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Brief Communication

No Interstitial Telomeres on Autosomes but Remarkable Amplification of Telomeric Repeats on the W Sex Chromosome in the Sand Lizard (*Lacerta agilis*)

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Abstract

Telomeres are repeat $(TTAGGG)_n$ sequences that form terminal ends of chromosomes and have several functions, such as protecting the coding DNA from erosion at mitosis. Due to chromosomal rearrangements through evolutionary history (e.g., inversions and fusions), telomeric sequences are also found between the centromere and the terminal ends (i.e., at interstitial telomeric sites, ITSs). ITS telomere sequences have been implicated in heritable disease caused by genomic instability of ITS polymorphic variants, both with respect to copy number and sequence. In the sand lizard (*Lacerta agilis*), we have shown that telomere length is predictive of lifetime fitness in females but not males. To assess whether this sex specific fitness effect could be traced to ITSs differences, we mapped $(TTAGGG)_n$ sequences using fluorescence in situ hybridization in fibroblast cells cultured from 4 specimens of known sex. No ITSs could be found on autosomes in either sex. However, females have heterogametic sex chromosomes in sand lizards (ZW, $2n = 38$) and the female W chromosome showed degeneration and remarkable $(TTAGGG)_n$ amplification, which was absent in the Z chromosomes. This work warrants further research on sex chromosome content, in particular of the degenerate W chromosome, and links to female fitness in sand lizards.

Subject area: Molecular adaptation and selection
Key words: interstitial telomeres, *Lacerta agilis*, sand lizard, telomeric repeats

Telomere sequences $(TTAGGG)_n$ form terminal ends of chromosomes and have several functions, such as protecting the coding DNA from erosion at mitosis and contribute to correct identification of double strand DNA repair sites. In many taxa, there is a negative correlation between age, telomere length, and the capacity of telomeres to perform these vital functions, and telomeres have therefore been implicated as strong candidates for dictating longevity and, consequently, life-history evolution (Monaghan and Haussmann 2006).

In recent years, the research community has seen a monumental increase in the research output on the roles telomeres play in a broad range of biomedical and biological situations, from cancer and disease research (Lin and Yan 2008), oxidative stress biochemistry (Von Zglinicki 2002), to life-history evolution (Monaghan and Haussmann 2006). Not the least in evolutionary ecology has this research interest increased with much focus on whether the telomere length, and the dynamics of terminal telomere restoration via telomerase, are "magic bullets" with respect to predicting life span and lifetime reproductive success (Pauliny et al. 2006; Olsson et al. 2011; Haussmann et al. 2003). The latter observation stems from the fact that, at least in longitudinal studies of species with negative age effects on telomere length [e.g., most homeotherms (Gomes et al. 2010)], telomere attrition, or the absolute length of the shortest telomeres, play a crucial role in cell, and perhaps, organismic senescence (Monaghan and Haussmann 2006). However, due to chromosomal rearrangements through evolutionary history (e.g., inversions and fusions), telomeric sequences are also found between the centromere and the terminal ends (i.e., at interstitial telomeric sites, ITS). ITS telomere sequences have been implicated in heritable disease caused by genomic instability of ITS polymorphic variants, both in terms of copy number and sequence (Bolzán and Bianchi 2006; Lin and Yan 2008). This diversity could be the result of ITSs acting as hotspots for breakage, recombination, rearrangement, and amplification, in addition to participating in DNA repair and regulation of gene expression. In evolutionary ecology, ITSs have so far largely been considered "noise" when estimating the length and attrition of terminal telomeres (Foote et al. 2013), whereas in biomedical research there has long been grave concern for the link between heritable disease and ITSs [but in some cases also with positive ITS effects on DNA repair and prevention of more serious DNA damage; (Yan et al. 2007)]. Interestingly, ITSs show remarkable taxonomic variation in number of sequence repeats and genome-wide distribution. In some squamate reptile species, such as *Varanus salvator macromaculatus* (Srikulnath et al. 2013) ITSs were not identified by fluorescence in situ hybridization (FISH), whereas the same technique revealed ITSs in the agamid lizard *Leiolepis reevesii rubritaeniata* (Srikulnath et al. 2009). In birds, interstitial telomeres appear widespread (Delany et al. 2000; Nanda et al. 2002; Foote et al. 2013), and in mammals 3 classes of commonly occurring ITSs have been described as short ITSs, long subtelomeric ITSs, and fusion ITSs (Lin and Yan 2008), with wide application value as genetic markers for disease caused by genetic instability (Lin and Yan 2008).

