

RESEARCH ARTICLE

Interstitial Telomeric Motifs in Squamate Reptiles: When the Exceptions Outnumber the Rule

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Abstract

Telomeres are nucleoprotein complexes protecting the physical ends of linear eukaryotic chromosomes and therefore helping to ensure their stability and integrity. Additionally, telomeric sequences can be localized in non-terminal regions of chromosomes, forming so-called interstitial telomeric sequences (ITSs). ITSs are traditionally considered to be relics of chromosomal rearrangements and thus very informative in the reconstruction of the evolutionary history of karyotype formation. We examined the distribution of the telomeric motifs (TTAGGG)_n using fluorescence *in situ* hybridization (FISH) in 30 species, representing 17 families of squamate reptiles, and compared them with the collected data from another 38 species from literature. Out of the 68 squamate species analyzed, 35 possess ITSs in pericentromeric regions, centromeric regions and/or within chromosome arms. We conclude that the occurrence of ITSs is rather common in squamates, despite their generally conserved karyotypes, suggesting frequent and independent cryptic chromosomal rearrangements in this vertebrate group.

Introduction

Telomeres are nucleoprotein complexes that protect the physical ends of linear eukaryotic chromosomes, playing a crucial role in maintaining chromosome stability and integrity [1]. In all vertebrates the DNA component of telomeres consists of the non-coding (TTAGGG)_n motif [2], which produce long tandem repetitions varying greatly in size between species, individuals and even cell types [3]. The telomeric motif demonstrates a remarkable evolutionary conservation across vertebrate species [4,5]. The telomere-specific complex associated with the telomeric sequence has been described as "shelterin" [6]. Shelterin is composed of three proteins (TRF1, TRF2 and POT1) that directly recognize the (TTAGGG)_n motif, and are interconnected by three additional proteins (TIN2, TPP1 and Rap1) to form a duplex structure [7] (for a review see [3]). The telomeric motif is synthesized by telomerase, a reverse transcriptase-like enzyme, which contains an RNA subunit and a catalytic protein subunit called telomerase

reverse transcriptase [8]. Telomerase uses the RNA template to add additional sequences directly to the telomeres [9]. In humans telomerase is expressed in embryonic tissues and specific germline cells whilst in adults, the enzyme can be detected mainly in the testis, and is absent in most normal somatic cells, in non-dividing oocytes and mature spermatozoa [10,11].

The main role of telomeres is to protect the edges of the linear chromosomes from degradation, recombination or fusion, preventing the chromosomal ends from being recognized as double-strand breaks by DNA repair machinery [3]. Furthermore, the DNA replication machinery cannot completely replicate the ends of linear chromosomes as there would not be any template strands to guide its synthesis ("end replication problem") [12]. In each cell division 50–200 bp are erased from the edges of the chromosomes decreasing the chromosome length and eventually affecting the inner genetic loci. Telomerase preserves the edge of the chromosomes by adding "expendable" telomeric motifs *de novo* [3]. However, in several cell types, such as human somatic cells, telomeres become shorter after subsequent replications [13], resulting in a minimum amount of telomeric sequence, leading to replicative senescence and ultimately cell death [14]. This phenomenon has been described as the "telomere hypothesis of cellular aging", a theory that proposes that telomeres serve as a "mitotic clock" controlling lifespan [15].

An additional role of telomeres is the maintenance of the chromosome topology in the nucleus matrix and the correct alignment of chromosomes for recombination during the first meiotic prophase [16–18]. Another important function of telomeres is the silencing of adjacent genes, a phenomenon known as "telomere position effect" [19,20].

As well as the crucial role of telomeres at the edges of chromosomes, non-terminal telomeric motifs known as interstitial telomeric sequences (ITSs) [21] or interstitial telomeric repeats (ITRs) [3], have been observed in many species. The pioneer publication by [5] provided the first cytogenetic evidence of this, reporting that 55 out of the 100 studied species of vertebrates had ITSs. Many more cases were described in the following years in vertebrates, including amphibians [22], fish [23], birds [24], rodents [25–27], marsupials [28–30] and primates [31].

