Part I

Sex determination and sex chromosome evolution in snakes

Chapter 1

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Evolutionary Dynamics of the W Chromosome in Caenophidian Snakes

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Abstract: The caenophidian (assigned also as "advanced") snakes are traditionally viewed as a group of reptiles with a limited karyotypic variation and stable ZZ/ZW sex chromosomes. The W chromosomes of the caenophidian snakes are heterochromatic, and pioneering studies demonstrated that they are rich in repetitive elements. However, a comparative study of the evolutionary dynamics of the repetitive content of the W chromosome across the whole lineage is missing. Using molecular-cytogenetic techniques, we explored the distribution of four repetitive motifs (microsatellites GATA, GACA, AG and telomeric-like sequences), which are frequently accumulated in differentiated sex chromosomes in vertebrates, in the genomes of 13 species of the caenophidian snakes covering a wide phylogenetic spectrum of the lineage. The results demonstrate a striking variability in the morphology and the repetitive content of the W chromosomes even between closely-related species, which is in contrast to the homology and long-term stability of the gene content of the caenophidian Z chromosome. We uncovered that the tested microsatellite motifs are accumulated on the degenerated, heterochromatic W chromosomes in all tested species of the caenophidian snakes with the exception of the Javan file snake representing a basal clade. On the other hand, the presence of the accumulation of the telomeric-like sequences on the caenophidian W chromosome is evolutionary much less stable. Moreover, we demonstrated that large accumulations of telomeric-like motifs on the W chromosome contribute to sexual differences in the number of copies of the telomeric and telomeric-like repeats estimated by quantitative PCR, which might be confusing and incorrectly interpreted as sexual differences in telomere length.

Keywords: heterochromatin; sex chromosomes; GATA; telomeres; microsatellites; FISH

1. Introduction

Snakes represent around one third of all current species of squamate reptiles. However, the reasons for their unusual evolutionary success are not known. Not all snake groups are equally diversified; nearly 90% of snake species (*ca*. 3000 out of 3400) belong to the group Caenophidia (advanced snakes), particularly to their subclade Colubroidea [1]. Thus, the key to understanding the evolutionary success of snakes is in uncovering the processes responsible for the superradiation of caenophidian/colubroid snakes in the last 80 million years (dating follows [2]). The colonization of new areas and the evolution of advanced venom-delivery systems were suggested to be responsible for the large diversity of caenophidian and colubroid snakes [3]. Nevertheless, it was also speculated that the enormous difference in the diversification rates between non-caenophidian and caenophidian snakes can be attributed to the differences in the degree of differentiation of sex chromosomes [4].

The snake karyotypes are usually very stable, and many the so-far studied species of non-caenophidian and caenophidian snakes possess the putative ancestral snake karyotype with

36 chromosomes (16 macro- and 20 micro-chromosomes) [5,6]. Nevertheless, only caenophidian snakes possess well-differentiated ZZ/ZW sex chromosomes with the highly heterochromatic W chromosome, which seems to be their important synapomorphy [4,7,8]. The Z chromosomes share similar gene content across 28 representative species from all caenophidian families (Acrochordidae, Colubridae, Elapidae, Homalopsidae, Lamprophiidae, Pareatidae, Viperidae, Xenodermatidae) [4], documenting the homology of sex chromosomes across this whole group. On the other hand, sex chromosomes of non-caenophidian snakes are only poorly differentiated and have not been properly identified in most lineages yet [4,9], with the important exception of the boa *Boa imperator* and the python *Python bivittatus*, which possess likely independently-evolved XX/XY sex chromosomes [10]. Most of the genes present in the caenophidian Z chromosome are lacking in their W chromosome [4,9], although certain genes are known from the W chromosome, as well [11,12]. The genes linked to the Z chromosome are evolving faster than autosomal genes [9]. Additionally, this "fast Z" effect was exactly suggested as the mechanism responsible for the faster evolution of caenophidian snakes reflected in their high diversification [4].

Tightly connected to this rapid evolution of Z-linked loci, but less studied, is the degeneration of the caenophidian W chromosome. Various oligonucleotide motifs are detected in long tandem repeats across vertebrate genomes, more commonly in heterochromatic regions such as (peri)centromeric regions and non-recombining regions of sex chromosomes [11,13]. For example, the accumulation of the $(AG)_n$ motif has been reported in the Y chromosomes of the skink *Bassiana duperreyi* and the turtle *Emydura macquarii*, as well in the W chromosomes of chicken [14] and the lacertid lizard *Eremias velox* [15]. The most common motifs accumulated in sex chromosomes are likely $(GATA)_n$ and $(GACA)_n$. In the caenophidian snakes, both of these motifs were detected in the heterochromatic parts of the W chromosomes as a part of the larger unit, the banded krait minor-satellite DNA (*Bkm*) repeat, documented in the members of the families Viperidae, Elapidae and Colubridae [16]. Beyond the caenophidian snakes, extensive $(GATA)_n$ accumulations have been reported for example in the Y chromosomes of the turtle *Chelodina longicollis*, the pygopodid gecko *Aprasia parapulchella*, the skink *Bassiana duperreyi* [14] and four species of the iguana genus *Anolis* [17]. The accumulation of the *Bkm* repeats was proposed as a pivotal event in the formation of sex-specific heterochromatin and hence differentiation of sex chromosomes [18,19]. However, the variability in the occurrence of the microsatellites on sex chromosomes across the phylogenetic spectrum probably reflects independent accumulations rather than common ancestry and questions the general involvement of the *Bkm*-like repeats in the heterochromatinization of sex chromosomes [14,15]. Recently, we found that $(GATA)_n$ accumulations are not present in the highly heterochromatic W chromosome of the Javan file snake (*Acrochordus javanicus*), a member of the family Acrochordidae, while it occurs in the heterochromatic W chromosome of the dragon snake (*Xenodermus javanicus*, Xenodermatidae) [7,8]. According to the recent phylogenetic hypotheses [2,20,21], the family Acrochordidae is a sister of all colubroid snakes, and the family Xenodermatidae is a sister of all other colubroid families. Therefore, we suggested that in snake evolution, the formation of the sex-specific heterochromatin preceded the emergence of GATA accumulations on the W chromosome, which would question the role of the *Bkm* repeats in the origin of sex-specific heterochromatin even in snakes. Nevertheless, we cannot exclude the alternative scenario, i.e., the secondary loss of the *Bkm* accumulations in the ancestor of *A. javanicus*. The later hypothesis would expect that the presence of the *Bkm* accumulations on the heterochromatic W is evolutionarily less stable than assumed across caenophidian snakes.

In addition to the *Bkm* repeats, the W chromosome in the dragon snake *X. javanicus* possesses extensive amplifications of the six-nucleotide motif $(TTAGGG)_n$ [7]. This motif is commonly amplified in the terminal position of vertebrate chromosomes by the cellular enzyme, telomerase, and has a crucial role in genome replication and chromosome stability (for reviews, see [22–24]). However, extensive amplifications of telomeric-like motifs have been documented in interstitial positions in many vertebrates, including mammals [25–27], fishes [28], amphibians [29,30] and reptiles [23,31,32]. Across squamate reptiles, the presence of interstitial telomeric repeats (ITRs) is a rule rather than

an exception [23]. Accumulations of telomeric-like motifs are often present also in differentiated sex chromosomes as for instance in the W chromosome of the gecko *Underwoodisaurus milii* [31], the lacertid lizard *Lacerta agilis* [33] or the dragon lizard *Pogona vitticeps* [34], but they are lacking in the W chromosome of the Javan file snake [8].

The variability in the distribution of telomeric-like motifs, as well the differences in the size and shape of the W chromosome among species [6,35] indicate that the repetitive content of the W chromosome across caenophidian snakes might be highly variable. However, a comparative study allowing determination of its evolutionary dynamics across caenophidian snakes is missing. Here, using molecular-cytogenetic techniques, we examined the heterochromatin distribution and partial repetitive content across six families of the caenophidian snakes: Acrochordidae, Colubridae, Homalopsidae, Lamprophiidae, Viperidae and Xenodermatidae. We supplemented these original data with the literature records to reconstruct the evolutionary dynamics and to explore the stability of the presence of the tested repetitive elements in the W chromosomes across caenophidian snakes.

2. Materials and Methods

2.1. Studied Material

We studied 13 forms of caenophidian snakes: *A. javanicus* (Acrochordidae), *Elaphe bimaculata*, *Lampropeltis ruthveni*, *Lampropeltis triangulum*, *Natrix natrix*, *Pantherophis guttatus*, *Zamenis situla* (Colubridae), *Homalopsis buccata* (Homalopsidae), *Boaedon* sp. 1 (brown colored, morphologically resembling *Boaedon fuliginosus*), *Boaedon* sp. 2 (black colored, morphologically resembling *Boaedon olivaceus*), *Boaedon* sp. 3 (green colored, morphologically resembling *Boaedon olivaceus*) (Lamprophiidae), *Crotalus durissus* unicolor (Viperidae) and *X. javanicus* (Xenodermatidae) (Table S1). The studied animals originated from the pet trade. We supplemented our dataset by the additional data on the repetitive content in the previously-studied species *A. javanicus* [8], *X. javanicus* [7], *Protobothrops flavoviridis* [11,36], *Notechis scutatus* [14,19,37,38], *Elaphe quadrivirgata* [14,39,40] and *Rhabdophis tigrinus* [14,39].

In the specimens we studied, we collected blood and used it for whole blood cell cultures and DNA isolation. The blood taking was approved by the Central Commission for Animal Welfare of the Czech Republic (Protocol No. 35484/2015-16). The genus *Boaedon* is likely a complicated species complex containing several undescribed taxa with not yet settled taxonomy [41,42]. Therefore, we amplified and sequenced mitochondrial DNA fragments from all studied specimens of the genus in order to "DNA barcode" our studied material for future proper determination. For genomic DNA isolation, we used the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA). We used the reptile-specific primers RepCOI-F/RepCOI-R for amplifying the standard barcoding region of mitochondrial gene *cytochrome oxidase* subunit I (COXI) [43]. The PCR reaction protocol and amplification conditions were reported previously [44]. In addition, we sequenced a fragment of the mitochondrial gene *cytochrome b* (cytb) using the universal primers H16064/L14910 [45]. The amplification conditions were described previously [41,42]. The PCR products were sent for bi-directional sequencing to Macrogen (Seoul, Korea). The sequences were aligned using BioEdit v5.0.9 [46] and subsequently analyzed in MEGA v6.0.5 [47] and DnaSP v5.10.1 [48]. A BLAST search was performed to compare our sequences with those from previous studies [41,42].

2.2. Chromosome Preparations and Staining

We used whole blood cell cultures for the preparation of metaphase chromosome spreads following the protocol described in [31]. The chromosomal preparations were stained by Giemsa. The C-banding was used for visualization of the constitutive heterochromatin and detection of heterochromatic sex chromosomes following [49], with a slight modification, as the chromosomes were counterstained by 4′,6-diamidino-2-phenylindole (DAPI) instead of Giemsa.

2.3. Fluorescence In Situ Hybridization with Telomeric Probe

Fluorescence in situ hybridization (FISH) with the telomeric probe was used to visualize the distribution of the telomeric-like motif $(TTAGGG)_n$. The probe was prepared by PCR using the primers $(TTAGGG)_5$ and $(CCCTAA)_5$ without genomic DNA template [50] and labelled in the same PCR reaction with dUTP-biotin. The PCR reaction mix and amplification conditions are presented in detail in [23]. The PCR product was precipitated and resuspended in 300 µL of hybridization buffer (50% formamide/2× saline-sodium citrate buffer, SSC) and stored in the freezer (−20 °C). Prior to hybridization, the probe was denatured at 73 °C for 6 min and chilled temporally on ice until use. The slides with chromosomal material were treated with RNAse and pepsin, fixed with 4% formaldehyde and then dehydrated through a series of 70%, 85% and 100% ethanol. They were subsequently denatured in 70% formamide/2× SSC at 75 °C for 4 min and dehydrated. We applied 11 µL of the telomeric probe per slide and incubated the slide overnight at 37 °C. The following day, the slides were washed three times in 50% formamide/2× SSC at 42 °C and in 2× SSC. We incubated the slides with 100 µL of 4× SSC/5% blocking reagent (Roche, Basel, Switzerland) at 37 °C for 45 min. For telomere detection, we used a modified avidin-FITC/biotinylated anti-avidin system (Vector Laboratories Burlingame, CA, USA) for an amplification of the fluorescent signals (for the detailed protocol, see [23]). Slides were stained with DAPI and mounted with the antifade medium Fluoroshield (Sigma-Aldrich, St. Louis, MO, USA).

2.4. Fluorescence In Situ Hybridization with Microsatellites

We used microsatellite probes $(GATA)_8$, $(GACA)_8$ and $(AG)_{15}$ to explore the accumulation of these microsatellite motifs in the W chromosome. The procedure was similar to FISH with the telomeric probe with slight differences in probe preparation and post-hybridization washes. The microsatellite probes were synthesized and 5′-end-labelled with biotin by Macrogen (Korea). Then, 30 pmol of microsatellite probe were resuspended in 11 µL hybridization buffer (50% formamide, 2× SSC, 10% sodium dodecyl sulfate, 10% dextran sulfate, 1× Denhard's buffer, pH 7). The post-hybridization washes were performed in 0.4× SSC/0.3% Nonidet P-40 (Sigma-Aldrich, St. Louis, MO, USA) at 40 °C for 2 min and in 2× SSC/0.1% Nonidet P-40 at room temperature for 30 s.

2.5. Microscopy and Image Analyses

The photos of metaphases were captured by the Provis AX70 (Olympus, Tokyo, Japan) fluorescence microscope equipped with a DP30BW digital camera (Olympus). The photos from FISH experiments were further processed using the DP manager imaging software (Olympus, Tokyo, Japan). Karyograms were constructed from Giemsa-stained preparations in the Ikaros karyotyping software (Metasystems, Altlussheim, Germany).

2.6. Estimation of Sexual Differences in the Number of Telomeric-Like Repeats

The accumulation of the telomeric-like repeats in the W chromosome should lead to the differences in repeats of telomeric-like sequences between sexes. Their number can be quantified by the quantitative polymerase chain reaction (qPCR) [31].

For the amplification of the telomeric-like sequences, we used the primers by [51]. In addition, we used the gene *MDS1 and EVI1 complex locus* (*mecom*) for normalization of the quantification cycle values (crossing point, Cp). The primers specific for the gene *mecom* [4] and the telomeric motifs [51], as well as the amplification conditions were described previously [4]. The qPCR was performed using the LightCycler II 480 (Roche Diagnostics, Basel, Switzerland). The samples were run in triplicate in a 15-µL reaction volume, containing 0.2 ng of genomic DNA, 7.5 µL SYBR Premix Ex Taq II (Takara Bio, Kusatsu, Japan) and 0.3 µL of each primer (10 pmol/µL stock solution). An estimation of the amount of the telomeric motifs was calculated from Cp values and was subsequently normalized to the dose of the reference gene *mecom* from the same DNA sample (for the formulas, see [52]). Subsequently, we

calculated the ratio of the relative numbers of copies of the telomeric-like repeats between sexes. A ratio around 1.0 is expected in species without ITRs on the W chromosome, while a much higher ratio is expected in species with the accumulation of telomeric-like sequences on the W chromosome. Our estimation of the W-linked ITRs' variation assumes that telomeres of the Z and W chromosomes are comparable in size and that there are no ITRs on the Z chromosomes. Moreover, it neglects that the number of telomeric motifs can vary between conspecific individuals due to an individual polymorphism typical for any repetitive sequences and also due to shortening of telomere with age [53]. Therefore, our approach is only a rough estimation of the size of the W-linked ITRs, but still, we expect that it should correspond to the results of the observation of ITRs in sex chromosomes from our FISH experiments.

3. Results

3.1. Species Identification in the Genus Boaedon

The sequences of *cytb* and COXI were successfully amplified in all our specimens of the genus *Boaedon* and compared with the sequences in GenBank [41,42] revealing three distinct haplotypes. The forms assigned as *Boaedon* sp. 2 and *Boaedon* sp. 3 have a genetic p-distance of 10.7% and 11.4%, respectively, in comparison to *Boaedon* sp. 1, and 8.9% to each other. Therefore, we assume that all three *Boaedon* forms correspond to distinct species. However, due to the unresolved taxonomy in this group of snakes (see [41,42]), we cannot safely assigned any valid species names to them. However, due to our DNA barcoding, the karyotype descriptions of our samples will be possible to attribute to species names in the future, after a careful taxonomic re-evaluation of this species complex. All haplotypes are deposited in GenBank.

3.2. Karyotypes of Previously Cytogenetically Unstudied Species

To our knowledge, the karyotypes of *L. ruthveni*, *L. triangulum*, *Z. situla* and our forms of the genus *Boaedon* have not been previously described. The karyotype of *E. bimaculata* will be described elsewhere. *L. ruthveni*, *L. triangulum* and *Z. situla* have similar karyotypes with 2n = 36 chromosomes; sex chromosomes are the fourth pair of the complement, which is frequent among caenophidian snakes [6]. The genus *Boaedon* exhibits unexpected variability in the karyotype. The karyotype of *Boaedon* sp. 1 consists of 42 chromosomes, that of *Boaedon* sp. 2 of 40 chromosomes and that of *Boaedon* sp. 3 of 34 chromosomes. The karyotype with 2n = 34 chromosomes was earlier described for *B. fuliginosus* [54]. The sex chromosomes are the fourth pair of the complement in *Boaedon* sp.2, but the first pair of the complement in *Boaedon* sp. 1 and *Boaedon* sp. 3.

Karyograms of all previously unstudied species are depicted in the Supplementary Material (Figure S1).

3.3. Morphology of W Chromosome and Distribution of Constitutive Heterochromatin

The W chromosome is subtelocentric or submetacentric in almost all studied species of the caenophidian snakes (Figure 1). We used C-banding to visualize constitutive heterochromatin. The heterochromatic regions on the W chromosome were present in all caenophidian species studied by us. The species differ in the size of the W-linked heterochromatic blocks (Figure 1). The heterochromatic blocks cover the whole W chromosome in *C. durissus unicolor, E. bimaculata*, *L. triangulum*, *L. ruthveni*, *N. natrix* and *Z. situla*, but only part of it in *H. buccata*, *P. guttatus* and all three *Boaedon* lineages (Figure 1). Wherever identifiable, the Z chromosomes do not include heterochromatic blocks, ITRs or accumulations of tested repetitive motifs.

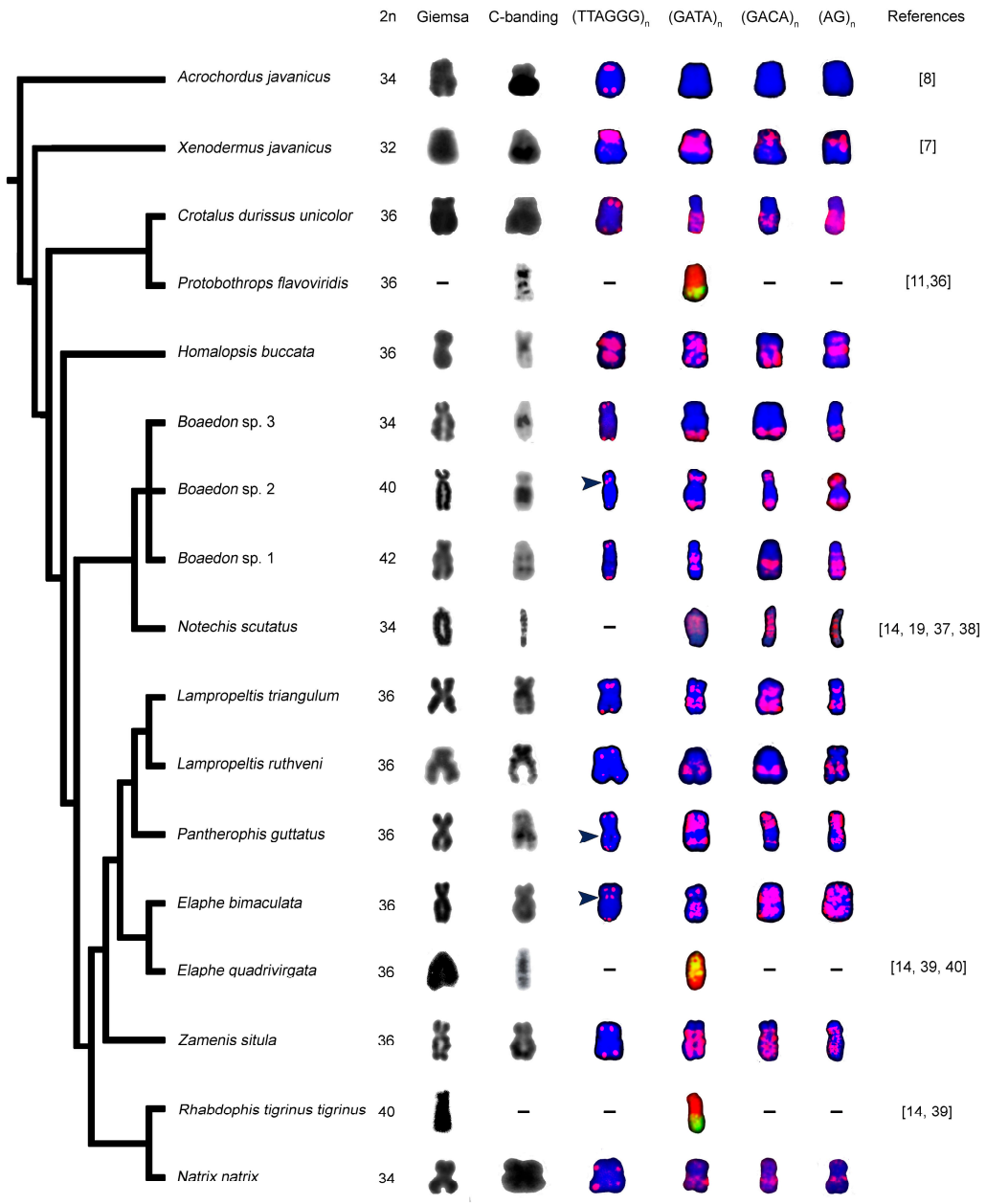


Figure 1. Comparative phylogenetic reconstruction of the heterochromatic content of the W chromosomes in Caenophidia. The phylogenetic tree of studied snakes was completed according to phylogenies by [2]. We compared the morphology of W chromosomes (Giemsa stain), the pattern of heterochromatin accumulation (C-banding) and the topology of four microsatellite repeats: $(TTAGGG)_n$, $(GATA)_n$, $(GACA)_n$ and $(AG)_n$ (FISH). The chromosomes from C-banding and FISH experiments were counterstained by 4′,6-diamidino-2-phenylindole (DAPI). Photos of C-banding were inverted. In the case of FISH experiments, the chromosomes with DAPI were stained blue, and the fluorescence signal of probes was pseudo-colorized in red. Dashes represent missing data. Interstitial telomeric repeats (ITRs) on W chromosomes are indicated by arrows when they are not easily visible. For data collected from the bibliography, see the references inside the figure.

3.4. Distribution of Telomeric-Like Sequence $(TTAGGG)_n$ in W Chromosomes

The telomeric repeats were detected in the terminal position of all chromosomes, including sex chromosomes in all studied species. ITRs were detected in the W chromosomes of *E. bimaculata*,

H. buccata, *P. guttatus* and in *Boaedon* sp. 2. In *X. javanicus*, the higher accumulation of ITRs is present around the centromeric region of the W chromosome [7].

The presence of the telomeric-like motif accumulation on the W chromosome was subsequently confirmed by the measurements of the relative number of the telomeric-like repeats by qPCR (Figure 2). Almost equal numbers of the telomeric-like repeats between sexes were estimated by qPCR in *A. javanicus*, *Boaedon* sp. 3 and *N. natrix*, i.e., in the three species where FISH with the telomeric probe did not reveal any ITRs in the W chromosomes (Figure 1). In addition, we did not detect any notable sex-specific difference in the amount of telomeric-like repeats in *P. guttatus*, which possesses rather small ITRs in the centromeric region of the W chromosome. In contrast, the significant differences between sexes were measured by qPCR in *H. buccata* and *X. javanicus* (see Figure 2), the species with extensive blocks of ITRs in their W chromosomes clearly visible in the results of the FISH with the telomeric probe (Figure 1).

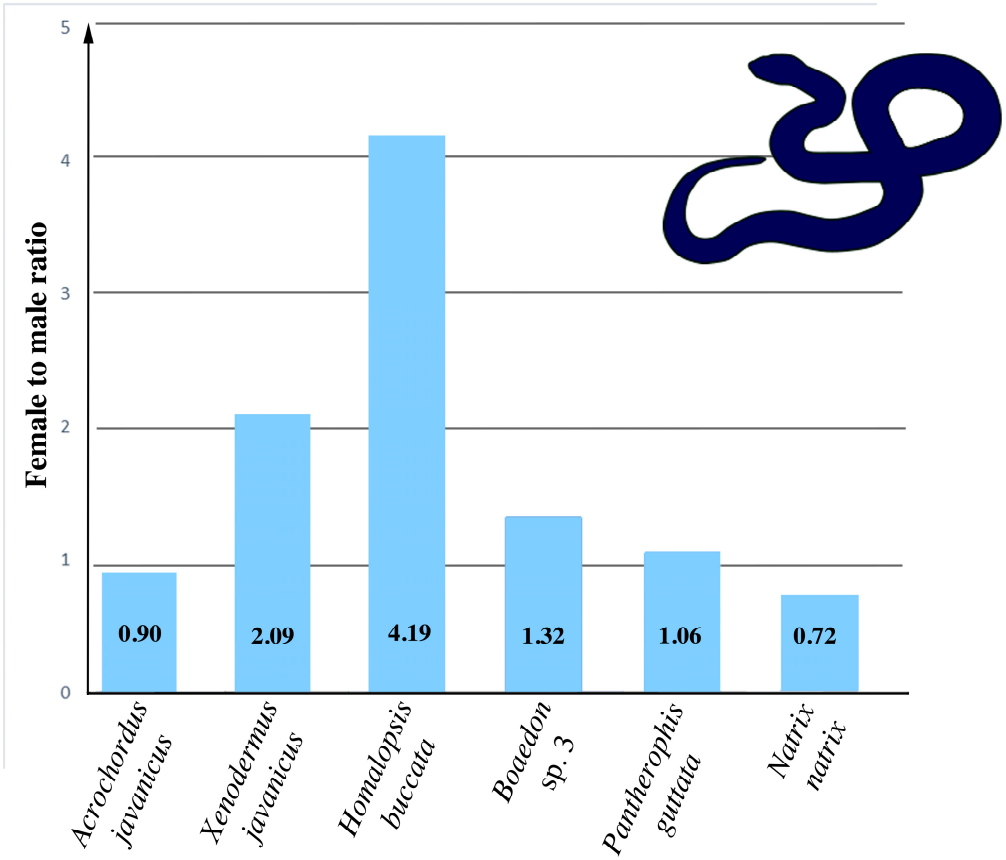


Figure 2. Female-to-male ratios of the number of telomeric-like motifs.

3.5. Accumulation of Microsatellites on W Chromosomes

We detected extensive accumulations of the repetitive microsatellite motifs $(GATA)_n$, $(GACA)_n$ and $(AG)_n$ on the W chromosomes in all herein studied species. However, the position and extent of the accumulations of the microsatellite motifs differ significantly, even in closely-related species (Figure 1).

4. Discussion

We can conclude that the distribution of the heterochromatin and the repetitive content of the W chromosome are highly variable among caenophidian snakes. The distribution of the accumulations of particular repetitive sequences in the W chromosome, as well as the presence of ITRs can be different even in closely-related species such as within the genus *Boaedon* (Figure 1). This supports the view that

differentiated and degenerated sex chromosomes are among the most evolutionary dynamic regions of the genome [14,15,55].

On the other hand, certain aspects of the W chromosomes across caenophidian snakes are remarkably stable. For example, we detected a heterochromatic region in the W chromosomes in every studied caenophidian snake (Figure 1), although heterochromatin seems to disappear completely from the degenerated Y chromosomes in certain lineages of iguanas, the other reptile lineage with a long-term stability of differentiated sex chromosomes [56]. Furthermore, up to now, all tested species of colubroid snakes, but not the single tested species of their sister lineage, the family Acrochordidae, possess the notable accumulations of the repetitive microsatellite motifs GATA, GACA and AG in their W chromosomes (Figure 1). Such stability in the presence of the accumulations of these motifs across colubroid snakes and the lack of the accumulation of microsatellite motifs in homomorphic sex chromosomes of the python *Liasis fuscus* [19] and other non-caenophidian snakes [57] suggest that the situation in the acrochordid represents a primitive state rather than a secondary loss of these accumulations. The presence of these accumulations is stable across a large colubroid radiation, which suggests that their loss is rare. It would be a strange coincidence if a rare loss not detected by us within Colubroidea happened just in the sister lineage of colubroid snakes. If the scenario of the emergence of W-linked heterochromatin in the common ancestor of the caenophidian snakes, but the emergence of the *Bkm*-repeats including GATA and GACA motifs only later in the evolution in the common ancestor of the colubroid snakes is correct, the *Bkm* repeats were not able to play an important role in the formation of sex heterochromatin in snakes as previously suggested [58]. It is also of interest that the distributions of the GATA and GACA accumulations on the snake W chromosomes slightly differ in some snake species (Figure 1), although they are supposed to be a part of the same repetitive element (*Bkm*). It is possible that these motifs occur also in other repetitive elements accumulated on the snake W chromosomes.

We demonstrated that the accumulations of the telomeric-like repeats on the W chromosomes occur relatively frequently among colubroid snakes (Figure 1). Their phylogenetic distribution suggests that they might evolve independently several times within Colubroidea as they did also in other squamate lineages [31,33,34]. These accumulations highly differ in size (Figure 1), and the most pronounced of them are easily measurable by qPCR detecting copy numbers of telomeric-like repeats in genomes. Interestingly, qPCR is commonly used for measurements of telomere length, although it cannot differentiate between true telomeric sequences and telomeric-like sequences producing ITRs [31]. A pronounced accumulation of telomeric-like repeats linked to non-recombining regions of a sex chromosome can lead to the finding of sex-specific differences in the copy number of telomeric-like repeats in qPCR, which can be interpreted as a sexual difference in telomere length. Sexual differences in telomere length were attributed to sexual differences in life-history including aging and sex-specific ecological or reproductive roles (e.g., [59] in a colubrid snake). Our study warns against premature adaptive explanations of sexual differences in estimated telomere lengths. We suggest that a careful examination of the genomic distribution of telomeric-like repeats in a studied species should be done before any interpretation of the causes of the variability in measured telomere lengths among sexes or individuals. It seems to us that it is very difficult to compare for example telomere shortening/aging by qPCR using mixed-sex samples and to interpret sexual differences in the measured copy numbers of telomeric or telomeric-like sequence in species with sex chromosomes.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4425/9/1/5/s1. Table S1: Summary of species and number of examined individuals used in the current study. Figure S1: Giemsa-stained karyotypes and C-banded metaphase spreads of unstudied species from female specimens of *Boaedon* sp.1 (a,b), *Boaedon* sp.2 (c,d), *Boaedon* sp.3 (e,f), *Lampropeltis ruthveni* (g,h), *Lampropeltis triangulum* (i,j) and *Zamenis situla* (k,l).

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Author Contributions: B.A., S.M. and M.R. performed most of the experimental work, M.R. and L.K. drafted the first version of the manuscript, all authors contributed to the final version of the manuscript.

Conflicts of Interest: The authors have no conflicts of interest to declare.

References

1. Uetz, P.; Freed, P.; Hošek, J. (Eds.) The Reptile Database. Available online: http://www.reptile-database.org (accessed on 6 August 2017).
2. Zheng, Y.; Wiens, J.J. Combining phylogenomic and supermatrix approaches, and a time-calibrated phylogeny for squamate reptiles (lizards and snakes) based on 52 genes and 4162 species. *Mol. Phylogenet. Evol.* **2016**, *94*, 537–547. [CrossRef] [PubMed]
3. Pyron, R.A.; Burbrink, F.T. Extinction, ecological opportunity, and the origins of global snake diversity. *Evolution* **2012**, *66*, 163–178. [CrossRef] [PubMed]
4. Rovatsos, M.; Vukić, J.; Lymberakis, P.; Kratochvíl, L. Evolutionary stability of sex chromosomes in snakes. *Proc. R. Soc. B Biol. Sci.* **2015**, *282*, 20151992. [CrossRef] [PubMed]
5. Olmo, E.; Signorino, G.G. Chromorep: A Reptile Chromosomes Database. 2005. Available online: http://chromorep.univpm.it (accessed on 26 August 2017).
6. Oguiura, N.; Ferrarezzi, H.; Batistic, R.F. Cytogenetics and molecular data in snakes: A phylogenetic approach. *Cytogenet. Genome Res.* **2009**, *127*, 128–142. [CrossRef] [PubMed]
7. Rovatsos, M.; Johnson Pokorná, M.; Kratochvíl, L. Differentiation of sex chromosomes and karyotype characterisation in the dragonsnake *Xenodermus javanicus* (*Squamata: Xenodermatidae*). *Cytogenet. Genome Res.* **2015**, *147*, 48–54. [CrossRef] [PubMed]
8. Rovatsos, M.; Altmanová, M.; Johnson Pokorná, M.; Augstenová, B.; Kratochvíl, L. Cytogenetics of the Javan file snake (*Acrochordus javanicus*) and the evolution of snake sex chromosomes. *J. Zool. Syst. Evol. Res.* **2017**. [CrossRef]
9. Vicoso, B.; Emerson, J.J.; Zektser, Y.; Mahajan, S.; Bachtrog, D. Comparative sex chromosome genomics in snakes: Differentiation, evolutionary strata, and lack of global dosage compensation. *PLoS Biol.* **2013**, *11*, e1001643. [CrossRef] [PubMed]
10. Gamble, T.; Castoe, T.A.; Nielsen, S.V.; Banks, J.L.; Card, D.C.; Schield, D.R.; Schuett, G.W.; Booth, W. The discovery of XY sex chromosomes in a boa and report the discovery of XY sex chromosomes in a boa and python. *Curr. Biol.* **2017**, *27*, 1–6. [CrossRef] [PubMed]
11. Matsubara, K.; Nishida, C.; Matsuda, Y.; Kumazawa, Y. Sex chromosome evolution in snakes inferred from divergence patterns of two gametologous genes and chromosome distribution of sex chromosome-linked repetitive sequences. *Zool. Lett.* **2016**, *2*, 19. [CrossRef] [PubMed]
12. Laopichienpong, N.; Muangmai, N.; Chanhome, L.; Suntrarachun, S.; Twilprawat, P.; Peyachoknagul, S.; Srikulnath, K. Evolutionary dynamics of the gametologous CTNNB1 gene on the Z and W chromosomes of snakes. *J. Hered.* **2017**, *108*, 142–151. [PubMed]
13. Cioffi, M.B.; Kejnovsky, E.; Bertollo, L.A.C. The chromosomal distribution of microsatellite repeats in the genome of the wolf fish *Hoplias malabaricus*, focusing on the sex chromosomes. *Cytogenet. Genome Res.* **2011**, *132*, 289–296. [CrossRef] [PubMed]
14. Matsubara, K.; O'Meally, D.; Azad, B.; Georges, A.; Sarre, S.D.; Graves, J.A.M.; Matsuda, Y.; Ezaz, T. Amplification of microsatellite repeat motifs is associated with the evolutionary differentiation and heterochromatinization of sex chromosomes in Sauropsida. *Chromosoma* **2016**, *125*, 111–123. [CrossRef] [PubMed]
15. Pokorná, M.; Kratochvíl, L.; Kejnovský, E. Microsatellite distribution on sex chromosomes at different stages of heteromorphism and heterochromatinization in two lizard species (Squamata: Eublepharidae: *Coleonyx elegans* and Lacertidae: *Eremias velox*). *BMC Genet.* **2011**, *12*, 90. [CrossRef] [PubMed]
16. Singh, L.; Purdom, F.; Jones, K.W. Satellite DNA and evolution of sex chromosomes. *Chromosoma* **1976**, *59*, 43–62. [CrossRef] [PubMed]
17. Gamble, T.; Geneva, A.J.; Glor, R.E.; Zarkower, D. *Anolis* sex chromosomes are derived from a single ancestral pair. *Evolution* **2014**, *68*, 1027–1041. [CrossRef] [PubMed]

18. Subramanian, S.; Mishra, R.K.; Singh, L. Genome-wide analysis of *Bkm* sequences (GATA repeats): Predominant association with sex chromosomes and potential role in higher order chromatin organization and function. *Bioinformatics* **2003**, *19*, 681–685. [CrossRef] [PubMed]
19. O'Meally, D.; Patel, H.R.; Stiglec, R.; Sarre, S.D.; Georges, A.; Marshall Graves, J.A.; Ezaz, T. Non-homologous sex chromosomes of birds and snakes share repetitive sequences. *Chromosom. Res.* **2010**, *18*, 787–800. [CrossRef] [PubMed]
20. Wiens, J.J.; Kuczynski, C.A.; Smith, S.A.; Mulcahy, D.G.; Sites, J.W.; Townsend, T.M.; Reeder, T.W. Branch lengths, support, and congruence: Testing the phylogenomic approach with 20 nuclear loci in snakes. *Syst. Biol.* **2008**, *57*, 420–431. [CrossRef] [PubMed]
21. Pyron, R.A.; Hendry, C.R.; Chou, V.M.; Lemmon, E.M.; Lemmon, A.R.; Burbrink, F.T. Effectiveness of phylogenomic data and coalescent species-tree methods for resolving difficult nodes in the phylogeny of advanced snakes (Serpentes: Caenophidia). *Mol. Phylogenet. Evol.* **2014**, *81*, 221–231. [CrossRef] [PubMed]
22. Ezaz, T.; Deakin, J.E. Repetitive sequence and sex chromosome evolution in vertebrates. *Adv. Evol. Biol.* **2014**, 1–9. [CrossRef]
23. Rovatsos, M.; Kratochvíl, L.; Altmanová, M.; Johnson Pokorná, M. Interstitial telomeric motifs in squamate reptiles: When the exceptions outnumber the rule. *PLoS ONE* **2015**, *10*, e0134985. [CrossRef] [PubMed]
24. Bolzán, A.D. Interstitial telomeric sequences in vertebrate chromosomes: Origin, function, instability and evolution. *Mutat. Res.* **2017**, *773*, 51–65. [CrossRef] [PubMed]
25. Rovatsos, M.T.; Marchal, J.A.; Romero-Fernández, I.; Fernández, F.J.; Giagia-Athanosopoulou, E.B.; Sánchez, A. Rapid, independent, and extensive amplification of telomeric repeats in pericentromeric regions in karyotypes of arvicoline rodents. *Chromosome Res.* **2011**, *19*, 869–882. [CrossRef] [PubMed]
26. Dumas, F.; Cuttaia, H.; Sineo, L. Chromosomal distribution of interstitial telomeric sequences in nine neotropical primates (Platyrrhini): Possible implications in evolution and phylogeny. *J. Zool. Syst. Evol. Res.* **2016**, *54*, 226–236. [CrossRef]
27. Mazzoleni, S.; Schillaci, O.; Sineo, L.; Dumas, F. Distribution of interstitial telomeric sequences in primates and the pygmy tree shrew (Scandentia). *Cytogenet. Genome Res.* **2017**, *151*, 141–150. [CrossRef] [PubMed]
28. Ocalewicz, K.; Furgala-Selezniow, G.; Szmyt, M.; Lisboa, R.; Kucinski, M.; Lejk, A.M.; Jankun, M. Pericentromeric location of the telomeric DNA sequences on the European grayling chromosomes. *Genetica* **2013**, *141*, 409–416. [CrossRef] [PubMed]
29. Bruschi, D.; Rivera, M.; Lima, A.; Zúñiga, A.; Recco-Pimentel, S. Interstitial Telomeric Sequences (ITS) and major rDNA mapping reveal insights into the karyotypical evolution of Neotropical leaf frogs species (*Phyllomedusa*, Hylidae, Anura). *Mol. Cytogenet.* **2014**, *7*, 22. [CrossRef] [PubMed]
30. Schmid, M.; Steinlein, C. Chromosome banding in Amphibia. XXXIV. Intrachromosomal telomeric DNA sequences in Anura. *Cytogenet. Genome Res.* **2016**, *148*, 211–226. [CrossRef] [PubMed]
31. Pokorná, M.; Rens, W.; Rovatsos, M.; Kratochvíl, L. A ZZ/ZW sex chromosome system in the *Thick-tailed gecko* (*Underwoodisaurus milii*; Squamata: Gekkota: Carphodactylidae), a member of the ancient gecko lineage. *Cytogenet. Genome Res.* **2014**, *142*, 190–196. [CrossRef] [PubMed]
32. Rovatsos, M.; Johnson Pokorná, M.; Altmanová, M.; Kratochvíl, L. Female heterogamety in Madagascar chameleons (Squamata: Chamaeleonidae: *Furcifer*): Differentiation of sex and neo-sex chromosomes. *Sci. Rep.* **2015**, *5*, 13196. [CrossRef] [PubMed]
33. Matsubara, K.; Uno, Y.; Srikulnath, K.; Matsuda, Y.; Miller, E.; Olsson, M. No interstitial telomeres on autosomes but remarkable amplification of telomeric repeats on the W sex chromosome in the sand lizard (*Lacerta agilis*). *J. Hered.* **2015**, *106*, 753–757. [CrossRef] [PubMed]
34. Young, M.J.; O'Meally, D.; Sarre, S.D.; Georges, A.; Ezaz, T. Molecular cytogenetic map of the central bearded dragon, *Pogona vitticeps* (*Squamata: Agamidae*). *Chromosome Res.* **2013**, *21*, 361–374. [CrossRef] [PubMed]
35. Beçak, W.; Beçak, M.L. Cytotaxonomy and chromosomal evolution in *Serpentes*. *Cytogenetics* **1969**, *8*, 247–262. [CrossRef] [PubMed]
36. Matsubara, K.; Uno, Y.; Srikulnath, K.; Seki, R.; Nishida, C.; Matsuda, Y. Molecular cloning and characterization of satellite DNA sequences from constitutive heterochromatin of the habu snake (*Protobothrops flavoviridis*, Viperidae) and the Burmese python (*Python bivittatus*, Pythonidae). *Chromosoma* **2015**, *124*, 529–539. [CrossRef] [PubMed]
37. Shine, R.; Bull, J.J. Skewed sex ratios in snakes. *Copeia* **1977**, 228–234. [CrossRef]