We have shown elsewhere (Olsson et al. 2011) that there is ongoing selection on telomere length in free-ranging sand lizards, with females having positive longevity and lifetime fitness benefits from having longer telomeres compared with males (Olsson et al. 2011). At the time of that work, it was unknown to us whether males and females differed with respect to distribution and abundance of ITSs and whether these constituted a confounding factor in our analyses of the effects of "telomere length" using Southern blotting (i.e., including all telomere repeat sequences, including ITSs). Our rationale for this study was therefore to 1) describe the chromosomal distribution of ITSs in males and females, and 2) discuss to what extent our results may help explain our previous link between telomere length and fitness in sand lizards.

Methods

Animals, Cell Culture, and Chromosome Preparation

Most of the methodology for FISH has been reported by us (and others) before (Matsuda and Chapman 1995; Srikulnath et al. 2009, 2013), and therefore here we only give a brief description. Four sand lizards (2 males, 2 females) were captured on a scientific license at our study population (Asketunnan) situated ca 50 km south of Gothenburg on the Swedish West coast (lat 57°22′, long 11°59′). The lizards were immediately exported to Nagoya University, Japan. After intraperitoneal injection of pentobarbital, the mesenteries were removed and used for cell culture. All experimental procedures in Japan using animals conformed to guidelines established by the Animal Care and Use Committee, Nagoya University, Japan. All procedures conducted in Sweden followed the guidelines established by the Animal Ethics Committee, Gothenburg University. The tissues were minced and cultured in Dulbecco's Modified Eagle's Medium (Life Technologies-Gibco), 100 µg/mL kanamycin, and 1% antibiotic-antimycotic (Life Technologies-Gibco). The cultures were incubated at 26 °C in a humidified atmosphere of 5% CO_2 in air. Primary cultured fibroblasts were harvested using trypsin and subcultured. Chromosome preparations were made following a standard air-drying method. The slides were kept at −80 °C until use.

C-banding and Molecular Cytogenetic Analyses

Chromosomal distribution of constitutive heterochromatin was examined by C-banding using the standard barium hydroxide/saline/Giemsa method (Sumner 1972).

The chromosomal locations of the telomeric $(TTAGGG)_n$ sequences were determined by FISH as previously described (Matsuda and Chapman 1995, Srikulnath et al. 2009). After hybridization with 250 ng of biotin-labeled 42-bp oligonucleotide complementary to $(TTAGGG)_n$ sequences, the probes were reacted with avidin labeled with fluorescein isothiocyanate (avidin-FITC; Vector Laboratories). To specify repeated sequence components of the heterochromatic W chromosome, we performed FISH analysis with fluorescein-labeled oligonucleotide probes of all possible mono-, di-, and trinucleotide microsatellite motifs (Pokorná et al. 2011), $(A)_{30}$, $(G)_{30}$, $(AC)_{15}$, $(AG)_{15}$, $(AT)_{15}$, $(CG)_{15}$, $(AAC)_{10}$, $(AAG)_{10}$, $(AAT)_{10}$, $(ACC)_{10}$, $(ACG)_{10}$, $(ACT)_{10}$, $(AGC)_{10}$, $(AGG)_{10}$, $(ATC)_{10}$, and $(CCG)_{10}$ and 2 tetranucleotide microsatellite motifs, $(AGAT)_8$ and $(ATCC)_8$, whose amplification has been found on the sex-specific chromosomes of many squamate reptiles in previous studies (Matsubara et al. 2013, 2015; Gamble et al. 2014). Slides were subsequently counterstained with 0.75 µg/mL propidium iodide (PI). To detect female-specific DNA sequences on the W chromosomes, we performed comparative genomic hybridization (CGH) as previously described with slight modification (Kawai et al. 2007). Genomic DNA was labeled by nick translation incorporating Cy3-dUTP (GE Healthcare) for males and FITC-dUTP (Life Technologies/Molecular Probes) for females. The labeled male and female DNA was coprecipitated, and dissolved in 20 µL hybridization buffer. The hybridization was carried out for 2 days. Fluorescent hybridization signals were captured using a cooled CCD camera mounted on a Leica DMRA microscope (Leica Microsystems, Wetzlar, Germany), and