Based on sequence organization and genomic location, Ruiz-Herrera et al. [21] identified two different types of ITSs: short ITSs (s-ITSs) and heterochromatic ITSs (het-ITSs). Other authors have classified ITSs in more detailed categories as short ITSs, long subtelomeric ITSs, fusion ITSs and pericentromeric ITSs [32]. S-ITSs are short sized telomeric repetitions located in internal sites of chromosomes, present in all completely sequenced mammalian genomes (at least 83 in human, 79 in chimpanzee, 244 in mouse and 250 in rat), but often not detectable by cytogenetic techniques such as fluorescent *in situ* hybridization (FISH) [21]. It was initially thought that s-ITSs were derived from the telomeric fusion of ancestral chromosomes [33]. However, recent studies concluded that s-ITSs are not in fact associated with chromosomal rearrangements [34] but instead were probably inserted by telomerase during the repair of DNA double-strand breaks [21,35,36]. This hypothesis is supported by the frequent association of transposable elements such as SINEs and LINEs with s-ITSs [37].

Het-ITSs are large stretches of telomeric sequences (up to hundreds of kb) localized mainly in heterochromatic chromosomal regions such as in centromeric or pericentromeric areas or within the chromosome arms. In contrast to s-ITSs, het-ITSs are only present in a limited number of species and it is widely believed that they correspond to the remnants of ancestral chromosomal rearrangements which occurred during karyotype evolution [38,39].

As far as we know, ITSs have been described in only 22 lizard species and never in snakes [5,40–53]. In general, squamate reptiles are often considered as a group with evolutionary conserved karyotypes. This view has been supported by classical cytogenetics techniques such as conventional staining and C-banding (e.g. [54–56]) as well as by chromosome painting [57–60], gene mapping [45,61,62], and qPCR mapping of genes linked to sex chromosomes [63–66].

Considering that particular types of ITSs represent relics of chromosome rearrangements, the conservation of karyotypes in squamates suggests that ITSs should be relatively rare in this group. In order to test this hypothesis, we reviewed published data on the occurrence of ITSs and supplemented it with our novel description of ITSs distribution in 13 species of squamates based on FISH experiments.

Material and Methods

Specimens and chromosomal preparations

The distribution pattern of telomeric motifs was studied in the karyotypes of 30 species of squamate reptiles (28 lizards and 2 snakes), belonging to 17 families (Fig 1) from our collection of metaphase chromosome spreads. The specimens originated from pet trade (the companies Animalfarm CZ, Zoopet Sandy, Happy Reptiles, B.A.R. and Zoo Shop Želvička) and were maintained in the reptile breeding laboratory of the Faculty of Science, Charles University in Prague, Czech Republic (accreditation No. 24773/2008-10001). Blood samples were taken from caudal or brachial vessels. The animal procedures were carried out under the supervision and with the approval of the Ethics Committee of the Faculty of Science, Charles University in Prague followed by the Committee for Animal Welfare of the Ministry of Agriculture of the Czech Republic (permission No. 29555/2006-30). Metaphase chromosome spreads were prepared from whole blood cell cultures following the previously described protocol [67] with slight modifications. Briefly, the small amount (approx. 40 μ l) of the peripheral blood was cultured for a week at 30°C in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich), enriched with 10% fetal bovine serum (Baria), 0.5% penicillin/streptomycin solution (Gibco), 1% L-glutamine (Sigma-Aldrich), 3% phytohaemagglutinin (Gibco), and 1% lipopolysaccharide (Sigma-Aldrich). Chromosome preparations were made following standard procedures including a 3.5 hours colcemid treatment, hypotonization, and fixation in 3: 1 methanol:acetic acid.

Fluorescent in situ hybridization (FISH)

A specific probe for the telomeric motif (TTAGGG)_n was produced and labelled with dUTP-biotin by PCR, using the primers (TTAGGG)₅ and (CCCTAA)₅, without a DNA template, according to the methodology of Ijdo et al. [68]. Briefly, a PCR reaction was performed in 50 μ l final volume, including 0.4 μ l of each primer (5 pmol/ μ l), 5 μ l of 10 \times PCR buffer (Bioline), 2.5 μ l MgCl₂ (50mM), 1 μ l dATP, dCTP, dGTP (10 mM each), 0.7 μ l dTTP (10 mM), 1 μ l dUTP-biotin (1 mM) and 1 μ l BioTaq DNA polymerase (5 U/ μ l, Bioline). The PCR cycling conditions were as follows: 5 min at 94°C, 10 cycles of 1 min at 94°C, 30 sec at 55°C and 1 min at 72°C, followed by 30 cycles of 1 min at 94°C, 30 sec at 60°C and 30 sec at 72°C, with a final step of 5 min at 72°C. The PCR product was precipitated and re-suspended in 300 μ l of hybridization buffer (50% formamide/2 \times SSC).