38. Mengden, G.A. Linear differentiation of the C-band pattern of the W chromosome in snakes and birds. *Chromosoma* **1981**, *83*, 275–287. [CrossRef] [PubMed]
39. Itoh, M.; Sasaki, M.; Makino, S. The chromosomes of some Japanese snakes, with special regard to sexual dimorphism. *Jpn. J. Genet.* **1970**, *45*, 121–128. [CrossRef]
40. Kawai, A.; Nishida-Umehara, C.; Ishijima, J.; Tsuda, Y.; Ota, H.; Matsuda, Y. Different origins of bird and reptile sex chromosomes inferred from comparative mapping of chicken Z-linked genes. *Cytogenet. Genome Res.* **2007**, *117*, 92–102. [CrossRef] [PubMed]
41. Kelly, C.M.R.; Branch, W.R.; Broadley, D.G.; Barker, N.P.; Villet, M.H. Molecular systematics of the African snake family *Lamprophiidae* Fitzinger, 1843 (Serpentes: Elapoidea), with particular focus on the genera *Lamprophis* Fitzinger 1843 and *Mehelya* Csiki 1903. *Mol. Phylogenet. Evol.* **2011**, *58*, 415–426. [CrossRef] [PubMed]
42. Greenbaum, E.; Portillo, F.; Jackson, K.; Kusamba, C. A phylogeny of Central African *Boaedon* (Serpentes: Lamprophiidae), with the description of a new cryptic species from the Albertine Rift. *Afr. J. Herpetol.* **2015**, *64*, 18–38. [CrossRef]
43. Nagy, Z.T.; Sonet, G.; Glaw, F.; Vences, M. First large-scale DNA barcoding assessment of reptiles in the biodiversity hotspot of Madagascar, based on newly designed COI primers. *PLoS ONE* **2012**, *7*. [CrossRef] [PubMed]
44. Koubová, M.; Pokorná, M.J.; Rovatsos, M.; Farkačová, K.; Altmanová, M.; Kratochvíl, L. Sex determination in Madagascar geckos of the genus *Paroedura* (*Squamata: Gekkonidae*): Are differentiated sex chromosomes indeed so evolutionary stable? *Chromosome Res.* **2014**, *22*, 441–452. [CrossRef] [PubMed]
45. Burbrink, F.T.; Lawson, R.; Slowinski, J.B. Mitochondrial DNA Phylogeography of the polytypic North American rat snake (*Elaphe obsoleta*): A critique of the subspecies concept. *Evolution* **2000**, *54*, 2107–2118. [CrossRef] [PubMed]
46. Hall, T. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* **1999**, *41*, 95–98.
47. Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A.; Kumar, S. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* **2013**, *30*, 2725–2729. [CrossRef] [PubMed]
48. Librado, P.; Rozas, J. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **2009**, *25*, 1451–1452. [CrossRef] [PubMed]
49. Sumner, A.T. A simple technique for demonstrating centromeric heterochromatin. *Exp. Cell Res.* **1972**, *75*, 304–306. [CrossRef]
50. IJdo, J.W.; Baldini, A.; Ward, D.C.; Reeders, S.T.; Wells, R.A. Origin of human chromosome 2: An ancestral telomere-telomere fusion. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 9051–9055. [CrossRef] [PubMed]
51. O'Callaghan, N.J.; Fenech, M. A quantitative PCR method for measuring absolute telomere length. *Biol. Proced. Online* **2011**, *13*, 3. [CrossRef] [PubMed]
52. Rovatsos, M.; Altmanová, M.; Pokorná, M.; Kratochvíl, L. Conserved sex chromosomes across adaptively radiated *Anolis lizards*. *Evolution* **2014**, *68*, 2079–2085. [CrossRef] [PubMed]
53. Cawthon, R.M. Telomere measurement by quantitative PCR. *Nucleic Acids Res.* **2002**, *30*, 47e. [CrossRef]
54. De Smet, W.H.O. Chromosomes of 23 species of snakes. *Acta Zool. Pathol. Antverp.* **1978**, *70*, 85–118.
55. Singh, L.; Purdom, I.F.; Jones, K.W. Sex chromosome associated satellite DNA: Evolution and conservation. *Chromosoma* **1980**, *79*, 137–157. [CrossRef] [PubMed]
56. Altmanová, M.; Rovatsos, M.; Kratochvíl, L.; Johnson Pokorná, M. Minute Y chromosomes and karyotype evolution in Madagascan iguanas (Squamata: Iguania: Opluridae). *Biol. J. Linn. Soc.* **2016**, *118*, 618–633. [CrossRef]
57. Augstenová, B. *Evolution of Karyotypes and Sex Chromosomes in Snakes*; Charles University: Prague, Czech Republic, 2017; pp. 1–70.
58. Singh, L. The charms of sex chromosomes in snakes. *J. Biosci.* **2011**, *36*, 17–21. [CrossRef] [PubMed]
59. Rollings, N.; Uhrig, E.J.; Krohmer, R.W.; Waye, H.L.; Mason, R.T.; Olsson, M.; Whittington, C.M.; Friesen, C.R. Age-related sex differences in body condition and telomere dynamics of red-sided garter snakes. *Proc. R. Soc. B Biol. Sci.* **2017**, *284*, 20162146. [CrossRef] [PubMed]



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Chapter 2

Rovatsos M., **Augstenová B**., Altmanová M., Sloboda M., Kodym P., Kratochvíl L. (2018): Triploid colubrid snake provides insight into the mechanism of sex determination in advanced snakes. *Sexual Development*, 12: 251-255.

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Triploid Colubrid Snake Provides Insight into the Mechanism of Sex Determination in Advanced Snakes

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Keywords
Female heterogamety · Reptiles · Sex chromosomes · Triploidy

Abstract
The advanced snakes (Caenophidia), the important amniote lineage encompassing more than 3,000 living species, possess highly conserved female heterogamety across all families. However, we still lack any knowledge on the gene(s) and the molecular mechanism controlling sex determination. Triploid individuals spontaneously appear in populations of diploid species and can provide an important insight into the evolution of sex determination. Here, we report a case of spontaneous triploidy in a male of the twin-spotted ratsnake (*Elaphe bimaculata*) with ZZW sex chromosomes. We speculate that as both ZZ and ZZW individuals develop male gonads, the ratio between the number of Z chromosomes and autosomes, and not the presence of the W chromosome in the genome, drives sex determination in the advanced snakes.

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The advanced snakes (Caenophidia), the important amniote lineage encompassing more than 3,000 living species, possess female heterogamety with ZZ/ZW sex chromosomes or derived multiple sex chromosomes [Matsubara et al., 2006; Oguiura et al., 2009; O'Meally et al., 2010; Pokorná et al., 2014]. Well-differentiated ZZ/ZW sex chromosomes are highly conserved for about 60 my across all families of caenophidian snakes [Rovatsos et al., 2015a]. The W chromosome is heterochromatic, and in most lineages it has strong accumulations of repetitive elements including the $(GATA)_n$ motif [Epplen et al., 1982; O'Meally et al., 2010; Augstenová et al., 2018; Rovatsos et al., 2018]. The gene content of the Z chromosome and to a lesser degree of the W chromosome is known [Matsubara et al., 2006; Vicoso et al., 2013; Rovatsos et al., 2015a]. However, we still lack any knowledge on the gene(s) and the molecular mechanism controlling sex determination in caenophidian snakes.

Two hypotheses, the 'Z Dosage' and 'Dominant W', have been proposed to explain sex determination in groups with female heterogamety [Clinton, 1998]. According to the 'Z Dosage' hypothesis, the sex determining locus is located on the Z chromosome, and the sex is determined by the ratio between the number of Z chromosomes and autosomes (Z:A ratio). In lineages without global dosage compensation, such as birds and snakes [Ellegren, 2002; Vicoso et al., 2013], the expression of the assumed masculinizing Z-specific sex determining gene should be correlated with gene copy numbers and hence considerably higher in males with 2 copies of the Z chromosome than in females with only a single copy.

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Under the 'Dominant W' hypothesis, a dominant ovary-determining locus should be linked to the W chromosome, and an individual should develop into a female when the W chromosome is present in the genome. Currently, the 'Z Dosage' hypothesis is more generally accepted in birds, as it was shown that *dmrt1*, the gene involved in the testis development pathway present on the avian Z and missing on their W, likely plays a crucial role in sex determination in chicken [Smith et al., 2009]. Nevertheless, the exact mechanism of avian sex determination is not totally clarified yet, and the existence of a W-linked gene that triggers femaleness is still possible [Arlt et al., 2004; Ellegren, 2011; Küpper et al., 2012]. Moreover, we lack evidence that all avian lineages share the same sex determination mechanism. Even less is known about the prevalent sex determination mechanism in advanced snakes.

Chromosomal rearrangements, such as polyploidy or aneuploidy of sex chromosomes, are especially informative for uncovering the mechanism of sex determination. Spontaneous triploidy caused by dispermic fertilization of an egg or abnormal meiosis during gametogenesis was reported in sauropsids including birds [Stenberg and Saura, 2013]. In the majority of cases, spontaneous triploidy in amniotes leads to death at a young age, and only a small percentage of triploids reach maturity [Bonaminio and Fechheimer, 1993; Baumer et al., 2000; Trukhina and Smirnov, 2014]. Adult triploid ZZZ and ZZW chicken and triploid ZZZ zebra finches usually develop a male phenotype often with abnormal gonads, although ovotestes were also reported [Ohno et al., 1963]. However, a triploid ZZW shorebird and triploid ZZW great reed warblers were fertile females [Arlt et al., 2004; Küpper et al., 2012]. These discrepancies can point to various systems of sex determination in birds. Nevertheless, we have to keep in mind that also the techniques of the detection of triploids varied across these studies.

In squamates (lizards and snakes), spontaneous triploid individuals were uncovered in the viperid snake *Agkistrodon piscivorus* [Tiersch and Figiel, 1991] and several iguanian species. Squamates include also several all-triploid asexual lineages, including the non-caenophidian blind snake *Indotyphlops braminus* [Olmo and Signorino, 2005 and citations therein]. In such cases, triploidy is often the result of mating between a female of diploid obligatory asexual hybrid species with a male of one of the sexual ancestor [Neaves and Baumann, 2011].

Here, we report the case of a spontaneous triploidy in a colubrid snake providing new insights into the evolution of sex determination in advanced snakes.

Material and Methods

During cytogenetic screening, chromosomal suspensions were prepared from 3 fully grown captive-bred individuals of the twin-spotted ratsnake (*Elaphe bimaculata*) by whole blood cultivation [Rovatsos et al., 2015b]. Chromosomes were stained by Giemsa, and karyograms were constructed using the Ikaros software (Metasystems). The W chromosome was verified by the presence of constitutive heterochromatin after C-banding and the accumulation of the $(GATA)_n$ microsatellite motif by FISH [Rovatsos et al., 2015b]. The phenotypic sex was tested by cloacal probing and in the case of one individual also by gonad inspection and histology.

We estimated the differences in gene copy numbers for 3 Z-linked genes (*adarb2*, *itgb1*, *tanc2*) in genomic DNA between individuals by qPCR using the single-copy gene *eef1a1* as a reference and the autosomal gene *mecom* as a control. The qPCR protocol and the calculations of the gene copy number were published previously [Rovatsos et al., 2015a].

We confirmed the species identity of the snakes by sequencing the 'barcoding' region of the mitochondrial gene *cytochrome c oxidase* subunit I (*cox1*) using the reptile-specific primers RepCOI-F/RepCOI-R [Nagy et al., 2012]. The PCR reaction protocol and amplification conditions were reported previously [Koubová et al., 2014]. PCR products were sequenced bi-directionally by Macrogen (Korea). The sequences were aligned using BioEdit v5.0.9 [Hall, 1999] and subsequently analyzed in MEGA v6.0.5 [Tamura et al., 2013] revealing a single haplotype. The haplotype sequence was deposited in GenBank under the accession number MH469728.

Results

The partial sequence of *cox1* confirmed that all 3 snakes are twin-spotted ratsnakes. A BLAST search was performed to compare our sequences with those from previous studies, revealing 99% similarity to the *cox1* sequence of the *E. bimaculata* deposited previously in GenBank (KM065513.1) [Yan et al., 2016]. In 2 individuals, the karyotype with 2n = 36 consists of 16 macrochromosomes (14 biarmed and 2 acrocentric) and 20 microchromosomes (Fig. 1a). The 4th largest chromosome pair is heteromorphic, consisting of 1 metacentric and 1 submetacentric, fully heterochromatic chromosome (Fig. 1b) with a strong accumulation of the $(GATA)_n$ microsatellite motif (Fig. 1c), which is typical for the snake W chromosome. A similar chromosome morphology was reported in other species of the genus *Elaphe* [Olmo and Signorino, 2005].

The third individual had an unexpected karyotype with 54 chromosomes in all examined metaphases (>40). All macrochromosomes can be grouped to triplets (Fig. 1d), revealing a rare case of spontaneous triploidy. The C-banding pattern (Fig. 1e) and the distribution of the $(GATA)_n$ microsatellite motif (Fig. 1f) showed a single W chromosome per metaphase. Two Z chromosomes were identified

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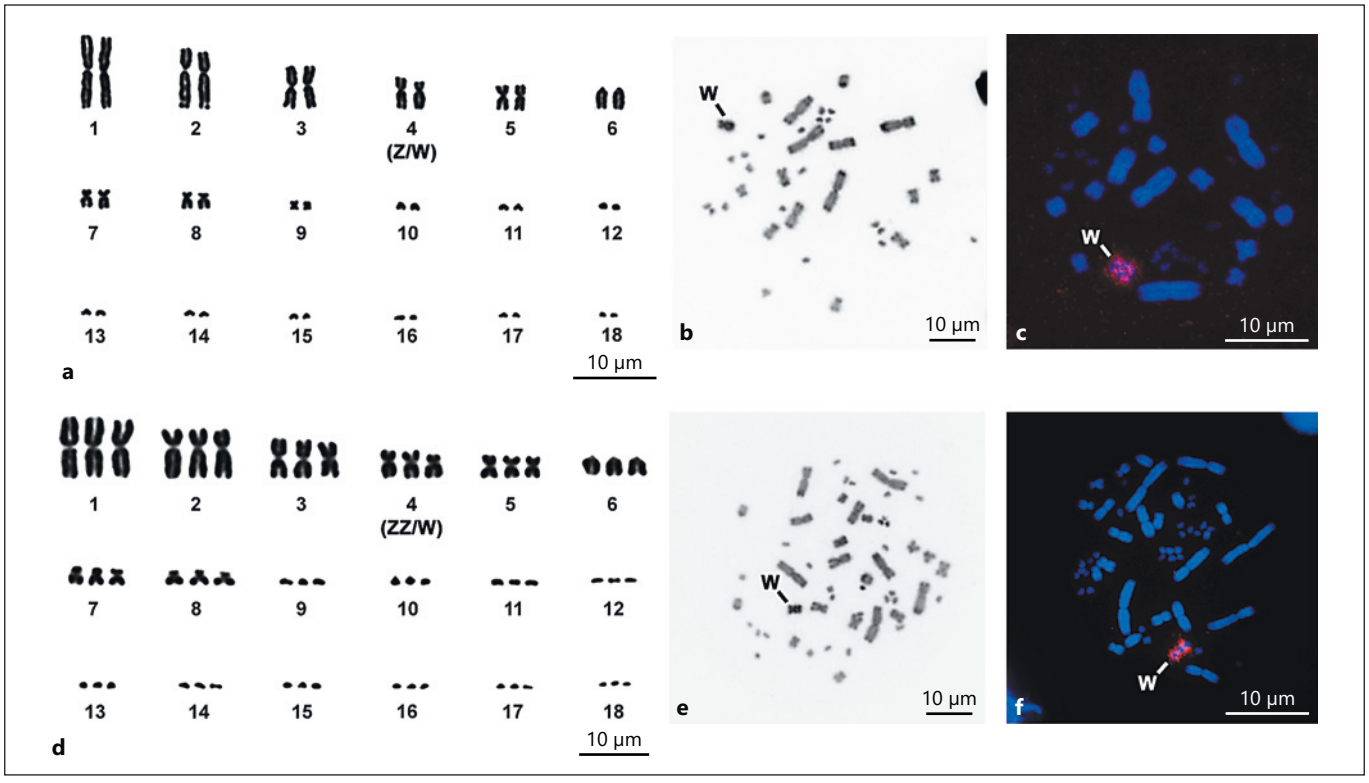


Fig. 1. Karyogram (**a**, **d**), C-banding (**b**, **e**), and in situ hybridization with $(GATA)_n$ microsatellite motif (**c**, **f**) on metaphases from diploid (2n) (**a–c**) and triploid (3n) (**d–f**) twin-spotted ratsnake (*E. bimaculata*), respectively. Sex chromosomes are indicated when recognizable.

based on the chromosome morphology per metaphase (Fig. 1d). The triploidy did not emerge during blood cell cultivation, as we repeated the whole procedure of chromosome preparation in this individual once more with the same results. The difference in Z chromosome copies in the genomes of the diploid and the triploid ratsnakes was detected by qPCR applied to DNA isolated from blood samples. We found a relatively lower gene copy ratio for all tested Z-linked genes in the genome of the diploid female snake (ZW) compared to the triploid (ZZW) (Fig. 2; Table 1).

The triploid twin-spotted ratsnake was born in captivity and was at least 6 years old when it died spontaneously. It was shorter than the other 2 individuals (females) of the same age kept together, and it had a swollen eye, notably shorter tail, and malformed vertebral column. During dissection, we found that its right kidney was untypically small (approximately 2.5 cm long), much smaller than its left kidney (approximately 7.5 cm in length). The right adrenal gland was detached from the kidney, placed approximately 14 cm more cranially. The hemi-

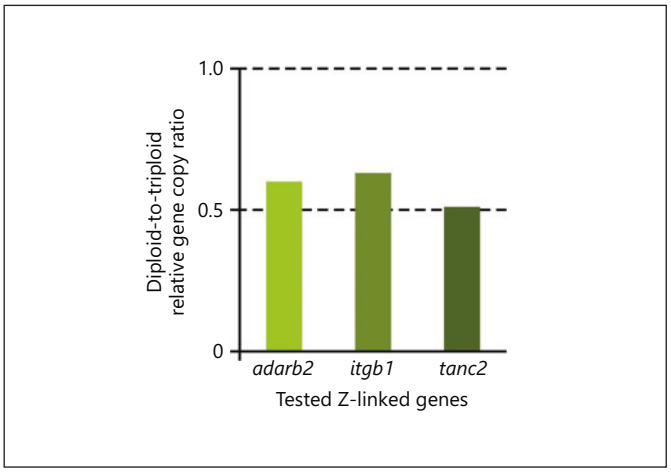


Fig. 2. Relative gene copy ratio between diploid (ZW) and triploid (ZZW) twin-spotted ratsnake (*E. bimaculata*) estimated by qPCR for the 3 Z-linked genes *adarb2*, *itgb1*, and *tanc2*.

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Table 1. The results of qPCR demonstrating differences in copy number of 3 Z-specific genes (*adarb2, itgb1, tanc2*) in blood cells between diploid ZW females and a triploid ZZW male relatively to *eef1a1*, the gene *mecom* serves as an autosomal control

Primer pair	Diploid ZW female			Triploid ZZW male			Diploid/triploid ratio in gene copy numbers
	mean Cp	St. Dev. Cp	rate for *EF1a3*	mean Cp	St. Dev. Cp	rate for *EF1a3*	
ef1a_1	29.28	0.15	–	28.69	0.14	–	–
mecom_5	25.39	0.11	14.84	24.84	0.18	14.44	1.03
adarb2_1	26.60	0.15	6.39	25.27	0.01	10.69	0.60
itgb1_3	33.52	0.32	0.05	32.26	0.09	0.08	0.63
tanc2_2	28.25	0.29	2.04	26.69	0.07	4.00	0.51

All samples were run in triplicates, average and standard deviation are given. Cp, crossing point; St. Dev. Cp, standard deviation of crossing point.

penes were relatively small (approximately 0.5 cm). The snake possessed only a single gonad resembling a testis in the left side of the body. Histological sections were performed by Exomed, Berlin, Germany and confirmed that the gonad was a malformed testis (Fig. 3). The organ consists of a network of connective tissue (rete testis) and lobular structures with channels lined with Leydig cells. Active spermatogenesis was not present.

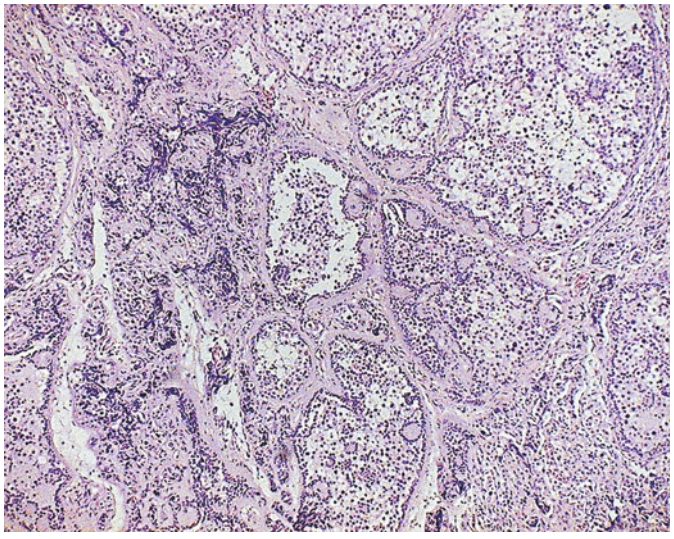


Fig. 3. Histological section of the testis of the triploid *E. bimaculata*. The organ consists of a network of connective tissue (rete testis) and lobular structures with channels lined with Leydig cells. Active spermatogenesis is not present.

Discussion

We provide first evidence of a triploid caenophidian snake with ZZW sex chromosomes supported by both cytogenetic and molecular data. The only other case of spontaneous triploidy in the caenophidian snakes was reported in a cottonmouth pit viper (*A. piscivorus*) based on flow cytometry, and no data on karyotype and phenotypic sex of this individual were presented there [Tiersch and Figiel, 1991].

Few spontaneous triploids reach adulthood in vertebrates [Bonaminio and Fechheimer, 1993; Baumer et al., 2000; Trukhina and Smirnov, 2014]. Although diploid-triploid mosaicism is known in other reptiles [Bezy and Camarillo, 2002], the qPCR results of blood and the untypical anatomy of several organs of the examined individual suggested that the individual could be totally triploid. The size of its hemipenes estimated by a cloacal probe was typical for females, but histology confirmed the gonad was undoubtedly a testis. This situation points out that cloacal probing, the standard procedure for snake sexing, might not be reliable for individuals with non-typical genotypes. Assuming triploidy in the gonads of this individual, we can speculate that since ZW individuals are females and the triploid ZZW individual possessed a testis, the ratio between the number of Z chromosomes and autosomes and not the presence of the W chromosome in the genome drives the sex determination in this species. As all families of advanced snakes share homologous Z chromosomes [Rovatsos et al., 2015a], they might share the same sex determination system with a contribution of the 'Z Dosage' mechanism. Nevertheless, as the gonad of our individual was not functional, we can speculate that sex determination even in *E. bimaculata* might be more complex, with a contribution of both 'Dominant W' and 'Z Dosage' mechanisms. It is notable that although polyploidy is quite frequent in squamates, there is only a single known polyploid species, *I. braminus*, among snakes

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[Wynn et al., 1987], which is not a member of the lineage Caenophidia. However, some non-caenophidian snakes possess a different sex determination system [Rovatsos et al., 2015a; Gamble et al., 2017]. In any case, the molecular mechanisms of sex determination in snakes deserve further study. Our contribution suggests that searching for a dominant regulatory W-linked factor might not be fruitful in at least some lineages of caenophidian snakes.

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Statement of Ethics

The work was approved by the Ethical Committee of the Czech Republic (protocol No. 35484/2015–16).

Disclosure Statement

The authors have no conflicts of interest to declare.

References

Arlt D, Bensch S, Hansson B, Hasselquist D, Westerdahl H: Observation of a ZZW female in a natural population: implications for avian sex determination. Proc R Soc B Biol Sci 271:S249–S251 (2004).

Augstenová B, Mazzoleni S, Kratochvíl L, Rovatsos M: Evolutionary dynamics of the W chromosome in caenophidian snakes. Genes 9:5 (2018).

Baumer A, Balmer D, Binkert F, Schinzel A: Parental origin and mechanisms of formation of triploidy: a study of 25 cases. Eur J Hum Genet 8:911–917 (2000).

Bezy RL, Camarillo JL: Systematics of xantusiid lizards of the genus *Lepidophyma*. Contrib Sci 493:1–41 (2002).

Bonaminio G, Fechheimer N: The gonadal histology of triploid chicken (*Gallus domesticus*) embryos. Genet Sel Evol 25:205–210 (1993).

Clinton M: Sex determination and gonadal development: a bird's eye view. J Exp Zool 281:457–465 (1998).

Ellegren H: Dosage compensation: do birds do it as well? Trends Genet 18:25–28 (2002).

Ellegren H: Sex-chromosome evolution: recent progress and the influence of male and female heterogamety. Nat Rev Genet 12:157–166 (2011).

Epplen JT, McCarrey JR, Sutou S, Ohno S: Base sequence of a cloned snake W-chromosome DNA fragment and identification of a male specific putative mRNA in the mouse. Proc Natl Acad Sci USA 79:3798–3802 (1982).

Gamble T, Castoe TA, Nielsen SV, Banks JL, Card DC, et al: The discovery of XY sex chromosomes in a boa and python. Curr Biol 27:2148–2153 (2017).

Hall T: BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98 (1999).

Koubová M, Johnson Pokorná M, Rovatsos M, Farkačová K, Altmanová M, Kratochvíl L: Sex determination in Madagascar geckos of the genus *Paroedura* (Squamata: Gekkonidae): are differentiated sex chromosomes indeed so evolutionary stable? Chromosome Res 22:441–452 (2014).

Küpper C, Augustin J, Edwards S, Székely T, Kosztolányi A, et al: Triploid plover female provides support for a role of the W chromosome in avian sex determination. Biol Lett 8:787–789 (2012).

Matsubara K, Tarui H, Toriba M, Yamada K, Nishida-Umehara C, et al: Evidence for different origin of sex chromosomes in snakes, birds, and mammals and step-wise differentiation of snake sex chromosomes. Proc Natl Acad Sci USA 103:18190–18195 (2006).

Nagy ZT, Sonet G, Glaw F, Vences M: First large-scale DNA barcoding assessment of reptiles in the biodiversity hotspot of Madagascar, based on newly designed COI primers. PLoS One 7:e34506 (2012).

Neaves WB, Baumann P: Unisexual reproduction among vertebrates. Trends Genet 27:81–88 (2011).

Oguiura N, Ferrarezzi H, Batistic RF: Cytogenetics and molecular data in snakes: a phylogenetic approach. Cytogenet Genome Res 127:128–142 (2009).

Ohno S, Kittrell WA, Christian LC, Stenius C, Witt GA: An adult triploid chicken (*Gallus domesticus*) with a left ovotestis. Cytogenetics 2:42–49 (1963).

Olmo E, Signorino GG: Chromorep: A reptile chromosomes database. http://chromorep.univpm.it (2005). Accessed 26 November 2016.

O'Meally D, Patel HR, Stiglec R, Sarre SD, Georges A, et al: Non-homologous sex chromosomes of birds and snakes share repetitive sequences. Chromosome Res 18:787–800 (2010).

Pokorná M, Altmanová M, Kratochvíl L: Multiple sex chromosomes in the light of female meiotic drive in amniote vertebrates. Chromosome Res 22:35–44 (2014).

Rovatsos M, Vukić J, Lymberakis P, Kratochvíl L: Evolutionary stability of sex chromosomes in snakes. Proc R Soc B Biol Sci 282:20151992 (2015a).

Rovatsos M, Johnson Pokorná M, Kratochvíl L: Differentiation of sex chromosomes and karyotype characterisation in the dragonsnake *Xenodermus javanicus* (Squamata: Xenodermatidae). Cytogenet Genome Res 147:48–54 (2015b).

Rovatsos M, Altmanová M, Johnson Pokorná M, Augstenová B, Kratochvíl L: Cytogenetics of the Javan file snake (*Acrochordus javanicus*) and the evolution of snake sex chromosomes. J Zool Syst Evol Res 56:117–125 (2018).

Smith CA, Roeszler KN, Ohnesorg T, Cummins DM, Farlie PG, et al: The avian Z-linked gene *DMRT1* is required for male sex determination in the chicken. Nature 461:267–271 (2009).

Stenberg P, Saura A: Meiosis and its deviations in polyploid animals. Cytogenet Genome Res 140:185–203 (2013).

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S: MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729 (2013).

Tiersch TR, Figiel CR: A triploid snake. Copeia 1991:838–841 (1991).

Trukhina AV, Smirnov AF: Problems of birds sex determination. Nat Sci 6:1232–1240 (2014).

Vicoso B, Emerson JJ, Zektser Y, Mahajan S, Bachtrog D: Comparative sex chromosome genomics in snakes: differentiation, evolutionary strata, and lack of global dosage compensation. PLoS Biol 11:e1001643 (2013).

Wynn AH, Cole CJ, Gardner AL: Apparent triploidy in the unisexual Brahminy blind snake, *Ramphotyphlops braminus*. Am Mus Nov 2868:1–7 (1987).

Yan L, Geng Z-Z, Yan P, Wu X-B: The complete mitochondrial genome of *Elaphe bimaculata* (Reptilia, Serpentes, Colubridae). Mitochondrial DNA 27:1285–1286 (2016).

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Chapter 3

Augstenová B., Johnson Pokorná M., Altmanová M., Frynta D., Rovatsos M., Kratochvíl L. (2018): ZW, XY, and yet ZW: Sex chromosome evolution in snakes even more complicated. *Evolution*, 72: 1701-1707.

BRIEF COMMUNICATION

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ZW, XY, and yet ZW: Sex chromosome evolution in snakes even more complicated

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Snakes are historically important in the formulation of several central concepts on the evolution of sex chromosomes. For over 50 years, it was believed that all snakes shared the same ZZ/ZW sex chromosomes, which are homomorphic and poorly differentiated in "basal" snakes such as pythons and boas, while heteromorphic and well differentiated in "advanced" (caenophidian) snakes. Recent molecular studies revealed that differentiated sex chromosomes are indeed shared among all families of caenophidian snakes, but that boas and pythons evolved likely independently male heterogamety (XX/XY sex chromosomes). The historical report of heteromorphic ZZ/ZW sex chromosomes in a boid snake was previously regarded as ambiguous. In the current study, we document heteromorphic ZZ/ZW sex chromosomes in a boid snake. A comparative approach suggests that these heteromorphic sex chromosomes evolved very recently and that they are poorly differentiated at the sequence level. Interestingly, two snake lineages with confirmed male heterogamety possess homomorphic sex chromosomes, but heteromorphic sex chromosomes are present in both snake lineages with female heterogamety. We point out that this phenomenon is more common across squamates. The presence of female heterogamety in non-caenophidian snakes indicates that the evolution of sex chromosomes in this lineage is much more complex than previously thought, making snakes an even better model system for the evolution of sex chromosomes.

KEY WORDS: **Heterochiasmy, heteromorphic sex chromosomes, reptiles, sex determination, Squamata.**

Amniotes, that is, mammals and sauropsids (= reptiles including birds), possess a wide diversity of sex determination systems. They include lineages where the sex of an individual is set according to environmental conditions as well as those possessing genotypic sex determination with male (XX/XY sex chromosomes) or female (ZZ/ZW) heterogamety. Knowledge on the distribution and phylogeny of sex determination systems across amniotes is still incomplete, preventing the reliable reconstruction of amniote ancestral sex determination and transitions among sex determination systems (cf. Ezaz et al. 2016; Johnson Pokorná and Kratochvíl 2016). However, there is robust molecular evidence that some lineages of amniotes such as viviparous mammals (Cortez et al. 2014), birds (Zhou et al. 2014), trionychid turtles (Rovatsos et al. 2017), iguanas (Rovatsos et al. 2014; Marin et al. 2017; Altmanová et al. 2018), and lacertid lizards (Rovatsos et al. 2016a) possess long-term stability of independently evolved sex chromosomes. For more than 50 years and until very recently, snakes were considered as a group with only a single sex-determination system, namely ZZ/ZW sex chromosomes (Ohno 1967). Molecular evidence for ZZ/ZW sex chromosomes was only available for caenophidian snakes (Vicoso et al. 2013; Rovatsos et al. 2015a), the group comprising of about 90% of extant snake species (Uetz and Hošek 2017). In this clade, all families share homologous highly differentiated ZZ/ZW sex

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chromosomes that evolved in their common ancestor at least 60 MYA (Rovatsos et al. 2015a, 2018; Augstenová et al. 2018). It was generally believed that in parallel to the situation in birds (Zhou et al. 2014), "basal" snakes such as pythons and boas share the same, albeit poorly differentiated, sex chromosomes (see, e.g., Ohno 1967; Matsubara et al. 2006, 2016; Vicoso et al. 2013). Recently, evidence for male, not female, heterogamety began to be accumulated from snakes outside Caenophidia. The inheritance of a color trait compatible with male heterogamety was reported in the ball python (*Python regius*) (Mallery 2014; Mallery and Carrillo 2016) and sex-linked loci were discovered in the boas *Boa constrictor* and *Boa imperator* and Burmese python *Python bivittatus* (Gamble et al. 2017). Interestingly, XX/XY sex chromosomes were found to be represented by different linkage groups in the python and the boa, and only the boa shares the gene content of sex chromosomes with caenophidian snakes (Gamble et al. 2017). The previous historical report of heteromorphic ZZ/ZW chromosomes in the other non-caenophidian, Dumeril´s boa (*Acrantophis dumerili*), was interpreted by Gamble et al. (2017) as dubious, potentially reflecting at that time the automatic expectation of the presence of ZZ/ZW sex chromosomes in all snakes.

We performed classical and molecular cytogenetic analyses across the Madagascan boas, the family Sanziniidae (alternatively assigned as the subfamily Sanziniinae of the family Boidae in Zheng and Wiens [2016]) to investigate whether differentiated sex chromosomes evolved in this lineage, which kind of heterogamety they possess, and to gain a greater understanding of the evolution of sex chromosomes in snakes.

Material and Methods

The family Sanziniidae contains four formally described species from two genera (Orozco-Terwengel et al. 2008). We based our study on *Sanzinia madagascariensis, Acrantophis madagascariensis, A. dumerili*, and *A.* sp. cf. *dumerili* (in total, we examined cytogenetically 23 individuals; see Table S1 for the list). The populations morphologically similar to *A. dumerili* were reported to be paraphyletic with respect to *A. madagascariensis* based on molecular evidence (Vences and Glaw 2003; Orozco-Terwengel et al. 2008; Nagy et al. 2012) and the lineage sister to *A. madagascariensis* was assigned as *A.* sp. cf. *dumerili* by Orozco-Terwengel et al. (2008). In this lineage, we sampled eight offspring (four males, four females) of a single female. In the other species, we examined animals with different origins based on their availability for cytogenetic examination (Table S1). For taxon identification, we applied DNA barcoding using partial sequences of mtDNA locus cytochrome oxidase subunit 1 (COI) following Nagy et al. (2012). Sequencing was performed bidirectionally in Macrogen, Korea (Macrogen, Inc., South Korea). The COI sequences were aligned using BioEdit version 5.0.9 (Hall 1999) and haplotypes were revealed in DnaSP version 5.10.1 (Librado and Rozas 2009). Genetic distances among haplotypes were calculated using p-distances in MEGA version 6.0.5 (Tamura et al. 2013). Sex was determined based on external morphology (pelvic spurs in males), cloacal probing, and in some cases, it was confirmed from the breeding history of the individual or through dissection.

Metaphase chromosomes were prepared from whole blood cell cultures according to the protocol described in Pokorná et al. (2014) and Koubová et al. (2014). The chromosomes were Giemsa-stained and photographed using a Provis AX70 fluorescence microscope equipped with a digital camera (Olympus DP30BW). C-banding was applied following Sumner (1972) to uncover heterochromatic regions, which are often accumulated on differentiated sex chromosomes. Karyograms were prepared using Ikaros software (MetaSystems, Germany). To identify sex-specific differences in sequence composition, we performed comparative genomic hybridization (CGH) using the protocol described in Pokorná et al. (2014) in *A.* sp. cf. *dumerili* and *S. madagascariensis*.

Results

The analysis of the COI sequences from snakes morphologically resembling *A. dumerili* revealed two haplotypes. A Basic Local Alignment Search (BLAST; Altschul et al. 1990) search showed that each haplotype has high similarity to *A. dumerili* and *A.* sp. cf. *dumerili*, respectively. The two haplotypes show net genetic p-distance of 5.1%, which is in accordance to a previous study (Nagy et al. 2012). Haplotype sequences are deposited in GenBank under the accession numbers MH469729 and MH469730.

Our cytogenetic analysis revealed that all studied species exhibited karyotypes with 34 chromosomes in a diploid state (Figs. 1, S1, and S2) as previously reported for *S. madagascariensis* and a member of the genus *Acrantophis*, probably *A.* sp. cf. *dumerili* (Mengden and Stock 1980). No heteromorphic pairs were found in *S. madagascariensis*, *A. madagascariensis*, and *A. dumerili.* The fourth largest pair in *A.* sp. cf. *dumerili* is polymorphic with metacentric chromosomes in all studied males, but with one metacentric and one subtelocentric chromosome in all females (Figs. 1 and S1). We identified the subtelocentric chromosome as the W chromosome based on its distribution indicating a significant linkage of this polymorphism to sex. Assuming 1:1 inheritance of the subtelocentric chromosome, the probability of finding the subtelocentric chromosome exclusively in examined females, not in males, only by chance is very small, $P = (1/2)^8 = 0.004$. The other chromosome of the pair was assigned as the Z chromosome. Also the two-tailed test of the sex

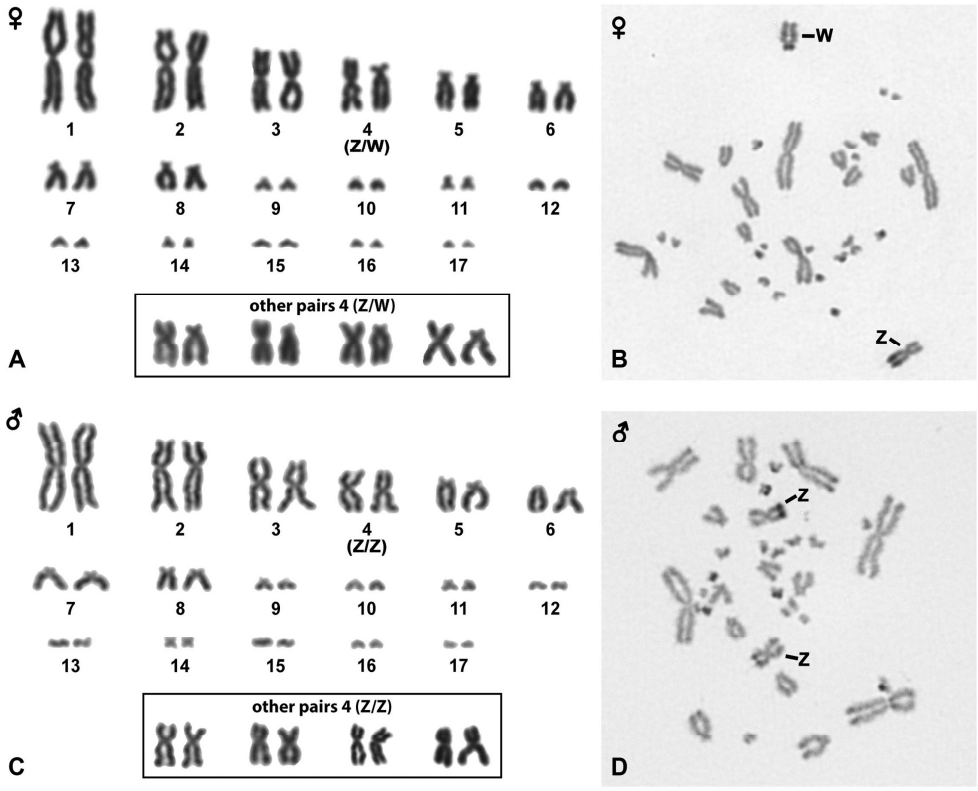


Figure 1. Giemsa-stained karyotype (a, c) and C-banded metaphases (b, d) of a female (a, b) and a male (c, d) of *Acrantophis* sp. cf. *dumerili*. Note the heteromorphic, partially heterochromatic sex chromosomes. Sex chromosomes from other metaphases are presented in each individual to demonstrate better the shape and size differences between Z and W.

linkage of the subtelomeric chromosome is significant even at the level of $\alpha = 0.01$ ($P = 2/256 = 0.0078$) and would stay significant after the correction for a multiple testing in four species (but any multiple testing did not happen in this case, as there is no comparable polymorphism to test in other three species). We also identified polymorphism of the Z chromosome (shorter/longer form of the chromosome) although the shorter form was rarer in our material and was observed only in one female individual (Fig. S1). The CGH experiments did not reveal any notable differences between male and female genomes at sequence level in *A*. sp. cf. *dumerili* nor in *S. madagascariensis* (Figs. S1 and S2).

