: of around -33 bp/yr for human leucocytes (Hastie et al. 1990), -515 bp/yr for zebra finch (*Taeniopygia guttata*), and -391 bp/yr for the tree swallows (*Tachycineta bicolor*; Haussmann et al. 2003). Adult colonies of scleractinian corals were reported to display telomerase activity (Zielk and Bodnar 2010), and there were no seasonal differences (Nakamichi et al. in press). *Cassiopea* jellyfish (Scyphozoa) exhibit telomerase activity throughout the polyp and medusa stages of their life cycle (Ojimi et al. 2009). If somatic tissues of most cnidarians display telomerase activity, the TROC might be affected by enzyme activity. Further studies are necessary in order to fully reveal the relationship between telomerase activity and TROC in corals.

This is the 1st report to measure telomere length of a solitary coral using the modified STELA. Although we did not find a significant decrease in telomere length as the coral weight increased, there was a significant difference in telomere lengths between sperm and somatic tissues. Findings from this study emphasize the need for molecular approaches when estimating coral age using somatic tissues.

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REFERENCES

Alverca EA, A Cuadrado, N Houve, S Franca, S Moreno Diaz de la Espina. 2007. Telomeric DNA localization on dinoflagellate chromosomes: structural and evolutionary implications. Cytogenet. Genome Res. **116:** 224-231.

Babcock RC. 1991. Comparative demography of three species of scleractinian corals using age-and size-dependent classifications. Ecol. Monogr. **61:** 225-244.

Baird DM, J Rowson, D Wynford-Thomas, D Kipling. 2003. Extensive allelic variation and ultrashort telomeres in senescent human cells. Nat. Genet. **33:** 203-207.

Blackburn EH. 1984. The molecular structure of centromeres and telomeres. Annu. Rev. Biochem. **53:** 163-194.

Colley SB, JS Feingold, J Pefia, PW Glynn. 2000. Reproductive ecology of *Diaseries distorta* (Michelin) (Fungiidae) in the Galapagos Islands, Ecuador. *In* MK Moosa, S Soemodihardjo, A Soegiarto, K Romimohtarto, A Nontji, Soekarno, Suharsono, eds. Proc. 9th Int. Coral Reef Symp. **1:** 373-381.

Hastie ND, M Dempster, MG Dunlop, AM Thompson, DK Green, RC Allshire. 1990. Telomere reduction in human colorectal carcinoma and with ageing. Nature **346:** 866-868.

Haussmann MF, DW Winkler, KM O'Reilly, CE Huntington, ICT Nisbet, CM Vieck. 2003. Telomeres shorten more slowly in long-lived birds and mammals than in shirt-lived ones. Proc. R. Soc. Lond. B Biol. Sci. **270:** 1382-1392.

Highsmith RC. 1982. Reproduction by fragmentation in corals. Mar. Ecol. Prog. Ser. **7:** 207-226.

Hughes TP, JH Connell. 1987. Population dynamics based on size or age? A reef-coral analysis. Am. Nat. **129:** 818-829.

Hughes TP, JBC Jackson. 1980. Do corals lie about their age? Some demographic consequences of partial mortality, fission, and fusion. Science **209:** 713-715.

Karmarsky-Winter E, M Fine, Y Loya. 1997. Coral polyp expulsion. Nature **387:** 137.

Kimura M, M Barbieri, JP Gardner, J Skurnick, X Cao, N van Riel et al. 2007. Leukocytes of exceptionally old persons display ultra-short telomeres. Am. J. Physiol.-Reg. I **293:** 2210-2217.

Krupp EA, PL Jokiel, TS Chartrand. 1992. Asexual reproduction by the solitary scleractinian coral *Fungia scutaria* on dead parent coralla in Kaneohe Bay, Oahu, Hawaiian Islands. Proc. 7th Int. Coral Reef Symp. **1:** 527-534.

LeBel C, JR Wellinger. 2005. Telomeres: What's new at your end? J. Cell Sci. **118:** 2785-2788.

Lian CL, M Abdul Wadud, Q Geng, K Shimatani, T Hogetsu. 2006. An improved technique for isolating codominant compound microsatellite markers. J. Plant Res. **119:** 415-417.

Loya Y, K Sakai. 2008. Bidirectional sex change in mushroom stony corals. Proc. R. Soc. Lond. B Biol. Sci. **275:** 2335-2343.

Nakagawa S, NJ Gemmell, T Burke. 2004. Measuring vertebrate telomeres: applications and limitations. Mol. Ecol. **13:** 2523-2533.

Nakamichi H, MC Ojimi, N Isomura, M Hidaka. 2012. Somatic tissues of the coral *Galaxea fascicularis* possess telomerase activity. Galaxea JCRS **14:** 53-59.

Nanda I, D Schrama, W Feichtinger, T Haaf, M Schartl, M Schmid. 2002. Distribution of telomeric (TTAGGG)n sequences in avian chromosomes. Chromosoma **111:** 215-227.

Nomoto Y, M Hirai, R Ueshima. 2001. Cloning of molluscan telomere DNA with (TTAGGG)n repeat and its chromosomal location in the freshwater snail *Biwamelania habei*. Zool. Sci. **18:** 417-422.

Ojimi MC, M Hidaka. 2010. Comparison of telomere length among different life cycle stages of the jellyfish *Cassiopea andromeda*. Mar. Biol. **157:** 2279-2287.

Ojimi MC, N Isomura, M Hidaka. 2009. Telomerase activity is not related to life history stage in the jellyfish *Cassiopea* sp. Comp. Biochem. Physiol. Part A **152:** 240-244.

Rinkevich B, Y Loya. 1986. Senescence and dying signals in a reef building coral. Experientia **43:** 320-322.

Sammarco PW. 1982. Polyp bail-out: an escape response to environmental stress and a new means of reproduction in corals. Mar. Ecol. Progr. Ser. **10:** 57-65.

Shawi M, C Autexier. 2008. Telomerase, senescence and ageing. Mech. Ageing Dev. **129:** 3-10.

Smith LD, TP Hughes. 1999. An experimental assessment of survival, re-attachment and fecundity of coral fragments. J. Exp. Mar. Biol. Ecol. **235:** 147-164.

Stoddart JA. 1983. Asexual production of planulae in the coral *Pocillopora damicornis*. Mar. Biol. **76:** 279-284.

Urquidi V, D Tarin, S Goodison. 2000. Role of telomerase in cell senescence and oncogenesis. Annu. Rev. Med. **51:** 65-79.

Vizel M, Y Loya, C Downs, E Kramarsky-Winter. 2011. A novel method for coral explant culture and micropropagation. Mar. Biotechnol. **13:** 423-432.

Wells JW. 1966. Evolutionary development in the scleractinian family Fungiidae. Symp. Zool. Soc. Lond. **16:** 223-246.

Yamashiro H, M Nishihira. 1998. Experimental study of growth and asexual reproduction in *Diaseris distorta* (Michelin, 1843), a free-living fungiid coral. J. Exp. Mar. Biol. Ecol. **225:** 253-267.

Zielke S, A Bodnar. 2010. Telomeres and telomerase activity in scleractinian corals and *Symbiodinium* spp. Biol. Bull. **218:** 113-121.