Prior to *in situ* hybridization, 10 μ l of the telomeric probe per slide was denatured at 75°C for 10 min and then chilled on ice for 10 min. In parallel, the metaphase slides were subsequently treated with RNase, pepsin, fixed with 4% formaldehyde, dehydrated through a series of 70%, 85% and 100% ethanol washes, denatured in 70% formamide/2 \times SSC at 75°C for 4 min, dehydrated again and air dried. Afterwards, the probe was applied to each slide and incubated at 37°C for 16–24 hours.

Post-hybridization washes were subsequently carried out in 50% formamide/2 \times SSC at 42°C (3 \times 5 min) and in 2 \times SSC (2 \times 5 min). The slides were incubated in 100 μ l of 4 \times SSC/5% blocking reagent (Roche Αρχήφόρμας Τέλοςφόρμας) at 37°C for 45 min. The telomeric signal was detected using a modified avidin-FITC/biotinylated anti-avidin protocol for FITC signal amplification. In detail, we prepared two different solutions: a primary antibody solution with

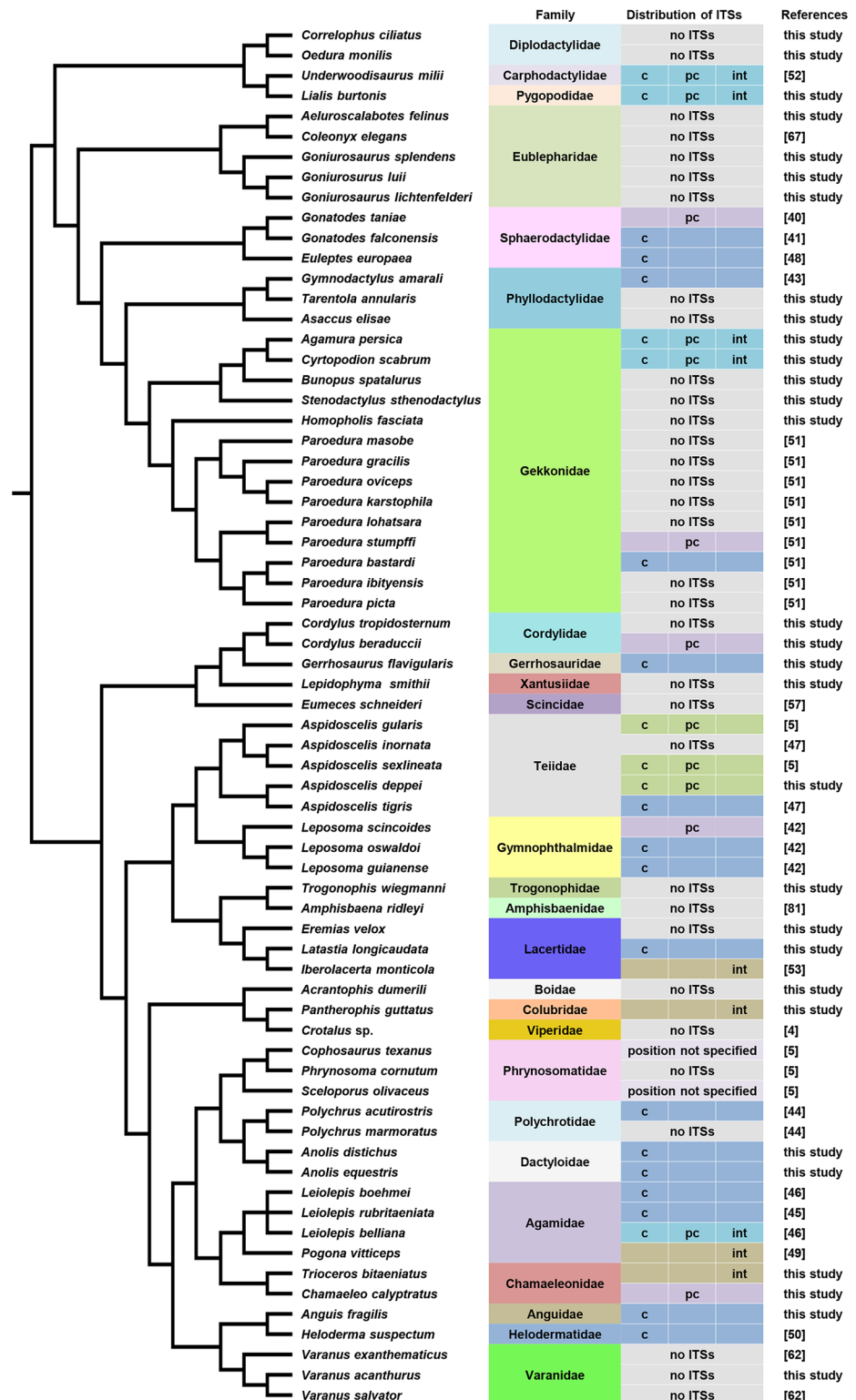


Fig 1. The position of telomeric sequences in 68 species of lizards and snakes. The telomeric motif (TTAGGG)_n was detected in the normal, terminal position of all chromosomes. In addition to the terminal topology, interstitial telomeric repeats (ITSs) were detected in several species within centromeric (c), pericentromeric (pc) and/or intermediate (int) chromosomal regions.

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300 μ l of 4 \times SSC/5% blocking reagent, including 0.3 μ l avidin-FITC per slide (Vector laboratories) and a secondary antibody solution with 200 μ l of 4 \times SSC/5% blocking reagent, including 2 μ l biotinylated anti-avidin per slide (Vector laboratories). The FITC signal was enhanced by five subsequent applications of the primary (three times) and the secondary (two times) antibody solutions at 37°C for 30 min each, using 100 μ l of each antibody solution per slide, with intermediate washes in 4 \times SSC/0.05% Tween20 (3 \times 5 min). Afterwards, the slides were dehydrated through an ethanol series, air dried, counterstained with 4',6-diamidino-2-phenylindole (DAPI) and mounted with Vectashield anti-fade medium (Vector Laboratories).

Microscopy and image analyses