Discussion

We found evidence for heteromorphic ZZ/ZW sex chromosomes in only one lineage of boas from the family Sanziniidae. The karyotype with the heteromorphic sex chromosomes corresponds to the historical record by Mengden and Stock (1980) published nearly 40 years ago. It is likely that these authors studied the still not formally described *A*. sp. cf. *dumerili*, which was at that time not distinguished from *A*. *dumerili.* The lack of heteromorphic sex chromosomes in other lineages of the family Sanziniidae, including *A. madagascariensis* and *A. dumerili*, suggests that

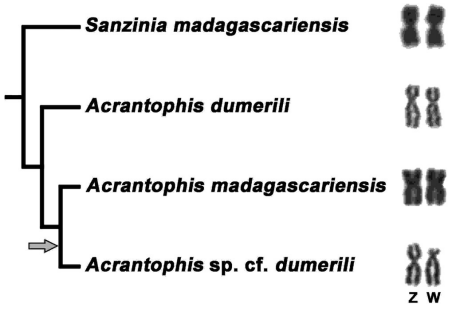


Figure 2. Phylogenetic relationships of the studied sanziniid snakes and morphology of their chromosomes of the fourth largest chromosomal pairs. The arrow indicates the origin of heteromorphic sex chromosomes. The phylogeny follows Orozco-Terwengel et al. (2008).

heteromorphic sex chromosomes are an apomorphy of *A*. sp. cf. *dumerili* (Fig. 2). We also noted a polymorphism in the Z chromosome. Its shorter form was found only in a single female (Fig. S1), which likely inherited it from her father. The unusual rare occurrence of this shorter form of the Z chromosome might point to multiple paternity in the clutch. The subtelocentric W chromosome evolved likely as an evolutionary novelty through pericentric inversion or another chromosomal rearrangement in this clade.

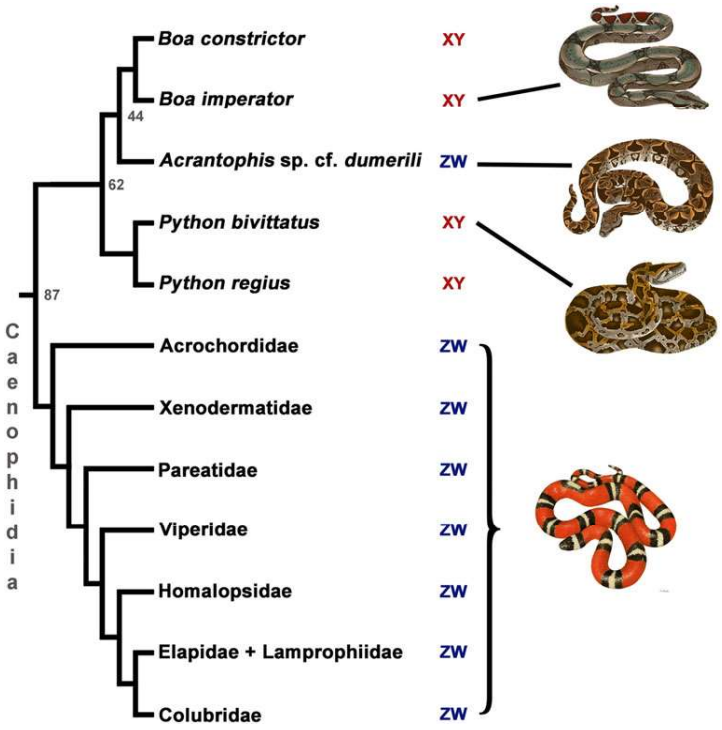


Figure 3. Phylogeny of snakes with genetic evidence for sex chromosomes. The numbers indicate the estimated divergence times in millions of years. The data are compiled from Rovatsos et al. (2015a), Mallery and Carillo (2016), Gamble et al. (2017), and this study. The phylogenetic relationships and divergence times are taken from Zheng and Wiens (2016).

This happened likely very recently, as the genetic distance between *A*. sp. cf. *dumerili* and other members of the genus *Acrantophis* is very small, only slightly above 1% in the fragment of 16S rRNA gene (Orozco-Terwengel et al. 2008). The location of the sex determining region in *A*. sp. cf. *dumerili* is not yet known, it can lie within, or be linked to the region morphologically differentiated between Z and W. Chromosomal rearrangements often prevent or at least impair recombination between homologous chromosomes. Recombination cessation leads progressively to gene loss and the accumulation of repetitive sequences such as telomeric and microsatellite motifs as documented in the W of caenophidian snakes (Augstenová et al. 2018). In *A*. sp. cf. *dumerili*, CGH did not detect any notable sequence dissimilarities between the Z and W (Fig. S1), which is in accordance with the recent origin of these highly heteromorphic sex chromosomes.

The discovery of female heterogamety in the Booidea superfamily indicates that the evolution of sex chromosomes in snakes is not only much more complex than it was thought for the last 50 years (Ohno 1967), but even much more so than reconstructed by Gamble et al. (2017). We can conclude that female heterogamety is present in two snake lineages (caenophidian snakes and *A*. sp. cf. *dumerili*) and male heterogamety in another two (*B. constrictor*/*B. imperator* and *P. bivittatus*/*P. regius*). The phylogenetic distribution (Fig. 3) suggests that there were at least two independent transitions between male and female heterogamety in snakes. Evidence from facultative parthenogenesis suggests that male heterogamety is more widespread in boid snakes and pythons. As far as is known, facultative asexuality in amniotes leads to progeny homozygous in all loci (Booth et al. 2014). Lineages with ZZ/ZW sex chromosomes and degenerated W thus reproduce via facultative asexuality male progeny, while exclusively female progeny can be expected under male heterogamety. Also, only all-female parthenogenetically produced progeny was reported in species of the genera *Python, Epicrates, Boa*, and *Eunectes* (Booth and Schuett 2016; Shibata et al. 2017, and other studies referred therein), which suggests that male heterogamety with poorly differentiated sex chromosomes might have been present in non-caenophidian snakes for at least 30 MY (the estimation of the split between the genera *Boa–Eunectes* and *Epicrates*; according to Zheng and Wiens [2016]). Current knowledge suggests that the XX/XY sex chromosomes of *P. bivittatus* and the ZZ/ZW sex chromosomes of the caenophidian snakes share gene content, whereas the gene content of the sex chromosomes of *B. constrictor*/*B. imperator* is different (Gamble et al. 2017). In most caenophidian snakes, sex chromosomes are the fourth largest pair in the karyotype (Augstenová et al. 2018), which we found also in *A*. sp. cf. *dumerili.* It is thus possible that the same sex chromosomes homologous to sex chromosomes of the caenophidian snakes were present in the common ancestor of Booidea and Caenophidia and due to the course of evolution, they

underwent shifts between male and female heterogamety. The sex chromosomes of *B. constrictor*/*B. imperator* would then evolve via sex chromosome turnover. This scenario needs to be tested in future studies.

Generally, the situation in snakes appears to support the model that poorly differentiated sex chromosomes are not stable and are prone to turnovers even between male and female heterogamety (Miura 2007), but once sex chromosomes cross the line in differentiation as in the common ancestor of caenophidian snakes, sex chromosomes become very evolutionarily stable (Bull 1983; Bull and Charnov 1985; but see Vicoso and Bachtrog 2015; Altmanová et al. 2018). It is interesting that both snake lineages with female heterogamety (*A.* sp. cf. *dumerili* and caenophidian snakes) have heteromorphic sex chromosomes, whereas snake lineages with male heterogamety (*B. constrictor*, *B. imperator*, and *P. bivittatus*) have homomorphic, cryptic sex chromosomes. Similar situations are known from other squamate lineages, for example, in chameleons it was recently reported that the species with male heterogamety have poorly differentiated homomorphic sex chromosomes, but two lineages with female heterogamety have heteromorphic sex chromosomes (Rovatsos et al. 2015b, Nielsen et al. 2018). Similarly, the majority of teiid lizards, presumably with male heterogamety, have only poorly differentiated sex chromosomes (Ezaz et al. 2009, Carvalho et al. 2015), whereas related lacertid lizards with female heterogamety have highly differentiated sex chromosomes (Pokorná et al. 2011; Rovatsos et al. 2016a,b). This observation is against the theoretical predictions that the Y chromosome should degenerate faster than the W chromosome, which could result from a stronger selection in males, male mutation bias, or a smaller effective population size of the Y chromosome with decreasing male to female ratio in a population (Bachtrog et al. 2011; Mank 2012; Wilson Sayres 2018). The phenomenon of an unequal rate of differentiation of sex chromosomes under male and female heterogamety deserves further experimental and theoretical work. One possible testable explanation is that the rate of sex chromosome differentiation might reflect a pattern of heterochiasmy (sexual differences in recombination rates) in a given section of the genome. This hypothesis predicts that the genome parts that later became sex chromosomes in chameleons and snakes recombined ancestrally more in males than in females and this ancestral situation led to a more rapid differentiation of sex chromosomes under female than under male heterogamety.

AUTHOR CONTRIBUTION

MJP and LK designed the study, which was later elaborated by all other coauthors. All authors contributed to experimental part and data acquisition. The cytogenetic analyses were performed by BA, MJP, MA, MR, and LK. MR analyzed sequences. All authors contributed to interpretation of the data. The first draft of the manuscript was written by BA and LK, all authors contributed to revisions of the draft and approved the final version.

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DATA ARCHIVING

Haplotype sequences are deposited in GenBank under the accession numbers MH469729 and MH469730.

LITERATURE CITED

Altmanová, M., M. Rovatsos, M. Johnson Pokorná, M. Veselý, F. Wagner, and L. Kratochvíl. 2018. All iguana families with the exception of basilisks share sex chromosomes. Zoology 126:98–102.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403–410.

Augstenová, B., S. Mazzoleni, L. Kratochvíl, and M. Rovatsos. 2018. Evolutionary dynamics of the W chromosome in caenophidian snakes. Genes 9:5. https://doi.org/10.3390/genes9010005.

Bachtrog, D., M. Kirkpatrick, J. Mank, J. C. Pires, W. Rice, and N. Valenzuela. 2011. Are all sex chromosomes created equal? Trends Genet. 27:350–357.

Booth, W., and G. W. Schuett. 2016. The emerging phylogenetic pattern of parthenogenesis in snakes. Biol. J. Linn. Soc. 118:172–186.

Booth, W., G. W. Schuett, A. Ridgway, D. W. Buxton, T. A. Castoe, G. Bastone, C. Bennett, and W. McMahan. 2014. New insights on facultative parthenogenesis in pythons. Biol. J. Linn. Soc. 112:461–468.

Bull, J. J. 1983. Evolution of sex determining mechanisms. Benjamin-Cummings Publishing, Menlo Park, CA.

Bull, J. J., and E. L. Charnov. 1985. On irreversible evolution. Evolution 39: 1149–1155.

Carvalho, N. D. M., J. A. Federico, F. A. da Silva, C. H. Schneider, and M. C. Gross. 2015. Cytogenetic analyses of five amazon lizard species of the subfamilies Teiinae and Tupinambinae and review of karyotyped diversity the family Teiidae. Comp. Cytogenet. 9:625–644.

Cortez, D., R. Marin, D. Toledo-Flores, L. Froidevaux, A. Liechti, P. D. Waters, F. Grützner, and H. Kaessmann. 2014. Origins and functional evolution of Y chromosomes across mammals. Nature 508:488–493.

Ezaz, T., S. D. Sarre, D. O'Meally, J. A. Marshall Graves, and A. Georges. 2009. Sex chromosome evolution in lizards: independent origins and rapid transitions. Cytogenet. Genome Res. 127:249–260.

Ezaz, T., K. Srikulnath, and J. A. Marshall Graves. 2016. Origin of amniote sex chromosomes: an ancestral super-sex chromosome, or common requirements? J. Hered. 108:94–105.

Gamble, T., T. A. Castoe, S. V. Nielsen, J. L. Banks, D. C. Card, D. R. Schield, G. W. Schuett, and W. Booth. 2017. The discovery of XY sex chromosomes in a boa and python. Curr. Biol. 27:2148–2153.

Hall, T. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41:95–98.

Johnson Pokorná, M., and L. Kratochvíl. 2016. What was the ancestral sex-determining mechanism in amniote vertebrates? Biol. Rev. 91:1–12.

Koubová, M., M. Johnson Pokorná, M. Rovatsos, K. Farkačová, M. Altmanová, and L. Kratochvíl. 2014. Sex determination in Madagascar geckos of the genus *Paroedura* (Squamata: Gekkonidae): are differentiated sex chromosomes indeed so evolutionary stable? Chromosome Res. 22:441–452.

Librado, P., and J. Rozas. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451–1452.

Mallery, C. S., Jr. 2014. Following the chromosomes of inheritance and sex-linkage: an explanation of coral glow/banana. Pp. 115–132 *in* K. McCurley, ed. The ultimate ball python: morph maker guide. Ecouniverse Herpetological Publishing and Distribution, Rodeo, NM.

Mallery, C. S., Jr., and M. M. Carrillo. 2016. A case study of sex-linkage in *Python regius* (Serpentes: Boidae), with new insights into sex determination in Henophidia. Phyllomedusa 15:29–42.

Mank, J. E. 2012. Small but mighty: the evolutionary dynamics of W and Y sex chromosomes. Chromosome Res. 20:21–33.

Marin, R., D. Cortez, F. Lamanna, M. M. Pradeepa, E. Leushkin, P. Julien, A. Liechti, J. Halbert, T. Brüning, K. Mössinger, et al. 2017. Convergent origination of a *Drosophila*-like dosage compensation mechanism in a reptile lineage. Genome Res. 27:1974–1987.

Matsubara, K., H. Tarui, M. Toriba, K. Yamada, C. Nishida-Umehara, K. Agata, and Y. Matsuda. 2006. Evidence for different origin of sex chromosomes in snakes, birds, and mammals and step-wise differentiation of snake sex chromosomes. Proc. Natl. Acad. Sci. USA 103:18190–18195.

Matsubara, K., C. Nishida, Y. Matsuda, and Y. Kumazawa. 2016. Sex chromosome evolution in snakes inferred from divergence patterns of two gametologous genes and chromosome distribution of sex chromosome-linked repetitive sequences. Zoological Lett. 2:19.

Mengden, G. A., and A. D. Stock. 1980. Chromosomal evolution in Serpentes; a comparison of G and C chromosome banding patterns of some colubrid and boid genera. Chromosoma 79:53–64.

Miura, I. 2007. An evolutionary witness: the frog *Rana rugosa* underwent change of heterogametic sex from XY male to ZW female. Sex. Dev. 1:323–331.

Nagy, Z.T., G. Sonet, F. Glaw, and M. Vences. 2012. First large-scale DNA barcoding assessment of reptiles in the biodiversity hotspot of Madagascar, based on newly designed COI primers. PLoS ONE 7: e34506.

Nielsen, S. V., J. L. Banks, R. E. Diaz Jr., P. A. Trainor, and T. Gamble. 2018. Dynamic sex chromosomes in Old World chameleons (Squamata: Chamaeleonidae). J. Evol. Biol. 31:484–490. https://doi.org/10.1111/jeb.13242.

Ohno, S. 1967. Sex chromosomes and sex-linked genes. Springer-Verlag, Berlin.

Orozco-Terwengel, P., Z. T. Nagy, D. R. Vieites, M. Vences, and E. Louis Jr. 2008. Phylogeography and phylogenetic relationships of Malagasy tree and ground boas. Biol. J. Linn. Soc. 95:640–652.

Pokorná, M., L. Kratochvíl, and E. Kejnovský. 2011. Microsatellite distribution on sex chromosomes at different stages of heteromorphism and heterochromatinization in two lizard species (Squamata: Eublepharidae: *Coleonyx elegans* and Lacertidae: *Eremias velox*). BMC Genet. 12:90.

Pokorná, M., W. Rens, M. Rovatsos, and L. Kratochvíl. 2014. A ZZ/ZW sex chromosome system in the thick-tailed gecko (*Underwoodisaurus milii*; Squamata: Gekkota: Carphodactylidae), a member of the ancient gecko lineage. Cytogenet. Genome Res. 142:190–196.

Rovatsos, M., M. Pokorná, M. Altmanová, and L. Kratochvíl. 2014. Cretaceous park of sex determination: sex chromosomes are conserved across iguanas. Biol. Lett. 10:20131093. https://doi.org/10.1098/rsbl.2013.1093.

Rovatsos, M., J. Vukić, P. Lymberakis, and L. Kratochvíl. 2015a. Evolutionary stability of sex chromosomes in snakes. Proc. Biol. Sci. 282:20151992. https://doi.org/10.1098/rspb.2015.1992.

Rovatsos, M., M. Johnson Pokorná, M. Altmanová, and L. Kratochvíl. 2015b. Female heterogamety in Madagascar chameleons (Squamata: Chamaeleonidae: *Furcifer*): differentiation of sex and neo-sex chromosomes. Sci. Rep. 5:13196. https://doi.org/10.1038/srep13196.

Rovatsos, M., J. Vukić, M. Altmanová, M. Johnson Pokorná, J. Moravec, and L. Kratochvíl. 2016a. Conservation of sex chromosomes in lacertid lizards. Mol. Ecol. 25:3120–3126.

Rovatsos, M., J. Vukić, and L. Kratochvíl. 2016b. Mammalian X homolog acts as sex chromosome in lacertid lizards. Heredity 117:8–13.

Rovatsos, M., P. Praschag, U. Fritz, and L. Kratochvíl. 2017. Stable Cretaceous sex chromosomes enable molecular sexing in soft-shell turtles (Testudines: Trionychidae). Sci. Rep. 7:42150. https://doi.org/10.1038/srep42150.

Rovatsos, M., M. Altmanová, M. Johnson Pokorná, B. Augstenová, and L. Kratochvíl. 2018. Cytogenetics of the Javan file snake (*Acrochordus javanicus*) and the evolution of snake sex chromosomes. J. Zool. Syst. Evol. Res. 56:117–125.

Shibata, H., S. Sakata, Y. Hirano, E. Nitasaka, and A. Sakabe. 2017. Facultative parthenogenesis validated by DNA analyses in the green anaconda (*Eunectes murinus*). PloS ONE 12:e0189654.

Sumner, A. T. 1972. A simple technique for demonstrating centromeric heterochromatin. Exp. Cell Res. 75:304–306.

Tamura, K., G. Stecher, D. Peterson, A. Filipski, and S. Kumar. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30:2725–2729.

Uetz, P., and J. Hošek. 2017. The reptile database. Available at http://reptile-database.org. Accessed February 1, 2018.

Vences, M, and F. Glaw. 2003. Phylogeography, systematics and conservation status of boid snakes from Madagascar (*Sanzinia* and *Acrantophis*). Salamandra 39:181–206.

Vicoso, B., and D. Bachtrog. 2015. Numerous transitions of sex chromosomes in Diptera. Plos Biology 13:e1002078.

Vicoso, B., J. J. Emerson, Y. Zektser, S. Mahajan, and D. Bachtrog. 2013. Comparative sex chromosome genomics in snakes: differentiation, evolutionary strata, and lack of global dosage compensation. PLoS Biol. 11:e1001643.

Wilson Sayres, M. A. 2018. Genetic diversity on the sex chromosomes. Genome Biol. Evol. 10:1064–1078.

Zheng, Y., and J. J. Wiens. 2016. Combining phylogenomic and supermatrix approaches, and a time-calibrated phylogeny for squamate reptiles (lizards and snakes) based on 52 genes and 4162 species. Mol. Phylogenet. Evol. 94:537–547.

Zhou, Q., J. Zhang, D. Bachtrog, N. An, Q. Huang, E. D. Jarvis, M. T. P. Gilbert, and G. Zhang. 2014. Complex evolutionary trajectories of sex chromosomes across bird taxa. Science 346:1246338. https://doi.org/10.1126/science.1246338.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Tables S1. Number and sex of cytogenetically studied individuals.

Figure S1. Giemsa-stained karyotypes of males and females of *Acrantophis* sp. cf. *dumerili* (a–f) and the results of comparative genomic hybridization (CGH) in the same species (g, female metaphase; h, male metaphase).

Figure S2. Giemsa-stained karyotypes and C-banded metaphases of a male and a female of *Acrantophis dumerili* (a, b, g, h), *Acrantophis madagascariensis* (c, d, i, j), and *Sanzinia madagascariensis* (e, f, k, l), and the results of comparative genomic hybridization (CGH) in *S. madagascariensis* (m, female metaphase; n, male metaphase).

Chapter 4

Augstenová B., Mazzoleni S., Kostmann A., Altmanová M., Frynta D., Kratochvíl L., Rovatsos M. (2019): Cytogenetic analysis did not reveal differentiated sex chromosomes in ten species of Boas and Pythons (Reptilia: Serpentes). *Genes*, 10*:* 934.

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Cytogenetic Analysis Did Not Reveal Differentiated Sex Chromosomes in Ten Species of Boas and Pythons (Reptilia: Serpentes)

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Abstract: Homologous and differentiated ZZ/ZW sex chromosomes (or derived multiple neo-sex chromosomes) were often described in caenophidian snakes, but sex chromosomes were unknown until recently in non-caenophidian snakes. Previous studies revealed that two species of boas (*Boa imperator*, *B. constrictor*) and one species of python (*Python bivittatus*) independently evolved XX/XY sex chromosomes. In addition, heteromorphic ZZ/ZW sex chromosomes were recently revealed in the Madagascar boa (*Acrantophis* sp. cf. *dumerili*) and putatively also in the blind snake *Myriopholis macrorhyncha*. Since the evolution of sex chromosomes in non-caenophidian snakes seems to be more complex than previously thought, we examined ten species of pythons and boas representing the families Boidae, Calabariidae, Candoiidae, Charinidae, Pythonidae, and Sanziniidae by conventional and molecular cytogenetic methods, aiming to reveal their sex chromosomes. Our results show that all examined species do not possess sex-specific differences in their genomes detectable by the applied cytogenetic methods, indicating the presence of poorly differentiated sex chromosomes or even the absence of sex chromosomes. Interestingly, fluorescence in situ hybridization with telomeric repeats revealed extensive distribution of interstitial telomeric repeats in eight species, which are likely a consequence of intra-chromosomal rearrangements.

Keywords: boa; comparative genomic hybridization; fluorescence in situ hybridization; evolution; karyotype; microsatellites; python; rDNA; sex chromosomes; sex determination; telomeres

1. Introduction

Snakes (Serpentes) are a clade of toxicoferan reptiles. With almost 3800 extant species, snakes represent about one third of all reptilian species [1]. Traditional taxonomy classifies snakes into three major lineages: Caenophidia, and the likely paraphyletic Scolecophidia and Henophidia [2–5]. Caenophidia is the most specious and diverse group, including more than 3100 species [1]. Scolecophidia is a group of approximately 400 species of blindsnakes with worm-like body shape and fossorial lifestyle. Henophidian snakes include about 200 species [1], recently divided into 21 families [3,6].

The diploid chromosomal number varies across snakes from 2n = 24 to 2n = 56 [7,8]. Phylogenetic reconstruction revealed that the ancestral snake karyotype consisted of 2n = 36 chromosomes with 16 macro- and 20 microchromosomes, which is the state commonly observed in the majority of the cytogenetically studied snake species [8].

Early pioneering cytogenetic studies revealed a ZZ/ZW sex determination system in several caenophidian snakes by conventional cytogenetic methods (e.g., karyotype reconstruction, C-banding) [7,8], which was later confirmed by molecular cytogenetic [9–12], bioinformatic [13], and molecular genetic [14] methods in all caenophidian families. The W chromosome of caenophidian snakes is highly degenerated and heterochromatic with accumulations of microsatellite motifs such as $(TTAGGG)_n$ and $(GATA)_n$, with variable topology and degree of accumulation among species [12]. On the contrary, sex-linked accumulation of repetitive sequences has not been reported so far in non-caenophidian snakes [11,15–17].

For a long time, it was assumed that non-caenophidian snakes exhibit ZZ/ZW sex determination with poorly differentiated sex chromosomes [13,18,19]. However, sporadic reports from non-caenophidian snakes, including the observations that (i) facultative parthenogenesis in pythons and boas leads to exclusively female progeny [20–23] and (ii) inheritance of a color mutation in the ball python, *Python regius* [24], indicate XX/XY sex determination. Recently, genomic studies confirmed the presence of XX/XY sex determination in three species of non-caenophidian snakes: the Burmese python *Python bivittatus,* the common boa *Boa constrictor,* and the northern boa *Boa imperator* [25]. Furthermore, the same study showed that the two boas and the python have non-homologous XX/XY sex chromosomes. However, a previous report of heteromorphic ZZ/ZW sex chromosomes in the Madagascar boa based on conventional cytogenetics [26] was recently confirmed by molecular cytogenetic methods in *Acrantophis* sp. cf. *dumerili* [27]. Another recent study showed that the scolecophidian long-nosed worm snake *Myriopholis macrorhyncha* may have heteromorphic ZZ/ZW sex chromosomes which are likely non-homologous to sex chromosomes of caenophidian snakes [28]. Therefore, in comparison to the long-term stability of the Z chromosome across all lineages of caenophidian snakes [14], the sex determination systems in non-caenophidian snakes are likely far less stable and more dynamic than was previously assumed.

In the present study, we examined the presence of differentiated sex chromosomes in ten species and one interspecific hybrid of snakes from six henophidian families: Boidae, Calabaridae, Candoiidae, Charinidae, Sanziniidae, and Pythonidae. We constructed karyograms and further explored their karyotypes by C-banding to reveal the distribution of constitutive heterochromatin. Furthermore, we applied comparative genomic hybridization (CGH) to identify sex-specific differences in the karyotypes and fluorescence in situ hybridization (FISH) with repetitive elements that often accumulate on the sex chromosomes of reptiles such as telomeric motifs, $(GATA)_n$ microsatellite repeats, and rDNA loci [12,29–31].

2. Materials and Methods

2.1. Studied Material, Chromosome Preparations, and Staining

We studied ten species belonging to six families of boas and pythons: *Chilabotrus angulifer*, *Eunectes notaeus* (Boidae), *Calabaria reinhardtii* (Calabariidae), *Candoia paulsoni* (Candoiidae), *Lichanura trivirgata* (Charinidae), *Morelia bredli*, *Morelia spilota* (Pythonidae), *Acrantophis dumerili*, *Acrantophis madagascariensis*, and *Sanzinia madagascariensis* (Sanziniidae) (Table 1). In addition, we studied an interspecific hybrid between *M. spilota* (father) and *M. bredli* (mother). Males and females were studied in all species with the exception of *M. bredli*, where we only had access to a female.

From all studied specimens, we collected 1–2 ml of peripheral blood which was used for DNA isolation and whole blood cell cultures. For DNA isolation, we used the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Mitotic chromosome suspensions were prepared from whole blood cell cultures according to the protocol described in Mazzoleni et al. [32].

Table 1. Summary of studied species and number of examined individuals.

Family	Species	♀	♂
Boidae	*Chilabothrus angulifer*	2	1
	Eunectes notaeus	1	2
Calabariidae	*Calabaria reinhardtii*	1	2
Candoiidae	*Candoia paulsoni*	1	1
Charinidae	*Lichanura trivirgata*	1	1
Sanziniidae	*Acrantophis dumerili*	7	3
	Acrantophis madagascariensis	2	1
	Sanzinia madagascariensis	1	1
Pythonidae	*Morelia bredli*	1	0
	Morelia spilota	3	4
	Morelia bredli × *Morelia spilota* hybrid	3	1

Chromosome preparations were stained by Giemsa for karyogram reconstruction. For visualization of constitutive heterochromatin, we applied C-banding following the protocol of Sumner [33] with slight modifications. Briefly, slides were treated in 0.2 N HCl for 30 minutes at room temperature, in 5% $Ba(OH)_2$ for 5 minutes at 45 °C, in 2× saline-sodium citrate (SSC) buffer for 1 h at 60 °C, washed with distilled water, and air-dried. The slides were stained by Fluoroshield with DAPI (4′,6-diamidino-2-phenylindole; Sigma-Aldrich, St. Louis, MO, USA). The results of C-banding from both sexes of *Acrantophis dumerili*, *Acrantophis madagascariensis*, and *Sanzinia madagascariensis* were published in the previous paper [27], and we did not repeat them here.

2.2. Fluorescence In Situ Hybridization with Telomeric Probe

We prepared the telomeric probe $(TTAGGG)_n$ by polymerase chain reaction (PCR) without a DNA template using the primers $(TTAGGG)_5$ and $(CCCTAA)_5$ according to the protocol of Ijdo et al. [34]. Primers were synthesized by Macrogen (Seoul, Korea). The probe was precipitated using salmon sperm, sodium acetate (3M), and ethanol. The precipitated probe was dissolved in hybridization buffer (50% formamide in 2× SSC).

Slides with chromosome spreads were treated according to our standard protocols described in Rovatsos et al. [35]. Briefly, the slides were washed in 2× SSC, treated with RNAse for 1 h at 37 °C, and washed in 2× SSC. The slides were subsequently treated with pepsin for 10 min at 37 °C, washed in phosphate buffered saline (PBS), incubated for 10 minutes in 1% solution of formaldehyde in 2× SSC, washed again in PBS, and then dehydrated using ethanol series. In the next step, the chromosomes were denatured in 70% formamide in 2× SSC at 75 °C for 4 min, washed in 2× SSC, and again dehydrated through ethanol series. Subsequently, we added 11 µL of probe to each slide and incubated the slides overnight at 37 °C. The following day, we washed the slides in 2× SSC and then in 50% formamide in 2× SSC at 40 °C (three times for 5 minutes). Slides were subsequently washed in 2× SSC and 4× SSC / 0.05% Tween20 (Sigma). Subsequently, the slides were incubated with 4× SSC / 5% blocking reagent (Roche, Basel, Switzerland) at 37 °C for 45 min. In the next step, we added 4× SSC / 5% blocking reagent containing avidin-FITC (Vector Laboratories, Burlingame, CA, USA). We amplified the fluorescence signal with a modified avidin-FITC/biotinylated anti-avidin system (Vector Laboratories, Burlingame, CA, USA). The slides were dehydrated by ethanol series and stained by Fluoroshield with DAPI.

2.3. Fluorescence In Situ Hybridization with rDNA Probe

The probe for FISH with rDNA loci 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* [36] and was labelled with dUTP-biotin using nick translation (Abbott Molecular, Lake Bluff, IL, USA). The rDNA probe was hybridized to chromosome preparations and detected according to the above described protocol.

2.4. Fluorescence In Situ Hybridization with $(GATA)_n$ Probe

The probe for the $(GATA)_n$ microsatellite motif was synthesized and biotin-labelled by Macrogen (Korea). The probe was resuspended in hybridization buffer (50% formamide, 10% sodium dodecyl sulphate (SDS), 10% dextran sulphate, Denhard's buffer, 2× SSC, pH 7). We followed the same protocol used for FISH with the telomeric probe, except that the post-hybridization washes were performed in 0.4% Nonidet P-40 in 2× SSC (Sigma-Aldrich) at 40 °C for 2 min and then in 0.1% Nonidet P-40 in 0.4× SSC at room temperature for 30s, instead of 50% formamide in 2× SSC.

2.5. Comparative Genomic Hybridization

The protocol followed Rovatsos et al. [37]. Briefly, male and female genomic DNA were labelled with biotin-deoxyuridine triphosphate (dUTP) and digoxigenin-dUTP, respectively, using a Nick Translation Kit (Abbott Laboratories, Lake Bluff, IL, USA). 1 µg of male and 1 µg of female labelled genomic DNA were co-precipitated overnight with 5 µL of salmon sperm DNA (10 mg/mL, Sigma), 10 µL of 3M sodium acetate, and 2.5× volume of ethanol. After precipitation, the dry pellets were resuspended in 22 µL of hybridization buffer (50% formamide, 2× SSC, 10% SDS, 10% dextran sulfate, 1× Denhardt's buffer, pH 7), denatured at 75 °C for 10 min, and subsequently chilled on ice for 10 min prior to hybridization. In parallel, the slides with chromosome preparations were treated with RNase and pepsin, fixed with 4% formaldehyde, dehydrated through a 70%, 85%, and 100% ethanol series, denatured in 70% formamide / 2× SSC at 75 °C for 3 min, and dehydrated again. 11 µL of the probe (concentration approximately 300–700 ng of labeled DNA) was applied to the slide per drop of chromosomal suspension and incubated at 37 °C for 48 h. Post-hybridization washes were performed in 50% formamide / 2× SSC at 42 °C and in 2× SSC. Each slide was incubated with 100 µL of 4× SSC / 5% blocking reagent (Roche) at 37 °C for 30 min and then with 100 µL of detection solution 4× SSC / 5% blocking reagent including 2 µL of avidin-FITC (Vector Laboratories, Burlingame, CA, USA) and 10 µL of anti-digoxigenin-rhodamine (Roche, Basel, Switzerland) at 37 °C for 30 min. The slides were washed in 4× SSC / 0.05% Tween 20, dehydrated through an ethanol series, and air dried. Finally, the slides were mounted with Fluoroshield antifade medium containing DAPI (Sigma-Aldrich, St. Louis, MO, USA). The results of CGH in *S. madagascariensis* and *Acrantophis* sp. cf. *dumerili* (closely related to *A. dumerili* and *A. madagascariensis*) were published by us in the previous paper [27]; therefore, we do not present the results of CGH for sanziniid snakes here. We were not able to perform CGH in *Morelia bredli*, where we had access to only a single sex; however, CGH was performed in the interspecific hybrids, whose mother was *M. bredli* and father *M. spilota*.

2.6. Microscopy and Image Analyses

Zeiss Axio Imager Z2, equipped with automatic Metafer-MSearch scanning platform (MetaSystems) and a CoolCube 1 b/w digital camera (MetaSystems, Altlussheim, Germany) was used to capture Giemsa-stained metaphases. Karyograms were prepared with software Ikaros (MetaSystems). A Provis AX70 (Olympus, Tokyo, Japan) fluorescence microscope equipped with a DP30BW digital camera (Olympus) was used to capture images from the FISH experiments, which were subsequently processed in DP MANAGER imaging software (Olympus).

3. Results

The results are presented in the figures according to the technique used in the order: Giemsa-stained karyograms of previously cytogenetically unstudied species (Figure 1), C-banding (Figure 2), distribution of $(TTAGGG)_n$ motifs (Figure 3), rDNA accumulations (Figure 4) and $(GATA)_8$ motifs, and CGH (Figure 6).

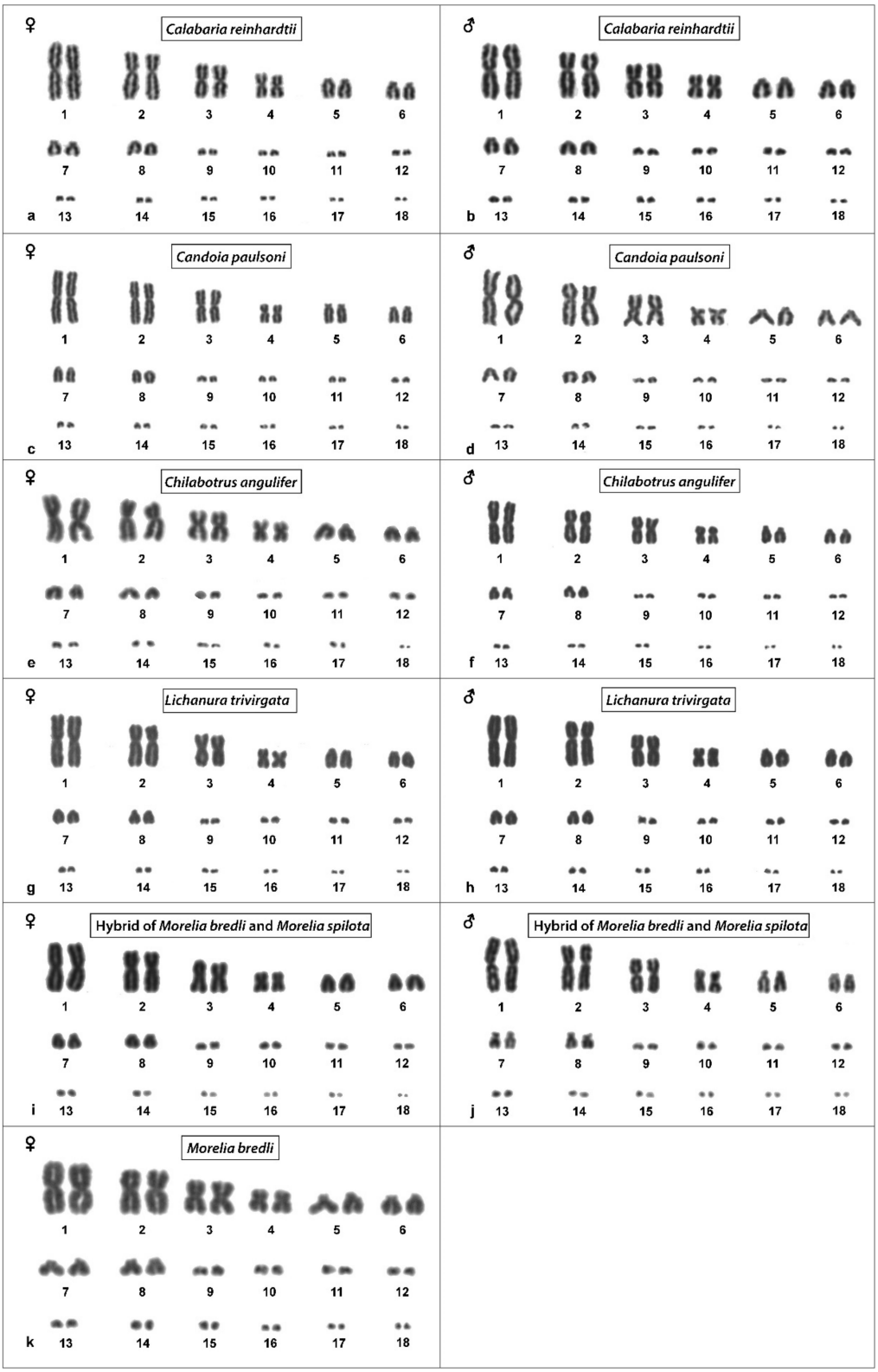


Figure 1. Giemsa-stained karyograms of *Calabaria reinhardtii* (**a**,**b**), *Candoia paulsoni* (**c**,**d**), *Chilabothrus angulifer* (**e**,**f**), *Lichanura trivirgata* (**g**,**h**), *Morelia bredli* × *M. spilota* hybrid (**i**,**j**) and *Morelia bredli* (**k**).

