Published in final edited form as:

Comp Biochem Physiol C Toxicol Pharmacol. 2012 January ; 155(1): 89–94. doi:10.1016/j.cbpc.2011.05.005.

Characterization of telomeres and telomerase expression in *Xiphophorus*[☆]

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Abstract

Research investigating telomere lengths and telomerase expression in vertebrates has progressively become important due to the association of these two biological endpoints with cellular aging and cancer in humans. Studies that rely upon the traditional use of laboratory mice have been faced with limitations largely due to inbred mice possessing large telomeres and ubiquitous expression of telomerase. Recently, a number of small fish species have been shown to provide potentially informative models for examining the role of telomeres and telomerase within intact vertebrate animals. *Xiphophorus* fishes represent a new world live-bearing genus that has not previously been assessed for telomere length or telomerase expression. To add to the knowledge base of telomere and telomerase biology in vertebrates we assessed telomere length and telomerase expression among several species of *Xiphophorus*. The telomere lengths in several organs (gill, brain, eyes, testis, ovary and liver) in three species (*X. hellerii*, *X. maculatus*, *X. couchianus*) and also in F₁ interspecies hybrids were approximately 2–6 kb. This size was consistent within the same organs of the same species, as well as between species and F₁ hybrids. Despite possessing relatively short telomere lengths compared to humans, the consistency of size among *Xiphophorus* species and organs may allow experimental detection of telomere shortening.

The relative expression of telomerase reverse transcriptase (TERT) was determined by quantitative real-time PCR. Expression levels of TERT was measured in seven organs (ovary, testis, liver, gill, brain, heart, skin) from *X. maculatus*, *X. hellerii* and in control and ultraviolet light (UVB) exposed skin samples from *X. maculatus*, *X. hellerii*, and F₁ interspecies hybrids. TERT gene expression was significantly higher in ovary and testis, while all other organs showed low relative TERT expression. Detectable increases in TERT expression were found in skin samples upon UVB exposure. Our findings suggest *Xiphophorus* may serve as a suitable model for future studies investigating the association of telomere length and telomerase expression in regard to aging and disease.

[☆]This paper is based on a presentation given at the *5th Aquatic Annual Models of Human Disease* conference: hosted by Oregon State University and Texas State University-San Marcos, and convened at Corvallis, OR, USA September 20–22, 2010.

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Keywords

Xiphophorus; telomerase; telomerase reverse transcriptase (TERT); induced gene expression; ultraviolet light (UVB); quantitative real time PCR

1. Introduction

Telomeres are regions of repetitive nucleotide structures located on the ends of chromosomes that, along with various associated proteins, function to protect and stabilize chromosomes ends (Blackburn 1991, Blackburn 2001). Most vertebrates possess telomeres made up of the tandem repeat TTAGGG that extends in the 5' to 3' direction toward the distal ends of each chromosome (Moyzis et al. 1988). The average number of telomeric repeat units in organisms can vary greatly. For example, human telomere lengths range from 8–12 kb, while some laboratory mice strains are close to 100 kb. Variations in telomere length have also been reported across tissues within the same organism and even within a single cell (De Lange et al. 1990). The shortening of telomeres is, in part, largely due to the inability of standard polymerases to fully replicate the ends of chromosomes, leading to a gradual loss of telomeric regions as the organism ages. Interestingly, certain cell types (i.e., germ line cells) are able to prevent telomere loss through the activation of an enzyme/RNA complex termed telomerase (Holt et al. 1999).

