First Report of Sex Chromosomes in Plated Lizards (Squamata: Gerrhosauridae)

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Keywords
CGH · FISH · rDNA · Sex chromosomes · Telomeres

Abstract
Squamate reptiles show high diversity in sex determination ranging from environmental sex determination to genotypic sex determination with varying degrees of differentiation of sex chromosomes. Unfortunately, we lack even basic information on sex determination mode in several lineages of squamates, which prevents full understanding of their diversity and evolution of sex determination. One of the reptilian lineages with missing information on sex determination is the family Gerrhosauridae, commonly known as the plated lizards. Several species of gerrhosaurids have been studied in the past by conventional cytogenetic methods, but sex-specific differences were not identified. In this study, we applied both conventional and molecular cytogenetic methods to metaphases from both sexes of the Peters’ keeled plated lizard (Tracheloptychus petersi). We identified accumulations of rDNA loci in a pair of microchromosomes in metaphases from males, but only in a single microchromosome in females. The restriction of the observed heterozygosity to females suggests a putative ZZ/ZW system of sex chromosomes, which represents the first report of sex chromosomes in a gerrhosaurid lizard. The lack of sex-specific signals in all other cytogenetic methods implies that the sex chromosomes of T. petersi are poorly differentiated in sequence content.

Introduction
Amniotes possess 2 major systems of sex determination: genotypic sex determination, where the sex of an individual is set by its sex-specific genotype, and environmental sex determination, where the sex is set by environmental factors, predominantly temperature [Bull, 1983]. Most of the variability in sex determination systems among amniotes is concentrated to squamate reptiles, but even within this lineage the variability is heavily unequally distributed. It is estimated that roughly 60% out of approximately 11,000 recent squamate species belong to 5 clades with highly evolutionary stable XX/XY (iguanas, skinks) and ZZ/ZW (caenophidian snakes, lacertids, monitors) sex chromosomes [Rovatsos et al., 2014, 2015a, 2016, 2019; Kostmann et al., 2021]. The majority of the variability is thus concentrated to the other squamate lin-
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In this context, sex determination is poorly studied in the lizards of the superfamily Scincioidea, which consists of 4 families: Scincidae, Xantusiidae, Cordylidae, and Gerrhosauridae [Pyron et al., 2013]. In skinks (family Scincidae), the far most diversified scincoid lineage, XX/XY sex chromosomes were identified cytogenetically in few species [Hardy, 1979; Donnellan, 1985; Caputo et al., 1994], while ZZ/ZW sex chromosomes were reported in a single species [Patawang et al., 2017]. In 2 recent studies using whole-genome sequencing and exploring the differences in read depth coverage between sexes, XX/XY sex chromosomes were revealed in 2 skink species: Scincus scincus [Kostmann et al., 2021] and Eulamprus heatwolei [Cornejo-Páramo et al., 2020]. In addition, our recent study using a qPCR-based method revealed that homologous XX/XY sex chromosomes are present in 13 species of skinks, covering most of the phylogenetic diversity of the family [Kostmann et al., 2021]. Furthermore, although sex chromosomes in skinks are poorly differentiated, they are phylogenetically stable for at least 85 million years [Kostmann et al., 2021].

Female heterogamety was identified by analysis of RADseq data in the xantusiid Xantusia henshawi [Nielsen et al., 2020]. In addition, female heterogamety was predicted for the xantusiid Lepidophyma smithii from the type of facultative parthenogenesis [Kratochvíl et al., 2020]. It was observed that females of this species can parthenogenetically produce offspring of both sexes, which is not compatible with male heterogamety [Kratochvíl et al., 2020]. Cytogenetic studies of species from the families Cordylidae and Gerrhosauridae were mostly performed by conventional cytogenetic methods, and sex-specific differences were not observed in their karyotypes [Odierna and Olmo, 1980; Odierna et al., 2002].

In this study, we applied conventional and molecular cytogenetic methods to metaphases from both sexes of the Malagasy Peters’ keeled plated lizard Tracheloptychus petersi, with the major aim to identify their sex chromosomes to fill the gap in our knowledge on the evolution of the sex determination in amniotes.

**Material and Methods**

**Samples and Species Verification**

Blood samples were collected from 2 males and 3 females of T. petersi originated from a legal import from Madagascar. All individuals were adults, and the sex was identified by external morphology (head shape, thickness of tail base) and evertting hemipenes in males by palpation. Total DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA). The standard DNA barcoding region of the mitochondrial gene cytochrome c oxidase I (COI) gene was amplified by PCR from DNA extracted from all 5 lizards, with primers optimized for reptiles [Nagy et al., 2012] and following a standard PCR protocol [Koubová et al., 2014]. The PCR products were purified and sequenced bi-directionally by Macrogen (Seoul, Korea). The COI sequences were subsequently trimmed in FinchTV, aligned in BioEdit v5.0.9 [Hall, 1999], analyzed in MEGA v10 [Kumar et al., 2018], and compared to sequences deposited in public databases by BLASTn [Altschul et al., 1990] to verify the taxon assessment. All sequences were deposited in GenBank under the accession numbers MW052709, MW052710 and MW326658.