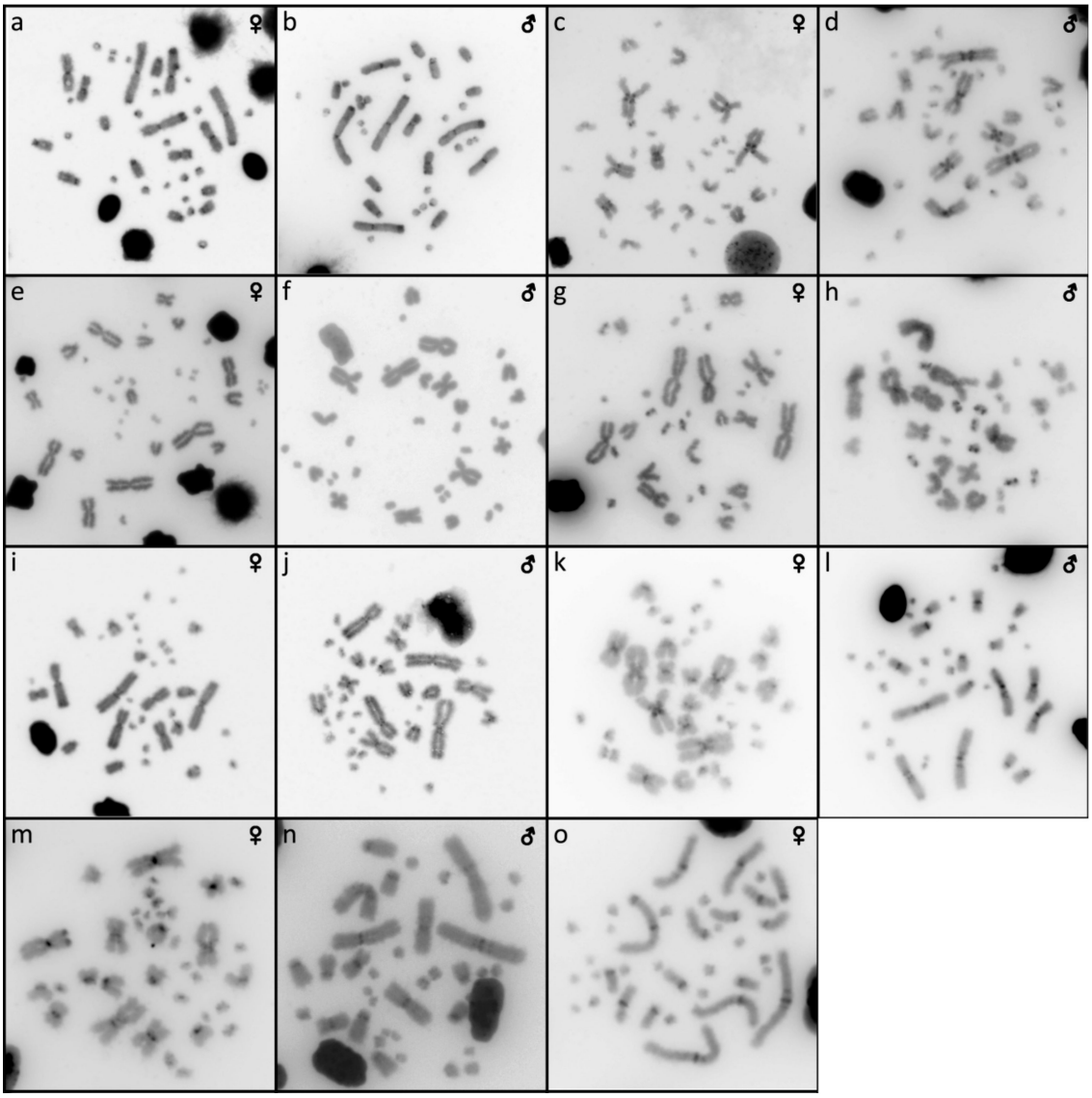


Figure 2. Distribution of heterochromatic blocks uncovered by C-banding in metaphases of *Calabaria reinhardtii* (**a**,**b**), *Candoia paulsoni* (**c**,**d**), *Chilabothrus angulifer* (**e**,**f**), *Eunectes notaeus* (**g**,**h**), *Lichanura trivirgata* (**i**,**j**), *Morelia spilota* (**k**,**l**), *Morelia bredli* × *M. spilota* hybrid (**m**,**n**) and *Morelia bredli* (**o**).

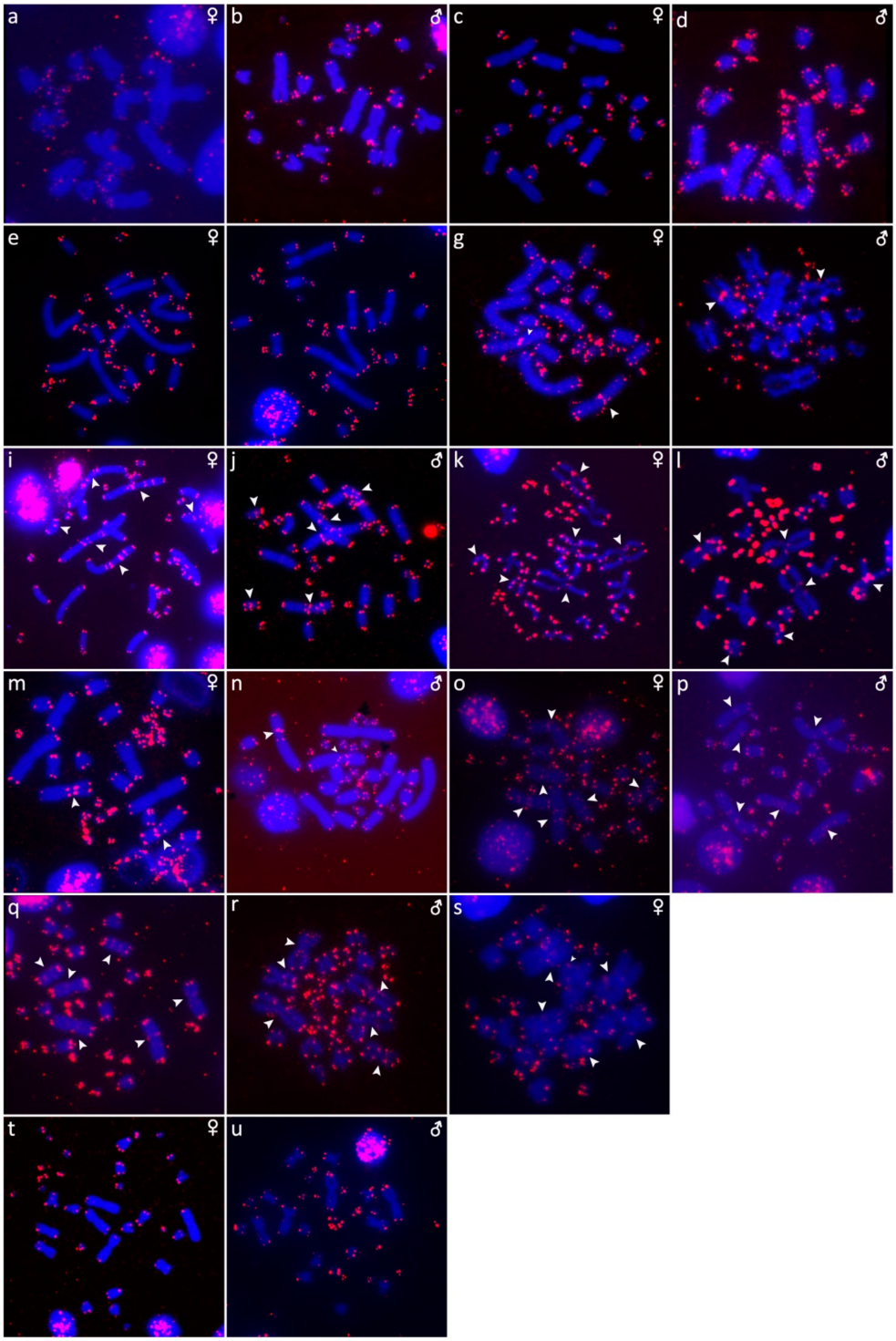


Figure 3. Distribution of $(TTAGGG)_n$ motifs in metaphases of *Acrantophis dumerili* (**a**,**b**), *Acrantophis madagascariensis* (**c**,**d**), *Calabaria reinhardtii* (**e**,**f**), *Candoia paulsoni* (**g**,**h**), *Chilabothrus angulifer* (**i**,**j**), *Eunectes notaeus* (**k**,**l**), *Lichanura trivirgata* (**m**,**n**), *Morelia spilota* (**o**,**p**), *Morelia bredli* × *M. spilota* hybrid (**q**,**r**), *Morelia bredli* (**s**), and *Sanzinia madagascariensis* (**t**,**u**). Chromosomes with interstitial telomeric repeats are shown by arrows.

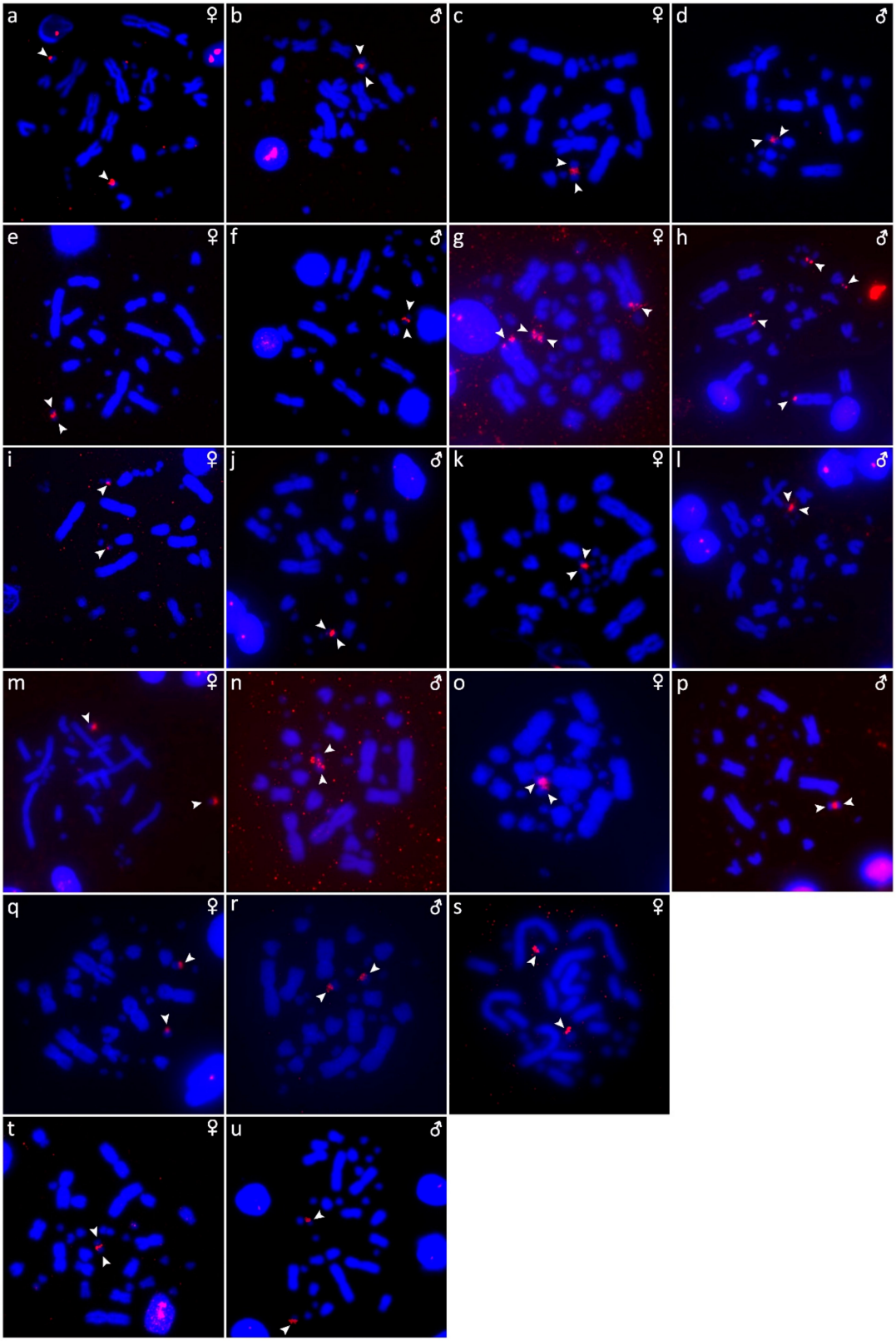


Figure 4. Topology of rDNA loci in metaphases of *Acrantophis dumerili* (**a**,**b**), *Acrantophis madagascariensis* (**c**,**d**), *Calabaria reinhardtii* (**e**,**f**), *Candoia paulsoni* (**g**,**h**), *Chilabothrus angulifer* (**i**,**j**), *Eunectes notaeus* (**k**,**l**), *Lichanura trivirgata* (**m**,**n**), *Morelia spilota* (**o**,**p**), *Morelia bredli* × *M. spilota* hybrid (**q**,**r**), *Morelia bredli* (**s**), and *Sanzinia madagascariensis* (**t**,**u**). Chromosomes with detected signal are shown by arrows.

3.1. *Acrantophis Dumerili*

The karyotype is composed of 2n = 34 chromosomes (16 macro- and 18 microchromosomes), as was previously described in Augstenová et al. [27], where the results of C-banding were presented. The telomeric sequences were detected only in terminal positions of the chromosomes (Figure 3a,b). FISH with rDNA probe revealed signals on a pair of microchromosomes (Figure 4a,b). FISH with $(GATA)_8$ motif revealed signals on the 5th pair and a pair of microchromosomes (Figure 5a,b).

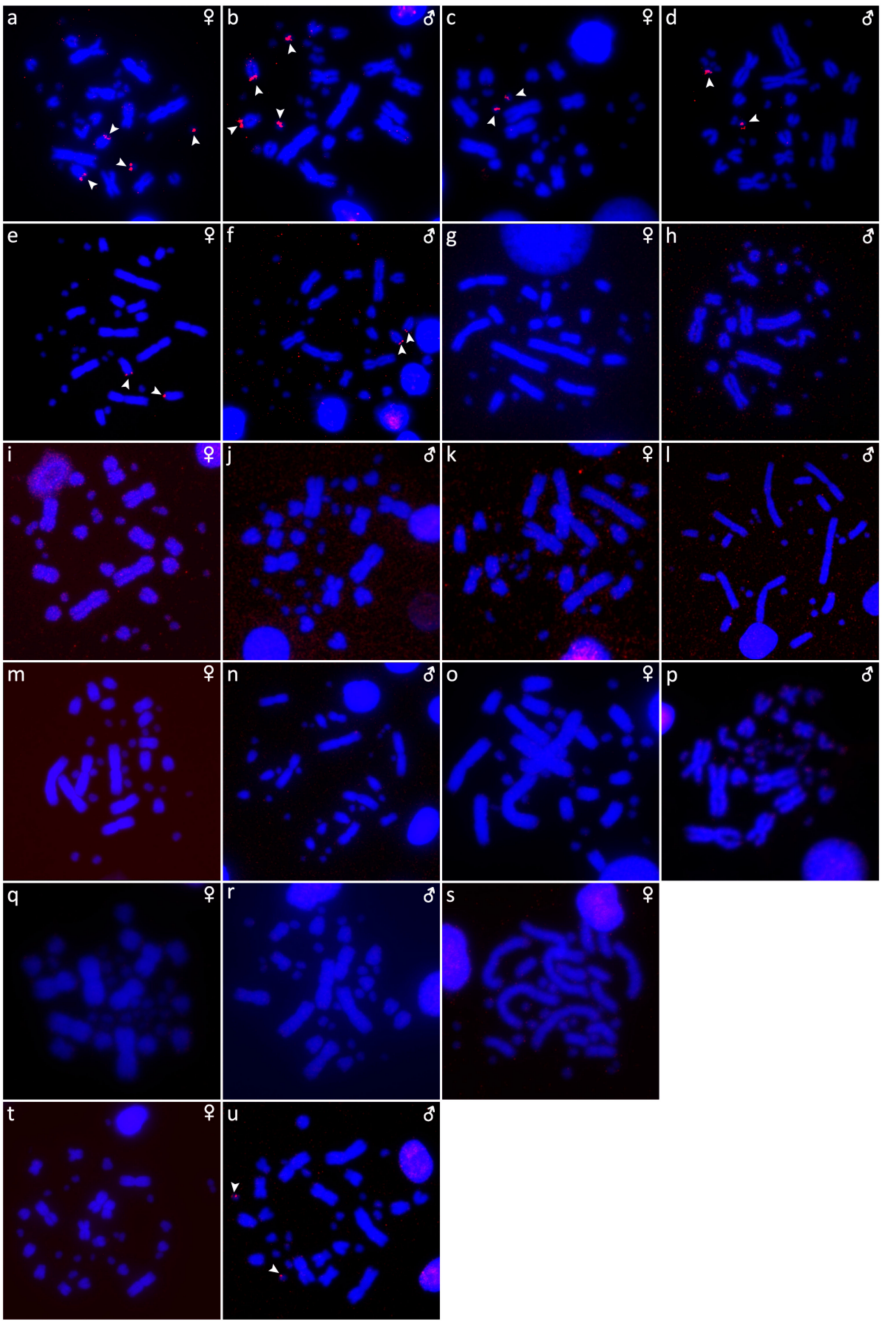


Figure 5. Distribution of $(GATA)_8$ motifs in metaphases of *Acrantophis dumerili* (**a**,**b**), *Acrantophis madagascariensis* (**c**,**d**), *Calabaria reinhardtii* (**e**,**f**), *Candoia paulsoni* (**g**,**h**), *Chilabothrus angulifer* (**i**,**j**), *Eunectes notaeus* (**k**,**l**), *Lichanura trivirgata* (**m**,**n**), *Morelia spilota* (**o**,**p**), *Morelia bredli* × *M. spilota* hybrid (**q**,**r**), *Morelia bredli* (**s**), and *Sanzinia madagascariensis* (**t**,**u**). Chromosomes with detected signal are shown by arrows.

3.2. *Acrantophis Madagascariensis*

The karyotype consists of 2n = 34 chromosomes (16 macro- and 18 microchromosomes) as was previously reported in Augstenová et al. [27], where the results of C-banding can be found. The telomeric sequences were detected only at terminal positions of the chromosomes (Figure 3c,d). FISH with rDNA probe revealed signals on a pair of microchromosomes (Figure 4c,d). FISH with $(GATA)_8$ motif revealed signals on a pair of microchromosomes (Figure 5c,d).

3.3. *Calabaria Reinhardtii*

The karyotype consists of 2n = 36 chromosomes (16 macro- and 20 microchromosomes). The pairs 1–4 consist of bi-armed chromosomes (metacentric or submetacentric), while the pairs 5–8 consist of acrocentric chromosomes (Figure 1a,b). C-banding revealed heterochromatin mainly in (peri-) centromeric regions (Figure 2a,b). The telomeric sequences were detected only at terminal positions of the chromosomes (Figure 3e,f). FISH with rDNA probe revealed signals on a pair of microchromosomes (Figure 4e,f). FISH with $(GATA)_8$ revealed signals on the 5th pair (Figure 5e,f). We did not detect any sex-specific differences at sequence level by CGH (Figure 6a,b).

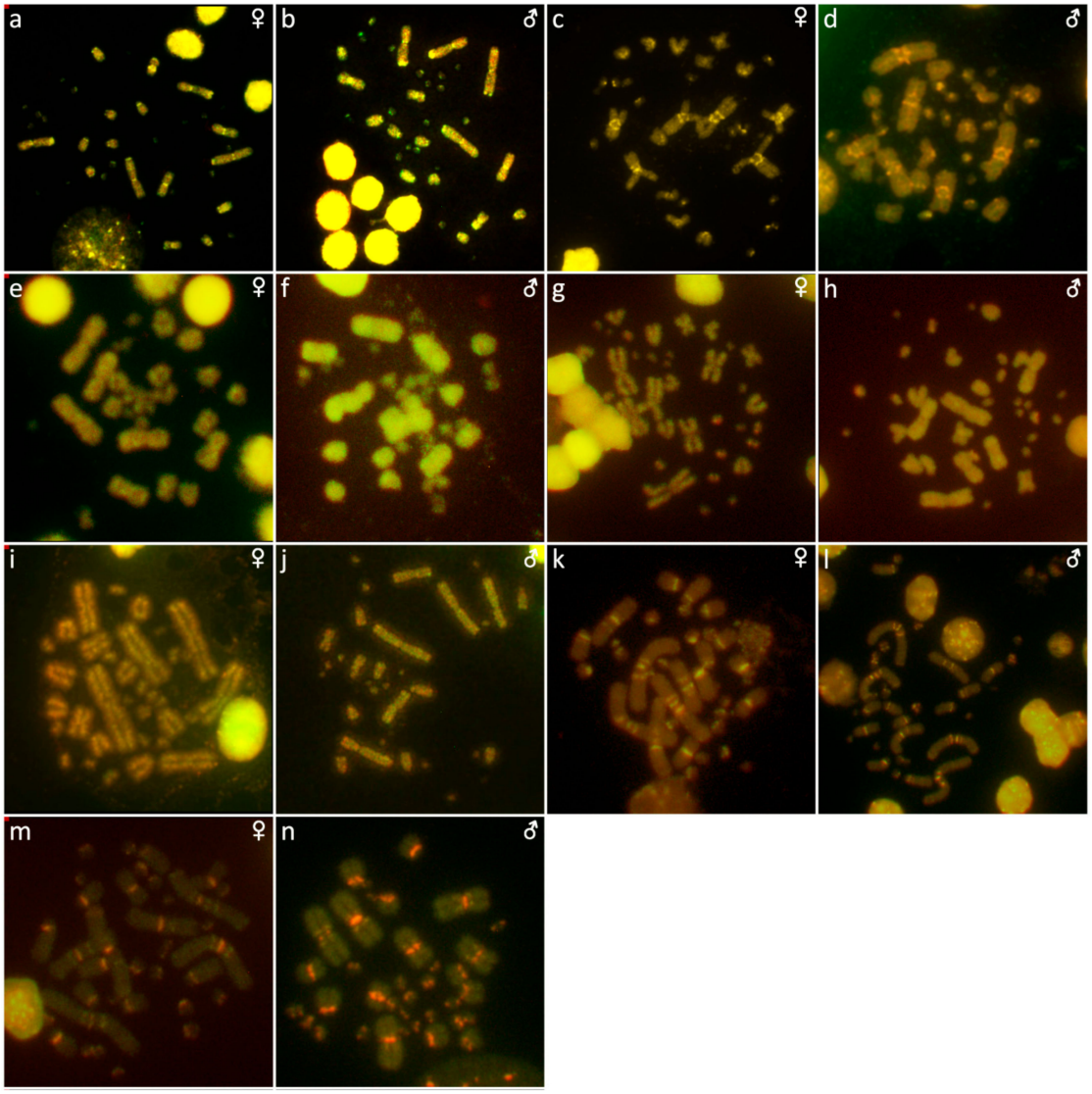


Figure 6. Comparative genomic hybridization in metaphases of *Calabaria reinhardtii* (**a**,**b**), *Candoia paulsoni* (**c**,**d**), *Chilabothrus angulifer* (**e**,**f**), *Eunectes notaeus* (**g**,**h**), *Lichanura trivirgata* (**i**,**j**), *Morelia spilota* (**k**,**l**), and *Morelia bredli* × *M. spilota* hybrid (**m**,**n**).

3.4. Candoia Paulsoni

The karyotype consists of 2n = 36 chromosomes (16 macro- and 20 microchromosomes). The pairs 1–4 are bi-armed chromosomes, while the pairs 5–8 are acrocentric (Figure 1c,d). C-banding revealed heterochromatin in pericentromeric regions of macrochromosomes (Figure 2c,d). The telomeric sequences were detected at terminal positions of the chromosomes. In addition, interstitial telomeric repeats (ITRs) were detected in the 2nd pair (Figure 3g,h). Accumulation of rDNA motifs was detected at terminal positions of the first pair and in a pair of microchromosomes (Figure 4g,h). FISH with $(GATA)_8$ did not reveal a signal on any chromosomes (Figure 5g,h). We did not detect any sex-specific differences at sequence level by CGH (Figure 6c,d).

3.5. Chilabothrus Angulifer

The karyotype is composed of 2n = 36 chromosomes (16 macro- and 20 microchromosomes). The pairs 1–4 consist of bi-armed chromosomes, while the pairs 5–8 consist of acrocentric chromosomes (Figure 1e,f). C-banding did not reveal any accumulations of heterochromatin (Figure 2e,f). The telomeric sequences were detected at terminal positions of the chromosomes. In addition, ITRs were detected in the 1st and 2nd chromosome pair (Figure 3i,j). FISH with rDNA probe revealed signals on a pair of microchromosomes (Figure 4i,j). FISH with $(GATA)_8$ motif did not reveal a signal on any chromosome (Figure 5i,j). We did not detect any sex-specific differences at sequence level by CGH (Figure 6e,f).

3.6. Eunectes Notaeus

The karyotype is composed of 2n = 36 chromosomes (16 macro- and 20 microchromosomes) as was previously described [17]. C-banding revealed a strong accumulation of heterochromatin in pericentromeric regions (Figure 2g,h). The telomeric sequences were detected in terminal positions of the chromosomes. FISH with telomeric probe uncovered ITRs on the 1st, 2nd, and 6th chromosomal pairs (Figure 3k,l). rDNA was accumulated in one pair of microchromosomes (Figure 4k,l). FISH with probe specific for $(GATA)_8$ motif did not reveal a signal on any chromosomes (Figure 5k,l). We did not detect any sex-specific differences at sequence level by CGH (Figure 6g,h).

3.7. Lichanura Trivirgata

The karyotype is composed of 2n = 36 chromosomes (16 macro- and 20 microchromosomes). The pairs 1–4 consist of bi-armed chromosomes, while the pairs 5–8 consist of acrocentric chromosomes (Figure 1g,h). C-banding revealed heterochromatin mainly in centromeric regions (Figure 2i,j). The telomeric sequences were detected in terminal positions of the chromosomes. In addition, ITRs were detected in the 2nd chromosome pair (Figure 3m,n). FISH with rDNA probe revealed signals on a pair of microchromosomes (Figure 4m,n). FISH with probe specific for $(GATA)_8$ motif did not reveal a signal on any chromosomes (Figure 5m,n). We did not detect any sex-specific differences at sequence level by CGH (Figure 6i,j).

3.8. Morelia Bredli

The karyotype consists of 36 chromosomes (16 macro- and 20 microchromosomes). The pairs 1–4 consist of bi-armed chromosomes, while the pairs 5–8 consist of acrocentric chromosomes (Figure 1k). C-banding revealed distribution of heterochromatin in pericentromeric regions (Figure 2o). The telomeric sequences were detected in terminal positions of the chromosomes. In addition, we detected ITRs in the 1st, 2nd, and 3rd pairs (Figure 3s). rDNA loci were accumulated on a pair of microchromosomes (Figure 4s). FISH with $(GATA)_8$ repetitive elements did not detect any sex-specific accumulation (Figure 5s). A male of this species was not available to us at the time of the study, therefore we did not apply the CGH method.

3.9. Morelia Spilota

The karyotype consists of 36 chromosomes with 16 macro- and 20 microchromosomes, as was previously described [7,38]. C-banding uncovered heterochromatin regions around centromeres (Figure 2k,l). The telomeric sequences were detected in terminal positions of the chromosomes. In addition, we detected ITRs on the 1st, 2nd, and 3rd pairs (Figure 3o,p). FISH with rDNA probe uncovered a signal on one pair of microchromosomes (Figure 4o,p). FISH with $(GATA)_8$ probe did not reveal any strong accumulation (Figure 5o,p). CGH did not revealed any sex-specific differences (Figure 6k,l).

3.10. Morelia Bredli × Morelia Spilota Hybrid

The karyotype of the interspecific hybrid between *Morelia bredli* and *Morelia spilota* is composed of 36 chromosomes with 16 macro- and 20 microchromosomes (Figure 1i,j). Heterochromatin regions are mostly restricted to pericentromeric regions as in the maternal species (Figure 2m,n). The telomeric sequences were detected in terminal positions of the chromosomes. In addition, we detected ITRs in the 1st, 2nd, and 3rd pair (Figure 3q,r). FISH with probe for rDNA loci revealed a signal on one pair of microchromosomes (Figure 4q,r). FISH with $(GATA)_8$ probe did not reveal any sex-specific accumulation (Figure 5q,r). CGH of maternal and paternal species detected no species-specific differences (Figure 6m,n).

3.11. Sanzinia Madagascariensis

The karyotype consists of 34 chromosomes with 18 macro- and 16 microchromosomes, as previously described [26,27]. The results of C-banding and CGH can be found in [27]. The telomeric sequences were detected in terminal positions of the chromosomes (Figure 3t,u). FISH with the rDNA probe uncovered a signal on one pair of microchromosomes (Figure 4t,u). Accumulation of $(GATA)_8$ microsatellite was detected in one pair of microchromosomes in the male, but not in the female (Figure 5t,u).

4. Discussion

According to our knowledge, we present the first description of karyotypes of five species, namely *Calabaria reinhardtii* (Calabariidae), *Candoia paulsoni* (Candoiidae), *Chilabothrus angulifer* (Boidae), *Lichanura trivirgata* (Charinidae), and *Morelia bredli* (Pythonidae) (Figure 1).

Cytogenetic analysis revealed variability among species in the topology of the telomeric repeats, rDNA loci, and $(GATA)_n$ motifs.

We detected interstitial telomeric repeats in six of the ten studied species (Figures 3 and 7). Notably, in contrast to a previous study that reported telomeric repeats only in terminal positions [17], we detected ITRs in three individuals of *Eunectes notaeus*. Boas and pythons have karyotypes with similar chromosome morphology (Figure 7). Therefore, we assume that interchromosomal rearrangements occur rarely in these groups, and that the ITRs in the six studied species emerged via intrachromosomal rearrangements, such as inversions, or retrotransposon activity. The extensive polymorphism in the topology of ITRs does not follow a notable phylogenetic pattern (Figure 7).

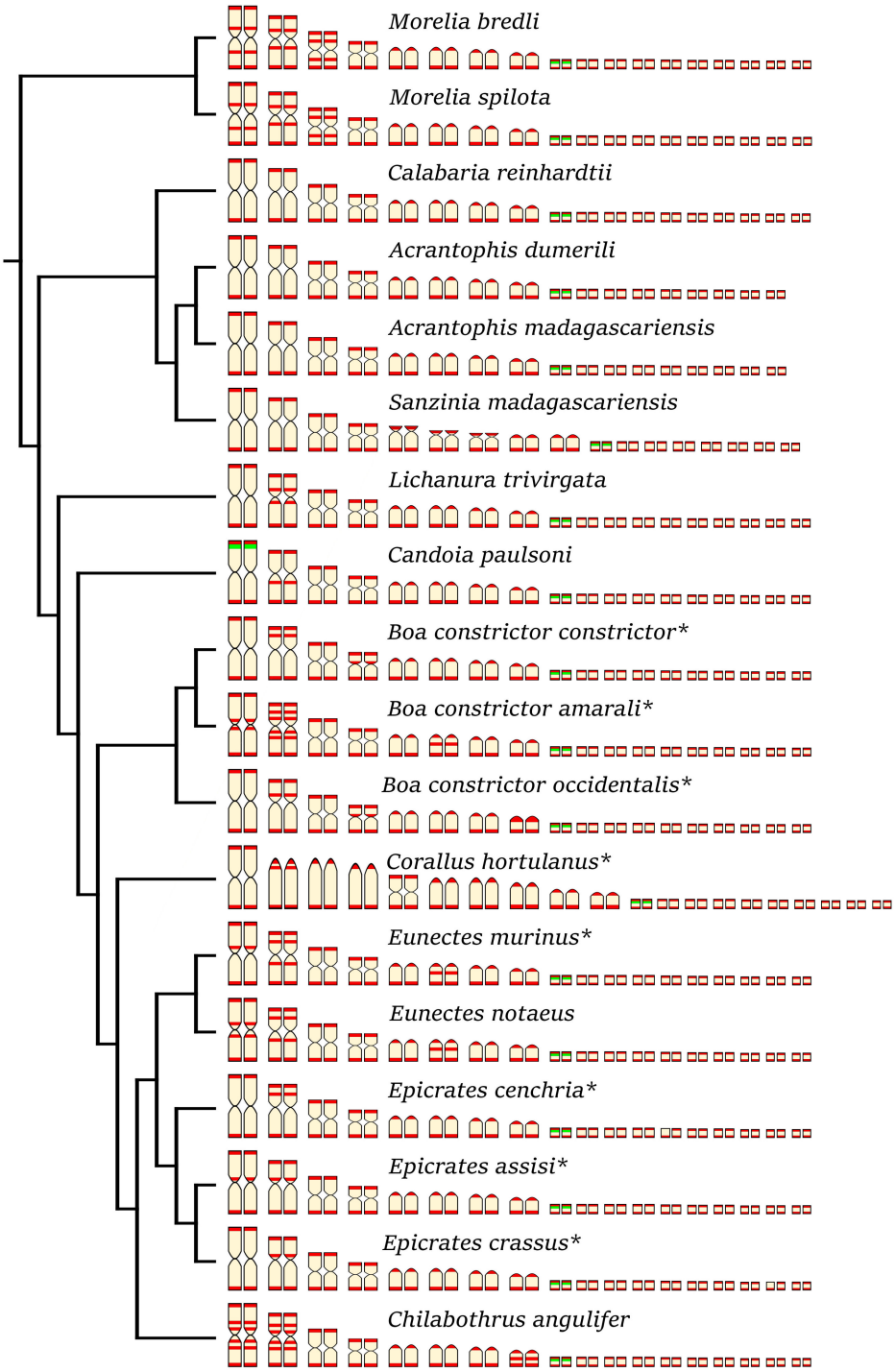


Figure 7. Idiogram showing the comparative topology of ITRs (in red) and rDNA loci (in green) on the chromosomes of 18 species of boas and pythons. The original data were extracted from Viana et al. [17] for species marked with asterisk (*). The phylogenetic relationships follow Reynolds et al. [39].

rDNA loci seem to accumulate on one pair of microchromosomes in all studied species (Figure 4), with the exception of *C. paulsoni*, which has an additional accumulation of rDNA on the 1st pair (Figure 4g,h).

The $(GATA)_n$ motif is accumulated in the genomes of boas and pythons only rarely (Figure 5). This accumulation was observed on the 5th chromosome pair in *Calabaria reinhardtii*, the 5th pair (Figure 5e,f) and a single pair of microchromosomes in *Acrantophis dumerili* (Figure 5a,b), and a pair of microchromosomes in *A. madagascariensis* (Figure 5c,d). The $(GATA)_n$ motif was observed on one pair

of microchromosomes in male metaphases of *Sanzinia madagascariensis*, but such accumulation was not detected in female metaphases (Figure 5t,u). It seems that this pattern reflects individual variability in *S. madagascariensis*.

We attempted to identify sex chromosomes in ten species of boas and pythons by both conventional and molecular cytogenetic methods. Sex-specific differences were not detected in the C-banded metaphases, indicating that sex chromosomes in the studied species are not heterochromatinized (Figure 2). Previous studies revealed an accumulation of telomeric-like sequences on the W sex chromosome in the lacertid lizard *Lacerta agilis* [40] and in the gecko *Underwoodisaurus milii* [41] as well as in several caenophidian snakes, including the dragon snake *Xenodermus javanicus* (Xenodermatidae) [9] and the masked water snake *Homalopsis buccata* (Homalopsidae) [12]. Some reptilian species, such as the red-bellied short-necked turtle *Emydura subglobosa* [42], the Chinese softshell turtle *Pelodiscus sinensis* [43], and the spiny softshell turtle *Apalone spinifera* [44], have accumulations of rDNA loci on sex chromosomes. Furthermore, the *Bkm* repeat, is composed of repetitions of $(GATA)_n$ and $(GACA)_n$ motifs [15,16], which commonly amplify in vertebrate sex chromosomes, including those of the vast majority of studied caenophidian snakes [12,30,31,45]. Our results did not reveal sex-specific accumulations of the telomeric repeats, rDNA loci, nor $(GATA)_n$ motifs in the ten studied species of boas and pythons, with a single exception being the dubious male-specific accumulation of the $(GATA)_n$ motif in *Sanzinia madagascariensis*. Comparative genomic hybridization (CGH) did not reveal sex-specific differences at the sequence level (up to the detection efficiency of in situ hybridization methodology) in all six tested species (Figure 6).

The differentiation process of sex chromosomes, including heterochromatinization, amplification of repetitive elements, and loss of functional genes in the heterogametic sex [46], seems to act as an "evolutionary trap", prohibiting sex chromosome turnover and thus stabilizing the sex determination system [47]. Snakes seem to support this hypothesis. The ZZ/ZW sex determination system of caenophidian snakes with highly differentiated sex chromosomes seems to have been stable for more than 60 million years [9]. In contrast, henophidian and scolecophidian snakes have poorly differentiated (if any) sex chromosomes [17,28,48,49] and demonstrate variability in sex determination systems [25,27,28].

The evolution of sex chromosomes and sex determination systems in henophidian and scolecophidian snakes should be further explored by genome-wide next generation sequencing approaches, such as restriction site associated DNA sequencing (RAD-seq) [25,50–52], which will allow the fine-tuned identification of sex-specific regions of the genome. Facultative parthenogenetic reproduction results in all-female homozygous progeny in *Epicrates maurus* [20], *Epicrates cenchria cenchria* [22], *Malayopython reticulatus,* and *Python regius* [23], which indicates that male heterogamety may be present in these species. Under male heterogamety, the mother with XX sex chromosome constitution could produce only female offspring from facultative asexual reproduction. In contrast, under female heterogamety, the ZW mother should produce offspring of both sexes, or exclusively ZZ males, depending on the degree of W chromosome degeneration and the viability of the WW individuals [53]. Despite genotypic sex determination being exclusively revealed in all studied snakes and commonly expected in this lineage [54,55], we cannot exclude the possibility that some non-caenophidian snakes might possess environmental sex determination. Temperature and hydric conditions can induce sex-differential mortality in snake embryos [56–58] and can alter neonate morphology, physiology, and behavior (reviewed in [55]). The sex determination mode is usually determined either by cytogenetic examination (restricted to species with differentiated sex chromosomes) or by incubating eggs using a range of temperatures in laboratory conditions and examining the variation in hatchlings' sex ratio [55]. However, most examined henophidian and scolecophidian snakes lack differentiated sex chromosomes and are not convenient for manipulative breeding experiments, as many of them are viviparous, lay small clutches, or rarely breed in captivity. We suggest that additional research is needed for uncovering the sex-determination mode in non-caenophidian snakes.