Telomerase synthesizes telomeric repeats at the ends of chromosomes using a catalytic protein subunit known as telomerase reverse transcriptase (TERT) and an integral RNA subunit as the template (Legassie et al. 2005). Although other proteins are involved in the function of the telomerase complex, these two essential components are conserved among all eukaryotes (Weinrich et al. 1997). In humans, it has been shown that telomeres shorten with age in somatic tissues due to a relative absence of telomerase activity, and that telomerase activity is limited to highly proliferative cell types, such as stem cells, germ cells, and tumor cells (Greider 1998). Interestingly, abnormally high expression of telomerase is present in a majority of cancer cell lines and associated with 80–90% of cancers (Stewart et al. 2006, Kim et al. 1994). To determine the mechanisms by which telomerase activation occurs during carcinogenesis, various studies investigating telomerase activity in skin cancer have been reported. In particular, several studies provided evidence that exposure to ultraviolet light (UV) induces telomerase expression in human skin, although the molecular mechanism by which this occurs is poorly understood (Taylor et al. 1996, Ueda et al. 1997). The human health conditions correlated with alterations in telomerase activity underscore the importance of developing vertebrate experimental models that may provide a more complete understanding of how this enzyme is regulated under various physiological conditions.

Limitations of *in vivo* studies associated with telomeres and telomerase expression has been largely due to lack of appropriate model organisms. To identify potential alternative vertebrate models, recent investigations have begun to examine the role of telomeres and telomerase in aquatic species. Studies with aquatic vertebrates such as zebrafish, flounder, pufferfish, and medaka have shown that telomerase expression is not limited to reproductive organs, but is ubiquitously expressed at high levels. Several aquatic species have also been found to possess telomere lengths that are similar to those in humans (McChesney et al. 2005). In addition, telomere shortening linked to aging appears to be present in fish such as the Japanese medaka (*Oryzias latipes*) and *Nothobranchius furzeri* despite ubiquitous telomerase expression (Hatakeyama et al. 2008, Hartman et al. 2009). The expression of telomerase in somatic tissues of fish is thought to mainly serve a role in tissue regeneration processes (Elmore et al. 2008, Lund et al. 2009).

An aquatic organism that has yet to be investigated in its potential to serve as a vertebrate model for studying telomeres and telomerase is *Xiphophorus*. *Xiphophorus* fishes comprise a freshwater genus composed of 27 species that are derived from Mexico, Guatemala, Belize, and Honduras. In the late 1920's it was recognized that various interspecies hybrids of *Xiphophorus* could be produced that were fertile. Subsequently, crossing of *X. maculatus* with the swordtail *X. hellerii* was shown to segregate backcross hybrid progeny (i.e., backcross to the *X. hellerii* parent) that developed melanoma according to Mendelian expectations (Gordon, 1927). In the years since these early experiments, *Xiphophorus* interspecies hybrids have developed into valuable experimental models to study the genetics underlying spontaneous and induced carcinogenesis (for review see; Walter and Kazianis, 2001).

Herein, we report telomere lengths determined by terminal restriction fragment (TRF) southern blot analysis across multiple organs in three different species of *Xiphophorus*, as well as F₁ interspecies hybrids. Telomere lengths were compared among the organs of each individual species and these data compared with telomere lengths between species. A portion of the TERT transcript and translated protein sequence was identified in *Xiphophorus* and compared to those previously found in other aquatic species including *Danio rerio*, *N. furzeri*, and *O. latipes*. Relative TERT expression was also determined by quantitative real-time PCR in multiple organs (testis, liver, gill, brain, heart, skin) of *X. maculatus* and *X. hellerii* as well as in UVB exposed skin from *X. maculatus*, *X. hellerii* and F₁ interspecies hybrids.

2. Materials and Methods

2.1. *Xiphophorus* strains and usage

Young adult *Xiphophorus* including *X. maculatus* Jp 163 A, *X. maculatus* Jp 163 B, *X. hellerii*, *X. couchianus*, and interspecies hybrids produced from crossing these parental lines were obtained from the *Xiphophorus* Genetic Stock Center (XGSC; Texas State University, San Marcos, TX, USA). All fish in the XGSC were maintained as pedigreed lines according to standard XGSC protocols (see www.xiphophorus.txstate.edu). The *X. maculatus* Jp 163 A and B lines, carrying the spot dorsal (*Sd*) and spot side (*Sp*) macromelanophore pigment patterns, were originally derived from a single female (*X. maculatus* Jp 163) collected from the wild. Upon establishment in the XGSC the *X. maculatus* Jp 163 line was split into Jp 163 A (*Sd*) and B (*Sp*) lines based on the pigment patterns and have been maintained as two independent pedigrees for over 100 generations (Kallman 2001, Kallman and Kazianis, 2006, Walter et al. 2006a,b). First generation hybrids (F₁) from three interspecies crosses were utilized in the studies presented. The interspecies crosses were as follows; (1) *X. maculatus* Jp 163 A (x) *X. couchianus* (herein designated, *Sd-Xc*), (2) *X. maculatus* Jp 163 A (x) *X. hellerii* (designated *Sd-Xh*), and (3) *X. maculatus* Jp 163 B (x) *X. hellerii* (designated *Sp-Xh*).