An Olympus Provis AX70 fluorescence microscope with a DP30BW digital camera was used to take grayscale images that were processed with DP manager imaging software (Olympus) to record the pattern of the telomeric repeats within the chromosomal metaphases.

Phylogenetic distribution

The phylogenetic distribution of the presence/absence of ITSs across squamate reptiles was visualized using Mesquite v.2.75 [69], based on the phylogenetic tree topology of Pyron et al. [70].

Results

FISH with telomeric probe proved to be a valuable tool in revealing the topology of the telomeric motif (TTAGGG)_n in the karyotypes of squamate reptiles. Based on the distribution and the putative origin of the telomeric sequences within the chromosomes we distinguished the following topologies in the karyotypes:

Karyotypes with only terminal distribution of telomeres

In 17 species we observed telomeric sequences only at the expected terminal positions at the ends of the chromosomes. Specifically, this group includes the species *Acrantophis dumerili* (Boidae), *Cordylus tropidosternum* (Fig 2a) (Cordylidae), *Correlophus ciliatus* (Fig 2b), *Oedura monilis* (Fig 2c) (Diplodactylidae), *Aeluroscalabotes felinus*, *Goniurosaurus lichtenfelderi*, *G. luui* (Fig 2d), *G. splendens* (Eublepharidae), *Bunopus spatalurus*, *Homopholis fasciata* (Fig 2e), *Stenodactylus sthenodactylus* (Gekkonidae), *Eremias velox* (Lacertidae), *Asaccus elisae*, *Tarentola annularis* (Phyllodactylidae), *Trogonophis wiegmanni* (Fig 2f) (Trogonophidae), *Varanus acanthurus* (Fig 2g) (Varanidae) and *Lepidophyma smithii* (Xantusiidae).

Karyotypes with ITSs in centromeric regions

Five species had karyotypes with telomeric motifs at the terminal positions of all chromosomes, and additional ITSs in centromeric regions of one or more chromosomal pairs. In detail, ITSs were detected at the centromeres of three submetacentric pairs in *Anguis fragilis* (Fig 2h) (Anguidae), seven chromosomal pairs in *Anolis distichus* (Fig 2i), five chromosomal pairs in *Anolis equestris* (Fig 2j) (Dactyloidae), two large metacentric pairs in *Gerrhosaurus flavigularis* (Fig 2k) (Gerrhosauridae) and five chromosomal pairs in *Latastia longicaudata* (Fig 2l) (Lacertidae).