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References

1. Uetz, P.; Freed, P.; Hošek, J. The Reptile Database. 2019. Available online: http://www.reptile-database.org (accessed on 6 October 2019).
2. Heise, P.J.; Maxson, L.R.; Dowling, H.G.; Hedges, S.B. Higher-level snake phylogeny inferred from mitochondrial DNA sequences of 12S rRNA and 16S rRNA genes. *Mol. Biol. Evol.* **1995**, *12*, 259–265. [CrossRef] [PubMed]
3. Pyron, R.A.; Burbrink, F.T.; Wiens, J.J. A phylogeny and revised classification of Squamata, including 4161 species of lizards and snakes. *BMC Evol. Biol.* **2013**, *13*, 93. [CrossRef] [PubMed]
4. Zheng, Y.; Wiens, J.J. Combining phylogenomic and supermatrix approaches, and a time-calibrated phylogeny for squamate reptiles (lizards and snakes) based on 52 genes and 4162 species. *Mol. Phylogenet. Evol.* **2016**, *94*, 537–547. [CrossRef] [PubMed]
5. Harrington, S.M.; Reeder, T.W. Phylogenetic inference and divergence dating of snakes using molecules, morphology and fossils: New insights into convergent evolution of feeding morphology and limb reduction. *Biol. J. Linn. Soc.* **2017**, *121*, 379–394. [CrossRef]
6. Pyron, R.A.; Hendry, C.R.; Chou, V.M.; Lemmon, E.M.; Lemmon, A.R.; Burbrink, F.T. Effectiveness of phylogenomic data and coalescent species-tree methods for resolving difficult nodes in the phylogeny of advanced snakes (Serpentes: Caenophidia). *Mol. Phylogenet. Evol.* **2014**, *81*, 221–231. [CrossRef]
7. Olmo, E.; Signorino, G.G. Chromorep: A Reptile Chromosomes Database. 2005. Available online: http://chromorep.univpm.it (accessed on 6 October 2019).
8. Oguiura, N.; Ferrarezzi, H.; Batistic, R.F. Cytogenetics and molecular data in snakes: A phylogenetic approach. *Cytogenet. Genome Res.* **2009**, *127*, 128–142. [CrossRef]
9. Rovatsos, M.; Johnson Pokorná, M.; Kratochvíl, L. Differentiation of sex chromosomes and karyotype characterisation in the dragonsnake *Xenodermus javanicus* (Squamata: Xenodermatidae). *Cytogenet. Genome Res.* **2015**, *147*, 48–54. [CrossRef]
10. Rovatsos, M.; Altmanová, M.; Johnson Pokorná, M.; Augstenová, B.; Kratochvíl, L. Cytogenetics of the Javan file snake (*Acrochordus javanicus*) and the evolution of snake sex chromosomes. *J. Zool. Syst. Evol. Res.* **2017**, *56*, 117–125. [CrossRef]
11. O'Meally, D.; Patel, H.R.; Stiglec, R.; Sarre, S.D.; Georges, A.; Marshall Graves, J.A.; Ezaz, T. Non-homologous sex chromosomes of birds and snakes share repetitive sequences. *Chromosome Res.* **2010**, *18*, 787–800. [CrossRef]
12. Augstenová, B.; Mazzoleni, S.; Kratochvíl, L.; Rovatsos, M. Evolutionary dynamics of the W chromosome in caenophidian snakes. *Genes* **2018**, *9*, 5. [CrossRef]
13. Vicoso, B.; Emerson, J.J.; Zektser, Y.; Mahajan, S.; Bachtrog, D. Comparative sex chromosome genomics in snakes: Differentiation, evolutionary strata, and lack of global dosage compensation. *PLoS Biol.* **2013**, *11*, e1001643. [CrossRef] [PubMed]
14. Rovatsos, M.; Vukić, J.; Lymberakis, P.; Kratochvíl, L. Evolutionary stability of sex chromosomes in snakes. *Proc. R. Soc. B Biol. Sci.* **2015**, *282*, 20151992. [CrossRef] [PubMed]
15. Singh, L.; Purdom, F.; Jones, K.W. Satellite DNA and evolution of sex chromosomes. *Chromosoma* **1976**, *59*, 43–62. [CrossRef] [PubMed]

16. Singh, L.; Purdom, I.F.; Jones, K.W. Sex chromosome associated satellite DNA: Evolution and conservation. *Chromosoma* **1980**, *79*, 137–157. [CrossRef] [PubMed]
17. Viana, P.F.; Ribeiro, L.B.; Souza, G.M.; Chalkidis, H.M.; Gross, M.C.; Feldberg, E. Is the karyotype of neotropical boid snakes really conserved? Cytotaxonomy, chromosomal rearrangements and karyotype organization in the Boidae family. *PLoS ONE* **2016**, *11*, e0160274. [CrossRef]
18. Ohno, S. *Sex. Chromosomes and Sex-Linked Genes*; Springer: Berlin, Germany, 1967; pp. 1–167.
19. Matsubara, K.; Tarui, H.; Toriba, M.; Yamada, K.; Nishida-Umehara, C.; Agata, K.; Matsuda, Y. Evidence for different origin of sex chromosomes in snakes, birds, and mammals and step-wise differentiation of snake sex chromosomes. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 18190–18195. [CrossRef]
20. Booth, W.; Million, L.; Reynolds, R.G.; Burghardt, G.M.; Vargo, E.L.; Schal, C.; Tzika, A.C.; Schuett, G.W. Consecutive virgin births in the New World boid snake, the Colombian rainbow boa, *Epicrates maurus*. *J. Hered.* **2011**, *102*, 759–763. [CrossRef]
21. Booth, W.; Johnson, D.H.; Moore, S.; Schal, C.; Vargo, E.L. Evidence for viable, non-clonal but fatherless *Boa constrictors*. *Biol. Lett.* **2011**, *7*, 253–256. [CrossRef]
22. Kinney, M.E.; Wack, R.F.; Grahn, R.A.; Lyons, L. Parthenogenesis in a Brazilian rainbow boa (*Epicrates cenchria cenchria*). *Zoo Biol.* **2013**, *32*, 172–176. [CrossRef]
23. Booth, W.; Schuett, G.W.; Ridgway, A.; Buxton, D.W.; Castoe, T.A.; Bastone, G.; Bennett, C.; McMahan, W. New insights on facultative parthenogenesis in pythons. *Biol. J. Linn. Soc.* **2014**, *112*, 461–468. [CrossRef]
24. Mallery, C.S., Jr.; Carrillo, M.M. A case study of sex-linkage in *Python regius* (Serpentes: Boidae), with new insights into sex determination in Henophidia. *Phyllomedusa* **2016**, *15*, 29–42. [CrossRef]
25. Gamble, T.; Castoe, T.A.; Nielsen, S.V.; Banks, J.L.; Card, D.C.; Schield, D.R.; Schuett, G.W.; Booth, W. The discovery of XY sex chromosomes in a boa and python. *Curr. Biol.* **2017**, *27*, 1–6. [CrossRef] [PubMed]
26. Mengden, G.A.; Stock, A.D. Chromosomal evolution in Serpentes; a comparison of G and C chromosome banding patterns of some colubrid and boid genera. *Chromosoma* **1980**, *79*, 53–64. [CrossRef]
27. Augstenová, B.; Johnson Pokorná, M.; Altmanová, M.; Frynta, D.; Rovatsos, M.; Kratochvíl, L. ZW, XY, and yet ZW: Sex chromosome evolution in snakes even more complicated. *Evolution* **2018**, *72*, 1701–1707. [CrossRef] [PubMed]
28. Matsubara, K.; Kumazawa, Y.; Ota, H.; Nishida, C.; Matsuda, Y. Karyotype analysis of four blind snake species (Reptilia: Squamata: Scolecophidia) and karyotypic changes in Serpentes. *Cytogenet. Genome Res.* **2019**, *157*, 98–106. [CrossRef] [PubMed]
29. Valenzuela, N.; Badenhorst, D.; Montiel, E.E.; Literman, R. Molecular cytogenetic search for cryptic sex chromosomes in painted turtles *Chrysemys picta*. *Cytogenet. Genome Res.* **2014**, *144*, 39–46. [CrossRef]
30. Matsubara, K.; Nishida, C.; Matsuda, Y.; Kumazawa, Y. Sex chromosome evolution in snakes inferred from divergence patterns of two gametologous genes and chromosome distribution of sex chromosome-linked repetitive sequences. *Zool. Lett.* **2016**, *2*, 19. [CrossRef]
31. Matsubara, K.; O'Meally, D.; Azad, B.; Georges, A.; Sarre, S.D.; Graves, J.A.M.; Matsuda, Y.; Ezaz, T. Amplification of microsatellite repeat motifs is associated with the evolutionary differentiation and heterochromatinization of sex chromosomes in Sauropsida. *Chromosoma* **2016**, *125*, 111–123. [CrossRef]
32. Mazzoleni, S.; Augstenová, B.; Clemente, L.; Auer, M.; Fritz, U.; Praschag, P.; Protiva, T.; Velenský, P.; Kratochvíl, L.; Rovatsos, M. Turtles of the genera *Geoemyda* and *Pangshura* (Testudines: Geoemydidae) lack differentiated sex chromosomes: The end of a 40-year error cascade for *Pangshura*. *PeerJ* **2019**, *7*, e6241. [CrossRef]
33. Sumner, A.T. A simple technique for demonstrating centromeric heterochromatin. *Exp. Cell Res.* **1972**, *75*, 304–306. [CrossRef]
34. Ijdo, J.W.; Baldini, A.; Ward, D.C.; Reeders, S.T.; Wells, R.A. Origin of human chromosome 2: An ancestral telomere-telomere fusion. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 9051–9055. [CrossRef] [PubMed]
35. Rovatsos, M.; Johnson Pokorná, M.; Altmanová, M.; Kratochvíl, L. Female heterogamety in Madagascar chameleons (Squamata: Chamaeleonidae: *Furcifer*): Differentiation of sex and neo-sex chromosomes. *Sci. Rep.* **2015**, *5*, 13196. [CrossRef] [PubMed]
36. Endow, S.A. Polytenization of the ribosomal genes on the X and Y chromosomes of *Drosophila melanogaster*. *Genetics* **1982**, *100*, 375–385. [PubMed]
37. Rovatsos, M.; Altmanová, M.; Johnson Pokorná, M.; Velenský, P.; Sánchez Baca, A.; Kratochvíl, L. Evolution of karyotypes in chameleons. *Genes* **2017**, *8*, 382. [CrossRef] [PubMed]

38. Mengden, G.A. Chromosomal Evolution and the Phylogeny of Elapid Snakes. Ph.D. Thesis, Australian National University, Canberra, Australia, 1982; pp. 1–221. [CrossRef]
39. Reynolds, R.G.; Niemiler, M.L.; Revell, L.J. Toward a Tree-of-Life for the boas and pythons: Multi locus species-level phylogeny with unprecedented taxon sampling. *Mol. Phylogenet. Evol.* **2014**, *71*, 201–213. [CrossRef]
40. Matsubara, K.; Uno, Y.; Srikulnath, K.; Matsuda, Y.; Miller, E.; Olsson, M. No interstitial telomeres on autosomes but remarkable amplification of telomeric repeats on the W sex chromosome in the sand lizard (*Lacerta agilis*). *J. Hered.* **2015**, *106*, 753–757. [CrossRef]
41. Pokorná, M.; Rens, W.; Rovatsos, M.; Kratochvíl, L. A ZZ/ZW sex chromosome system in the thick-tailed gecko (*Underwoodisaurus milii*; Squamata: Gekkota: Carphodactylidae), a member of the ancient gecko lineage. *Cytogenet. Genome Res.* **2014**, *142*, 190–196. [CrossRef]
42. Lee, L.; Montiel, E.E.; Valenzuela, N. Discovery of putative XX/XY male heterogamety in *Emydura subglobosa* turtles exposes a novel trajectory of sex chromosome evolution in *Emydura*. *Cytogenet. Genome Res.* **2019**, *158*, 160–169. [CrossRef]
43. Kawai, A.; Nishida-Umehara, C.; Ishijima, J.; Tsuda, Y.; Ota, H.; Matsuda, Y. Different origins of bird and reptile sex chromosomes inferred from comparative mapping of chicken Z-linked genes. *Cytogenet. Genome Res.* **2007**, *117*, 92–102. [CrossRef]
44. Literman, R.; Badenhorst, D.; Valenzuela, N. qPCR-based molecular sexing by copy number variation in rRNA genes and its utility for sex identification in soft-shell turtles. *Methods Ecol. Evol.* **2014**, *5*, 872–880. [CrossRef]
45. Viana, P.F.; Ezaz, T.; Cioffi, M.B.; Almeida, B.J.; Feldberg, E. Evolutionary insights of the ZW sex chromosomes in snakes: A new chapter added by the amazonian puffing snakes of the genus *Spilotes*. *Genes* **2019**, *10*, 288. [CrossRef] [PubMed]
46. Charlesworth, B. The evolution of sex chromosomes. *Science* **1991**, *251*, 1030–1033. [CrossRef] [PubMed]
47. Pokorná, M.; Kratochvíl, L. Phylogeny of sex-determining mechanisms in squamate reptiles: Are sex chromosomes an evolutionary trap? *Zool. J. Linn. Soc.* **2009**, *156*, 168–183. [CrossRef]
48. Gorman, G.C.; Gress, F. Chromosome cytology of four boid snakes and a varanid lizard, with comments on the cytosystematics of primitive snakes. *Herpetologica* **1970**, *26*, 308–317.
49. Mezzasalma, M.; Andreone, F.; Glaw, F.; Petraccioli, A.; Odierna, G.; Guarino, F.M. A karyological study of three typhlopid species with some inferences on chromosome evolution in blindsnakes (Scolecophidia). *Zool. Anz.* **2016**, *264*, 34–40. [CrossRef]
50. Gamble, T.; Zarkower, D. Identification of sex-specific molecular markers using restriction site-associated DNA sequencing. *Mol. Ecol. Res.* **2014**, *14*, 902–913. [CrossRef]
51. Gamble, T.; Coryell, J.; Ezaz, T.; Lynch, J.; Scantlebury, D.P.; Zarkower, D. Restriction site-associated DNA sequencing (RAD-seq) reveals an extraordinary number of transitions among gecko sex-determining systems. *Mol. Biol. Evol.* **2015**, *32*, 1296–1309. [CrossRef]
52. Wilson, C.A.; Titus, T.; Batzel, P.; Postlethwait, J.H.; Raman, R. A search for sex-linked loci in the agamid lizard, *Calotes versicolor*. *Sex. Dev.* **2019**, *13*, 143–150. [CrossRef]
53. Watts, P.C.; Buley, K.R.; Sanderson, S.; Boardman, W.; Ciofi, C.; Gibson, R. Parthenogenesis in Komodo dragons. *Nature* **2006**, *444*, 1021–1022. [CrossRef]
54. Solari, A.J. *Sex. Chromosomes and Sex. Determination in Vertebrates*; CRC Press: London, UK, 1993; pp. 1–336.
55. Valenzuela, N.; Lance, V.A. *Temperature-Dependent Sex Determination in Vertebrates*; Smithsonian Books: Washington, DC, USA, 2004; pp. 1–194.
56. Burger, J.; Zappalorti, R.T. Effects of incubation temperature on sex ratios in pine snakes: Differential vulnerability of males and females. *Am. Natur.* **1988**, *132*, 492–505. [CrossRef]
57. Dunlap, K.D.; Lang, J.W. Offspring sex ratio varies with maternal size in the common garter snake, *Thamnophis sirtalis*. *Copeia* **1990**, *1990*, 568–570. [CrossRef]
58. Reichling, S.B.; Gutzke, W.H. Phenotypic consequences of incubation environment in the African elapid genus *Aspidelaps*. *Zoo Biol.* **1996**, *15*, 301–308. [CrossRef]



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Part II

Sex determination and sex chromosome evolution in chameleons

Chapter 5

Rovatsos M., Altmanová M., **Augstenová B.**, Mazzoleni S., Velenský P., Kratochvíl L. (2019): ZZ/ZW sex determination with multiple neo-sex chromosomes is common in Madagascan chameleons of the genus *Furcifer* (Reptilia: Chamaeleonidae). *Genes*, 10: 1020.

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ZZ/ZW Sex Determination with Multiple Neo-Sex Chromosomes is Common in Madagascan Chameleons of the Genus *Furcifer* (Reptilia: Chamaeleonidae)

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Abstract: Chameleons are well-known, highly distinctive lizards characterized by unique morphological and physiological traits, but their karyotypes and sex determination system have remained poorly studied. We studied karyotypes in six species of Madagascan chameleons of the genus *Furcifer* by classical (conventional stain, C-banding) and molecular (comparative genomic hybridization, in situ hybridization with rDNA, microsatellite, and telomeric sequences) cytogenetic approaches. In contrast to most sauropsid lineages, the chameleons of the genus *Furcifer* show chromosomal variability even among closely related species, with diploid chromosome numbers varying from $2n = 22$ to $2n = 28$. We identified female heterogamety with cytogenetically distinct Z and W sex chromosomes in all studied species. Notably, multiple neo-sex chromosomes in the form $Z_1Z_1Z_2Z_2/Z_1Z_2W$ were uncovered in four species of the genus (*F. bifidus*, *F. verrucosus*, *F. willsii*, and previously studied *F. pardalis*). Phylogenetic distribution and morphology of sex chromosomes suggest that multiple sex chromosomes, which are generally very rare among vertebrates with female heterogamety, possibly evolved several times within the genus *Furcifer*. Although acrodontan lizards (chameleons and dragon lizards) demonstrate otherwise notable variability in sex determination, it seems that female heterogamety with differentiated sex chromosomes remained stable in the chameleons of the genus *Furcifer* for about 30 million years.

Keywords: comparative genome hybridization (CGH); evolution; fluorescence in situ hybridization (FISH); heterochromatin; karyotype; Madagascar; microsatellites; rDNA; sex chromosomes; telomeres

1. Introduction

Sex determination systems are variable in amniotes; however, this variability is unequally distributed across clades. Several clades such as viviparous mammals, caenophidian snakes, iguanas, softshell turtles, lacertids, and varanids and their relatives exhibited notable stability of sex chromosomes for several dozens of millions of years [1–7]. As far as is known, the variability in sex determination is concentrated to a few clades such as emydid and geoemydid turtles, geckos, and acrodontan lizards [8–10]. The reasons for the differences in the stability of sex determination systems among clades are still not fully understood. Some models suggest that the variability in sex determination is a consequence of the ancestral environmental sex determination (ESD) in a group and multiple

independent transitions from ESD to genotypic sex determination (GSD), which is then largely resistant to transitions to ESD [9–12]. Other scenarios assume that transitions between ESD and GSD are possible in both directions [13,14]. In any case, it seems that highly differentiated sex chromosomes tend to be evolutionary stable and recent molecular evidence disputed earlier reported transitions from GSD with highly differentiated sex chromosomes to ESD [15,16]. On the other hand, poorly differentiated sex chromosomes in reptiles, e.g., in boas and pythons [17,18], tend to be prone to sex chromosome turnovers, but it is not yet clear whether they can be replaced only with other GSD systems or with ESD as well. More accurate testing of these scenarios is currently prevented by the lack of primary data on sex determination in most reptilian lineages.

Iguania is an important squamate group with approximately 1935 species [19]. The two iguanian sublineages, Pleurodonta and Acrodonta, differ in the stability of sex determination. As far as is known, all pleurodontan lizards share male heterogamety, with sex chromosomes being homologous across all iguana families with the exception of basilisks [4,20]. In contrast, species with either ESD, female heterogamety, or male heterogamety were previously reported in Acrodonta (chameleons and agamids). Our knowledge of sex determination in chameleons has, until now, been scarce. Earlier reports on ESD in this group appear questionable [21–23]. Male heterogamety with poorly differentiated XX/XY sex chromosomes was found in *Chamaeleo calyptratus* [10], while female heterogamety was reported in *Furcifer oustaleti* and *Furcifer pardalis* [24]. In both species of the genus *Furcifer*, the sex chromosomes are heteromorphic and the W chromosomes are heterochromatic. Notably, *F. pardalis* has multiple neo-sex chromosomes of the type $Z_1Z_1Z_2Z_2/Z_1Z_2W$ [24].

Here, we present the results of our cytogenetic examination based on classical (conventional stain, C-banding) and molecular (comparative genomic hybridization, in situ hybridization with rDNA, microsatellites, and telomeric sequences) cytogenetic approaches in six species of the genus *Furcifer* with the aim of expanding our knowledge of the evolution of karyotypes and sex determination in this group.

2. Materials and Methods

2.1. Samples and Species Verification

Blood samples were collected from six species of chameleons from the genus *Furcifer*, to prepare mitotic chromosome suspensions and DNA isolation (Table 1). Experimental design was performed from accredited researcher (MR: CZ03540, MA: CZ01223, LK: CZ02535), while animals were temporarily kept, when needed, in the animal facility of the Faculty of Science, Charles University (accreditation No. 37428/2019-MZE-18134). 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). All chameleons are kept in Zoo Plzeň, Prague Zoo, and Zoopark Zájezd (Czech Republic).

Table 1. Number of individuals per species and sex, analyzed in this study.

Species	Sex	
	♂	♀
Furcifer antimena	4	0
Furcifer bifidus	2	2
Furcifer lateralis	2	2
Furcifer minor	3	0
Furcifer verrucosus	3	4
Furcifer willsii	2	2

2.2. DNA Isolation and Species Identification

Total DNA was isolated from all examined individuals 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 with primers optimized for reptiles [25] and following standard protocol [26]. The PCR products were purified and sequenced bi-directionally by Macrogen (Seoul, Korea). The obtained *COI* sequences were trimmed in FinchTV [27], aligned in BioEdit v5.0.9 [28], analysed in MEGA v10 [29] and DnaSP v6 [30], and compared to sequences deposited in public databases by BLASTn [31] for accurate taxon identification. All sequences were deposited in GenBank under the accession numbers MN757875–MN757882.

2.3. Chromosome Preparation and Staining

The mitotic chromosomal suspensions were prepared by leucocyte cultivation from fresh peripheral blood. Our protocol was previously described in Mazzoleni et al. [32]. Briefly, the cultivation medium (100 mL) was prepared by using 90 mL of commercially prepared D-MEM medium (Sigma-Aldrich, St. Louis, MO, USA), enriched with 10 mL of fetal bovine serum (Gibco, Carlsbad, CA, USA), 3 mL of phytohemagglutinin M (Gibco, Carlsbad, CA, USA), 1 mL of penicillin/streptomycin solution (10000 units/mL; Gibco, Carlsbad, CA, USA), 1 mL L-glutamine solution (200 mM; Sigma-Aldrich, St. Louis, MO, USA), and 1 mL lipopolysaccharide solution (10 mg/mL; Sigma-Aldrich, St. Louis, MO, USA). Then, 100–200 µL of blood were cultivated in 5 mL of the medium for one week at 30 °C. Afterwards, 35 µL colcemid (Roche, Basel, Switzerland) was added 3.5 h before harvesting. The cells were hypotonized in pre-warmed 0.075 M KCl solution for 30 min at 30 °C. Subsequently, the cells were washed four times and stored in Carnoy's fixative solution (methanol: acetic acid, 3:1).

Chromosomal preparations were dropped to slides and stained by Giemsa for karyotype reconstruction and C-banding for visualization of constitutive heterochromatin, following the protocol of Sumner [33] modified according to Mazzoleni et al. [32].

2.4. Fluorescence In Situ Hybridization (FISH) with Telomeric Probe

We applied in situ hybridization with telomeric probe with the aim of uncovering the distribution of telomeric-like sequence $(TTAGGG)_n$ in the genomes of the chameleons of the genus *Furcifer*. The probe was prepared and labelled by PCR according to the protocols of Ijdo et al. [34] and Rovatsos et al. [35]. The probe was diluted in hybridization buffer (50% formamide in 2 × saline-sodium citrate (SSC), pH 7.0).

Slides with chromosomal spreads were washed in 2 × SSC solution and subsequently treated with RNase A (100 µg/mL) for 1 h at 37 °C. The slides were again washed in 2 × SSC and treated by 0.01% pepsin for 10 min at 37 °C. Subsequently, the slides were washed in phosphate buffered saline (PBS) solution, post-fixed in 1% formaldehyde solution for 10 min, washed again in PBS, dehydrated in ethanol series and air-dried. The slides were treated in 70% formamide for 4 min at 75 °C, washed in 2 × SSC and again dehydrated in ethanol series. Once the slides were dried, we added the telomeric probe and incubated them overnight at 37 °C. The second day started with washing the slides in 2 × SSC and then three times in 50% formamide for 5 min at 37 °C. In the next step, the slides were washed twice in 2 × SSC and once in 4 × SSC/0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) solution. After washing, we incubated the slides in 4 × SSC/5% blocking reagent (Roche, Basel, Switzerland) for 45 min at 37 °C. The slides were washed in 4 × SSC/0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 30 min with avidin-FITC (Vector Laboratories, Burlingame, CA, USA) at 37 °C. The fluorescence signal was twice amplified by the avidin-FITC/biotinylated anti-avidin system (Vector Laboratories, Burlingame, CA, USA). At the end the slides were washed twice in 4 × SSC/0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA), once in PBS and dehydrated by ethanol series. Dried slides were stained by using Fluoroshield with DAPI (4', 6-diamidino-2-phenylindole) (Sigma-Aldrich, St. Louis, MO, USA).

2.5. Fluorescence In Situ Hybridization with rDNA Probe

The probe for FISH with rDNA loci 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* [36] and labelled by dUTP-biotin using nick translation (Abbott Laboratories, Lake Bluff, IL, USA). The rDNA probe was hybridized to chromosome suspensions and detected according to the protocol for FISH with telomeric probes, described above.

2.6. Fluorescence In Situ Hybridization with Probes for Repetitive Elements

We tested the distribution of repetitive elements in sex chromosomes of three microsatellite motifs: GATA, AG, and TAC in the species where we had female individuals. Those microsatellite motifs often accumulate in the heterochromatic regions of sex chromosomes in reptiles, including caenophidian snakes [37], chameleons [24], geckos [38], and turtles [39]. For the FISH experiment, we used commercially-synthesized and biotin-labelled probes with sequences $(GATA)_8$, $(AG)_{15}$, and $(TAC)_8$ from Macrogen (Seoul, Korea). The probes were diluted in hybridization buffer (50% formamide in 2 × SSC; 10% dextran sulfate; 10% sodium dodecyl sulfate; and Denhard's buffer, pH 7.0). Slide treatment, hybridization conditions, and signal detection followed the protocol of FISH with telomeric probe with the only difference in post-hybridization washes, where we used 0.4% Nonidet P-40 (Sigma-Aldrich, St. Louis, MO, USA)/2 × SSC and 0.1% Nonidet P-40 (Sigma-Aldrich, St. Louis, MO, USA)/0.4 × SSC solutions instead of 50% formamide/2 × SSC.

2.7. Comparative Genome Hybridization (CGH)

The probes for CGH were prepared by nick translation (Abbott Laboratories, Lake Bluff, IL, USA), according to the manufacturer's protocol. The probe specific for male genome was labeled by dUTP-biotin (Roche, Basel, Switzerland), while the probe specific for female genome by dUTP-digoxigenin (Roche, Basel, Switzerland). Both probes were mixed in equal concentration and diluted in hybridization buffer (50% formamide in 2 × SSC; 10% dextran sulfate; 10% sodium dodecyl sulfate; and Denhard's buffer, pH 7.0). The FISH protocol follows the protocol of the FISH with telomeric probe for the slide preparation. The slides were incubated for 48 h at 37 °C. The probe was washed in 50% formamide/2 × SSC three times for 5 min at 37 °C. In the next step, the slides were washed in 2 × SSC at room temperature and 4 × SSC/0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA). After the washes, we incubated the slides in 4 × SSC/5% blocking reagent for 30 min at 37 °C. Subsequently, we incubated the slides with avidin-FITC (Vector Laboratories, Burlingame, CA, USA) and anti-digoxigenin-Rhodamin (Roche, Basel, Switzerland) for 30 min at 37 °C. The slides were washed in 4 × SSC/0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) and PBS solution and dehydrated in ethanol series. The slides were stained by Fluoroshield with DAPI (Sigma-Aldrich, St. Louis, MO, USA).

2.8. Microscopy and Image Analyses

We captured Giemsa-stained metaphases with an Axio Imager Z2 microscope (Zeiss, Oberkochen, Germany), equipped with automatic Metafer-MSearch scanning platform and a CoolCube 1 b/w digital camera (MetaSystems, Altlussheim, Germany). Karyograms were assembled using the software Ikaros (MetaSystems, Altlussheim, Germany). Fluorochrome-stained metaphases were examined under Provis AX70 microscope (Olympus, Tokyo, Japan) equipped with DP30BW camera (Olympus, Tokyo, Japan). The photos from FISH experiments were pseudo-colored and merged in DP Manager (Olympus, Tokyo, Japan). The color of C-banded metaphases was inverted.

3. Results

3.1. Furcifer antimena

The karyotype consists of $2n = 24$ chromosomes (Figure 1a), in accordance with the results of Bourgat [40]. C-banding revealed heterochromatin in the centromeric and pericentromeric chromosome regions of many chromosomes (Figure 2a). Only males were examined and no sign of sex chromosomes were identified. The telomeric sequences were detected in terminal positions of all chromosomes and in the centromeric, pericentromeric, and interstitial regions of the sixth largest chromosome pairs (Figure 3a). rDNA loci were located in terminal position of the q arm of the third chromosome pair (Figure 4a).

3.2. Furcifer bifidus

The karyotype consists of $2n = 24$ chromosomes in males, but $2n = 23$ chromosomes in females (Figure 1c,d). C-banding revealed distribution of heterochromatin in the centromeric and pericentromeric regions in all autosomes with a notable accumulation on the W chromosome (Figure 2c,d). The sex chromosomes are of $Z_1Z_1Z_2Z_2/Z_1Z_2W$ constitution. Both Z_1 and Z_2 chromosomes are microchromosomes, while the W chromosome is medium-sized, subtelocentric, and partially heterochromatic, with a prominent C-positive band in the centromere (Figure 1c,d and Figure 2c,d). The telomeric sequences were detected in the terminal positions of all chromosomes and in the centromeric regions of all chromosomes, except the first pair and the W chromosome (Figure 3c). rDNA loci were located in the terminal position of the q arm of the second chromosome pair (Figure 4c). GATA motif accumulates in three pairs of autosomes, but not in the sex chromosomes (Figure 5a). AG motif accumulates in three pairs of autosomes and in the centromeric region of the W chromosome (Figure 5e). TAC motif accumulates in three pairs of autosomes and in the interstitial regions of the W chromosome (Figure 5i). CGH revealed strong sex-specific differences only in the female in the short arm and/or pericentromeric region of the W chromosome (Figure 5m,q).

3.3. Furcifer lateralis

The karyotype consists of $2n = 24$ chromosomes in both sexes (Figure 1e,f). C-banding revealed distribution of heterochromatin in (peri)centromeric regions in most autosomes and across the whole W chromosome (Figure 2e,f). The sex chromosomes are of the ZZ/ZW type. Both Z and W chromosomes are microchromosomes and the W chromosome is heterochromatic (Figure 2e). The telomeric sequences were detected in the terminal positions of all chromosomes and in the centromeric, pericentromeric and interstitial regions of five chromosome pairs (Figure 3d). rDNA loci were located in the terminal position of the q arm of the third chromosome pair (Figure 4d). GATA motif accumulates in two pairs of microchromosomes and in both sex chromosomes (Figure 5b). AG and TAC motifs accumulate in all autosomes and in both sex chromosomes (Figure 5f,j). CGH revealed strong sex-specific differences only in the female across the whole W chromosome (Figure 5n,r).

3.4. Furcifer minor

The karyotype consists of $2n = 22$ chromosomes (Figure 1b). C-banding revealed distribution of heterochromatin in the (peri)centromeric chromosome regions (Figure 2b). Sex chromosomes were not identified; however, only males were examined in this species. The telomeric sequences were detected in the terminal positions of all chromosomes, and in the centromeric, pericentromeric, and interstitial regions of five chromosome pairs (Figure 3b). rDNA loci were located in terminal position of the q arm of the second chromosome pair (Figure 4b).

3.5. *Furcifer verrucosus*

The karyotype consists of $2n = 22$ chromosomes in males, but $2n = 21$ chromosomes in females (Figure 1g,h). Karyotype with $2n = 22$ chromosomes was previously reported for this species from Bourgat [41]. C-banding revealed distribution of heterochromatin in the centromeric and pericentromeric regions in many autosomes and notable block on the W chromosome (Figure 2g,h). The sex chromosomes are of the $Z_1Z_1Z_2Z_2/Z_1Z_2W$ type. Both Z_1 and Z_2 chromosomes are microchromosomes, while the W chromosome is small-sized, subtelocentric and partially heterochromatic (Figures 1g and 2g). The telomeric sequences were detected in the terminal positions of all chromosomes and in the centromeric, pericentromeric and interstitial regions of six chromosome pairs (Figure 3e). rDNA loci were located in the terminal position of the q arm of the 4th chromosome pair (Figure 4e). GATA motif accumulates in the W chromosome (Figure 5c). AG motif accumulates in all chromosomes, including the sex chromosomes (Figure 5g). TAC motif accumulates in the W chromosome (Figure 5k). CGH revealed strong sex-specific differences only in females in the short arm and part of the long arm of the W chromosome (Figure 5o,s).

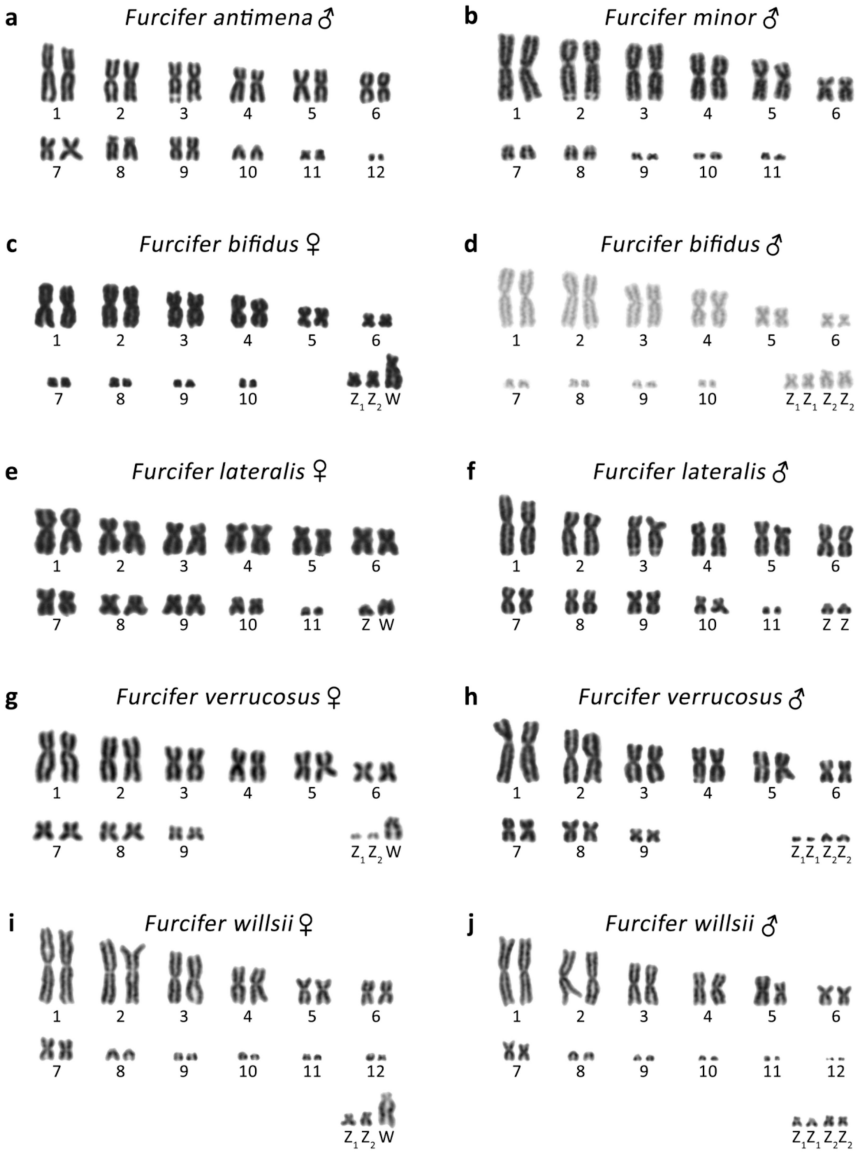


Figure 1. Giemsa-stained karyograms of *Furcifer antimena* (**a**), *F. minor* (**b**), *F. bifidus* (**c**,**d**), *F. lateralis* (**e**,**f**), *F. verrucosus* (**g**,**h**), and *F. willsii* (**i**,**j**). Sex chromosomes are indicated.

3.6. *Furcifer willsii*

The karyotype consists of $2n = 28$ chromosomes in males, but $2n = 27$ chromosomes in females (Figure 1i,j). A karyotype with $2n = 28$ chromosomes was previously reported for this species [42]. C-banding revealed distribution of heterochromatin in the (peri)centromeric regions in all autosomes and a large accumulation in the W chromosome (Figure 2i,j). The sex chromosomes are of the $Z_1Z_1Z_2Z_2/Z_1Z_2W$ type. Both Z_1 and Z_2 chromosomes are microchromosomes, while the W chromosome is large, subtelocentric, and partially heterochromatic (Figures 1i and 2i). The telomeric sequences were detected in terminal positions of all chromosomes and in the centromeric regions of the first and second chromosome pair and in the W chromosome (Figure 3e). rDNA loci are located in the terminal positions of four chromosome pairs in five clusters (Figure 4f). GATA, AG, and TAC motifs accumulate in the centromeric regions of the first and second chromosome pair and in the p arm of the W chromosome (Figure 5d,h,l). CGH revealed strong sex-specific differences only in the central part of the long arm of the W chromosome (Figure 5p,t).

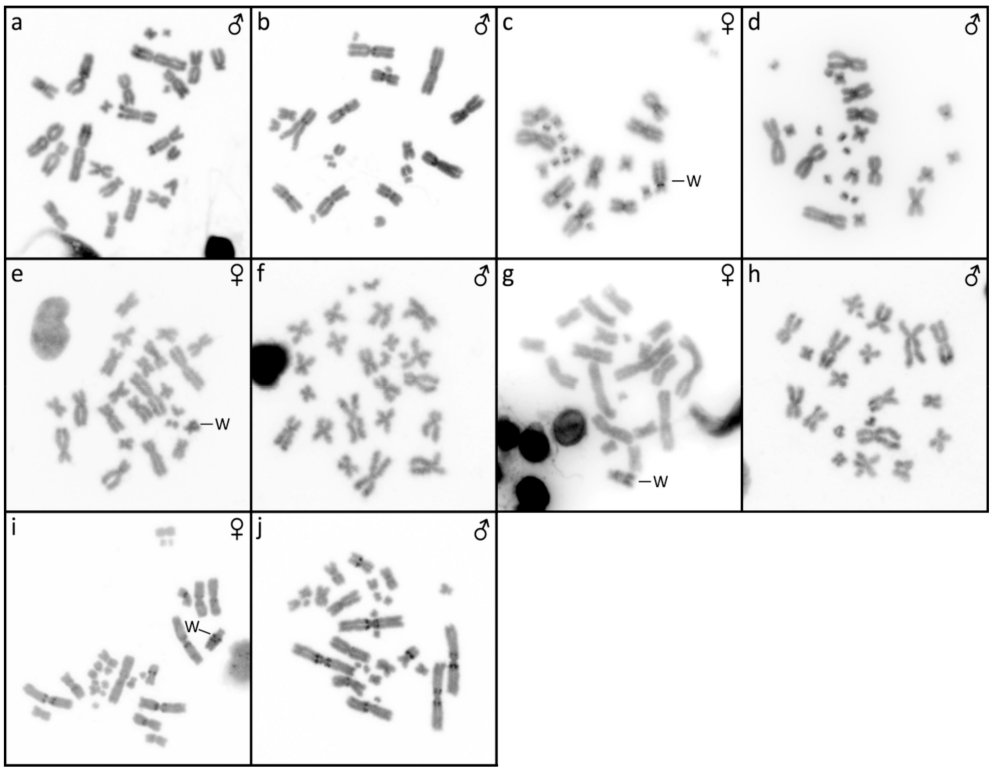


Figure 2. C-banded metaphases of *Furcifer antimena* (**a**), *F. minor* (**b**), *F. bifidus* (**c**,**d**), *F. lateralis* (**e**,**f**), *F. verrucosus* (**g**,**h**), and *F. willsii* (**i**,**j**). W chromosome is marked in females.

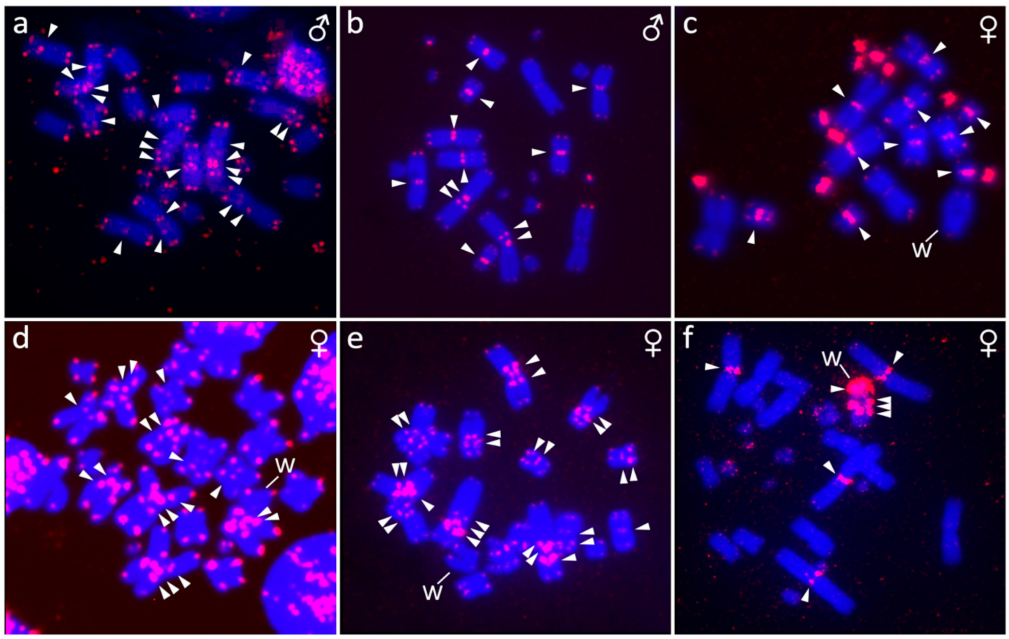


Figure 3. Position of telomeric repeats in metaphases from *Furcifer antimena* (**a**), *F. minor* (**b**), *F. bifidus* (**c**), *F. lateralis* (**d**), *F. verrucosus* (**e**), and *F. willsii* (**f**). Arrowheads point to signals detected by FISH on both arms of a pair of macrochromosomes. W chromosome is marked in females.