processed using 550CW-QFISH software by Leica Microsystems Imaging Solutions Ltd. (Cambridge, UK).

Data Archiving

In fulfilment of data archiving guidelines (Baker 2013), the data in this work is presented in the micrographs in Figure 1.

Results

C-banding revealed that C-positive heterochromatin was present in the centromeric and telomeric regions of most chromosomes; the micro-W chromosome exhibited C-positive heterochromatin in the entire region (Figure 1; $2n$ = 38, with a ZZ/ZW male/female sex chromosome system). The chromosomal locations of the $(TTAGGG)_n$ repeated sequences showed the terminal ends on all autosomes and the Z chromosome with no interstitial telomeric sequences in 2 males and 2 females examined (Figure 2, Supplementary Figure 1 online). We observed FISH signals for more than 20 metaphase spreads in each individual. There were no differences in the intensity and distribution of the sequence on autosomes between cells examined and between individuals. The hybridization signals of the $(TTAGGG)_n$ repeated sequences were observed in the entire region of the degenerate micro-W sex chromosome, suggesting that the $(TTAGGG)_n$ sequence has been amplified site-specifically. There was no difference in the signal intensity on the W chromosomes between the 2 females examined. A karyological FISH analysis was published in Srikulnath et al. (2014), including a cytogenetic map of 86 functional genes (this is outside the scope of this study). No hybridization signals were observed on the W chromosome for 18 microsatellite motifs (data not shown). In CGH analysis, differential hybridization signals between

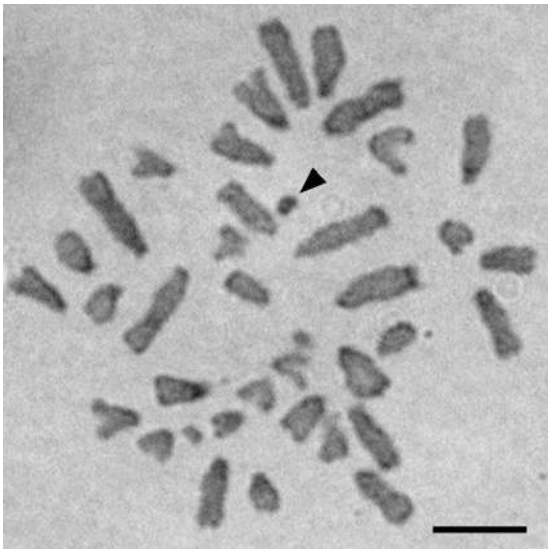

Figure 1. C-banded metaphase spread of a female *Lacerta agilis* (#F1). An arrowhead indicates the micro-W sex chromosome. Scale bar represents 10 μm.