**Chromosome Preparation and Staining**

Whole-blood cell cultures were set up to obtain mitotic chromosome suspensions as described in Mazzoleni et al. [2019]. Karyograms were constructed from Giemsa-stained metaphase chromosomes. Heterochromatic regions were visualized by C-banding according to Sumner [1972] with slight modifications.

**Fluorescence in situ Hybridization with Probes for Repetitive Elements**

We used fluorescence in situ hybridization (FISH) to visualize the topology of ribosomal DNA loci and telomeric sequences (TTAGGG)n. The rDNA probe was prepared from a plasmid (pDmr.a 51#1) with an 11.5-kb insert encoding the 18S and 28S ribosomal units of Drosophila melanogaster [Endow, 1982] and labeled by dUTP-biotin using nick translation. The telomeric probe was prepared according to Ijdo et al. [1991] and Rovatsos et al. [2011]. The probes were hybridized to chromosome preparations following the protocol of Rovatsos et al. [2011].

**Comparative Genome Hybridization**

Comparative genome hybridization (CGH) was applied to both male and female metaphase chromosomes to reveal sex-specific genomic regions. The detailed protocol can be found in Rovatsos et al. [2015b].

**Microscopy and Image Analyses**

Images from at least 10 metaphases per method and sex were captured using either a Zeiss Axio Imager Z2 equipped with automatic Metafer-MSearch scanning platform (MetaSystems, Altusheim, Germany) or a CoolCube 1 b/w digital camera (Meta-Systems) or a Provis AX70 fluorescence microscope (Olympus, Tokyo, Japan) equipped with a DP30BW digital camera (Olympus).

**Results and Discussion**

The haplotype analysis revealed 3 haplotypes of COI gene. The BLASTn revealed 98.19, 98.79 and 98.95% similarity of sequences from our sequenced individuals to those from the specimens of T. petersi previously studied by [Nagy et al., 2012] across an alignment of 664 bp confirming the expected taxon assessment of our material.
Two different haplotypes were found in each sex pointing that individuals within a given sex are not siblings, and the variability in cytogenetic markers between sexes thus cannot be attributed to kinship.

The examination of metaphases from 5 individuals revealed a diploid number of $2n = 34$, with 12 macrochromosomes and 22 microchromosomes (Fig. 1A, B). The macrochromosomes are bi-armed, either metacentric or submetacentric, but the morphology of microchromosomes is not possible to identify. Prominent accumulations of heterochromatin were identified in the pericentric regions of the 4 larger chromosome pairs, but no sex-specific accumulation was observed (Fig. 1C, D). The karyotype of *T. petersi* is similar to its closest relative *Tracheloptychus madagascariensis* previously reported by Odierna et al. [2002]. A diploid chromosome number of $2n = 34$ was observed in the majority of gerrhosaurids ($2n = 34–36$) and cordylids ($2n = 32–46$) [Odierna and Olmo, 1980; Odierna et al., 2002; Olmo and Signorino, 2016], suggesting that the karyotype consisting of 12 macrochromosomes and 22 microchromosomes was probably present already in the common ancestor of

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**Fig. 1.** Giemsa-stained karyograms (A, B), distribution of C-banding (C, D), rDNA loci (E, F) and telomeric motifs (G, H), and comparative genome hybridization (I, J) in metaphases from both sexes of the Peters’ keeled plated lizard (*T. petersi*). Z chromosomes are indicated when recognizable. ITRs are indicated by yellow arrows.
these families. On the contrary, diploid chromosome number is quite variable in skinks (2n = 22–36) and xantusiids (2n = 24–40) [Hass and Hedges, 1992; Olmo and Signorino, 2016].

FISH with the rDNA probe showed strong signals in the centromeric region of the fourth largest chromosome pair in both sexes of T. petersi (Fig. 1E, F). An additional signal was found on 1 microchromosome in females, but on 2 microchromosomes in males. The occurrence of the heterozygous state only in females indicates that this species possesses female heterogamety (ZZ/ZW sex chromosomes) with the Z chromosome having rDNA accumulation missing on the W. Sex-specific differences in accumulations of rDNA loci has been previously reported in many phylogenetically distant taxa. Within Scincioidea, the rDNA loci are accumulated in the X chromosome, but not in the Y in the common sandfish (S. scincus) [Caputo et al., 1994; Kostmann et al., 2021]. In an analogous case to T. petersi, rDNA loci are accumulated in the Z chromosome of the Kajika frog (Buergeria buergeri), but are missing in its W chromosome [Schmid et al., 1993]. In other cases, both sex chromosomes possess accumulations of rDNA loci, but they differ at their extent as in softshell turtles, where rDNA accumulations are usually much more expanded on the W [Badenhorst et al., 2013; Literman et al., 2014; Rovatsos et al., 2017].