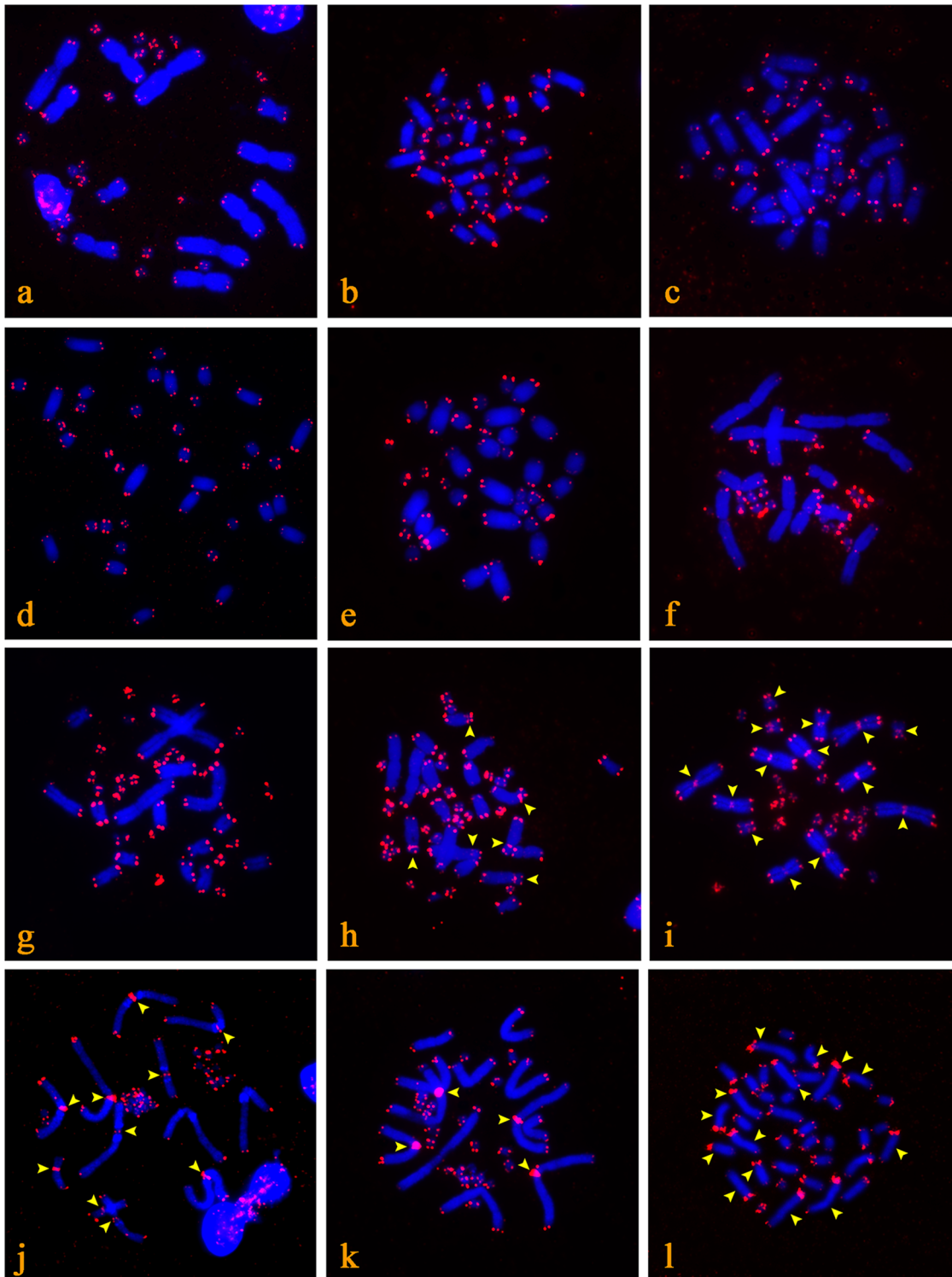


Fig 2. The position of the telomeric sequences in the chromosomal spreads, as revealed by FISH. Exclusively terminal distribution: *Cordylus tropidosternum* (a), *Correlophus ciliatus* (b), *Oedura monilis* (c), *Goniurosaurus luii* (d), *Homopholis fasciata* (e), *Trogonophis wiegmanni* (f), *Varanus acanthurus* (g). ITSs in centromeric regions: *Anguis fragilis* (h), *Anolis distichus* (i), *Anolis equestris* (j), *Gerrhosaurus flavigularis* (k), *Latastia longicaudata* (l). Chromosomes are stained with DAPI (blue), while the FITC signal from the telomeric probe is red. Chromosomes with ITSs are indicated by arrows.

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Karyotypes with ITSs in pericentromeric regions

Two lizard species, *Chamaeleo calyptratus* (Fig 3a) (Chamaeleonidae) and *Cordylus beraduccii* (Fig 3b) (Cordylidae), had telomeric motifs at the terminal positions of all chromosomes, and additional ITSs in pericentromeric regions of the largest metacentric chromosome pair.

Karyotypes with ITSs within chromosome arms

Two species exhibit ITSs signals between terminal telomeres and the centromeric/pericentromeric regions. In *Trioceros bitaeniatus* (Fig 3c) (Chamaeleonidae), ITSs are present at intermediate positions on six chromosomal pairs, and in *Pantherophis guttatus* (Fig 3d) (Colubridae) there is an interstitial telomeric band on a medium sized metacentric chromosome.

Karyotypes with ITSs in numerous positions

Finally, in four species we observed ITSs in numerous positions on chromosomes including all of the above mentioned categories (centromeric, pericentromeric and within the chromosome arms). This extensive accumulation was observed in *Aspidoscelis deppei* (Fig 3e) (Teiidae), *Lialis burtonis* (Fig 3f) (Pygopodidae), *Cyrtopodion scabrum* (Fig 3g) and *Agamura persica* (Fig 3h) (Gekkonidae).

The phylogenetic distribution of ITSs (Fig 1) suggests that in general ITS emergence/loss is evolutionary dynamic across squamates. A high incidence of ITSs is present in the sister families Teiidae and Gymnophthalmidae, while Iguania also possess the tendency to accumulate ITSs in their genomes, yet ITSs appear to have a rather random distribution across the other squamate lineages.