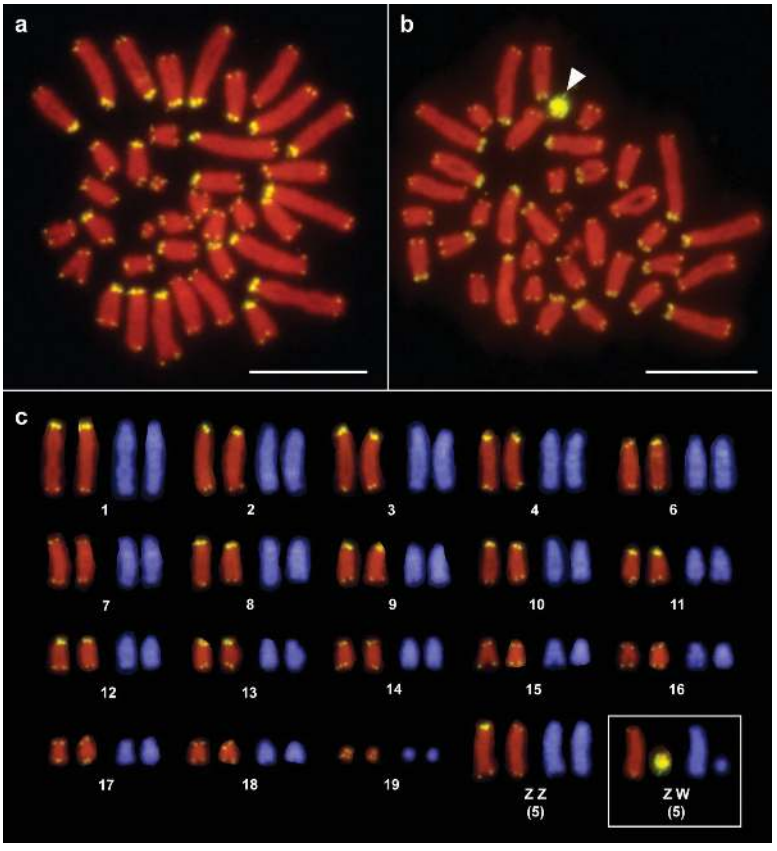

Figure 2. Chromosomal locations of the (TTAGGG)*n* repeated sequence in a male (#M2) (**a**) and a female (#F1) (**b**) *Lacerta agilis*, and aligned chromosomes (**c**). The arrowhead indicates the hybridization signals of the (TTAGGG)*n* sequence on the W chromosome (b). PI-stained chromosomes with signals (right) and the Hoechst-stained same chromosomes (left) of the male metaphase are aligned according to our previous study (Srikulnath et al. 2014), and the Z and W chromosomes isolated from the female metaphase are shown in an inset (c). Scale bars represent 10 μm.