Odierna et al. [2002] examined the position of active rDNA loci by silver-staining across different species of cordylids and gerrhosaurids including T. madagascariensis, but did not find sex-specific differences in any of them. In T. madagascariensis, the accumulations of rDNA loci are localized in 2 microchromosomes, which could be homologous to the sex chromosomes of T. petersi. It seems that the complete or partial loss of rDNA accumulation from the W chromosome is an apomorphy of T. petersi. The signal intensity of the rDNA loci varies significantly even in T. petersi males between the 2 putative Z chromosomes (Fig. 1E). Notably, rDNA loci seem to be variable in number of repeats and chromosome positions at both inter- and intraspecific levels [Porter et al., 1991; Stults et al., 2008; Altmannová et al., 2016; reviewed by Sochorová et al., 2017; Mazzoleni et al., 2018; Degrandi et al., 2020]. The high mobility of rDNA clusters among genome regions could be caused by insertion of rDNA genes into chromosomes via translocation followed by amplification in the new sites and elimination in the old location [Dubcovsky and Dvořák, 1995]. The functional and evolutionary significance of the variability in the amount and position of rDNA loci still remains unclear. In humans, rDNA loci seem to act as “recombination hotspots” resulting in extensive variability in rDNA quantity and genomic organization [Stults et al., 2008]. In some cases, like in fruit flies from the genus Drosophila, rDNA loci on the X and Y chromosomes seem to have a vital role in meiosis I [McKee and Karpen, 1990]. This variability in the amount and chromosomal topology of rDNA loci can be to a large extent neutral, and it can be partially tolerated by the cellular mechanism. For example, it was demonstrated that only 25–50% of the diploid number of rDNA loci are necessary for normal development in wildtype and mutant individuals of Xenopus laevis [Knolvand and Miller, 1970]. Nevertheless, the different numbers of repeats of rDNA loci might be viable, but still could be connected with negative consequences, such as the development of cancer [Gibbons et al., 2015; Xu et al., 2017] and genome instability [Kobayashi, 2011].

In T. petersi, FISH with the telomeric probe revealed the expected terminal signals in all chromosomes and additional interstitial telomeric sequences (ITRs) in the centromeric and/or pericentromeric regions of all 12 macrochromosomes (Fig. 1G, H). ITRs are generally common in squamate reptiles with centromeric ITRs being the most common [Rovatsos et al., 2015c]. ITRs are often remnants of past chromosomal rearrangements, such as chromosome fusions and inversions [Rovatsos et al., 2015c]. Accumulations of telomeric sequences in reptiles also often occur on differentiated sex chromosomes [Matsubara et al., 2015], e.g., in caenophidian snakes [Augstenová et al., 2017] and a carphodactylid gecko [Pokořná et al., 2014]. Nevertheless, no sex-specific accumulations were detected in T. petersi (Fig. 1G, H).

CGH did not show accumulations of female-specific sequences on the W chromosome, indicating that the sex chromosomes of T. petersi are poorly differentiated in sequences (Fig. 1I, J). The results of CGH suggests that most of the W chromosome is pseudoautosomal and the W-specific region is small, below the detection limit of this method, which is estimated to approximately 2–3 megabases [Schoumans et al., 2004]. Nevertheless, previous studies revealed that CGH shows strong signals when the sex-specific chromosomal region is heterochromatic or enriched in repetitive elements, otherwise even bigger sex-specific regions might not be visualized by this method [Altmannová et al., 2016; Kostmann et al., 2021].

In summary, our study provides the first karyotype description of T. petersi and the first report of sex chromosomes in the gerrhosaurid lizards, expanding our knowledge on the evolution of sex determination systems in vertebrates. Future studies should focus on the identification of sex chromosome gene content, for example, by

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DOI: 10.1159/000513764
next generation sequencing methodologies, and to explore the homology of sex chromosomes across scincioideal lizards. Next to skinks and xanthusiids, gerrhosaurids are another scincioideal lineage with poorly differentiated sex chromosomes, which indicates that recombination suppression on sex chromosomes might be slower in this group than in other reptiles, which often have highly differentiated sex chromosomes [Rovatsos et al., 2015a, 2016, 2019]. Comparison of the rates of recombination between scincioideal lizards and other lineages will allow us to explore the effect of recombination on the emergence and stability of sex chromosomes and sex determination systems.

Acknowledgement

We would like to express our gratitude to Petr Ráb and members of his laboratory for their support and to Jana Thomayerová, Anna Bauerová, and Nuria Viñuela Rodriguez for technical assistance.

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Statement of Ethics

All experimental procedures were carried out under the supervision and with the approval of the Ethics Committee of the Faculty of Science, Charles University, followed by the Committee for Animal Welfare of the Ministry of Agriculture of the Czech Republic (permissions No. 35484/2015-16 and 8604/2019-7).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

This study was funded by the Czech Science Foundation project No. 20-27236J, the Charles University Grant Agency project 1518119, Charles University projects PRIMUS/SCI/46 and the Research Centre program 204069.

Author Contributions

A.K. performed the cytotgenetic analyses; L.K. and M.R. contributed to the experimental part. All authors drafted and approved the final manuscript.

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Sex Dev 2020;14:60–65
DOI: 10.1159/000513764
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