2.1. UVB Exposure

X. maculatus Sd, *X. maculatus Sp*, *X. hellerii*, and the F₁ interspecies hybrid (*Sp-Xh*) male fish were exposed to 300 J/m²/day (8:45 min. exposure time) for 5 days resulting in a total dose of 1500 J/m². The UVB exposure chamber allowed free-swimming fish to be irradiated concurrently on both sides within UV transparent chambers. UV exposure was performed using Phillips TL20W/01 RS UVB lamps filtered through Kodacel (K-6805). Fish were maintained in the dark 24 h before the first exposure and after each exposure. After the final UVB exposure the fish were dissected and select organs immediately flash frozen for RNA isolation.

2.3. Telomere length measurements

Genomic DNA was extracted from the liver, gill, brain, eyes, and testis of male and ovaries of female tissue from *X. maculatus Sd*, *X. hellerii*, *X. couchianus* and F₁ interspecies hybrids, *Sd-Xc* and *Sd-Xh*, using the Genomic-tip system (Qiagen). For each assay, 2 µg of genomic DNA was digested with restriction enzymes RsaI and HinfI for ~2 h at 37 °C. Digested DNA was resolved by electrophoresis (30 V) on 0.8% agarose gels using TAE buffer (40 mM Tris-acetate, pH 7.5, 1 mM EDTA). DNA fragments in the agarose gel were transferred by capillary transfer and UV cross-linked to a Hybond-N+ membrane. Immobilized DNA fragments were incubated with a digoxigenin (DIG)-labeled probe specific for telomeric repeats (Roche), washed, and then incubated with a DIG-specific antibody covalently coupled to alkaline phosphatase (Roche). A highly sensitive chemiluminescent substrate, CDP-Star (Roche), was used to visualize telomeres on these blots. The average terminal restriction fragment (TRF) length was determined by comparison of the intensity of telomere sample to a DIG-labeled molecular weight standard run on the same gel.

2.4. Retrieval of *Xiphophorus* telomerase (TERT) sequence

RNA-seq data derived from *X. maculatus* Jp 163 A testis and ovaries mRNA were assembled *de novo* (unpublished results) using the Velvet/Oases short read assembler package (Zirbino and Birney 2008). Raw data comprised ~42 million 60 nt read pairs sequenced on the Illumina GAII platform. The assembly contained 112,822 contigs with an N50 (length-weighted median) of 1,000 bp with an average length of 613.9 bp. The longest contig in the assembly was 15,566 bp. Using TERT mRNA sequences obtained from the public databases from *Oryzias latipes* (**DQ248968.1**), *Takifugu rubripes* (**AY861384**), and *Danio rerio* (**BC163300**) we performed a similarity search (Blast) of the *X. maculatus* assembly to identify a set of contigs with high similarity to the queries. This set of *Xiphophorus* TERT contigs were used to manually splice together a consensus draft of TERT mRNA.

2.5. RT-PCR and PCR amplification of TERT

Total RNA was extracted from each organ using TRIZOL (Invitrogen) and first strand cDNA was synthesized with Multiscribe Reverse Transcriptase (Applied Biosystems). Gene specific primers homologous to the medaka TERT sequence (NCBI, NM_001104816) were designed using the *X. maculatus* transcriptome based draft TERT sequence. Standard PCR and qRT-PCR were performed. The sequences of these primers are: TERT-F (5'-CTGGGACATGGCTCAGTATGC-3'; sense) and TERT-R (5'-AGCTCCACCGCCTCGTATTGC-3'; antisense).