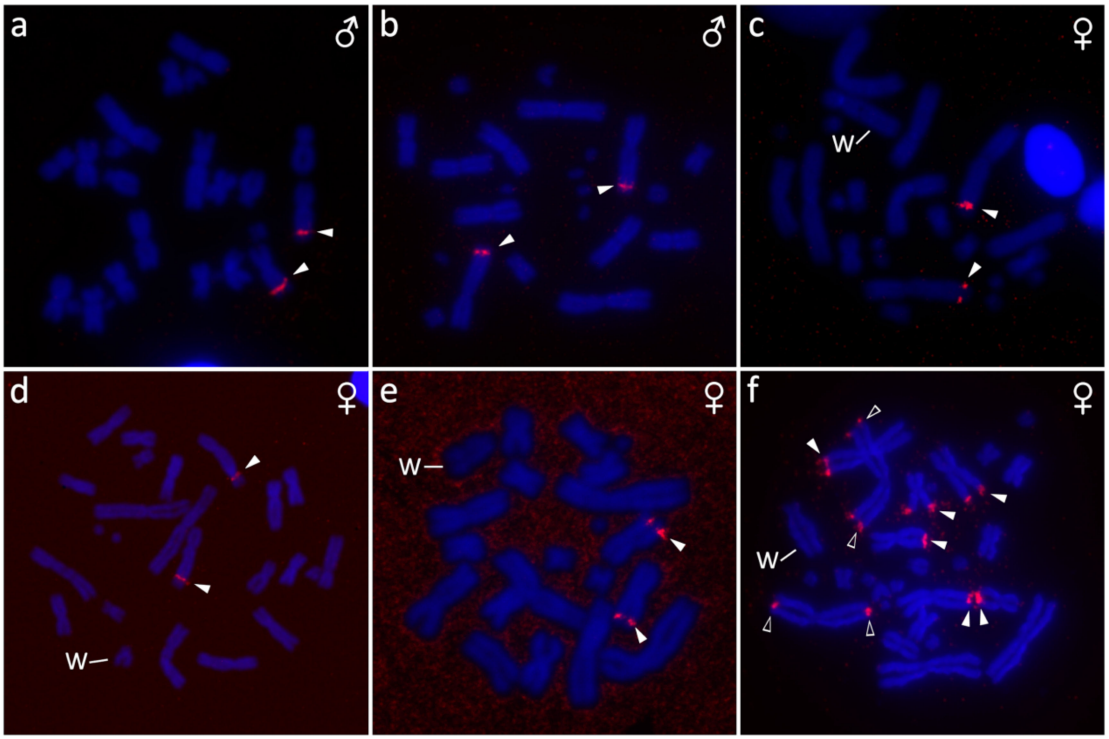


Figure 4. Position of rDNA loci in metaphases of *Furcifer antimena* (**a**), *F. minor* (**b**), *F. bifidus* (**c**), *F. lateralis* (**d**), *F. verrucosus* (**e**) and *F. willsii* (**f**). Arrowheads point to signals detected by fluorescence in situ hybridization (FISH) with empty arrowheads marking signals on both arms of a pair of macrochromosomes. W chromosome is marked in females.

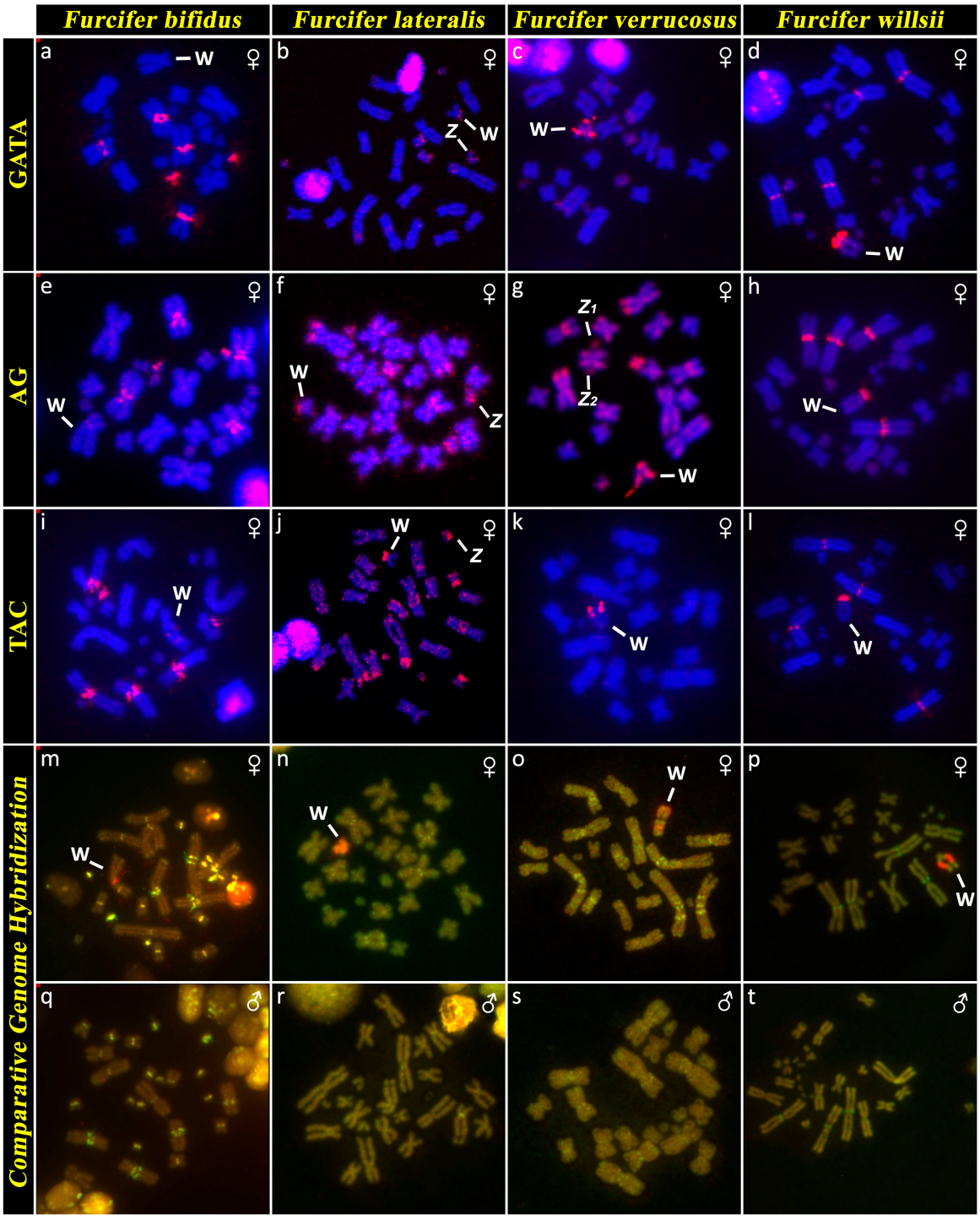


Figure 5. Distribution of the microsatellite motifs GATA (**a**–**d**), AG (**e**–**h**), and TAC (**i**–**l**), and comparative genome hybridization (CGH) (**m**–**t**) in *Furcifer bifidus*, *F. lateralis*, *F. verrucosus*, and *F. willsii*. Sex chromosomes with signal from in situ hybridization are marked. Note that there are no visible sex-specific regions in males (**q**–**t**), in comparison to females, where a female-specific region was detected in the W chromosome (**m**–**p**).

4. Discussion

Chromosome numbers revealed by us in *F. antimena*, *F. verrucosus*, and *F. willsii* agree with the pioneering descriptions of their male karyotypes performed before the onset of advanced cytogenetic techniques [40–42]. According to our knowledge, karyotypes of three species (*F. bifidus*, *F. lateralis*, and

F. minor) are described here for the first time. Our study increased the number of the chameleons of the genus *Furcifer* with described karyotypes to 14 (reviewed in [43]) and confirmed that the members of this genus have rather variable karyotypes, with diploid chromosome numbers ($2n$) in males being $2n$ = 22, 24, 26, and 28. Notably, chameleons range in diploid chromosome numbers from $2n$ = 20 to $2n$ = 62 [43] and have generally higher variability in comparison to other closely-related squamate lineages, such as iguanas ($2n$ = 19–48) [44–46], snakes ($2n$ = 24–56) [44,47], and varanids ($2n$ = 40 in all studied species) [48]. This variability is visible not only in the chromosomal numbers, but also in differences in size and shape of chromosomes as can be exemplified in the variation of the position of interstitial telomeric repeats (Figure 3) and rDNA loci (Figure 4). Such variation could be the result of multiple inter- and intra-chromosomal rearrangements.

Before our present study, a chromosome system of sex determination was known in only three species of chameleons (*F. oustaleti, F. pardalis,* and *C. calyptratus*) [10,24]. Our data show that female heterogamety is common in the genus *Furcifer*, as we detected cytogenetically distinguishable sex chromosomes in all four species (*F. bifidus*, *F. lateralis*, *F. verrucosus,* and *F. willsii*), with available female specimens. The W chromosomes in all these species contain notable heterochromatic blocks (Figure 2) and CGH revealed that their genetic content is at least partially female-specific (Figure 5) in accordance with the previously published results in *F. oustaleti* and *F. pardalis* [24]. The female-specific signals in CGH co-localize with heterochromatin in the W chromosomes. On the contrary, the XX/XY sex chromosomes in *C. calyptratus* are euchromatic and not detectable by CGH [43,49]. C-banding, CGH, and FISH with telomeric repeats did not detect any clear sex-specific differences in karyotypes of several other species of chameleons from the genera *Calumma, Rieppeleon,* and *Trioceros* [43]. It seems that female heterogamety with a distinct W sex chromosome is an apomorphy of the clade containing the studied members of the genus *Furcifer* [50–53]. Only limited (AG and TAC) or even lack (GATA) of accumulations of microsatellite motifs were observed on the W chromosome in *F. bifidus*, but notable accumulation of all three motifs was observed in *F. verrucosus*, *F. lateralis*, and *F. willsii*, suggesting that the W chromosomes are homologous in these three species, with the accumulations emerging in their common ancestor after their split from *F. bifidus*. Despite this variability in the W chromosomes, the Z chromosomes seem to have similar morphology in all examined species. Therefore, we assume that female heterogamety was already present in the common ancestor of the studied members of the genus *Furcifer* living approximately 30 million years ago (dating follows [51]).

In vertebrates, multiple neo-sex chromosomes evolve frequently under male heterogamety, but rarely under female heterogamety as documented in mammals, birds, reptiles, and fishes [54,55]. Several hypotheses were suggested to explain this pattern, including stronger sexual selection in males reducing effective population size for the Y, male-biased mutation rates [55], and the preferential segregation against neo-sex chromosomes from meiotic drive in female meiosis [54]. The latter hypothesis suggests that rearrangements connected with the emergence of multiple neo-sex chromosomes under female heterogamety could result in a biased sex ratio due to the female meiotic drive, which should be penalized by selection for equal sex ratio. According to this hypothesis, the fixation of the rearrangements of the Y chromosome leading to multiple neo-sex chromosomes can be fixed in a population more frequently, as this male-specific chromosome is not involved in female meiosis and is therefore sheltered against the effects of the female meiotic drive affecting the X, Z, and W chromosomes and autosomes. Interestingly, four species of the genus *Furcifer* (*F. bifidus*, *F. verrucosus*, *F. willsii*, and *F. pardalis*) with female heterogamety possess multiple sex chromosomes. Multiple sex chromosomes can arise either by fusion of the ancestral W chromosome with an autosome or by fission of the ancestral Z chromosome. In the members of the genus *Furcifer* with multiple sex chromosomes, the W is always a large chromosome, therefore, we assume that their multiple sex chromosomes were formed by fusions. Even more interestingly, four species with multiple neo-sex chromosomes do not form a monophyletic clade in respect to the two species with simple ZZ/ZW sex chromosomes (*F. lateralis* and *F. oustaleti*) in the reconstructed phylogeny of the genus (Figure 6). We assume that the $Z_1Z_1Z_2Z_2/Z_1Z_2W$ neo-sex chromosomes were formed independently several, possibly even four times,

from the ancestral simple ZZ/ZW system. However, we cannot exclude a scenario that simple ZZ/ZW sex chromosome systems are derived and emerged within the genus via chromosomal fissions from the ancestral multiple neo-sex chromosomes. These alternatives should be investigated in future by identification of the gene content of the sex/neo-sex chromosomes, allowing the assessment of their homology. The genus *Furcifer* together with elapid sea snakes, the other reptilian lineage with multiple emergences of neo-sex chromosomes under female heterogamety [54], can serve as excellent models to investigate evolutionary hypotheses on the origin of multiple neo-sex chromosomes.

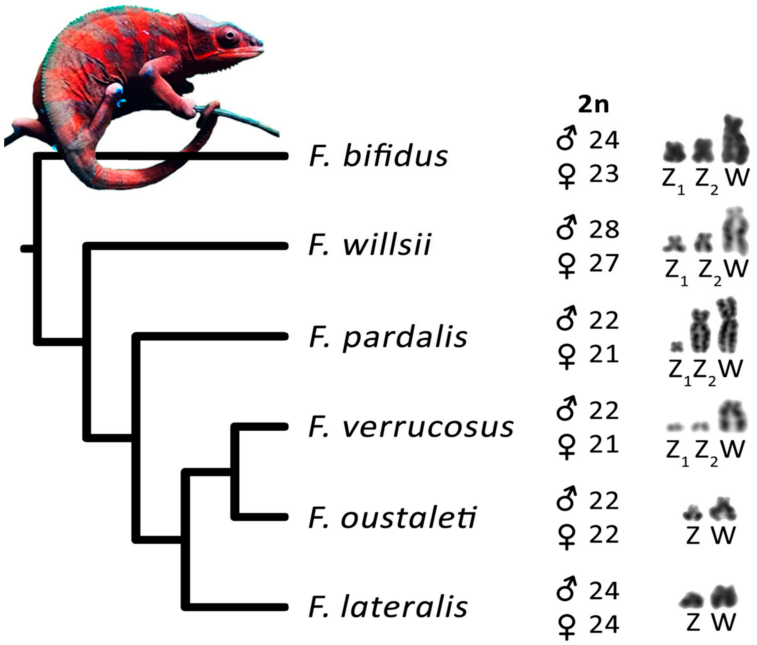


Figure 6. Phylogenetic relationships of the studied species of the genus *Furcifer* following Pyron and Burbrink [51]. Diploid chromosome numbers and sex chromosome constitution are illustrated.

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References

1. Waters, P.D.; Wallis, M.C.; Graves, J.A.M. Mammalian sex origin and evolution of the Y chromosome and SRY. *Semin. Cell Dev. Biol.* **2007**, *18*, 389–400. [CrossRef] [PubMed]
2. Gamble, T.; Geneva, A.J.; Glor, R.E.; Zarkower, D. *Anolis* sex chromosomes are derived from a single ancestral pair. *Evolution* **2014**, *68*, 1027–1041. [CrossRef] [PubMed]
3. Rovatsos, M.; Altmanová, M.; Pokorná, M.; Kratochvíl, L. Conserved sex chromosomes across adaptively radiated *Anolis* lizards. *Evolution* **2014**, *68*, 2079–2085. [CrossRef] [PubMed]
4. Rovatsos, M.; Pokorná, M.; Altmanová, M.; Kratochvíl, L. Cretaceous park of sex determination: sex chromosomes are conserved across iguanas. *Biol. Lett.* **2014**, *10*, 20131093. [CrossRef] [PubMed]
5. Rovatsos, M.; Vukić, J.; Lymberakis, P.; Kratochvíl, L. Evolutionary stability of sex chromosomes in snakes. *Proc. R. Soc. B.* **2015**, *282*, 20151992. [CrossRef]

6. Rovatsos, M.; Vukić, J.; Altmanová, M.; Johnson Pokorná, M.; Moravec, J.; Kratochvíl, L. Conservation of sex chromosomes in lacertid lizards. *Mol. Ecol.* **2016**, *25*, 3120–3126. [CrossRef]
7. Rovatsos, M.; Praschag, P.; Fritz, U.; Kratochvíl, L. Stable Cretaceous sex chromosomes enable molecular sexing in softshell turtles (Testudines: Trionychidae). *Sci. Rep.* **2017**, *7*, 42150. [CrossRef]
8. Valenzuela, N.; Adams, D.C. Chromosome number and sex determination coevolve in turtles. *Evolution* **2011**, *65*, 1808–1813. [CrossRef]
9. Gamble, T.; Coryell, J.; Ezaz, T.; Lynch, J.; Scantlebury, D.P.; Zarkower, D. Restriction site-associated DNA sequencing (RAD-seq) reveals an extraordinary number of transitions among gecko sex-determining systems. *Mol. Biol. Evol.* **2015**, *32*, 1296–1309. [CrossRef]
10. Nielsen, S.V.; Banks, J.L.; Diaz, R.E., Jr.; Trainor, P.A.; Gamble, T. Dynamic sex chromosomes in Old World chameleons (Squamata: Chamaeleonidae). *J. Evol. Biol.* **2018**, *31*, 484–490. [CrossRef]
11. Pokorná, M.; Kratochvil, L. Phylogeny of sex-determining mechanisms in squamate reptiles: are sex chromosomes an evolutionary trap? *Zool. J. Linn. Soc.* **2009**, *156*, 168–183. [CrossRef]
12. Johnson Pokorná, M.; Kratochvíl, L. What was the ancestral sex-determining mechanism in amniote vertebrates? *Biol. Rev.* **2016**, *91*, 1–12. [CrossRef] [PubMed]
13. Holleley, C.E.; O'Meally, D.; Sarre, S.D.; Graves, J.A.M.; Ezaz, T.; Matsubara, K.; Azad, B.; Zhang, X.; Georges, A. Sex reversal triggers the rapid transition from genetic to temperature-dependent sex. *Nature* **2015**, *523*, 79. [CrossRef] [PubMed]
14. Ezaz, T.; Srikulnath, K.; Graves, J.A.M. Origin of amniote sex chromosomes: an ancestral super-sex chromosome, or common requirements? *J. Hered.* **2016**, *108*, 94–105. [CrossRef]
15. Rovatsos, M.; Vukić, J.; Mrugała, A.; Suwala, G.; Lymberakis, P.; Kratochvíl, L. Little evidence for switches to environmental sex determination and turnover of sex chromosomes in lacertid lizards. *Sci. Rep.* **2019**, *9*, 7832. [CrossRef]
16. Rovatsos, M.; Rehák, I.; Velenský, P.; Kratochvíl, L. Shared ancient sex chromosomes in varanids, beaded lizards, and alligator lizards. *Mol. Biol. Evol.* **2019**, *36*, 1113–1120. [CrossRef]
17. Gamble, T.; Castoe, T.A.; Nielsen, S.V.; Banks, J.L.; Card, D.C.; Schield, D.R.; Schuett, G.W.; Booth, W. The discovery of XY sex chromosomes in a boa and python. *Curr. Biol.* **2017**, *27*, 2148–2153. [CrossRef]
18. Augstenová, B.; Johnson Pokorná, M.; Altmanová, M.; Frynta, D.; Rovatsos, M.; Kratochvíl, L. ZW, XY, and yet ZW: Sex chromosome evolution in snakes even more complicated. *Evolution* **2018**, *72*, 1701–1707. [CrossRef]
19. Uetz, P.; Freed, P.; Hošek, J. The Reptile Database. 2019. Available online: http://www.reptile-database.org (accessed on 23 October 2019).
20. Altmanová, M.; Rovatsos, M.; Johnson Pokorná, M.; Veselý, M.; Wagner, F.; Kratochvíl, L. All iguana families with the exception of basilisks share sex chromosomes. *Zoology* **2018**, *126*, 98–102. [CrossRef]
21. Viets, B.E.; Ewert, M.A.; Talent, L.G.; Nelson, C.E. Sex-determining mechanisms in squamate reptiles. *J. Exp. Zool.* **1994**, *270*, 45–56. [CrossRef]
22. Harlow, P.S. Temperature-dependent sex determination in lizards. In *Temperature-dependent Sex Determination in Vertebrates*; Valenzuela, N., Lance, V.A., Eds.; Smithsonian Institution: Washington, DC, USA, 2004; pp. 11–20.
23. Andrews, R.M. Incubation temperature and sex ratio of the veiled chameleon (*Chamaeleo calyptratus*). *J. Herpetol.* **2005**, *39*, 515–518. [CrossRef]
24. Rovatsos, M.; Pokorná, M.J.; Altmanová, M.; Kratochvíl, L. Female heterogamety in Madagascar chameleons (Squamata: Chamaeleonidae: *Furcifer*): differentiation of sex and neo-sex chromosomes. *Sci. Rep.* **2015**, *5*, 13196. [CrossRef] [PubMed]
25. Nagy, Z.T.; Sonet, G.; Glaw, F.; Vences, M. First large-scale DNA barcoding assessment of reptiles in the biodiversity hotspot of Madagascar, based on newly designed COI primers. *PLoS ONE* **2012**, *7*, e34506. [CrossRef] [PubMed]
26. Koubová, M.; Johnson Pokorná, M.; Rovatsos, M.; Farkačová, K.; Altmanová, M.; Kratochvíl, L. Sex determination in Madagascar geckos of the genus *Paroedura* (Squamata: Gekkonidae): are differentiated sex chromosomes indeed so evolutionary stable? *Chromosome Res.* **2014**, *22*, 441–452. [CrossRef] [PubMed]
27. *FinchTV*; Version 1.4.0.; Geospiza, Inc.: Seattle, WA, USA, 2019.

28. Hall, T.A. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In *Nucleic Acids Symposium Series*; Information Retrieval Ltd.: London, UK, 1999; Volume 41, pp. 95–98.
29. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [CrossRef] [PubMed]
30. Rozas, J.; Ferrer-Mata, A.; Sánchez-DelBarrio, J.C.; Guirao-Rico, S.; Librado, P.; Ramos-Onsins, S.; Sánchez-Gracia, A. DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Mol. Biol. Evol.* **2017**, *34*, 3299–3302. [CrossRef]
31. Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, D.J. Basic local alignment search tool. *J. Mol. Biol.* **1990**, *215*, 403–410. [CrossRef]
32. Mazzoleni, S.; Augstenová, B.; Clemente, L.; Auer, M.; Fritz, U.; Praschag, P.; Protiva, T.; Velenský, P.; Kratochvíl, L.; Rovatsos, M. Turtles of the genera *Geoemyda* and *Pangshura* (Testudines: Geoemydidae) lack differentiated sex chromosomes: The end of a 40-year error cascade for *Pangshura*. *PeerJ* **2019**, *7*, e6241. [CrossRef]
33. Sumner, A.T. A simple technique for demonstrating centromeric heterochromatin. *Exp. Cell Res.* **1972**, *75*, 304–306. [CrossRef]
34. Ijdo, J.W.; Baldini, A.; Ward, D.C.; Reeders, S.T.; Wells, R.A. Origin of human chromosome 2: An ancestral telomere-telomere fusion. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 9051–9055. [CrossRef]
35. Rovatsos, M.T.; Marchal, J.A.; Romero-Fernández, I.; Fernández, F.J.; Giagia-Athanosopoulou, E.B.; Sánchez, A. Rapid, independent, and extensive amplification of telomeric repeats in pericentromeric regions in karyotypes of arvicoline rodents. *Chromosome Res.* **2011**, *19*, 869–882. [CrossRef] [PubMed]
36. Endow, S.A. Polytenization of the ribosomal genes on the X and Y chromosomes of *Drosophila melanogaster*. *Genetics* **1982**, *100*, 375–385. [PubMed]
37. Augstenová, B.; Mazzoleni, S.; Kratochvíl, L.; Rovatsos, M. Evolutionary dynamics of the W chromosome in caenophidian snakes. *Genes* **2018**, *9*, 5. [CrossRef] [PubMed]
38. Matsubara, K.; Knopp, T.; Sarre, S.D.; Georges, A.; Ezaz, T. Karyotypic analysis and FISH mapping of microsatellite motifs reveal highly differentiated XX/XY sex chromosomes in the pink-tailed worm-lizard (*Aprasia parapulchella*, Pygopodidae, Squamata). *Mol. Cytogenet.* **2013**, *6*, 60. [CrossRef]
39. Matsubara, K.; O'Meally, D.; Azad, B.; Georges, A.; Sarre, S.D.; Graves, J.A.M.; Matsuda, Y.; Ezaz, T. Amplification of microsatellite repeat motifs is associated with the evolutionary differentiation and heterochromatinization of sex chromosomes in Sauropsida. *Chromosoma* **2016**, *125*, 111–123. [CrossRef]
40. Bourgat, R.M. New karyotypes of Malagasian Chamaeleonidae. Confirmation of the existence of 2 types of chromosomal patterns. *C.R. Seances Soc. Biol.* **1971**, *165*, 1200–1203.
41. Bourgat, R.M. Cytogénétique des caméléons de Madagascar. Incidences taxonomiques, biogéographiques et phylogénétiques. *Bull. Soc. Zool. France* **1973**, *98*, 81–90.
42. Matthey, R.; Van Brink, J. Nouvelle contribution à la cytologie comparée des Chamaeleontidae (Reptilia-Lacertilia). *Bull. Soc. Vaud. Sci. Nat.* **1960**, *67*, 333–348.
43. Rovatsos, M.; Altmanová, M.; Johnson Pokorná, M.; Velenský, P.; Sánchez Baca, A.; Kratochvíl, L. Evolution of karyotypes in chameleons. *Genes* **2017**, *8*, 382. [CrossRef]
44. Olmo, E.; Signorino, G.G. Chromorep: A Reptile Chromosomes Database. 2005. Available online: http://chromorep.univpm.it (accessed on 23 October 2019).
45. Bertolotto, C.E.V.; Rodrigues, M.T.; Yonenaga-Yassuda, Y. Banding patterns, multiple sex chromosome system and localization of telomeric (TTAGGG)n sequences by FISH on two species of *Polychrus* (Squamata, Polychrotidae). *Caryologia* **2001**, *54*, 217–226. [CrossRef]
46. Webster, T.P.; Hall, W.P.; Williams, E.E. Fission in the evolution of a lizard karyotype. *Science* **1972**, *177*, 611–613. [CrossRef] [PubMed]
47. Oguiura, N.; Ferrarezzi, H.; Batistic, R.F. Cytogenetics and molecular data in snakes: A phylogenetic approach. *Cytogenet. Genome Res.* **2009**, *127*, 128–142. [CrossRef] [PubMed]
48. Iannucci, A.; Altmanová, M.; Ciofi, C.; Ferguson-Smith, M.; Milan, M.; Pereira, J.C.; Pether, J.; Rehák, I.; Rovatsos, M.; Stanyon, R.; et al. Conserved sex chromosomes and karyotype evolution in monitor lizards (Varanidae). *Heredity* **2019**, *123*, 215–227. [CrossRef] [PubMed]

49. Pokorná, M.; Giovannotti, M.; Kratochvíl, L.; Kasai, F.; Trifonov, V.A.; O'Brien, P.C.M.; Caputo, V.; Olmo, E.; Ferguson-Smith, M.A.; Rens, W. Strong conservation of the bird Z chromosome in reptilian genomes is revealed by comparative painting despite 275 million years divergence. *Chromosoma* **2011**, *120*, 455–468. [CrossRef] [PubMed]
50. Townsend, T.M.; Tolley, K.A.; Glaw, F.; Böhme, W.; Vences, M. Eastward from Africa: paleocurrent-mediated chameleon dispersal to the Seychelles islands. *Biol. Lett.* **2010**, *7*, 225–228. [CrossRef] [PubMed]
51. Pyron, R.A.; Burbrink, F.T. Early origin of viviparity and multiple reversions to oviparity in squamate reptiles. *Ecol. Lett.* **2014**, *17*, 13–21. [CrossRef] [PubMed]
52. Zheng, Y.; Wiens, J.J. Combining phylogenomic and supermatrix approaches, and a time-calibrated phylogeny for squamate reptiles (lizards and snakes) based on 52 genes and 4162 species. *Mol. Phylogenet. Evol.* **2016**, *94*, 537–547. [CrossRef]
53. Tonini, J.F.R.; Beard, K.H.; Ferreira, R.B.; Jetz, W.; Pyron, R.A. Fully-sampled phylogenies of squamates reveal evolutionary patterns in threat status. *Biol. Conserv.* **2016**, *204*, 23–31. [CrossRef]
54. Pokorná, M.; Altmanová, M.; Kratochvíl, L. Multiple sex chromosomes in the light of female meiotic drive in amniote vertebrates. *Chromosome Res.* **2014**, *22*, 35–44. [CrossRef]
55. Pennell, M.W.; Kirkpatrick, M.; Otto, S.P.; Vamosi, J.C.; Peichel, C.L.; Valenzuela, N.; Kitano, J. Y fuse? Sex chromosome fusions in fishes and reptiles. *PLoS Genet.* **2015**, *11*, e1005237. [CrossRef]



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Part III

Sex determination and sex chromosome evolution in anguimorphan lizards

Chapter 6

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Cytogenetic Evidence for Sex Chromosomes and Karyotype Evolution in Anguimorphan Lizards

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Abstract: Anguimorphan lizards are a morphologically variable group of squamate reptiles with a wide geographical distribution. In spite of their importance, they have been cytogenetically understudied. Here, we present the results of the cytogenetic examination of 23 species from five anguimorphan families (Anguidae, Helodermatidae, Shinisauridae, Varanidae and Xenosauridae). We applied both conventional (Giemsa staining and C-banding) and molecular cytogenetic methods (fluorescence *in situ* hybridization with probes for the telomeric motifs and rDNA loci, comparative genome hybridization), intending to describe the karyotypes of previously unstudied species, to uncover the sex determination mode, and to reveal the distribution of variability in cytogenetic characteristics among anguimorphan lizards. We documented that karyotypes are generally quite variable across anguimorphan lineages, with anguids being the most varying. However, the derived chromosome number of 2n = 40 exhibits a notable long-term evolutionary stasis in monitors. Differentiated ZZ/ZW sex chromosomes were documented in monitors and helodermatids, as well as in the anguids *Abronia lythrochila*, and preliminary also in *Celestus warreni* and *Gerrhonotus liocephalus*. Several other anguimorphan species have likely poorly differentiated sex chromosomes, which cannot be detected by the applied cytogenetic methods, although the presence of environmental sex determination cannot be excluded. In addition, we uncovered a rare case of spontaneous triploidy in a fully grown *Varanus primordius*.

Keywords: evolution; CGH; FISH; karyotype; rDNA; sex chromosomes; telomeres

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1. Introduction

Sex chromosomes have evolved independently across vertebrates multiple times [1–3]. They mostly evolve from a pair of autosomes by the emergence of a sex-determining gene, and may further differentiate via the suppression of recombination leading to the loss of functional genes, accumulation of heterochromatin and/or repetitive elements in the sex-specific chromosome [4–6]. For decades, conventional and, more recently, molecular cytogenetic methods have been used to identify sex chromosomes and the degree of their differentiation. Cytogenetic analysis showed that the process of sex chromosome differentiation varies significantly among lineages: some lineages such as skinks possessed for a long evolutionary time relatively poorly differentiated, cytogenetically hardly distinguishable sex chromosomes [7], while others such as the chameleons of the genus *Furcifer* evolved heteromorphic sex chromosomes in a much shorter evolutionary time than, e.g., skinks [8,9]. Sex chromosomes are often detectable by cytogenetic techniques due to their unusual content of repetitive sequences. Extensive accumulation of various repetitive elements such as rDNA loci, microsatellite motifs, and retrotransposons has been detected in sex chromosomes of many animal and plant species, often in heterochromatic regions [10–19].

Anguimorpha is a group of squamate reptiles distributed in all continents except Antarctica, containing around 240 extant species [20] divided into seven families: Anguidae, Anniellidae, Helodermatidae, Lanthanotidae, Shinisauridae, Varanidae, and Xenosauridae [21]. The most species-rich anguimorphan groups are the families Anguidae (slow

worms, glass lizards, and alligator lizards) and Varanidae (monitors) [20]. Monitors are also the cytogenetically most studied group of the anguimorphan clade. The karyotype has been so far described in 30 species [16,22–33]. All species of monitors show karyotypes with the 2n = 40 chromosomes (16 macro- and 24 microchromosomes) and similar morphology of chromosomes. A notable variability has been detected in them only in the morphology of the chromosome pairs 5–8, which can be attributed to intrachromosomal rearrangements according to the results of chromosome painting [33]. All studied species of monitors show ZZ/ZW system of sex determination [33,34]. The sex chromosomes were cytogenetically identified in several species of monitors as a pair of microchromosomes, where the W is usually heterochromatic [16,25,33]. Comparative chromosome painting [33] and comparison of the differences in gene copy numbers of Z-specific genes between sexes by quantitative PCR (qPCR) revealed an extensive homology of the sex chromosomes across monitors [34]. The Z chromosome of monitors is largely homologous to chicken chromosome 28, and the gene *anti-Müllerian hormone* (*amh)* was proposed as a candidate sex-determining locus [34,35].

In addition to monitors, sex chromosomes were uncovered by cytogenetic methods in *Heloderma suspectum* (Helodermatidae) [36]. The sex chromosomes of this species are microchromosomes with highly heterochromatic W [36]. The qPCR test of sex chromosome homology revealed that three tested species of helodermatids (*Heloderma suspectum, Heloderma horridum* and *Heloderma exasperatum*) as well as the alligator lizard *Abronia lythrochila* (Anguidae) share the same sex chromosomes with monitors [34]. The homology of the Z chromosomes among the families Anguidae, Helodermatidae, and Varanidae suggests that the age of the anguimorphan sex chromosomes can be estimated to 115–180 MY [34]. Interestingly, the tested Z-specific genes of monitors and helodermatids were pseudoautosomal or autosomal in the slow worm *Anguis fragilis* (Anguidae) suggesting that the sex chromosomes of certain species of anguids might not be homologous to those of the other studied anguimorphan lizards [34].

Previous studies described the karyotypes of one unspecified species of the genus *Xenosaurus* (Xenosauridae), three species of the family Anniellidae (*Anniella geronimensis, Anniella pulchra, Anniella stebbinsi*) and 12 species of the family Anguidae (*Abronia monticola, Anguis fragilis, Anguis veronensis, Celestus costatus, Diploglossus fasciatus, Diploglossus millepunctatus, Elgaria coerulea, Elgaria multicarinata, Elgaria paucicarinata, Ophiodes striatus, Ophisaurus ventralis, Pseudopus apodus* [37–43]. Sex chromosomes were not identified in any of these species; however, they might escape detection, as in some cases individuals of single or unknown sex were studied and mostly only conventional cytogenetic methods were applied.

Our current knowledge of the sex determination systems in anguimorphan lizards has been limited to monitors, helodermatids, and a single species of alligator lizard [33,34,36]. In the current study, we examined anguimorphan lizards from five families (Anguidae, Helodermatidae, Shinisauridae, Xenosauridae, and Varanidae) by both conventional (Giemsa staining, C-banding) and molecular cytogenetic methods (fluorescence *in situ* hybridization with probes for telomeric motifs and rDNA loci, comparative genome hybridization).

2. Materials and Methods

2.1. Studied Material, Chromosome Preparations and Staining

We studied 23 species from five families of Anguimorpha: Abronia campbelli, Abronia deppii, Abronia graminea, Abronia lythrochila, Abronia mixteca, Abronia smithi, Abronia taeniata, Barisia rudicollis, Celestus warreni, Gerrhonotus liocephalus (Anguidae), Heloderma exasperatum, Heloderma horridum (Helodermatidae), Shinisaurus crocodilurus (Shinisauridae), Varanus auffenbergi, Varanus cumingi, Varanus kordensis, Varanus olivaceus, Varanus primordius, Varanus salvadorii, Varanus salvator komaini (Varanidae), Xenosaurus grandis, Xenosaurus platyceps, Xenosaurus rectocollaris (Xenosauridae) (Table S1).

We collected 0.2–2 mL of peripheral blood, which was subsequently used for DNA isolation and preparation of mitotic chromosome suspensions. DNA was isolated using the

DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. We used whole blood cell cultures to prepare mitotic chromosome suspensions according to our standard protocol described in [44].

We used C-banding to determine the distribution of constitutive heterochromatin. Slides were treated according to the protocol of Sumner [45] with slight modifications: first, we treated the slides with 0.2 N HCl for 35 min at room temperature, then in 5% $Ba(OH)_2$ for 9 min at 45 °C, and subsequently in 2× SSC for 1 h at 60 °C. In a final step, the slides were washed with distilled water, air-dried, and stained with Fluoroshield mounting medium with DAPI (4′, 6-diamidino-2-phenylindole; Sigma-Aldrich, St. Louis, MO, USA).

2.2. *Fluorescence* In Situ *Hybridization with Probe for Telomeric Sequences*

For the Fluorescence *in situ* Hybridization (FISH) with telomeric probes, we used standard protocols of Ijdo et al. [46] and Mazzoleni et al. [44]. The telomeric probe $(TTAGGG)_n$ was prepared by Polymerase Chain Reaction (PCR) without DNA template using the primers $(TTAGGG)_5$ and $(CCCTAA)_5$. The probe was precipitated overnight at −20 °C by sodium acetate (3M), salmon sperm, and ethanol and subsequently diluted in 50% formamide in 2× SSC (pH 7).

The chromosomal spreads were treated according to the protocol described in [44]. Briefly, the slides were treated with RNAse (100 µg/mL) for 60 min at 37 °C, and washed in 2× saline–sodium citrate (2× SSC) buffer. They were subsequently treated with 0.01% pepsin for 10 min at 37 °C, washed in phosphate-buffered saline (PBS), then treated with the solution of 1% formaldehyde in 2× SSC, and afterwards washed again in PBS. Slides were dehydrated in ethanol series and air-dried. Dried slides were denatured in 70% formamide in 2× SSC at 70 °C for 3 min, washed in 2× SSC, and again dehydrated in ethanol series and air-dried. The probe was applied to the chromosome spreads for overnight hybridization at 37 °C.

The following day the slides were washed three times in 50% formamide in 2× SSC at 37 °C for 5 min, then in 2× SSC and subsequently in 4× SSC/0.05% Tween20 (Sigma-Aldrich) for 5 min. Afterwards, we applied 4× SSC/5% blocking reagent (Roche, Basel, Switzerland) and incubated the slides for 45 min at 37 °C. The slides were washed in 4× SSC/0.05% Tween20 and incubated with 4× SSC/5% blocking reagent with avidin-FITC (Vector Laboratories, Burlingame, CA, USA) for 30 min at 37 °C. The signal was amplified by using avidin-FITC/biotinylated anti-avidin system (Vector Laboratories Burlingame, CA, USA). Subsequently, the slides were washed in 4× SSC/0.05% Tween20 and PBS solution, dehydrated in ethanol series and air-dried. The slides were stained by Fluoroshield mounting medium with DAPI (Sigma-Aldrich, St. Louis, MO, USA).

2.3. *Fluorescence* In Situ *Hybridization with Probe for 18S/28S rDNA Loci*

A plasmid (pDmr.a 51#1) with an 11.5-kb insert encoding the 18S and 28S ribosomal units of *Drosophila melanogaster* [47] was used to prepare the probe for the rDNA loci, according to the protocol of Rovatsos et al. [8]. The probe was labeled with dUTP-biotin (Roche, Basel, Switzerland) by nick translation using the manufacturer's protocol of Nick Translation Kit (Abbott Laboratories, Lake Bluff, IL, USA). The probe was precipitated and the FISH was performed according to the protocol described above.

2.4. *Comparative Genome Hybridization*

For comparative genome hybridization (CGH) we used a standard protocol described in Rovatsos et al. [9]. The probes were labeled by the Nick Translation Kit (Abbott Laboratories, Lake Bluff, IL, USA) according to the manufacturer's protocol. The female-specific probe was labeled by dUTP-digoxigenin (Roche, Basel, Switzerland) and the male-specific probe by dUTP-biotin (Roche, Basel, Switzerland). We prepared the probes for both male and female genome per species with equal concentrations and mixed them during the probe precipitation. The probes were precipitated as described above. Chromosome preparation and denaturation were the same as in the FISH experiments. The probes hybridized

for 48 h. For the signal detection, the slides were first washed three times in 50% formamide in 2× SSC at 37 °C for 5 min, subsequently twice in 2× SSC and once in 4× SSC/0.05% Tween20 for 5 min. In the next step, we applied 4× SSC/5% blocking reagent (Roche, Basel, Switzerland) for 30 min at 37 °C. Afterwards, we incubated the slides with 4× SSC/5% blocking reagent with avidin-FITC (Vector Laboratories Burlingame, CA, USA) and anti-digoxigenin-Rhodamine (Roche, Basel, Switzerland) for 30 min at 37 °C. In the end, the slides were washed twice in 4× SSC/0.05% Tween20, once in PBS solution, and dehydrated in ethanol series. The slides were air-dried and stained by Fluoroshield with DAPI (Sigma-Aldrich, St. Louis, MO, USA).

2.5. Microscopy and Image Analyses

The Giemsa-stained slides were scanned with an Axio Imager Z2 microscope (Zeiss, Oberkochen, Germany) equipped with Metafer-MSearch scanning platform and a CoolCube 1 b/w digital camera (MetaSystems, Altlussheim, Germany). The karyograms were constructed in the program Ikaros (MetaSystems). The photos of slides stained by DAPI (slides from C-banding and FISH experiments) were taken by a Provis AX70 microscope (Olympus, Tokyo, Japan) equipped with a DP30BW camera (Olympus). The photos were subsequently processed in DP Manager (Olympus).

2.6. qPCR Test for Sex Chromosome Constitution in the Triploid Varanus primordius

The cytogenetic analysis revealed a spontaneous triploid *Varanus primordius*. We applied a qPCR approach, based on Rovatsos et al. [34], to estimate the number of Z chromosomes per cell and, therefore, to reveal the sex chromosome constitution. For the qPCR test, we used primers for two autosomal genes (*adarb2*, *eef1a*, *mecom*) and one Z-specific gene (*grin3b*), designed by Rovatsos et al. [34] (Table S2).

3. Results

Out of 23 species of anguimorphan lizards examined in the current study, 22 species have been to our knowledge cytogenetically studied for the first time here. We performed karyogram reconstruction from Giemsa-stained metaphases (Figures 1–3), C-banding (Figures 4 and 5), and FISH with probes for telomeric motifs (Figures 6 and 7) and rDNA loci (Figures 8 and 9) in all species, except *Abronia smithi*, *Abronia taeniata*, and *Xenosaurus grandis*, where only karyograms were prepared due to the small amount of the obtained chromosomal material (Figures 1 and 3). In addition, CGH was performed in selected species (Figure 10).