Discussion

ITSs are present in members of all major lineages of squamates (Fig 1). Taking into account previous publications and our results, only 48.5% of squamates ($n = 33$ species) demonstrate the normal, expected distribution of telomeres at the edges of all chromosomes. Surprisingly, around half of the studied squamate species (51.5%, $n = 35$) show ITSs in centromeric regions, pericentromeric regions and/or within chromosomal arms. It therefore appears that the existence of ITSs in squamate genomes is not an exception, but rather a common event (Fig 1). The species with karyotypes demonstrating ITSs seem to be more or less randomly distributed across the phylogeny of squamates, with several families including species with both normal terminal telomeres and ITSs, while a higher incidence of ITSs typifies sister families Teiidae and Gymnophthalmidae and the lineage Iguania (Fig 1). Nevertheless, it should be noted that although our sampling includes members of most major lineages of Squamata, the total number of species tested for the presence of ITSs is still quite small and somewhat patchy, which precludes detailed statistical analyses of ITSs correlations and phylogenetic distribution.

ITSs are commonly observed in centromeric regions of both bi-armed and acrocentric chromosomes. ITSs in the centromeres of bi-armed chromosomes might have originated from the remains of “old” terminal telomeres after Robertsonian fusion (e.g. in the gecko *Gymnodactylus amarali*; [43]), while the ITSs on the centromeres of acrocentrics may be the result of extensive amplification due to their proximity to satellite sequences. It is well-documented that telomeres can be part of centromere repetitive elements [26,71]. In a recent study [71], it was demonstrated that all centromeres of a vole species exhibit co-localization of ITSs with three other satellite sequences. These ITSs were cloned and sequenced, demonstrating 87% to 94% similarity to the terminal telomeric motif (TTAGGG)_n [26]. The extensive amplification of ITSs in the centromeres of acrocentric chromosomes covering a large part of the centromeric region can