PCR reactions were carried out at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 56 °C for 45 s, 72 °C for 45 s and a final extension step of 72 °C for 5 min. The PCR products were resolved in a 2.5% agarose gel with TAE buffer at 125 V. A 50 bp Ladder (Invitrogen) was used for determining product sizes. Visible amplification products were gel extracted and purified using the Wizard SV Gel and PCR Clean-Up System (Promega). Sequencing of the purified DNA was performed by Davis Sequencing (Davis, CA, USA).

2.6 Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was used to assess the relative gene expression levels of TERT within various tissues of *X. maculatus Sd* and *X. hellerii*. Primer sequences used in these reactions included gene specific primers TERT-F and TERT-R, 18S rRNA normalization primers (18S-F 5'-CGGAAAGGATTGACAGATTGA-3' and 18S-R 5'-CTCAATCTCGTGTGGCTGAA-3') and MAPK14A normalization primers (MAPK-F 5'-

CCTCTTGCTGTAGACTTGCTGG-3' and MAPK-R 5'-GCTCTTCCACTCCTCGATTTC-3'). Real-time PCR reactions were performed with a 7500 Fast Real-Time PCR System using the Fast SYBR Green Master Mix detection method (Applied Biosystems). For each assay a total of 100 ng cDNA was used in triplicate. To normalize target gene expression for differences in cDNA input, cDNA was diluted an additional 1/500 for measuring 18S rRNA levels. Diluted cDNAs (8 µL aliquots) were added to the wells of a 96-well plate with 10 µL of Fast SYBR Green Master Mix and 0.5 µM of each primer pair. For quantification of PCR results cycle threshold (C_T) values were determined for each reaction. A standard curve was constructed from data derived using a dilution series of ovary cDNA, a tissue expected to possess significant TERT expression. The standard curve allowed assessment of the relationship between the quantity of starting material and the C_T values as an indicator of the efficiency of TERT amplification. Relative TERT expression was determined using the comparative ΔC_T method by comparing the TERT C_T values for each tissue sample with the 18S or MAPK14A rRNA C_T values. For each assay, the tissue sample with the lowest normalized TERT expression values was chosen for calculating the fold change in expression.

3. Results

3.1 Telomere length analysis

The lengths of telomeres in six organs (gill, liver, eye, brain, testis, ovary) of three species of *Xiphophorus* (*X. maculatus* Jp Sd, *X. hellerii*, and *X. couchianus*), as well as eye and liver organs derived from two F_1 interspecies hybrids (*Sp-Xc* and *Sp-Xh*) were determined using terminal restriction fragment analysis (Fig. 1). Average telomere lengths were determined to be between 2 to 6 kb in each of the six organs from each *Xiphophorus* species examined (Table 1). Relatively low heterogeneity in telomere lengths was observed when comparing individual organs within each species (Table 1). Tissues from *X. hellerii* (e.g., eyes) possessed the smallest range of telomere lengths (3.5–5 kb) relative to those observed for *X. maculatus* Sd and *X. couchianus* (2.7–5 kb and 2.5–5 kb, respectively).

3.2 Comparison of *Xiphophorus* TERT transcript and protein

The *O. latipes* TERT sequence (ABB76648.1) was utilized to search (tBlastn) for the *Xiphophorus* TERT mRNA in the assembled contigs. Comparison of the translated *Xiphophorus* TERT protein sequence with other fish species; *E. coioides* (ABC47309.1, 78% identity), *O. latipes* (ABB76648.1, 71% identity), *T. rubripes* (AAX59693.1, 72% identity), *N. furzeri* (ACN38321.1, 68% identity), *D. rerio* (AAI63300.1, 49% identity) and *O. melastigma* (ABB92622.1, 63% identity) revealed $\geq 49\%$ conservation at the protein level. Conserved TERT motifs were identified in *Xiphophorus* by multiple sequence alignments with other fish TERTs and relatively high ($>50\%$) sequence identities were revealed (Fig. 2).