3.1. *Abronia campbelli* (Brodie & Savage, 1993)

We examined only a male individual. Karyotype with 2n = 30 chromosomes (20 macro- and 10 microchromosomes). Among macrochromosomes, the pairs 1–3 and 7–10 are bi-armed, while the pairs 4–6 are acrocentric. The morphology of the microchromosomes cannot be determined (Figure 1a). The C-banding revealed heterochromatin mainly in centromeric regions of all macrochromosomes (Figure 4a). The telomeric sequences were detected only in the terminal positions of the chromosomes (Figure 6a). The FISH with rDNA probe revealed a signal on the 10th pair of macrochromosomes (Figure 8a).

3.2. *Abronia deppii* (Wiegmann, 1828)

Karyotype with 2n = 30 chromosomes (20 macro- and 10 microchromosomes). The pairs 1–3 and 7–10 are bi-armed, while the pairs 4–6 are acrocentric (Figure 1b,c). The C-banding revealed heterochromatin mainly in the centromeric regions of all macrochromosomes (Figure 4b,c). The telomeric sequences were detected only in the terminal positions of the chromosomes (Figure 6b,c). The FISH with rDNA probe revealed a signal on the 10th pair of macrochromosomes (Figure 8b,c). CGH did not detect any sex-specific differences (Figure 10a,b).

3.3. *Abronia graminea* (Cope, 1864)

We examined only a male individual. Karyotype with 2n = 30 chromosomes (20 macro- and 10 microchromosomes). The pairs 1–3, 8 and 10 are bi-armed, while the pairs 4–7 and 9 are acrocentric. Polymorphism was detected in the chromosomal pair 10, which consists of one submetacentric and one subtelocentric chromosome (Figure 1d). The C-banding revealed heterochromatin in the telomeric and centromeric regions of all macrochromosomes and in some microchromosomes (Figure 4d). The telomeric sequences were detected only in the terminal positions of the chromosomes (Figure 6d). The FISH with rDNA probe revealed a signal on the 10th pair of the karyogram (Figure 8d).

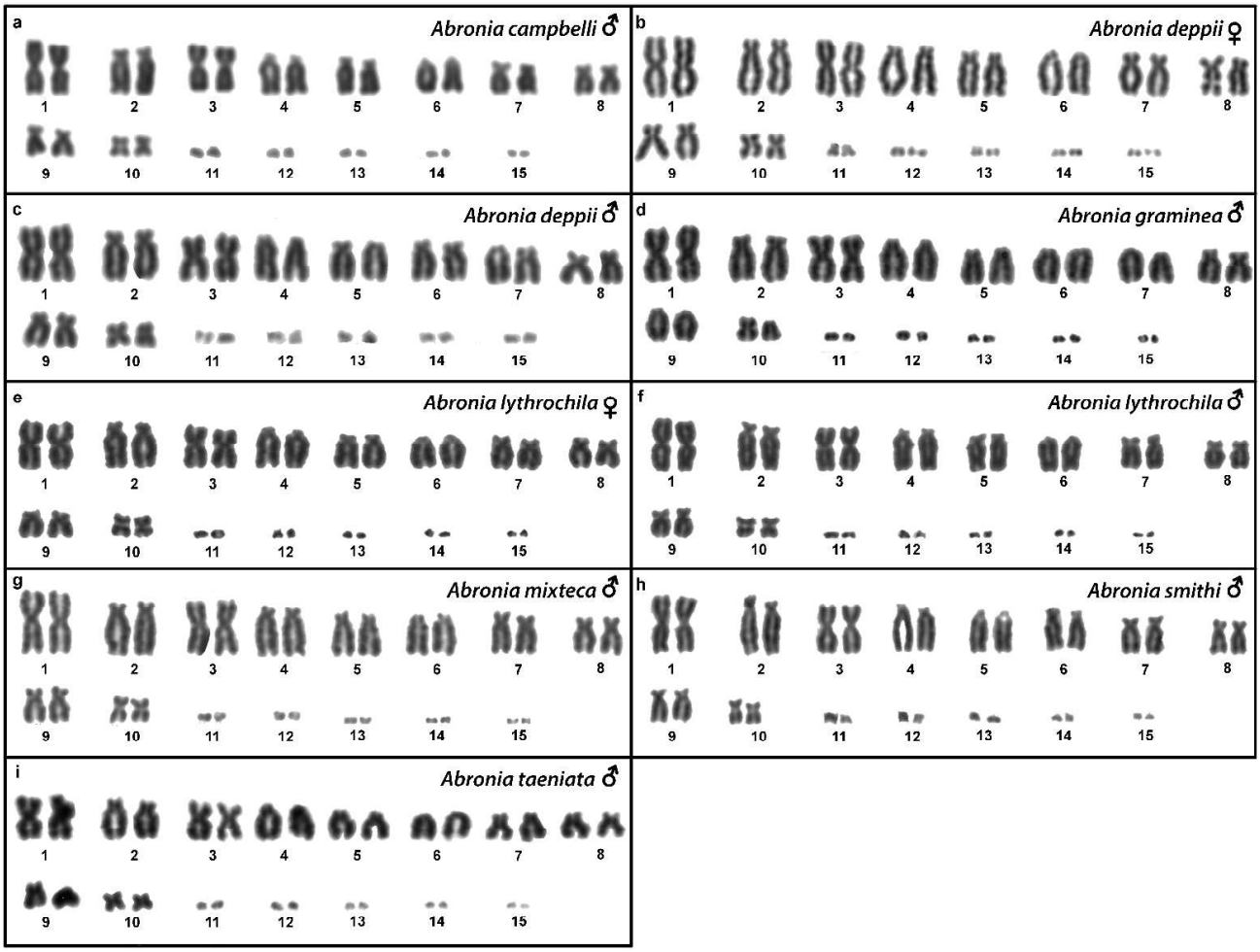


Figure 1. Giemsa-stained karyograms of *Abronia campbelli* (**a**), *Abronia deppii* (**b**,**c**), *Abronia graminea* (**d**), *Abronia lythrochila* (**e**,**f**), *Abronia mixteca* (**g**), *Abronia smithi* (**h**) and *Abronia taeniata* (**i**).

3.4. *Abronia lythrochila* Smith & Alvarez del Toro, 1963

Karyotype with 2n = 30 chromosomes (20 macro- and 10 microchromosomes). The pairs 1–3 and 7–10 are bi-armed, while the pairs 4–6 are acrocentric (Figure 1e,f). The C-banding revealed the accumulation of heterochromatin in the centromeric regions of all macrochromosomes and in the telomeric regions of some macrochromosomes (Figure 4e,f). A prominent accumulation was revealed on one of the microchromosomes in the female (Figure 4c). The telomeric sequences were detected only in the terminal positions of all chromosomes (Figure 6e,f). The FISH with rDNA probe revealed a signal on the 10th

macrochromosome pair (Figure 7e,f). CGH did not detect any sex-specific differences (Figure 10c,d).

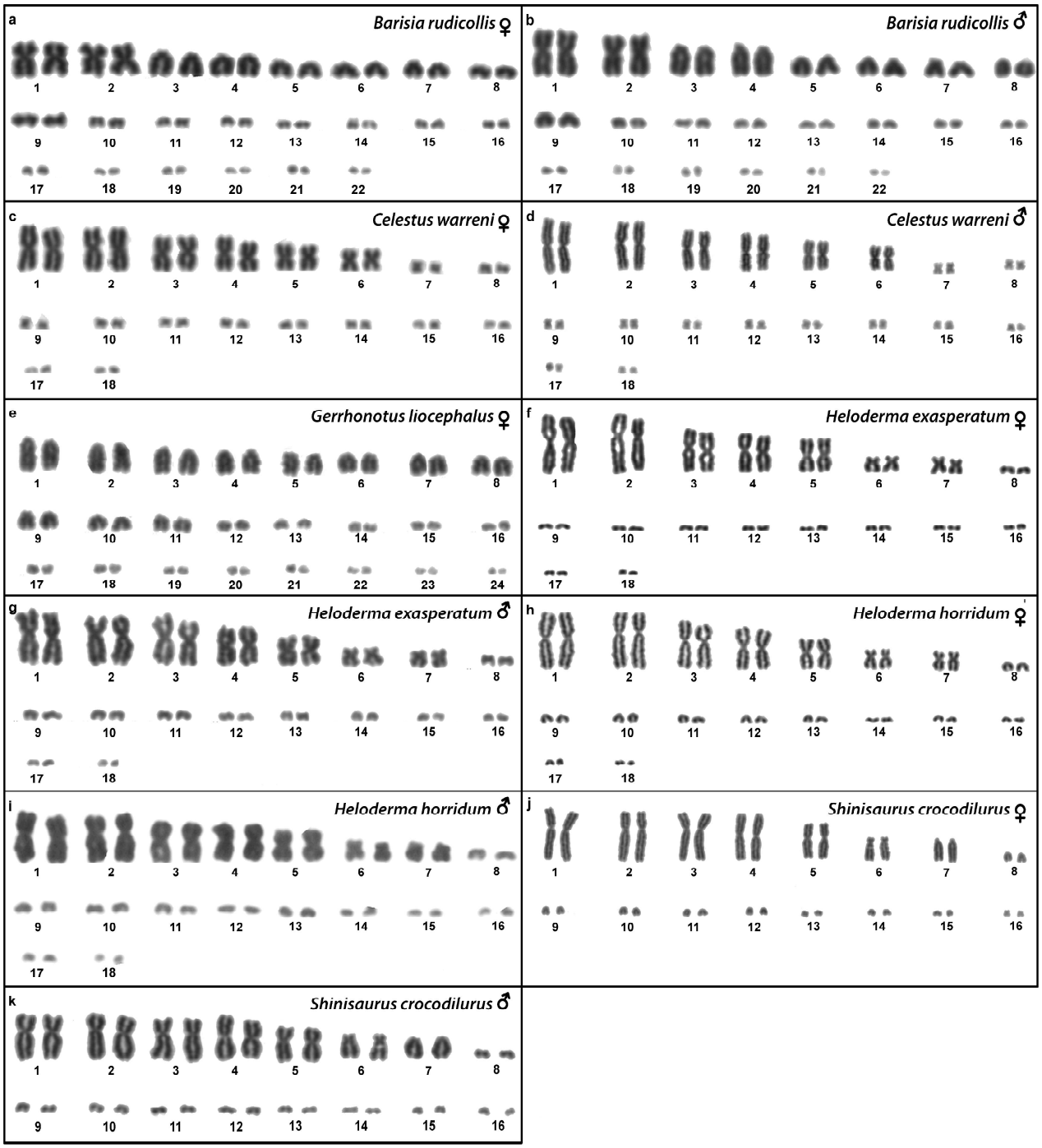


Figure 2. Giemsa-stained karyograms of *Barisia rudicollis* (**a**,**b**), *Celestus warreni* (**c**,**d**), *Gerrhonotus liocephalus* (**e**), *Heloderma exasperatum* (**f**,**g**), *Heloderma horridum* (**h**,**i**) and *Shinisaurus crocodilurus* (**j**,**k**).

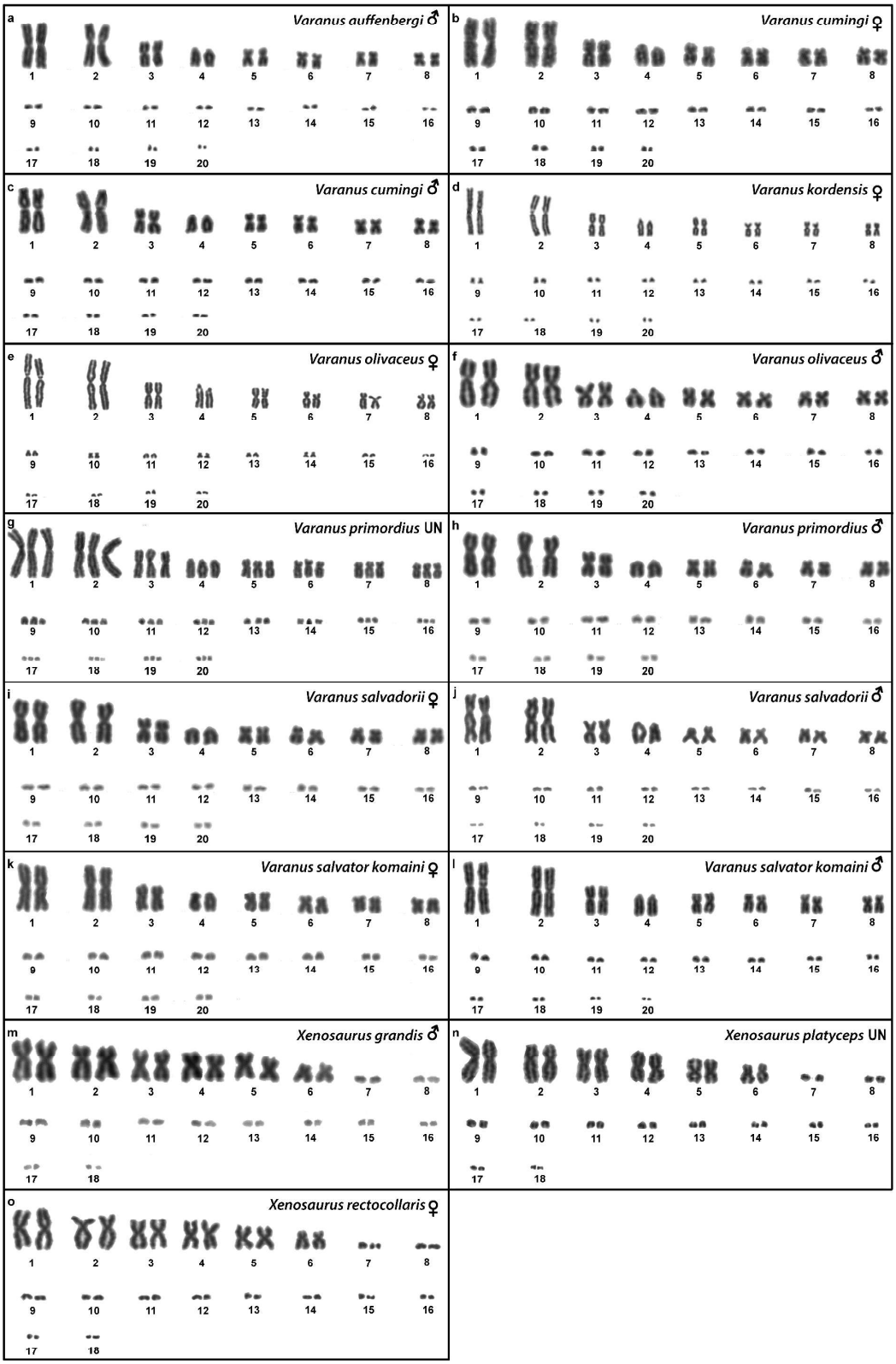


Figure 3. Giemsa-stained karyograms of *Varanus auffenbergi* (**a**), *Varanus cumingi* (**b**,**c**), *Varanus kordensis* (**d**), *Varanus olivaceus* (**e**,**f**), *Varanus primordius* (**g**,**h**), *Varanus salvadorii* (**i**,**j**), *Varanus salvator komaini* (**k**,**l**), *Xenosaurus grandis* (**m**), *Xenosaurus platyceps* (**n**) and *Xenosaurus rectocollaris* (**o**). UN: sex not known.

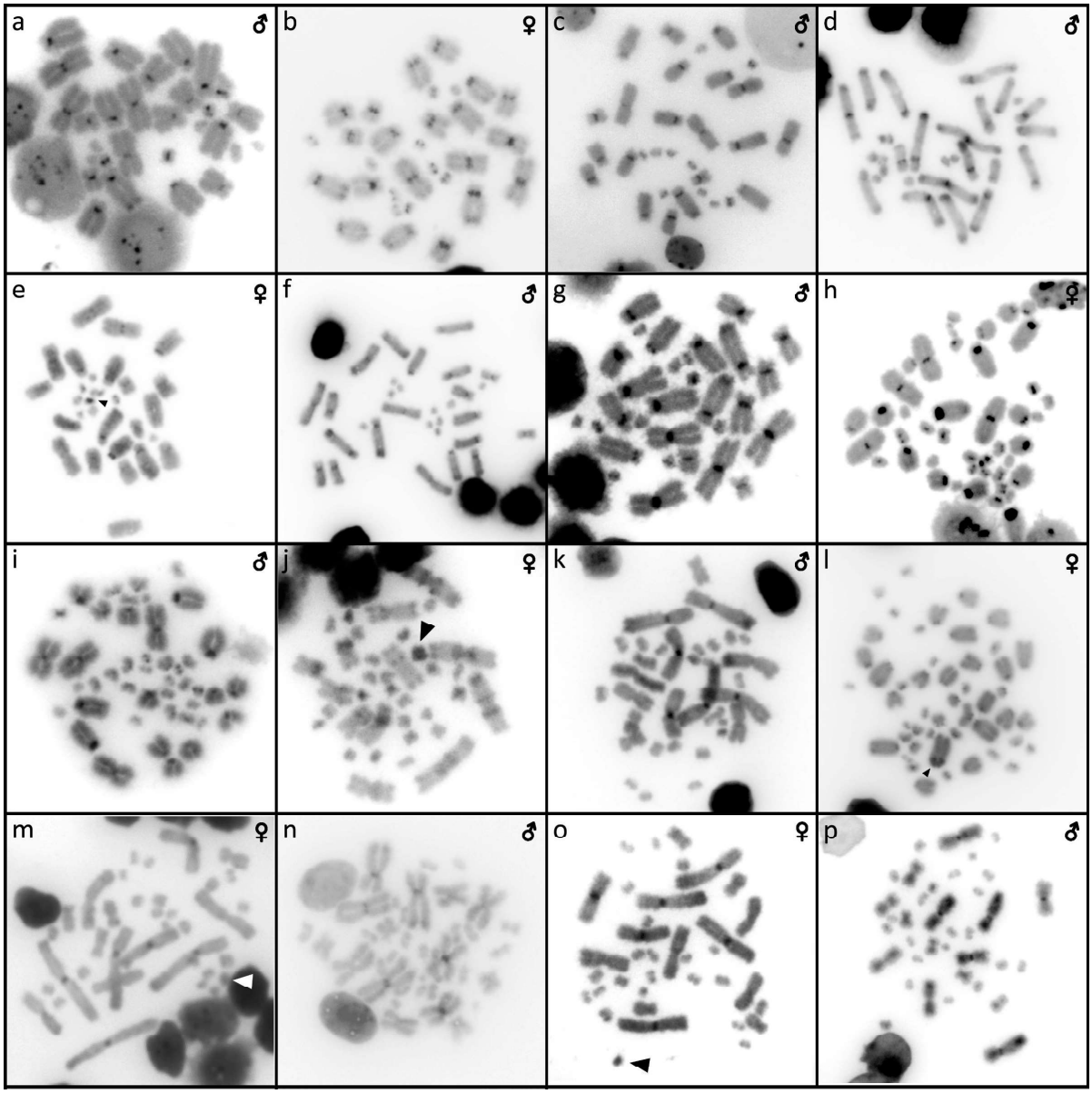


Figure 4. C-banded metaphases of *Abronia campbelli* (**a**), *Abronia deppii* (**b**,**c**), *Abronia graminea* (**d**), *Abronia lythrochila* (**e**,**f**), *Abronia mixteca* (**g**), *Barisia rudicollis* (**h**,**i**), *Celestus warreni* (**j**,**k**), *Gerrhonotus liocephalus* (**l**), *Heloderma exasperatum* (**m**,**n**) and *Heloderma horridum* (**o**,**p**). When visible, the W chromosomes are indicated by arrows.

3.5. *Abronia mixteca* Bogert & Porter, 1967

We examined only a male individual. The karyotype with 2n = 30 chromosomes (20 macro- and 10 microchromosomes). The pairs 1–3 and 7–10 are bi-armed, while the pairs 4–6 are acrocentric (Figure 1g). The C-banding revealed an accumulation of heterochromatin in the centromeric regions of the macrochromosomes and in the telomeric region of a pair of macrochromosomes (Figure 4g). Some microchromosomes are also enriched in heterochromatin. The telomeric sequences were detected only in the terminal positions of all chromosomes (Figure 6g). The FISH with rDNA probe revealed a signal on the 10th pair of macrochromosomes (Figure 8g).

3.6. *Abronia smithi* Campbell & Frost, 1993

We examined only a male individual. Karyotype with 2n = 30 chromosomes (20 macro- and 10 microchromosomes). The chromosome pairs 1–3 and 7–10 are bi-armed, while the pairs 4–6 are acrocentric (Figure 1h).

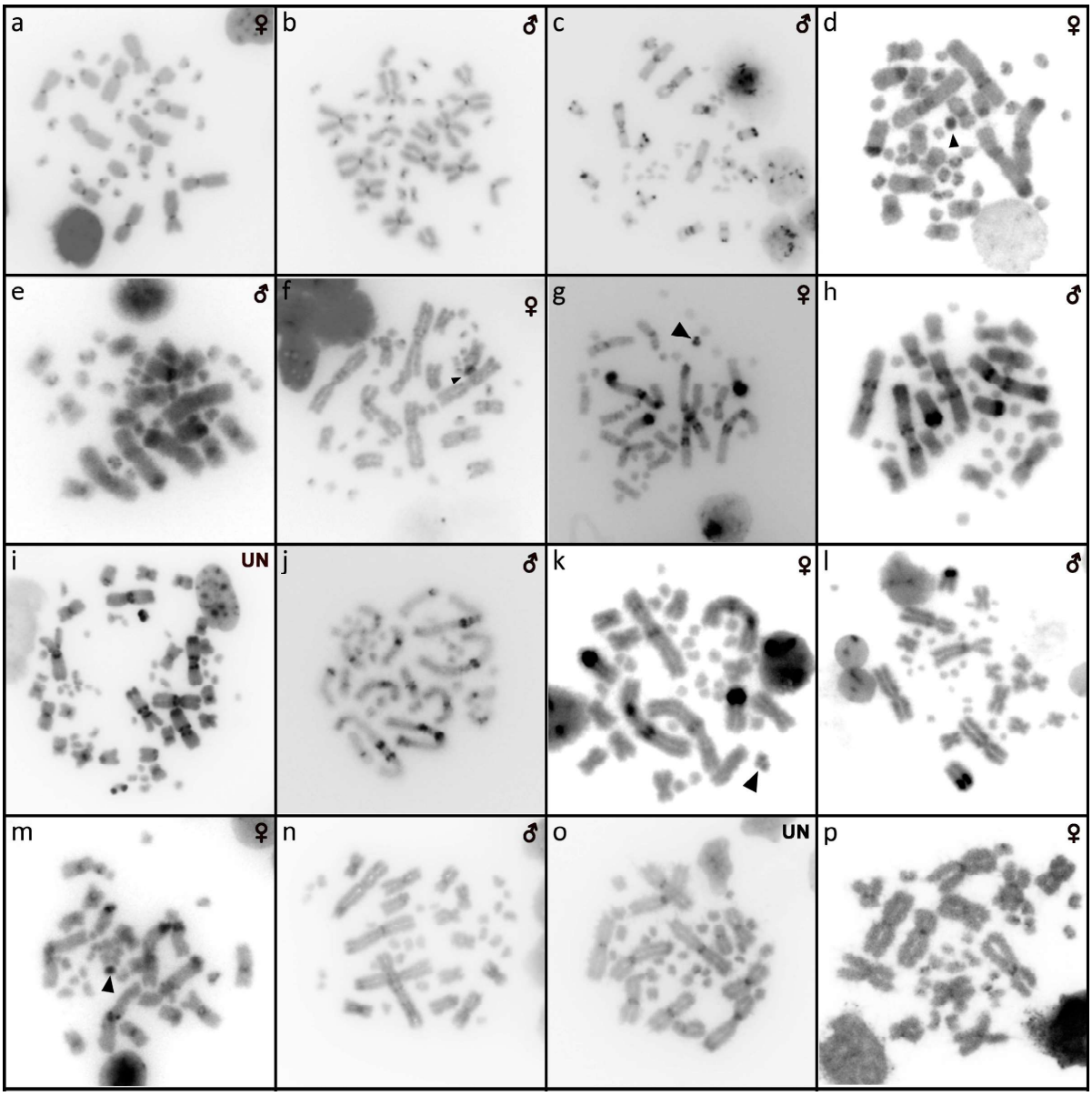


Figure 5. C-banded metaphases of *Shinisaurus crocodilurus* (**a**,**b**), *Varanus auffenbergi* (**c**), *Varanus cumingi* (**d**,**e**), *Varanus kordensis* (**f**), *Varanus olivaceus* (**g**,**h**), *Varanus primordius* (**i**,**j**), *Varanus salvadorii* (**k**,**l**), *Varanus salvator komaini* (**m**,**n**), *Xenosaurus platyceps* (**o**), and *Xenosaurus rectocollaris* (**p**). When visible, the W chromosomes are indicated by arrows. UN: sex not known.

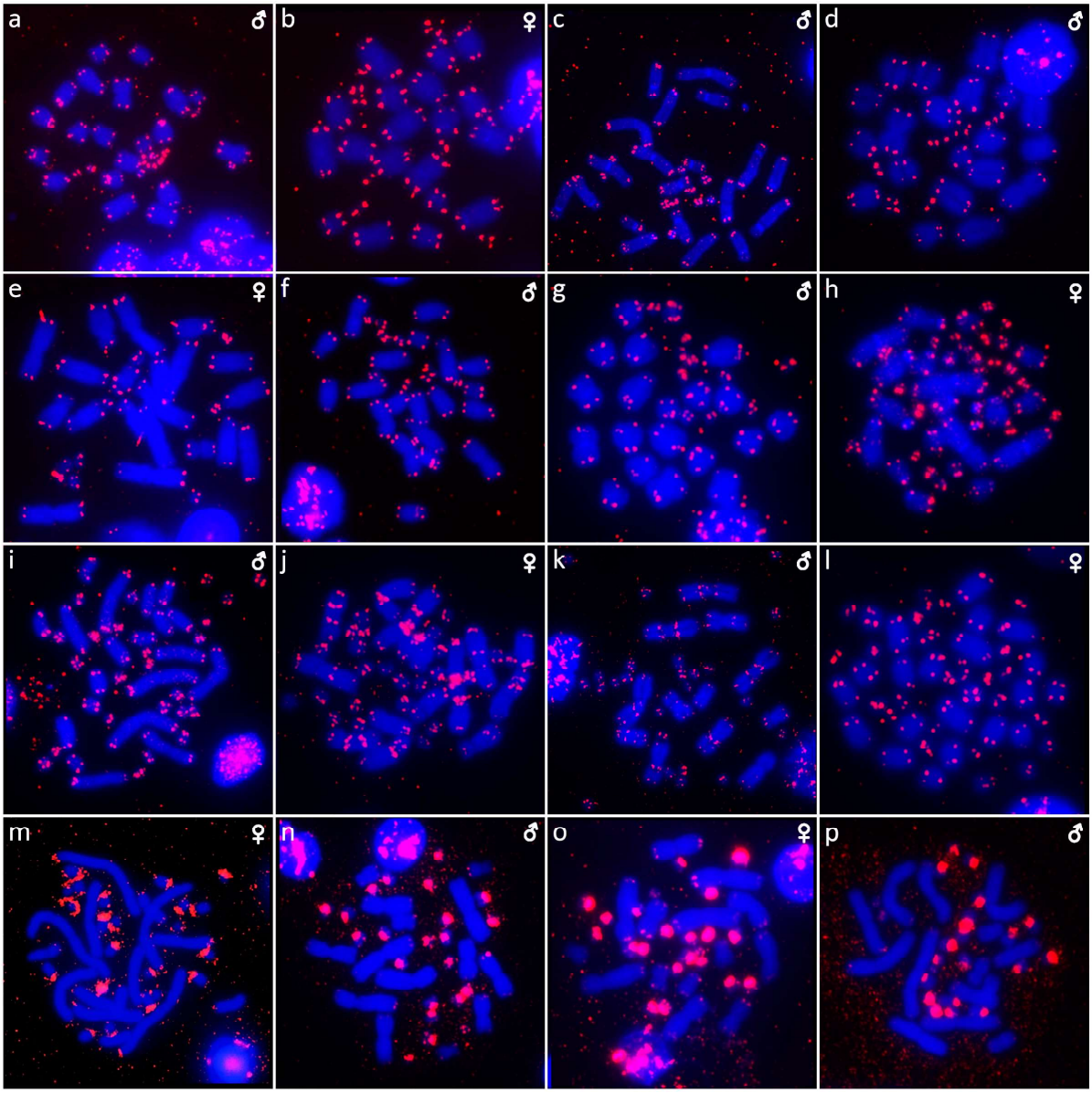


Figure 6. *In situ* hybridization with probe for telomeric sequences in *Abronia campbelli* (**a**), *Abronia deppii* (**b**,**c**), *Abronia graminea* (**d**), *Abronia lythrochila* (**e**,**f**), *Abronia mixteca* (**g**), *Barisia rudicollis* (**h**,**i**), *Celestus warreni* (**j**,**k**), *Gerrhonotus liocephalus* (**l**), *Heloderma exasperatum* (**m**,**n**) and *Heloderma horridum* (**o**,**p**).

3.7. *Abronia taeniata* (Wiegmann, 1828)

We examined only a male individual. Karyotype with 2n = 30 chromosomes (20 macro- and 10 microchromosomes). The chromosome pairs 1–3 and 7–10 are bi-armed, while the pairs 4–6 are acrocentric (Figure 1i).

3.8. *Barisia rudicollis* (Wiegmann, 1828)

Karyotype with 2n = 44 chromosomes (18 can be classified as macro- and 26 as microchromosomes, but the distinction is not prominent). The 1st and 2nd chromosome pairs are metacentric, the pairs 3–9 are acrocentric (Figure 2a,b). Heterochromatin is accumulated in the centromeric regions (Figure 4h,i). The telomeric sequences were detected only in the terminal positions (Figure 6h,i). The FISH with rDNA probe revealed

a signal on a pair of microchromosomes (Figure 8h,i). CGH did not reveal any sex-specific differences (Figure 10e,f).

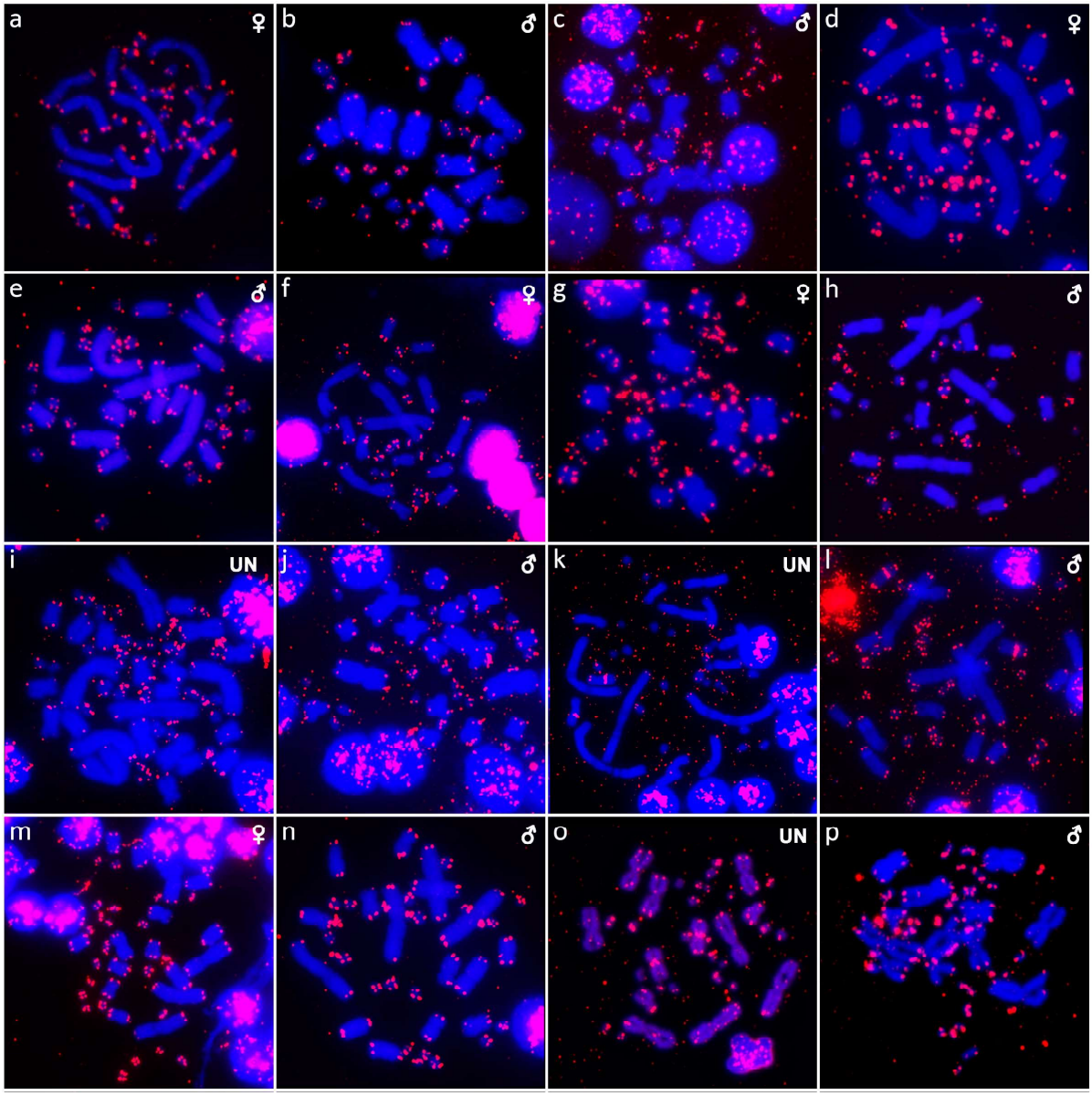


Figure 7. *In situ* hybridization with probe for telomeric sequences in *Shinisaurus crocodilurus* (**a**,**b**), *Varanus auffenbergi* (**c**), *Varanus cumingi* (**d**,**e**), *Varanus kordensis* (**f**), *Varanus primordius* (**g**,**h**), *Varanus olivaceus* (**i**,**j**), *Varanus salvadorii* (**k**,**l**), *Varanus salvator komaini* (**m**,**n**), *Xenosaurus platyceps* (**o**) and *Xenosaurus rectocollaris* (**p**). UN: sex not known.

3.9. *Celestus warreni* (Schwartz, 1970)

Karyotype with 2n = 36 chromosomes (12 macro- and 14 microchromosomes). All macrochromosomes are bi-armed (Figure 2c,d). The C-banding revealed heterochromatin in the pericentromeric as well as telomeric regions of the macrochromosomes. The telomeric sequences were detected in the terminal positions of the chromosomes, in some of them also in the telomeric positions. A single microchromosome in the female possesses a notable accumulation of heterochromatin, which is not present in the male (Figure 4j,k). Interstitial telomeric repeats (ITRs) were detected in the pericentromeric regions of the chromosome pairs 1–5 (Figure 6j,k). The FISH with rDNA probe revealed a signal in the telomeric region

of the 2nd chromosome pair (Figure 8j,k). CGH did not reveal any sex-specific differences (Figure 10g,h).

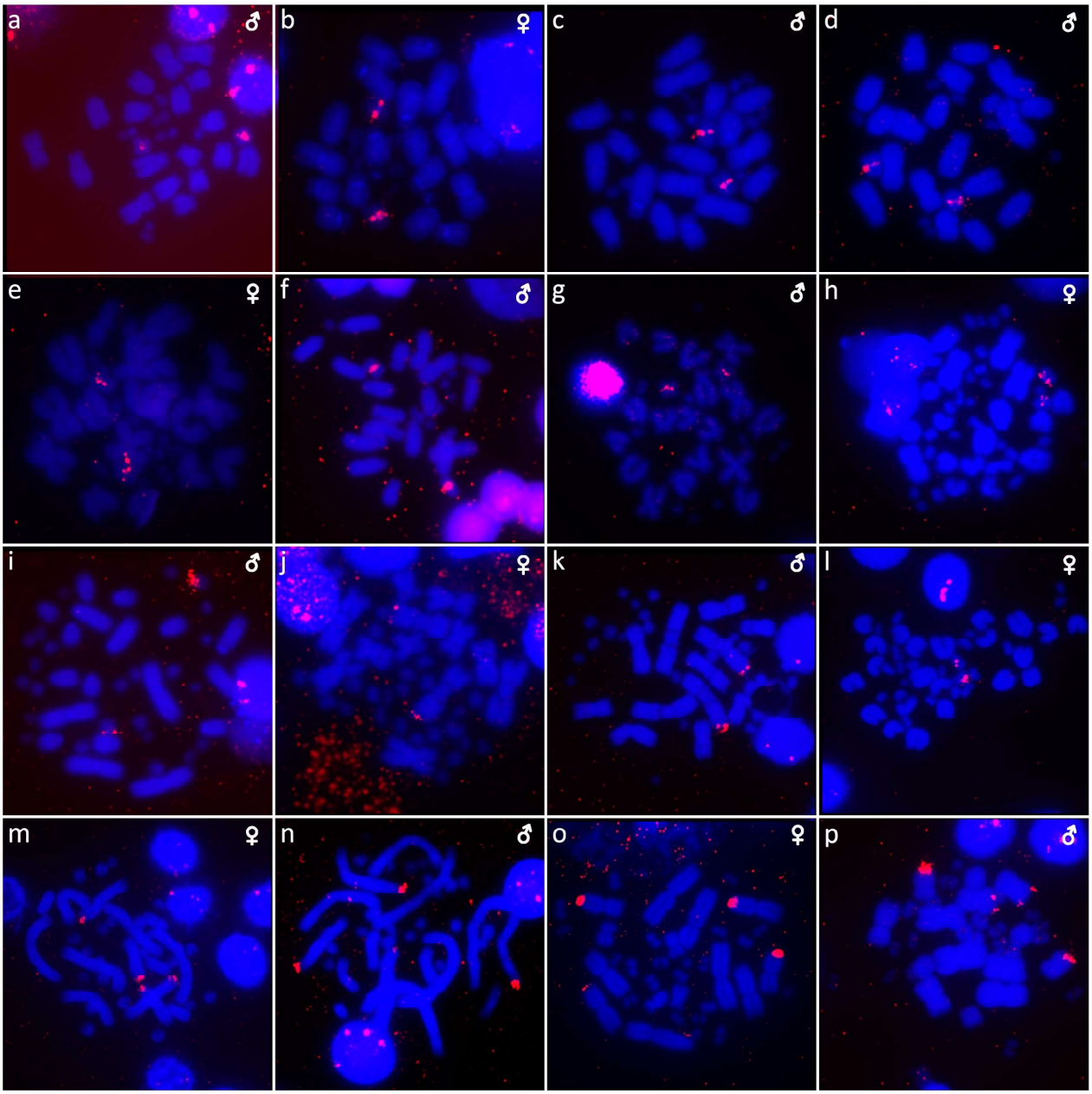


Figure 8. *In situ* hybridization with probe for 18S/28S rDNA loci in *Abronia campbelli* (**a**), *Abronia deppii* (**b**,**c**), *Abronia graminea* (**d**), *Abronia lythrochila* (**e**,**f**), *Abronia mixteca* (**g**), *Barisia rudicollis* (**h**,**i**), *Celestus warreni* (**j**,**k**), *Gerrhonotus liocephalus* (**l**), *Heloderma exasperatum* (**m**,**n**) and *Heloderma horridum* (**o**,**p**).

3.10. *Gerrhonotus liocephalus* (Wiegmann, 1828)

We examined only a female individual. Karyotype with 2n = 48 chromosomes (22 macro- and 26 microchromosomes, but the differences in size are more gradual). All macrochromosomes are acrocentric (Figure 2e). The C-banding revealed heterochromatic regions in the centromeric region of all chromosomes. Notably, a single chromosome from the first pair has a prominent accumulation of heterochromatin in comparison to other chromosomes (Figure 4l). The telomeric sequences were detected only in the terminal

positions of all chromosomes (Figure 6l). The FISH with rDNA probe revealed a signal on one pair of microchromosomes (Figure 8l). Although the chromosomal material from the male individual was not available to us, we isolated DNA from a male, which we used for CGH. By using CGH method we detected a female-specific region in the female metaphases. The signal was detected in one of the chromosomes from the 1st chromosome pair (Figure 10i).