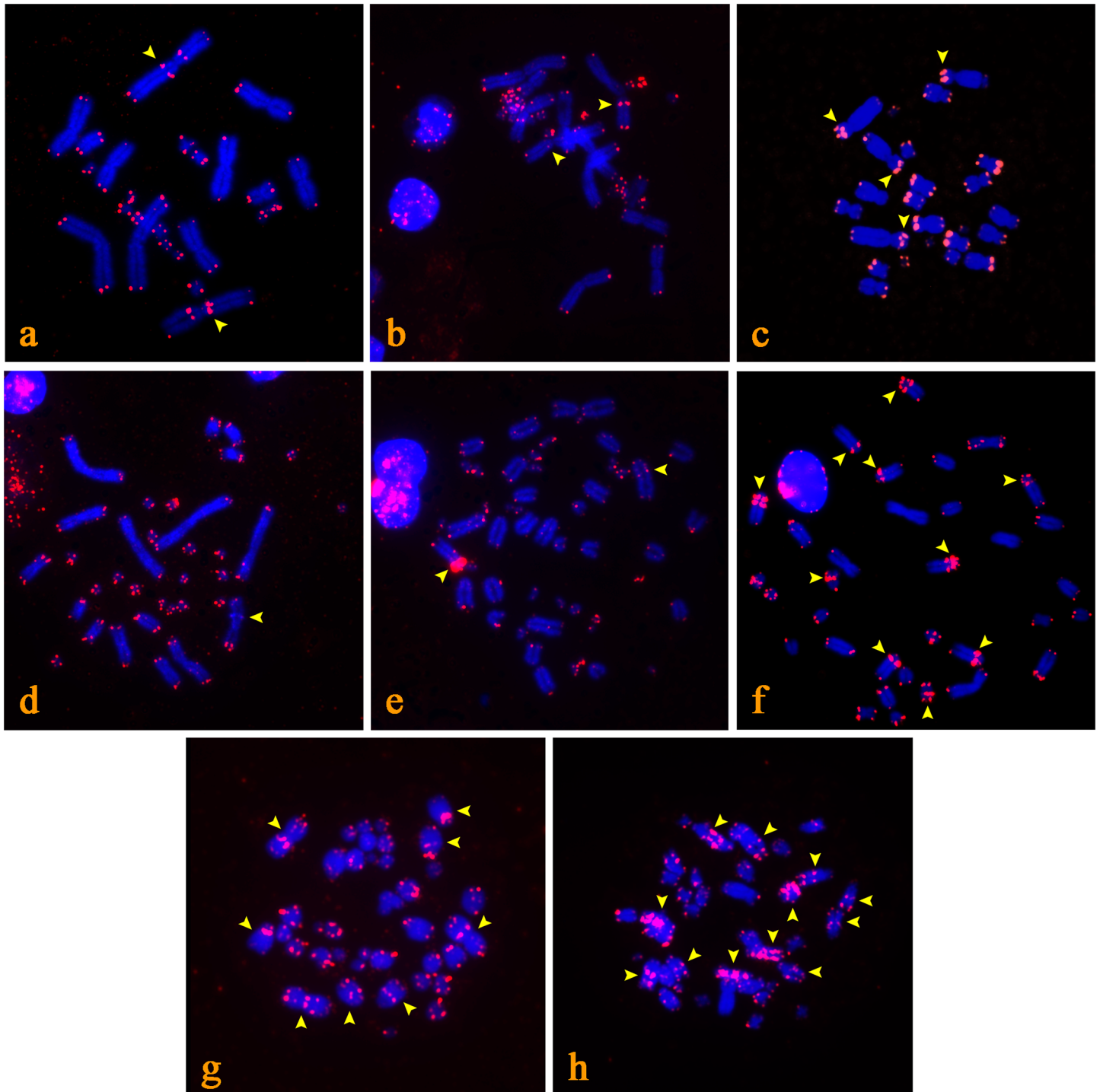


Fig 3. The position of the telomeric sequences in the chromosomal spreads, as revealed by FISH. ITSs in pericentromeric regions: *Chamaeleo calypttratus* (a), *Cordylus beraduccii* (b). ITSs in intermediate positions: *Trioceros bitaeniatus* (c), *Pantherophis guttatus* (d). ITSs in numerous positions: *Aspidoscelis deppei* (e), *Lialis burtonis* (f), *Cyrtopodion scabrum* (g), *Agamura persica* (h). Chromosomes are stained with DAPI (blue), while the FITC signal from the telomeric probe is red. Chromosomes with ITSs are indicated by arrows.