3.3 Telomerase expression in *Xiphophorus*

Telomerase gene expression was assessed by PCR and qRT-PCR of RNA samples isolated from multiple organs (brain, liver, skin, gill, heart, testis, ovary) of adult *X. maculatus* and *X. hellerii* as well as skin samples from *X. maculatus* (Sd and Sp), *X. hellerii* and F_1 interspecies hybrids (*Sp-Xh*). TERT PCR products were only observed in the testis and ovaries samples (results not shown). qRT-PCR expression analysis revealed significant levels (≥ 85 -fold normalized to liver) of TERT in both testis and ovaries for *X. maculatus* Sd and *X. hellerii* (Fig. 3, Table 2). In contrast, very low levels of detectable gene expression ($< 3\%$ relative to ovaries) were observed in the RNA derived from liver, brain, gills, and skin organs for either species. Surprisingly, *X. maculatus* Sd heart tissue appeared to have high gene expression compared to the other organs but was still very low compared to expression

in testis and ovaries (8.7% relative to ovaries). Expression of *X. maculatus Sd* TERT in multiple organs (brain, skin, gill, liver, heart) was also compared to the expression of another gene, mitogen-activated protein kinase 14a (MAP14KA). Previous work in our laboratory had determined the MAP14KA transcript to be ubiquitously expressed at relatively high and balanced levels in most all *Xiphophorus* tissues (unpublished observations) and so we used expression of MAP14KA as a second gene target to ensure our reactions were valid. Normalization of the two genes (TERT and MAP14KA) to 18S rRNA levels revealed significantly lower expression of telomerase compared to MAPK14A in most tissues. An average difference in expression between TERT and MAPK14A was determined at about 30 fold for expression in liver, brain, skin, and heart of *X. maculatus Sd*. However, the expression of MAPK14A and TERT in testis and ovaries were determined to be about equal. Comparison of relative telomerase and MAPK14A expression supports the observation that TERT is expressed at very low levels in most *X. maculatus* organs.

Detectable increases in relative TERT expression was observed in RNA isolated from skin of fish exposed to UVB radiation (Fig. 4). As shown in Table 3, the largest induction of TERT expression in UVB exposed skin was observed in *X. maculatus Sp* (7.2 ± 1.0 fold) while significant but smaller changes were found for *X. maculatus Sd* (5.0 ± 0.3 fold), *X. hellerii* (2.7 ± 0.3 fold), and the *Sp-Xh* interspecies hybrid between these two parental lines (4.2 ± 0.9 fold).

4. Discussion

The association of telomere shortening in human aging and disease has led to an interest in developing translational models for studying telomere molecular biology. Limitations of *in vitro* culture systems and laboratory mice strains are implied by their extremely large telomeres lengths compared to human telomeres. This has led investigators to search for alternative models among aquatic species (McChesney et al. 2005). Several aquatic species including *D. rerio*, *O. latipes*, *O. melastigma*, and *N. furzeri* (Au et al. 2008, Hartmann et al. 2009) have been shown to possess telomere lengths similar to human lengths.

In this report we show results indicating telomere length in various organs and species of *Xiphophorus* average about 2 to 6 kb. Compared to other fish species (e.g., *O. latipes* and *D. rerio*) the average telomere lengths observed in *Xiphophorus* were more homogenous across organs and between species. For example, similar telomere lengths were observed for liver in *X. maculatus Sd* (2.5 to 5.5 kb), *X. hellerii* (3.5 to 6.0 kb), *X. couchianus* (2.5 to 5.5 kb), and for two F₁ interspecies hybrids (Fig. 1, Table 1). This is in contrast to previous reports showing telomere lengths to be highly heterogeneous in the liver of two medaka species *O. latipes* (6–12 kb) and *O. melastigma* (0.5–12 kb; Au et al. 2009). Telomere lengths observed in different tissues of zebrafish have also been found to possess highly variable lengths as well (McChesney et al. 2005). The consistency of telomere length in *Xiphophorus* may be due the pedigree breeding program maintained at the XGSC (Walter et al. 2006a). The *X. maculatus* and *X. couchianus* lines are highly inbred, over 100 and 76 generations, respectively, while the *X. hellerii* line used in these studies (i.e., Sarabia) has been maintained in the XGSC since 1963 (Walter et al. 2006b). The relatively constant telomere lengths found in *Xiphophorus* suggests these fish may be a good model to assess the effects of age or environmental perturbation (e.g., oxidative stress) on telomere length.