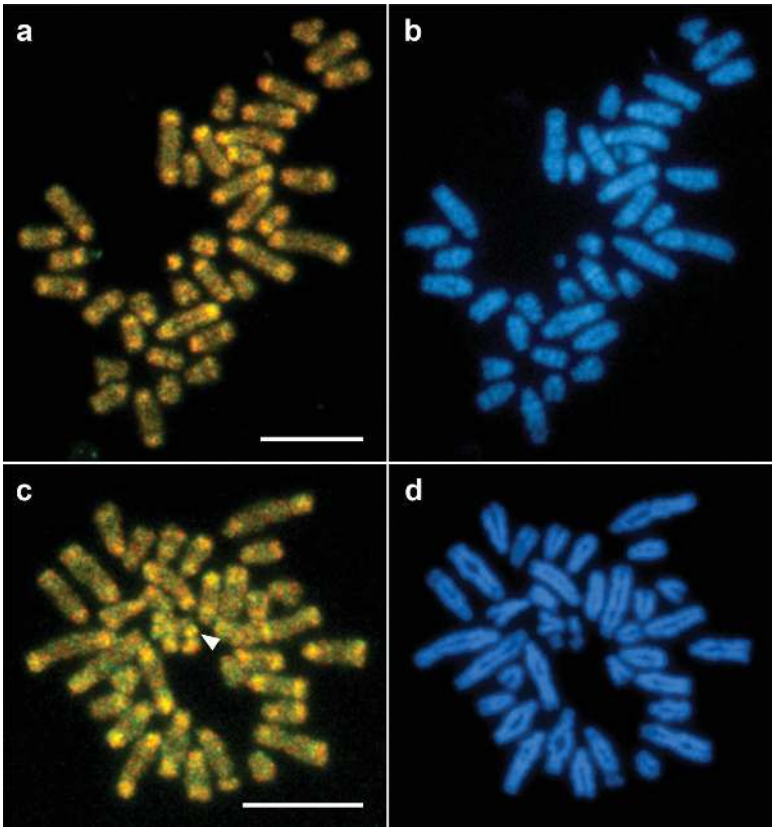

Figure 3. CGH in a male (#M2) (**a**) and a female (#F1) (**c**) *Lacerta agilis*. CGH with male (red) and female (green) genomic DNA show no obvious sex-specific sequences in both sexes (a, c). DAPI staining of the same metaphases (**b**, **d**). An arrowhead indicates the W chromosome (c). Scale bars represent 10 μm.

male- and female-genomic DNAs were not observed on the W chromosomes (Figure 3).

Discussion

Our FISH analysis showed no interstitial $(TTAGGG)_n$ repeated sequences on any autosomes in either sex in sand lizards using a protocol that identifies such ITSs in other reptilian species (Srikulnath et al. 2009). This variation among reptilian species suggests taxon specific evolutionary histories of genomic and/or chromosomal reorganization with respect to ITSs generating events. This lack of ITSs in sand lizards also suggests that our previously described sex-specific links to proximate (Olsson et al. 2010) and ultimate (Olsson et al. 2011) dynamics of telomere length is not confounded by ITSs on autosomes. However, our molecular analysis also describes the first example in a reptilian species of extreme degeneration of a sex chromosome (W), with remarkably amplified $(TTAGGG)_n$ repeated sequences in the entire region. No hybridization signals of microsatellite motifs were detected on the *L*acerta *agilis* W chromosome, although the amplification of microsatellite motifs on the heterochromatic sex-specific Y or W chromosomes have been generally reported in sauropsids (Pokorná et al. 2011; Matsubara et al. 2013, 2014, 2015; Gamble et al. 2014). CGH showed equal intensities of hybridization signals between male and female-genomic DNAs on the W chromosomes. These suggest that the micro-W sex chromosome is largely composed of the $(TTAGGG)_n$ sequences but not the microsatellite motifs and the female-specific sequences. Remarkable amplification of the $(TTAGGG)_n$ sequences are found in autosomal microchromosomes in many avian and squamate species (Nanda et al. 2002; Srikulnath et al. 2011), whereas the amplification on the sex-specific chromosome as found in *L. agilis* has not been reported in sauropsids except for a gecko species, *Underwoodisaurus milii* (Pokorná et al. 2014). No gene has been identified on the W chromosome of *L. agilis* (Srikulnath et al. 2014). Thus, whether these $(TTAGGG)_n$ sequences have any fitness-influencing effects on W-linked genes (e.g., by regulating gene expression) is still unknown. Given that these W chromosomal features are female-specific, it is note-worthy that our previous work has shown higher mortality risks at the production of daughters rather than sons (Olsson et al. 2004, 2005). This agrees with a higher risk of expressing deleterious recessives on the Z chromosome in the heterogametic (ZW) rather than homogametic (ZZ) sex. However, this does not preclude other deleterious effects resulting from potentially epistatic effects between degenerate W gene content and other parts of the genome in females.

In summary, we have shown that sand lizards lack interstitial telomere sequences that could otherwise have interfered with estimates of terminal telomere length, and that sex chromosomes may contain telomere repeats to a hereto unappreciated level (although this constitutes less than 1/38 chromosomes, or <<2.6% of the genome, given that the W is on a par in size with the second smallest chromosome, No. 18). Some of our previous fitness effects of telomeres, and their differences in such effects between males and females, may depend on independent or epistatic W telomere sequence effects. We also remind researchers that ITSs may have profound fitness consequences (e.g., by acting as mutation hot spots and DNA breakage points) as suggested by the biomedical literature, which may further warrant

their investigation from an evolutionary perspective in free-ranging populations.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/

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