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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No interstitial telomeres on autosomes but remarkable amplification of telomeric repeats on the W sex chromosome in the sand lizard (Lacerta agilis)

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.

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No interstitial telomeres
on sand lizard autosomes *(Lacerta agilis)*

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Telomeres are repeat (TTAGGG)\textsuperscript{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 Terminal Sites, ITSs). ITS telomeres 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 (\textit{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)\textsuperscript{n} sequences using fluorescence in situ hybridization (FISH) in fibroblast cells cultured from specimens of known sex. No ITSs could be found on autosomes in either sex. However, females are the heterogametic sex in sand lizards (ZW, 2N = 38) and female W chromosomes showed remarkable degeneration and (TTAGGG)\textsuperscript{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.
1. Introduction

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 a strong candidate for dictating longevity and, consequently, life history evolution [1].

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 [2], oxidative stress biochemistry [3], to life history evolution [1]. 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 [4-6]. The latter observation stems from the fact that, at least in species with negative age effects on telomere length (e.g., most homeotherms [9]), telomere attrition, or the absolute length of the shortest telomeres, play a crucial role in cell, and perhaps, organismic senescence [1]. 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 Terminal Sites, ITS). ITS telomeres have been implicated in heritable disease caused by genomic instability of ITS polymorphic variants, both in terms of copy number and sequence [2, 10]. 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 [11], whereas in biomedical research there has long been grave concern for the link between heritable disease and ITs (but in some cases also with positive ITS effects on DNA repair and prevention of more serious DNA damage; [12]). Interestingly, ITSs show remarkable taxonomic variation in number of sequence repeats and genome-wide distribution. In some reptile species, such as Varanus salvator macromaculatus [13] ITSs were not identified by FISH, whereas the same technique revealed ITSs in the agamid lizard Leiolepis belliana belliana and Leiolepis reevesii rubritaeniata [14]. In birds, interstitial telomeres appear widespread [11, 15], and in mammals three classes of commonly
occurring ITSs have been described as short ITSs, long subtelomeric ITSs, and fusion ITSs [2], with wide application value as genetic markers for disease caused by genetic instability [2].

We have shown elsewhere [5] 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 to males [5]. 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 rational for this study was therefore to (i) describe the chromosomal distribution of ITSs in males and females, and (ii) discuss to what extent our results may help explain our previous link between telomere length and fitness in sand lizards.

2. Material and methods

Most of the methodology for fluorescence in situ hybridization has been reported by us (and others) before [13, 14, 16], and we here therefore only give a brief description. Four sand lizards (two males, two females) were captured on scientific license (XXXXXXXX) 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 heart, lungs, and 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 (permit number XXXXX). The tissues were minced and cultured in Gibco® Dulbecco’s Modified Eagle’s Medium (Life Technologies Corporation), 100 µg/ml kanamycin, and 1 % Gibco® Antibiotic-Antimycotic (Life Technologies Corporation). The cultures were incubated at 26 °C in a humidified atmosphere of 5% CO₂ in air. Primary cultured fibroblasts were harvested using trypsin and subcultured. Chromosome preparations were made following a standard air-drying method; after staining the chromosome slides with Hoechst 33258 (1 µg/ml) for 8 minutes, the slides
were heated at 65 °C for 3 minutes and exposed to ultraviolet light at 65 °C for another 6 minutes [16]. The slides were kept at -80°C until use.

The chromosomal locations of the telomeric (TTAGGG)n sequences were determined by FISH as previously described [16]. We labelled 250 ng of cDNA or 5S rDNA probe with biotin-16-dUTP (Roche Diagnostics, Basel, Switzerland) by nick translation following the manufacturer’s protocol. After hybridization, the probes were reacted with goat antibiotin (Vector Laboratories, Burlingame, CA, USA), and stained with Alexa Fluor® 488 rabbit anti-goat IgG (H+L) conjugate (Molecular Probes®, Life Technologies Corporation). Slides were subsequently counterstained with 0.75 µg/ml propidium iodide (PI). Hybridization signals were captured using a cooled charge-coupled device (CCD) camera mounted on a Nikon microscope (Nikon Corporation, Tokyo, Japan), and processed using software by Nikon Microsystems Managing Solutions Ltd. (Tokyo, Japan). Dual-color FISH was performed to identify the chromosomal locations of the telomeric (TTAGGG)n sequences. A biotin-labelled 42-bp oligonucleotide complementary to (TTAGGG)n sequences (Sigma-Aldrich Corporation) was used as a probe. After hybridization, the biotin-labelled probe was stained with anti-digoxigenin-rhodamine Fab fragments (Roche Diagnostics) and avidin labelled with fluorescein isothiocyanate (avidin-FITC; Vector Laboratories). Fluorescence 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).

3. Results

The chromosomal locations of the (TTAGGG)n repeated sequences in male (Fig. 1a) and female (Fig. 1b) *Lacerta agilis*, show the terminal locations of the telomeric sequences on all autosomes with no interstitial telomeres, with the arrowheads indicating the hybridization signals of the (TTAGGG)n repeated sequences on the highly degenerate W sex chromosome (Fig. 1b; 2N = 38, with a ZZ/ZW male/female sex chromosome system). A karyologic FISH analysis will be submitted elsewhere, including a linkage map of 56 functional genes (this is outside the scope of this study).
4. Discussion

Our FISH analysis showed that interstitial telomeres do not occur on autosomes in either sex in sand lizards using a protocol that identified such ITSs in other reptilian species [14]. This variation among reptilian species suggests taxon specific evolutionary histories with respect to ITSs generating events. This lack of ITSs in sand lizards also suggests that our previously described sex-specific links to proximate [17] and ultimate [5] 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 a remarkable (TTAGGG)n repeated sequence. To the best of our knowledge, no one has to date mapped any genes to lizard W chromosomes [but some in snakes, 18]. Thus, whether these (TTAGGG)n sequences have any fitness-influencing effects on W-genes (e.g., by regulating gene expression) is still unknown. Given that these W chromosomes are female-specific, it is noteworthy that our previous work has shown higher mortality risks at the production of daughters rather than sons [18-19]. 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 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, or << 2.6% of the genome, given that W is on a par in size with the second smallest chromosome, No. 18). We also remind our colleagues 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.

Acknowledgements

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Data accessibility
The data in this work is presented in the micrographs in Figure 1.

References


Legend to figures:

Figure 1. Chromosomal locations of the (TTAGGG)$n$ repeated sequences in male (a) and female (b) *Lacerta agilis* and shown as hybridization patterns of the (TTAGGG)$n$ repeat. Arrows and arrowheads indicate the hybridization signals of the (TTAGGG)$n$ repeated sequences in the W sex chromosome (g). Scale bars represent 10 µm.
Figure 1 a.

Figure 1 b.