The expression of telomerase in humans is largely restricted to germ line cells and stem cells, and tends toward a gradual telomere shortening in somatic tissues. The activation of telomerase has been found associated with 85% of cancers and up to 99% of tumor derived cell lines, although the exact mechanism by which telomerase becomes activated is not well understood. In contrast to absence of telomerase expression in most human tissues,

ubiquitous telomerase expression has been reported in many tissues of various fish species (McChesney et al. 2005). Specifically, ubiquitous expression of the catalytically active portion of telomerase (TERT) has been detected in all tissues of zebrafish, medaka, and *N. furzeri*. The ability of zebrafish to regenerate damaged tissues at an exceedingly high rate has been suggested as an explanation for the relatively high expression of telomerase. Relatively high telomerase activity has also been observed in the liver, kidney, intestine, muscle, gonad, heart, brain, spleen, and gill of medaka irrespective of age (Pfennig et al. 2008). Furthermore, telomerase activity in the majority of medaka samples examined was 2 to 4 times higher than that of a human cervical cancer cell line (Siha cells; Hatakeyama et al. 2008). Telomerase is highly expressed in the testes and ovaries of *X. maculatus Sd* and *X. hellerii* while other tissues including skin, liver, brain, and gill exhibited very low levels of expression. Although studies in other fish species have indicated that telomerase expression is normally higher in testes and ovaries compared to other tissues, we observed a more drastic difference of expression ratio between gonads and somatic organs for *Xiphophorus*. Even when TERT expression is normalized to heart, the somatic tissue that displayed the highest levels of TERT expression in *X. maculatus Sd*, the relative change in TERT expression for testis and ovaries is 84 and 192 fold, respectively. This pattern more resembles mammalian TERT expression than that of other fishes.

The effects of UVB exposure on TERT expression was investigated in skin samples obtained from *X. maculatus (Sd and Sp)*, *X. hellerii*, and an F₁ interspecies hybrid (*Sp-Xh*). We observed UVB induction of TERT expression in skin samples after 5 days of UVB exposure. The UVB exposure protocol utilized mirrored that often employed in UV induced melanoma studies (Nairn et al., 2001, Mitchell et al., 2007). The levels of TERT induction by UVB ranged from 5 to 7 fold in skin samples from *X. maculatus Sd* and *Sp*, respectively. A ~3 fold UVB induction was observed for *X. hellerii* TERT. The F₁ interspecies hybrid (*Sp-Xh*) exhibited an intermediate response (~4 fold) between the two parental phenotypes for UVB induced TERT expression. In previous DNA repair studies it was that F₁ interspecies hybrids may exhibit the repair phenotype of one parent, or the other, and in some cases exhibit DNA repair capability much different than either parent. Further, these DNA repair phenotypes were shown to be different and variable between organs in the same fish (David et al., 2004). UV exposure studies in the hairless mouse model showed significant increases in telomerase activity (12 fold) occurred 4 weeks after the UVB exposure (Balasubramanian et al. 1999). In humans, evidence of increased telomerase activity has also been found in malignant skin conditions and areas of skin exposed to higher levels of sunlight (Ueda et al. 1997). The mechanisms of UV induction of telomerase and what affects this may have on cellular damage, repair, and downstream induction of carcinogenesis should be further explored in future work. *Xiphophorus* offers several UVB induced cancer models that may be informative for such studies (Mitchell et al. 2007, Nairn et al. 2001). Thus, *Xiphophorus* appears to be a unique and applicative model when compared to other aquatic species for the functional study of telomeres and telomerase.