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be observed in several squamates, such as *Latastia longicaudata* (Fig 2). However, further studies are needed to reveal the type of repetitive elements co-localized with ITSs in squamates and to show if species with ITSs share a similar content of satellite sequences within their centromeres.

Extended ITSs in intermediate positions in several lizard species may reflect remnants of past intrachromosomal rearrangements. Although sauropsids possess a relatively low rate of interchromosomal rearrangements, it has been shown in birds that intrachromosomal rearrangements occur rather frequently [72–74]. In fact, whereas no interchromosomal rearrangements have been documented in the microchromosomes of the chicken, turkey or zebra finch, there have been numerous intrachromosomal rearrangements recorded in these species [74]. In the same context, several pericentromeric inversions have been discovered in the chromosomal pairs 1–4 of *Anolis carolinensis* using *in situ* hybridization with BACs [75]. Furthermore, the comparison between the homologous part of chromosome 15 of the chicken and chromosome X of *Anolis carolinensis* revealed extensive synteny of the gene content, and numerous intrachromosomal, but few interchromosomal rearrangements in the studied chromosomal region [66]. Evidence for numerous intrachromosomal, but rare interchromosomal rearrangements based on interspecific chromosome painting was recently presented in geckos [76].

In some cases telomeric-like sequences appear to accumulate at the heterochromatic part of sex chromosomes. The exact role of the accumulation of ITSs and satellite sequences (for a review see [77]) on the highly heterochromatic W (e.g. in the gecko *Underwoodisaurus milii*; [52]) or Y chromosomes remains unclear. It has been speculated that the accumulation of repetitive sequences on one pair facilitates the suppression of recombination between sex chromosome homologues, enabling the accumulation of sexually beneficial mutations on respective sex chromosomes. Some authors however suggest that the repetitive sequences may accumulate near the sex determining locus as a result of the suppression of recombination rather than inducing it ([77] and references within).

Finally, closely related species with similar chromosome morphology seem to possess different patterns of ITSs distribution, e.g. species of the genus *Anolis*, *Cordylus*, *Paroedura* [51] and *Leiolepis* [45,46] (Fig 1). Such differences could be explained either by the dynamic nature of ITSs (e.g. as part of satellite DNA or transposable elements) or cryptic rearrangements. Many reptile lineages, with the exception of their avian clade, show persistent telomerase activity in the somatic tissues of adults which might not only explain their extensive tissue regeneration potential [78], but also the existence of ITSs accumulation at numerous positions in their genome (Fig 1). In fact, telomerase appears to be active in all of the tissues of adult *Aspidoscelis sexlineata* [78], a species with ITSs accumulation (Fig 1; [5]). Furthermore, skin fibroblasts from the blue racer snake (*Coluber constrictor*) show increased telomerase activity after a high number of generations *in vitro* [79]. Moreover, telomere length does not decrease with age in the water python (*Liasis fuscus*), but instead increases from approximately 7 kb at hatching to 28 kb at adult age [80], providing another exception to the hypothesis of “cellular aging”.

In summary, we detected ITSs for the first time in the genomes of 13 species of squamate reptiles and documented that ITSs were observed in approximately half (35 out of 68) of the species of lizards, snakes and amphisbaenians, e.g. [81], studied so far. Therefore we can conclude that the occurrence of ITSs is surprisingly high in this group of vertebrates which has otherwise stable and conserved karyotypes. This discrepancy suggests that, similar to birds, squamate reptiles may have a rather high rate of intrachromosomal rearrangement and a low rate of interchromosomal rearrangement. The origin of ITSs in some species of squamate reptiles may however be attributed to other factors such as high telomerase activity and/or the repair mechanisms of double-strand breaks, e.g. triggered by the activity of transposable elements. Future studies should be devoted to increasing the taxonomic scope of the testing of

ITSs distribution across squamates and to address questions regarding the functional importance of these unusually frequent elements in squamate genomes.

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Author Contributions

Conceived and designed the experiments: MR LK MA MJP. Performed the experiments: MR MA MJP. Analyzed the data: MR LK MA MJP. Contributed reagents/materials/analysis tools: MR LK MA MJP. Wrote the paper: MR LK MA MJP.

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