Acknowledgments

The authors would like to thank the other employees of the *Xiphophorus* Genetic Stock Center, Texas State University, for maintaining the fish lines. The work was supported from an NIH, National Center for Research Resources grant award R24-RR024790 and support from Texas State University

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Figure 1.

Telomere length assay in *Xiphophorus* species. Telomere length representation of *X. hellerii* (A:1,5 B:3,6), *X. maculatus* (A:3,7 B:1,4), *X. couchianus* (B:2,5), F₁ interspecies hybrids *Sp-Xc* (A:2,6) and *Sp-Xh* (A:4,8) by terminal restriction fragment (TRF) Southern blot analysis.

	T motif	motif 1
O_latipes	WLLDGFVVGVLVRACFYATESVGQKNAIRFYRQEVWSKLQDLAFRRHIA	AQVASLPKGTVI
E_coioides	WLLDGYVVS LVRACFYVTESVGQKNAVRFYRQEVWAKLQDLAFRGHLS	AQVASLPKTTVI
X_maculatus	WLLDGFVVGVLVRACFYATESVGQKNAIRFYRQEVWTKLQDLAFKGHLS	AQAASLPKGTVI
N_furzeri	WLLDGYVVGVLVRACFYATEGMRQKCCIRFYRREVWAKLQDLAFKDYLE	AEVASFPKDTV
T_rubripes	WLLDGFVVGVLVRACFYATESMGQKNAIRFYRQEVWAKLQDLAFRSHIS	KQVAALPKSTII
D_rerio	WMLSGFVVGVLVRAQFYITESMGHKHTLRFYRGDVWSRLQDQAFRAHLP	SQALKVPNSAVT
	:.:*.**** ** *: : * :**** :*::** ** : :. :. .*: :	
	motif 2	A motif
O_latipes	SQLRFIPKTDG MRPITRVIGADSNTLRHKKRIR	KPQPLYFVKVDVSGAYDSLPHDKLKEV
E_coioides	SRLRFIPKTDG MRPITRVVGADAKTRVYRGRVR	KPQPLYFVKVDVSGAYESLPHDKLIEV
X_maculatus	SRLRFIPKSDG MRPITRVIGTDPKTRLFQARVR	RPQPLYFVKVDVSGAYESLPHDKLIEV
N_furzeri	SRLRFIPKTDG MRPITRFIGRDTKTCLYQAKAR	KPQHLYFVKVDVSSAYESLPHDKLMEV
T_rubripes	SRLRFIPKTDG MRPITRVIGADAKTRLYQSHVR	KPQPLYFVKMDVSGAYESLPHNKLIEV
D_rerio	SRIRFIPKTSS MRPITRLSGSRDTLQYFQSCVR	SPRPLYFVKVDVSGAYDSLPHLKLVEV
	*:*****:.. *****. * . : :. * *: *****:***.***:***** ** **	
	B motif	C motif
O_latipes	ITEALSP KTYRQCRGIPQGSVVSLLCCLCYGHMENVLFRD	LMRLVDDFLLITPDRNQ
E_coioides	IGQALSP KTYRQCRGIPQGSVVSLLCCLCYGHMENVLFKD	LMRLVDDFLLITPDLQE
X_maculatus	IGQALCP KTYHQCRGIPQGSVVSLLCCLCYGHMENVLFKD	LMRLVDDFLLITPDQHQ
N_furzeri	IGQVLSP KFYRRCRGIPQGSIMSSLLCCLCYGHMERVLFT	LMRLVDDFLLITPDQRQ
T_rubripes	INQVLTP KTYRQCQGVPOGSVSVLCCCLCYGHMENVLFKD	LMRLVDDFLLITPNLHD
D_rerio	LKEVLGP QMFRQVCIGIPQGSVVSLLCNLCYGHMEKALLKD	LMRLIDDFLLITPHLSK
	: :.* * : :. : * :***** :*.:** *****.***: : *****:*****. .	
	D motif	E motif
O_latipes	AQSFLSILLAGVPQYGVVANPQKV V	HCLFPWCGLLL
E_coioides	AQSFLRILLAGVPQYGLVVNPQKVAV	HCLFPWCGLLL
X_maculatus	AQTFLKTLLAGVPQYGLMVNPQKVAV	HSLFPWCGLLL
N_furzeri	AHTFLKTLLAGVPQYGLVVNPQKV V	HCLFPWCGLLL
T_rubripes	AQTFLKILLAGVPQYGLVVNPQKV V	NCLFPWCGLLL
D_rerio	ATEFLTTLLSGVPDYGCQINPQKVAV	NCLFPWCGLMI
	* ** *:***:** *****.* :.*****: :	

Figure 2.

Telomerase reverse transcriptase motif alignments across fish species. Alignment includes TERT amino acid motifs from *X. maculatus* (translated), *O. latipes* ([ABB76648.1](#)), *E. coioides* ([ABC47309.1](#)), *D. rerio* ([AAI63300.1](#)), *T. rubripes* ([AAX59693.1](#)), and *N. furzeri* ([ACN38321.1](#)).

Figure 3. Relative gene expression of TERT in multiple tissues of adult *X. maculatus Sd* (A) and *X. hellerii* (B) determined by qRT-PCR. Expression values of TERT were normalized against 18s rRNA expression and relative fold changes $\pm$ SEM (n=3) were calculated with respect to expression in liver.

Figure 4.

Relative expression of TERT in UVB exposed skin samples from *X. hellerii* (XH), *X. maculatus* Sd (XM163A), *X. maculatus* Sp (XM163B), and an interspecies hybrid made by crossing parental *X. maculatus* Sp and *X. hellerii* (Sp-Xh). Expression values of TERT were normalized against 18s rRNA expression levels. Relative fold changes $\pm$ SEM (n=3) for UV treated samples were calculated by normalization to un-exposed skin expression for each species.

Table 1

Telomere lengths in *X. hellerii*, *X. maculatus* *Sd*, and *X. couchianus* tissues determined by terminal restriction fragment Southern blot analysis.

Tissue	<i>X. maculatus</i> (kb)	<i>X. couchianus</i> (kb)	<i>X. hellerii</i> (kb)
Gill	2–5	2.5–6	3–6
Liver	2–5	2–5.5	3.5–6
Brain	2–5	2–5	2.7–5
Eyes	2.7–5	2.5–5	3.5–5
Testis	2–5.5	2–5	2.5–5
Ovary	2–5	2–5	2.7–5

Table 2

Relative gene expression of TERT in *X. maculatus* Sd and *X. hellerii* tissues. Values were determined by qRT-PCR repeated with three technical repeats and two biological repeats. ND = not determined

Tissue	<i>X. hellerii</i> fold change	<i>X. maculatus</i> fold change
Ovary	1000.0 $\pm$ 13.3	196.0 $\pm$ 5.8
Testis	162.0 $\pm$ 9.4	85.0 $\pm$ 4.3
Liver	1.0	1.0
Gill	20.0 $\pm$ 2.0	4.0 $\pm$ 0.4
Brain	15.0 $\pm$ 1.4	1.0 $\pm$ 0.1
Skin	22.0 $\pm$ 0.5	6.0 $\pm$ 1.1
Heart	ND	17.0 $\pm$ 0.1

Table 3

Relative gene expression of TERT in untreated and UVB treated skin from *X. hellerii*, *X. maculatus Sd*, *X. maculatus Sp*, and an interspecies hybrid made by crossing parental *X. maculatus Sp* and *X. helleri* (*Sp-Xh*). Values were determined by qRT-PCR repeated with three technical repeats and two biological repeats.

Species	normal skin	UV treated skin
<i>X. hellerii</i>	1.0	2.7 ± 0.3
<i>X. maculatus Sd</i>	1.0	5.0 ± 0.3
<i>X. maculatus Sp</i>	1.0	7.2 ± 1.0
<i>Sp-Xh</i>	1.0	4.2 ± 0.9