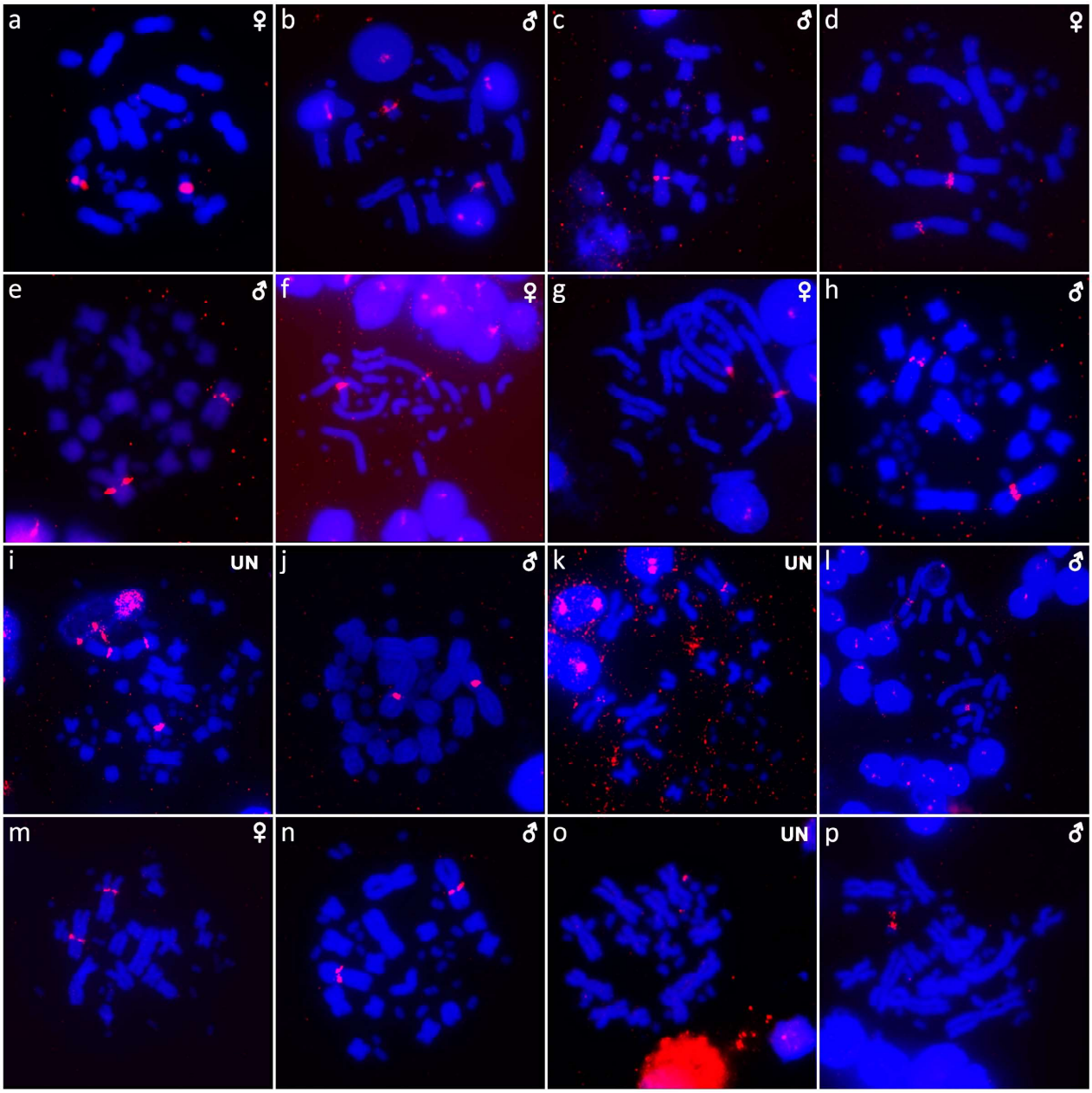


Figure 9. *In situ* hybridization with probe for 18S/28S rDNA loci in *Shinisaurus crocodilurus* (**a**,**b**), *Varanus auffenbergi* (**c**), *Varanus cumingi* (**d**,**e**), *Varanus kordensis* (**f**), *Varanus primordius* (**g**,**h**), *Varanus olivaceus* (**i**,**j**), *Varanus salvadorii* (**k**,**l**), *Varanus salvator komaini* (**m**,**n**), *Xenosaurus platyceps* (**o**) and *Xenosaurus rectocollaris* (**p**). UN: sex not known.

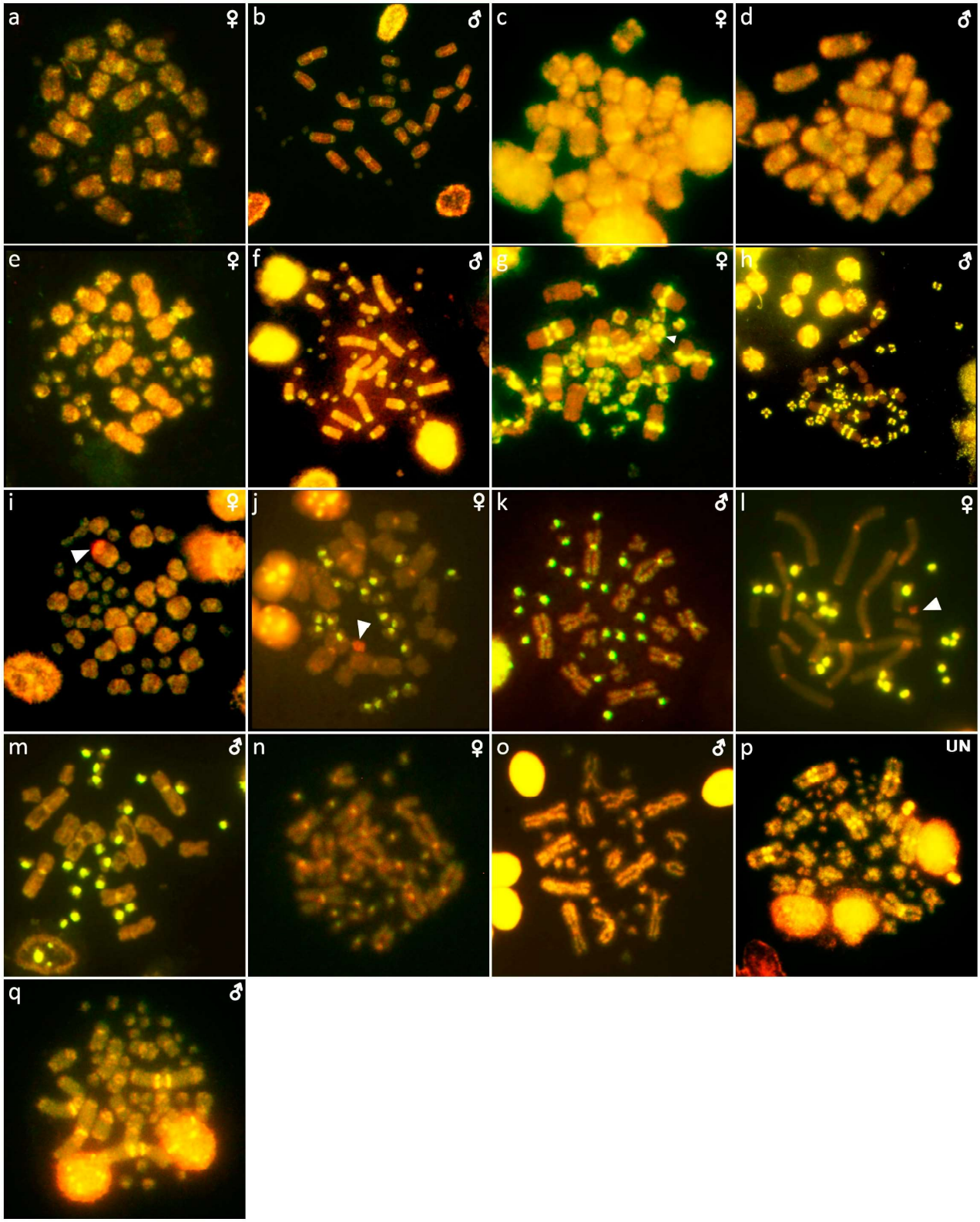


Figure 10. Comparative genome hybridization in *Abronia deppii* (**a**,**b**), *Abronia lythrochila* (**c**,**d**), *Barisia rudicollis* (**e**,**f**), *Celestus warreni* (**g**,**h**), *Gerrhonotus liocephalus* (**i**), *Heloderma exasperatum* (**j**,**k**), *Heloderma horridum* (**l**,**m**), *Shinisaurus crocodilurus* (**n**,**o**) and *Varanus primordius* (**p**,**q**). When visible, the W chromosomes are indicated by arrows. UN: sex not known.

3.11. *Heloderma exasperatum* Bogert & Martin del Campo, 1956

Karyotype with 2n = 36 chromosomes (14 macro- and 22 microchromosomes). All macrochromosomes are bi-armed (Figure 2f,g). C-banding revealed heterochromatin mainly in the centromeric regions, in female animals the heterochromatin was accumulated also on the W chromosome (Figure 4m,n). ITRs were detected in 10 pairs of microchromosomes (Figure 6m,n). The FISH with rDNA probe revealed a signal at the telomeric region of the 3rd or 4th (both chromosome pairs have the same morphology) and the 5th chromosome pairs (Figure 8m,n). CGH revealed a female-specific signal in a single microchromosome in females, which corresponds to the W chromosome (Figure 10j,k).

3.12. *Heloderma horridum* (Wiegmann, 1828)

Karyotype with 2n = 36 chromosomes (14 macro- and 22 microchromosomes). All pairs of macrochromosomes are bi-armed (Figure 2h,i). The C-banding revealed heterochromatin in the centromeric region of all macrochromosomes and in the W chromosome of the females (Figure 4o,p). ITRs were detected in 10 pairs of microchromosomes (Figure 6o,p). The FISH with rDNA probe revealed a signal at the telomeric region of the 3rd or 4th (not possible to distinguish, as both chromosome pairs have the same morphology) and the 5th chromosome pairs (Figure 8o,p). CGH revealed a female-specific signal in a single microchromosome in the metaphases from the female specimen, which corresponds to the W chromosome (Figure 10l,m).

3.13. *Shinisaurus crocodilurus* Ahl, 1930

Karyotype with 2n = 32 chromosomes (14 macro- and 18 microchromosomes) (Figure 2j,k). The chromosome pairs 1–5 are bi-armed, while the pairs 6 and 7 are acrocentric. The C-banding revealed accumulation of heterochromatin in the centromeric region of the chromosomes, with a similar pattern in both sexes (Figure 5a,b). The telomeric sequences were detected only at the terminal positions of all chromosomes (Figure 7a,b). The FISH with rDNA probe revealed a signal at the pericentromeric region of the 6th chromosomal pair (Figure 9a,b). CGH method did not reveal any sex-specific differences (Figure 10n,o).

3.14. *Varanus auffenbergi* Sprackland, 1999

We examined only a male individual. Karyotype with 2n = 40 chromosomes (16 macro- and 24 microchromosomes). All macrochromosomes are bi-armed with the exception of the 4th chromosomal pair which is acrocentric (Figure 3a). The C-banding revealed the accumulation of heterochromatin in the pericentromeric and telomeric regions of the macrochromosomes (Figure 5c). The telomeric sequences were detected only in the terminal positions of all chromosomes (Figure 7c). FISH with rDNA probe revealed a signal in the pericentromeric region of the 1st chromosome pair (Figure 9c).

3.15. *Varanus cumingi* Martin, 1893

Karyotype with 2n = 40 chromosomes (16 macro- and 24 microchromosomes). The 4th chromosomal pair is acrocentric, while the other macrochromosomes are bi-armed (Figure 3b,c). The C-banding revealed the accumulation of heterochromatin in the pericentromeric region of all chromosomes and the telomeric region of the second chromosome pair. The W chromosomes in females are heterochromatic (Figure 5d,e). The telomeric sequences were detected only in the terminal positions of all chromosomes (Figure 7d,e). FISH with the rDNA probe revealed a signal in the pericentromeric region of the 1st chromosome pair (Figure 9d,e).

3.16. *Varanus kordensis* (Meyer, 1874)

We examined only a female individual. Karyotype with 2n = 40 chromosomes (16 macro- and 24 microchromosomes). All macrochromosomes are bi-armed except the 4th chromosome pair which is acrocentric (Figure 3d). The C-banding revealed accumulation of heterochromatin in the pericentromeric region of the chromosomes and in the W

chromosome in females (Figure 5f). The telomeric sequences were detected only in the terminal positions of all chromosomes (Figure 7f). FISH with rDNA probe revealed a signal in the pericentromeric region of the 1st chromosome pair (Figure 9f).

3.17. *Varanus olivaceus* Hallowell, 1857

Karyotype with 2n = 40 chromosomes (16 macro- and 24 microchromosomes). All macrochromosomes are bi-armed, except the 4th pair which is acrocentric (Figure 3e,f). The C-banding revealed accumulation of heterochromatin mainly in the pericentromeric and telomeric regions of macrochromosomes and in the W chromosome in females (Figure 5g,h). The telomeric sequences were detected only in the terminal positions of all chromosomes (Figure 7g,h). The FISH with rDNA probe revealed a signal in the pericentromeric region of the 1st chromosome pair (Figure 9g,h).

3.18. *Varanus primordius* Mertens, 1942

We examined only a male individual. Karyotype with 2n = 40 chromosomes (16 macro- and 24 microchromosomes). The macrochromosome pairs 1–3 and 5–8 are bi-armed, the 4th pair is acrocentric (Figure 3h). An additional animal of unknown sex was identified as a triploid with 3n = 60 (Figure 2g). The C-banding revealed accumulation of heterochromatin mainly in the pericentromeric region of the chromosomes (Figure 5i,j). The telomeric sequences were detected only in the terminal positions of all chromosomes (Figure 7i,j). The FISH with rDNA probe revealed a signal in the pericentromeric region of the 1st chromosome pair (Figure 9i,j). The qPCR test revealed that the triploid individual had an average of 0.71 gene dose ratio for the Z-specific genes in comparison to the male, which corresponds to ZZW sex chromosome constitution (Table S2). The W chromosome cannot be accurately identified in the metaphases by the C-banding (Figure 5i,j) or CGH (Figure 10p,q), due to its small size and the bright signals on the other microchromosomes.

3.19. *Varanus salvadorii* (Peters & Doria, 1878)

Karyotype with 2n = 40 chromosomes (16 macro- and 24 microchromosomes). The chromosomal pairs 1–3 and 5–8 are bi-armed, while the pair 4 is acrocentric (Figure 3i,j). The C-banding revealed the accumulation of heterochromatin in the pericentromeric region of the chromosomes and in the W chromosome in females (Figure 5k,l). The telomeric sequences were detected in the terminal positions of all chromosomes. ITRs were detected in the pericentromeric region of the 6th chromosome pair (Figure 7k,l). The FISH with rDNA probe revealed a signal in the pericentromeric region of the 1st chromosome pair (Figure 9k,l).

3.20. *Varanus salvator komaini* Nutaphand 1987

Karyotype with 2n = 40 chromosomes (16 macro- and 24 microchromosomes). All macrochromosomes are bi-armed, except the 4th pair which is acrocentric (Figure 3k,l). The C-banding revealed accumulation of heterochromatin in the pericentromeric region of all chromosomes, in the telomeric region of the second chromosomal pair, and in the W of the female (Figure 5m,n). The telomeric sequences were detected only in the terminal positions of all chromosomes (Figure 7m,n). The FISH with rDNA probe revealed a signal in the pericentromeric region of the 1st chromosome pair (Figure 9m,n).

3.21. *Xenosaurus grandis* (Gray, 1856)

We examined only a single male individual. Karyotype with 2n = 36 chromosomes (12 macro- and 24 microchromosomes). All macrochromosomes are bi-armed (Figure 3m).

3.22. *Xenosaurus platyceps* King & Thompson, 1968

Two individuals of unknown sex were examined. Karyotype with 2n = 36 chromosomes (12 macro- and 24 microchromosomes). All macrochromosomes are bi-armed (Figure 3n). The C-banding revealed the accumulation of heterochromatin in the pericen-

tromeric regions (Figure 5o). The telomeric sequences were detected in the terminal positions of all chromosomes and ITRs were detected on the 5th chromosomal pair (Figure 7o). The FISH with rDNA probe revealed a signal on one pair of microchromosomes (Figure 9o).

3.23. Xenosaurus rectocollaris Smith & Iverson, 1993

Only female individuals were examined. Karyotype with 2n = 36 chromosomes (12 macro- and 24 microchromosomes). All macrochromosomes are bi-armed (Figure 3o). C-banding revealed heterochromatin in the centromeric regions of all macrochromosomes and in some microchromosomes (Figure 5p). The telomeric sequences were detected only in the terminal positions of all chromosomes (Figure 7p). The FISH with rDNA probe revealed a signal on one pair of the microchromosomes (Figure 9p).

4. Discussion

The diploid chromosome numbers are in general variable in Anguimorpha (Figure 11), from 2n = 20 in *Anniella stebbinsi* (reported by Bezy et al. [40] as *Anniella pulchra*; [43]) to 2n = 48 in *Elgaria multicarinata* [37] and *Gerrhonotus liocephalus* (current study). However, the variability in chromosome number is highly unequally distributed among anguimorphan families, with most of the variability concentrated in the families Anguidae and Anniellidae. On the contrary, other anguimorphan lineages have much more stable chromosome numbers. Karyotypes with 2n = 40 chromosomes were identified in all 36 studied species of monitors (family Varanidae), 2n = 36 chromosomes in three examined species of helodermatids (family Helodermatidae), and in three species of the knob-scaled lizards (family Xenosauridae). The karyotype with 2n = 32 chromosomes was revealed in the current study in the only species of the family Shinisauridae (Figure 11).

Notably, 2n = 36 chromosomes is the most common diploid chromosome number in the species of the clade Toxicofera, and it is considered the ancestral state for caenophidian snakes [48], chameleons [49], and potentially also in iguanas and agamid lizards [29,50]. These estimations were largely based on the distribution of diploid chromosome number variation across the phylogenetic spectrum of each lineage, and at least in the case of snakes and iguanas, the reconstructed ancestral karyotypes differ in several interchromosomal rearrangements and thus in chromosome morphology. We think that the ancestral karyotype of the anguimorphan reptiles cannot be accurately reconstructed at the current state of knowledge, due to the extensive variability in diploid chromosome numbers and chromosome morphology. The karyotype evolution of the anguimorphan reptiles should be reconstructed in future studies either by comparative chromosome painting as applied in monitors [33] or by comparing chromosome level assemblies, which are currently available only for the Komodo dragon, *Varanus komodoensis* [35] and the Chinese crocodile lizard, *Shinisaurus crocodilurus* [51]. We are confident that the recent advances in next-generation sequencing technologies will make chromosome level assemblies feasible for additional phylogenetically informative species in the near future.

In addition to the terminal position (Figures 6 and 7), interstitial telomeric-like repeats were detected in five out of 20 studied species, namely, in *Celestus warreni, Heloderma exasperatum, Heloderma horridum, Varanus salvadorii,* and *Xenosaurus platyceps*. In both species of the genus *Heloderma,* extensive amplification of telomeric repeats were identified in several pairs of microchromosomes. Telomeric repeats often tend to accumulate in the microchromosomes of sauropsids, as was documented in birds [52], turtles [44,53,54], and snakes [55]. Microchromosomes have generally higher rates of recombination in comparison to macrochromosomes [56–58]; therefore, we can speculate that telomeres extensively amplify in microchromosomes as a consequence of the DNA repair mechanism occurring after recombination events [59,60]. ITRs were detected in a single pair of macrochromosomes in *Xenosaurus platyceps* and *Varanus salvadorii*, and in five pairs of macrochromosomes in *Celestus warreni*. The origin of the ITRs is not clear, it might be a result of chromosomal rearrangements, such as chromosomal fusions or inversions, as well as activity of retrotransposons [61–64]. In addition, telomeric-like sequences are often part

of the (peri)centromeric satellite motifs [65,66]. Overall, the presence of ITRs is quite rare in anguimorphan reptiles, in comparison to other toxicoferan reptiles, such as "haenophidian" snakes [67] and chameleons [49].

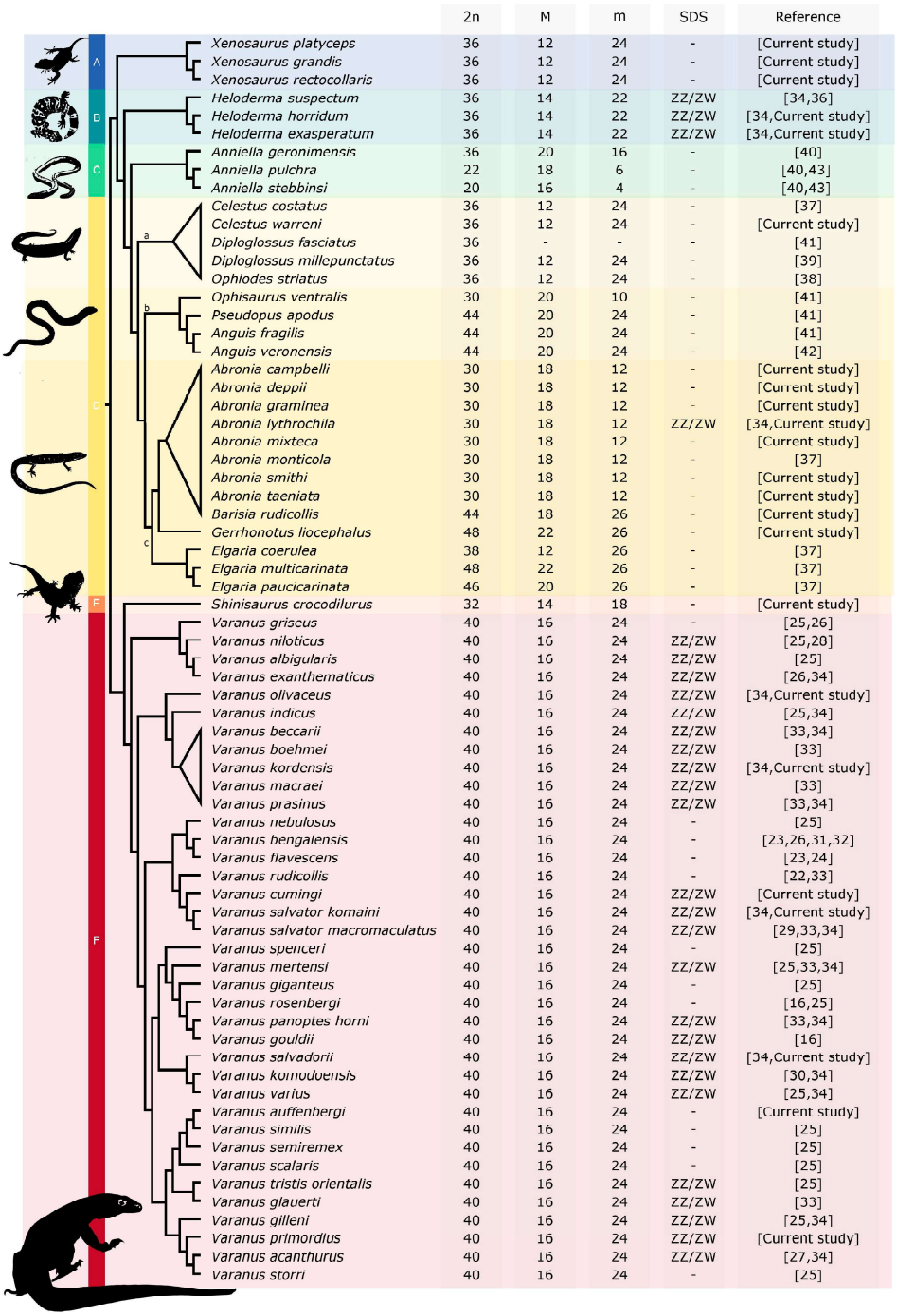


Figure 11. Evolution of karyotypes in anguimorphan reptiles. 2n: diploid chromosome number, M: number of macrochromosomes, m: number of microchromosomes, SDS: sex determination system. A: Xenosauridae, B: Helodermatidae, C: Anniellidae, D: Anguidae, E: Shinisauridae, F: Varanidae, a: Diploglossinae, b: Anguinae, c: Gerrhonotinae. The phylogenetic relationships follow [21,68–71].

Animals often show variability in the chromosomal position and the amount of accumulation of rDNA loci among and within species, e.g. [72–78]. The rDNA loci were detected only in one pair of chromosomes in most of the studied anguimorphan species, but their topology varies from a pair of microchromosomes in four species (*Barisia rudicollis*, *Gerrhonotus liocephalus*, *Xenosaurus platyceps*, *Xenosaurus rectocollaris*) to a pair of macrochromosomes in the remaining studied species (Figures 8 and 9). Notably, rDNA loci were detected in two pairs of macrochromosomes in *Heloderma horridum* and *H. exasperatum* (Figure 8). The presence of rDNA loci in multiple chromosome pairs is rather uncommon in reptiles, and it was previously reported in few species, such as the boa *Candoia paulsoni* [67] and the iguanas *Oplurus cyclurus* [74], *Leiocephalus carinatus* and *Leiocephalus raviceps* [72].

In addition, rDNA loci often accumulate on the sex chromosomes of reptiles. Notable differences in the copies of rDNA loci between the Z/W and X/Y sex chromosomes were previously reported in several species of trionychid and chelid turtles [53,79–81]. On the other hand, the rDNA loci are not detected on the Y chromosome of the common sandfish *Scincus scincus* (Scincidae) and the W chromosome of the Peters' keeled plated lizard *Tracheloptychus petersi* (Gerrhosauridae), although they contain their X and Z chromosomes, respectively [7,82]. Notably, we did not detect any sex-specific accumulation on the sex chromosomes of anguimorphan reptiles (Figures 8 and 9).

The chromosomal regions with rDNA loci tend to act as "hotspots" for recombination, which can explain, at least partially, the large variability in the accumulation and chromosome position of rDNA loci [73]. Such variability can be neutral and tolerated by the cell. In support, the comparison of silver-stained NORs (i.e., transcriptionally active rDNA loci) and rDNA-FISH (i.e., all chromosome positions with rDNA loci) revealed variability in the activity of rDNA loci [83–85]. Therefore, we speculate that the genome of some species might have more copies of rDNA loci than the minimum requirement for proper cellular function. For example, only 25–50% of rDNA loci are needed for normal development in the African clawed frog, *Xenopus laevis* [86]. On the other hand, the variability in rDNA loci can cause the development of cancer [87,88] and genome instability [89].

During the cytogenetic analysis, we identified a triploid individual of *Varanus primordius* (3n = 60) with the ZZW combination of sex chromosomes revealed by qPCR (Table S2). The sex of the individual was not possible to identify accurately by morphology. Another case of triploidy in the family Varanidae was previously reported in a male individual of *Varanus albigularis* with ZZZ sex chromosomes [33]. The spontaneous triploidy occurs rarely in reptilian species with a typical diploid karyotype. In addition to the above-mentioned cases of monitors, to the best of our knowledge, spontaneous triploidy was previously described in two species of snakes, *Elaphe bimaculata* [90] and *Agkistrodon piscivorus* [91]. Among reptiles, several species of obligatory parthenogens of hybrid origin are triploids [92–100]. The identification of spontaneous triploidy in adults and the existence of obligatory parthenogenetic triploid species show that triploid individuals are viable in many reptile lineages.

The C-banding revealed heterochromatin typically in the (peri)centromeric regions, and occasionally in the telomeric regions (Figures 6 and 7). The W chromosomes were detected by C-banding, in all studied species of helodermatids and monitors, except *V. primordius* (Figures 4, 5 and 10). Putative W chromosomes were detected in *Abronia lythrochila*, *Celestus warreni,* and *Gerrhonotus liocephalus*. In *Abronia lythrochila,* the C-banding (Figure 4e,f) revealed a stronger accumulation of the heterochromatin in a single microchromosome, which agrees with the previous report of a ZZ/ZW system in this species [34]; however, no sex-specific differences were detected by CGH (Figure 10c,d). Notably, C-banding and CGH did not reveal any sex-specific pattern in the closely related species *Abronia deppii* (Figure 4b,c and Figure 10a,b), which indicates that the species of the genus *Abronia* might not share homologous, or at least not equally differentiated sex chromosomes. In *Celestus warreni*, female-specific signal was detected in a single microchromosome by both C-banding (Figure 4j,k) and CGH (Figure 10g,h). In *Gerrhonotus liocephalus*, female-specific signal was detected in a single chromosome from the 1st pair by both C-banding

(Figure 4l) and CGH (Figure 10i). However, chromosomal material was not available from a male in *Gerrhonotus liocephalus*, and we were thus not able to compare both sexes to validate the presence of a ZZ/ZW sex determination system.

We assume that sex chromosomes are likely homomorphic and poorly differentiated in the rest of the studied anguimorphan species, although we cannot exclude that perhaps some of these species might have environmental sex determination (ESD). To expand our knowledge on sex determination and sex chromosome evolution in the anguimorphan lizards, we will need to use advanced genomic/bioinformatic analysis (such as RAD-seq, genome coverage analysis), as well as incubation experiments for the detection of possible ESD.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cells10071612/s1, Table S1: Summary of studied species and number of examined individuals, Table S2: Primers and results of the qPCR test for estimating the sex chromosome constitution in the triploid *Varanus primordius*.

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References

1. Wilson, M.A.; Makova, K.D. Genomic analyses of sex chromosome evolution. *Rev. Genom. Hum. Genet.* **2009**, *10*, 333–354. [CrossRef] [PubMed]
2. Bachtrog, D.; Mank, J.E.; Peichel, C.L.; Kirkpatrick, M.; Otto, S.P.; Ashman, T.L.; Hahn, M.W.; Kitano, J.; Mayrose, I.; Ming, R.; et al. Tree of sex consortium. Sex determination: Why so many ways of doing it? *PLoS Biol.* **2014**, *12*, e1001899. [CrossRef]
3. Pennell, M.W.; Mank, J.E.; Peichel, C.L. Transitions in sex determination and sex chromosomes across vertebrate species. *Mol. Ecol.* **2018**, *27*, 3950–3963. [CrossRef] [PubMed]
4. Ohno, S. *Sex Chromosomes and Sex-Linked Genes*; Springer: Berlin/Heidelberg, Germany, 1967.
5. Rice, W.R. The accumulation of sexually antagonistic genes as a selective agent promoting the evolution of reduced recombination between primitive sex chromosomes. *Evolution* **1987**, *41*, 911–914. [CrossRef] [PubMed]
6. Charlesworth, B. The evolution of chromosomal sex determination and dosage compensation. *Curr. Biol.* **1996**, *6*, 149–162. [CrossRef]
7. Kostmann, A.; Kratochvíl, L.; Rovatsos, M. Poorly differentiated XX/XY sex chromosomes are widely shared across skink radiation. *Proc. R. Soc. B* **2021**, *288*, 20202139. [CrossRef]
8. Rovatsos, M.; Johnson Pokorná, M.; Altmanová, M.; Kratochvíl, L. Female heterogamety in Madagascar chameleons (Squamata: Chamaeleonidae: *Furcifer*): Differentiation of sex and neo-sex chromosomes. *Sci. Rep.* **2015**, *5*, 13196. [CrossRef] [PubMed]
9. Rovatsos, M.; Altmanová, M.; Augstenová, B.; Mazzoleni, S.; Velenský, P.; Kratochvíl, L. ZZ/ZW sex determination with multiple neo-sex chromosomes is common in Madagascan chameleons of the genus *Furcifer* (Reptilia: Chamaeleonidae). *Genes* **2019**, *10*, 1020. [CrossRef]

10. Epplen, J.T.; McCarrey, J.R.; Sutou, S.; Ohno, S. Base sequence of a cloned snake W-chromosome 65DNA fragment and identification of a male-specific putative mRNA in the mouse. *Proc. Natl. Acad. Sci. USA* **1982**, *79*, 3798–3802. [CrossRef] [PubMed]
11. Nanda, I.; Deubelbeiss, C.; Guttenbach, M.; Epplen, J.T.; Schmid, M. Heterogeneities in the distribution of $(GACA)_n$ simple repeats in the karyotypes of primates and mouse. *Hum. Genet.* **1990**, *85*, 187–194. [CrossRef]
12. Steinemann, S.; Steinemann, M. Retroelements: Tools for sex chromosome evolution. *Cytogenet. Genome Res.* **2005**, *110*, 134–143. [CrossRef] [PubMed]
13. Kejnovsky, E.; Hobza, R.; Cermak, T.; Kubat, Z.; Vyskot, B. The role of repetitive DNA in structure and evolution of sex chromosomes in plants. *Heredity* **2009**, *102*, 533–541. [CrossRef] [PubMed]
14. O'Meally, D.; Patel, H.R.; Stiglec, R.; Sarre, S.D.; Georges, A.; Marshall Graves, J.A.; Ezaz, T. Non-homologous sex chromosomes of birds and snakes share repetitive sequences. *Chromosome Res.* **2010**, *18*, 787–800. [CrossRef]
15. Pokorná, M.; Kratochvíl, L.; Kejnovský, E. Microsatellite distribution on sex chromosomes at different stages of heteromorphism and heterochromatinization in two lizard species (Squamata: Eublepharidae: *Coleonyx elegans* and Lacertidae: *Eremias velox*). *BMC Genet.* **2011**, *12*, 90. [CrossRef] [PubMed]
16. Matsubara, K.; Sarre, S.D.; Georges, A.; Matsuda, Y.; Graves, J.A.M.; Ezaz, T. Highly differentiated ZW sex microchromosomes in the Australian *Varanus* species evolved through rapid amplification of repetitive sequences. *PLoS ONE* **2014**, *9*, e95226. [CrossRef]
17. Matsubara, K.; O'Meally, D.; Azad, B.; Georges, A.; Sarre, S.D.; Graves, J.A.M.; Matsuda, Y.; Ezaz, T. Amplification of microsatellite repeat motifs is associated with the evolutionary differentiation and heterochromatinization of sex chromosomes in Sauropsida. *Chromosoma* **2016**, *125*, 111–123. [CrossRef]
18. Augstenová, B.; Mazzoleni, S.; Kratochvíl, L.; Rovatsos, M. Evolutionary dynamics of the W chromosome in caenophidian snakes. *Genes* **2018**, *9*, 5. [CrossRef]
19. Suwala, G.; Altmanová, M.; Mazzoleni, S.; Karameta, E.; Pafilis, P.; Kratochvíl, L.; Rovatsos, M. Evolutionary variability of W-linked repetitive content in Lacertid lizards. *Genes* **2020**, *11*, 531. [CrossRef]
20. Uetz, P.; Freed, P.; Hošek, J. (Eds.) The Reptile Database. 2020. Available online: http://www.reptile-database.org (accessed on 5 March 2021).
21. Pyron, R.A.; Burbrink, F.T.; Wiens, J.J. A phylogeny and revised classification of Squamata, including 4161 species of lizards and snakes. *BMC Evol. Biol.* **2013**, *13*, 1–54. [CrossRef]
22. Gorman, G.C.; Gress, F. Chromosome cytology of four boid snakes and a varanid lizard, with comments on the cytosystematics of primitive snakes. *Herpetologica* **1970**, *26*, 308–317.
23. Singh, L.; Sharma, T.; Ray-Chaudhu, S.P. Chromosome numbers and sex chromosomes in few Indian species of amphibia and reptiles. *Mamm. Chrom. News* **1970**, *11*, 91–94.
24. Singh, L. Study of mitotic and meiotic chromosomes in seven species of lizards. *Proc. Zool. Soc.* **1974**, *27*, 57–79.
25. King, M.; King, D. Chromosomal evolution in the lizard genus *Varanus* (Reptilia). *Aust. J. Biol. Sci.* **1975**, *28*, 89–108. [CrossRef]
26. De Smet, W.H.O. Description of the orsein stained karyotypes of 136 lizard species (Lacertilia, Reptilia) belonging to the families Teiidae, Scincidae, Lacertidae, Cordylidae and Varanidae (Autarchoglossa). *Acta Zool. Pathol. Antverp.* **1981**, *76*, 407–420.
27. King, M.; Mengden, G.A.; King, D. A pericentric-inversion polymorphism and a ZZ/ZW sex-chromosome system in *Varanus acanthurus* Boulenger analysed by G- and C-banding and Ag staining. *Genetica* **1982**, *58*, 39–45. [CrossRef]
28. Porter, C.; Haiduk, M.; De Queiroz, K. Evolution and phylogenetic significance of ribosomal gene location in chromosomes of squamate reptiles. *Copeia* **1994**, *1994*, 302–313. [CrossRef]
29. Srikulnath, K.; Uno, Y.; Nishida, C.; Matsuda, Y. Karyotype evolution in monitor lizards: Cross-species chromosome mapping of cDNA reveals highly conserved synteny and gene order in the Toxicofera clade. *Chromosome Res.* **2013**, *21*, 805–819. [CrossRef]
30. Johnson Pokorná, M.; Altmanová, M.; Rovatsos, M.; Velenský, P.; Vodička, R.; Rehák, I.; Kratochvíl, L. First description of the karyotype and sex chromosomes in the Komodo dragon (*Varanus komodoensis*). *Cytogenet. Genome Res.* **2016**, *148*, 284–291. [CrossRef]
31. Patawang, I.; Tanomtong, A. Constitutive heterochromatin observed on metaphase chromosome of *Varanus bengalensis* by C-banding and DAPI methods. *Cytologia* **2017**, *82*, 1. [CrossRef]
32. Patawang, I.; Tanomtong, A.; Getlekha, N.; Phimphan, S.; Pinthong, K.; Neeratanaphan, L. Standardized karyotype and idiogram of bengal monitor lizard, Varanus bengalensis (Squamata, Varanidae). *Cytologia* **2017**, *82*, 75–82. [CrossRef]
33. Iannucci, A.; Altmanová, M.; Ciofi, C.; Ferguson-Smith, M.; Milan, M.; Pereira, J.C.; Pether, J.; Rehák, I.; Rovatsos, M.; Stanyon, R.; et al. Conserved sex chromosomes and karyotype evolution in monitor lizards (Varanidae). *Heredity* **2019**, *123*, 215–227. [CrossRef] [PubMed]
34. Rovatsos, M.; Rehák, I.; Velenský, P.; Kratochvíl, L. Shared ancient sex chromosomes in varanids, beaded lizards, and alligator lizards. *Mol. Biol. Evol.* **2019**, *36*, 1113–1120. [CrossRef] [PubMed]
35. Lind, A.L.; Lai, Y.Y.; Mostovoy, Y.; Holloway, A.K.; Iannucci, A.; Mak, A.C.Y.; Fondi, M.; Orlandini, V.; Eckalbar, W.L.; Milan, M.; et al. Genome of the Komodo dragon reveals adaptations in the cardiovascular and chemosensory systems of monitor lizards. *Nat. Ecol. Evol.* **2019**, *3*, 1241–1252. [CrossRef]
36. Johnson Pokorná, M.; Rovatsos, M.; Kratochvíl, L. Sex chromosomes and karyotype of the (nearly) mythical creature, the Gila monster, *Heloderma suspectum* (Squamata: Helodermatidae). *PLoS ONE* **2014**, *9*, e104716.
37. Bury, R.B.; Gorman, G.C.; Lynch, J.F. Karyotypic data for five species of anguid lizards. *Experientia* **1969**, *25*, 314–316. [CrossRef]

38. Beçak, M.L.; Beçak, W.; Denaro, L. Chromosome polymorphism, geographical variation and karyotypes in Sauria. *Caryologia* **1972**, *25*, 313–326. [CrossRef]
39. Stamm, B.; Gorman, G.C. Notes on the chromosomes of Anolis agassizi (Sauria: Iguanidae) and Diploglossus millepunctatus (Sauria: Anguidae). In *The Biological Investigation of Malpelo Island*; Graham, J.B., Ed.; Smithsonian Contributions to Zoology Colombia: Washington, DC, USA, 1975; Volume 176, pp. 52–56.
40. Bezy, R.L.; Gorman, G.C.; Kim, Y.J.; Wright, J.W. Chromosomal and genetic divergence in the fossorial lizards of the family Anniellidae. *Syst. Biol.* **1977**, *26*, 57–71. [CrossRef]
41. Olmo, E.; Signorino, G.G. Chromorep: A Reptile Chromosomes Database. 2005. Available online: http://chromorep.univpm.it (accessed on 4 March 2021).
42. Mezzasalma, M.; Guarino, F.M.; Aprea, G.; Petraccioli, A.; Crottini, A.; Odierna, G. Karyological evidence for diversification of Italian slow worm populations (Squamata, Anguidae). *Comp. Cytogenet.* **2013**, *7*, 217. [CrossRef] [PubMed]
43. Papenfuss, T.J.; Parham, J.F. Four new species of California legless lizards (Anniella). *Breviora* **2013**, *536*, 1–17. [CrossRef]
44. Mazzoleni, S.; Augstenová, B.; Clemente, L.; Auer, M.; Fritz, U.; Praschag, P.; Protiva, T.; Velenský, P.; Kratochvíl, L.; Rovatsos, M. Turtles of the genera Geoemyda and Pangshura (Testudines: Geoemydidae) lack differentiated sex chromosomes: The end of a 40-year error cascade for Pangshura. *PeerJ* **2019**, *7*, e6241. [CrossRef]
45. Sumner, A.T. A simple technique for demonstrating centromeric heterochromatin. *Exp. Cell Res.* **1972**, *75*, 304–306. [CrossRef]
46. Ijdo, J.W.; Baldini, A.; Ward, D.C.; Reeders, S.T.; Wells, R.A. Origin of human chromosome 2: An ancestral telomere-telomere fusion. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 9051–9055. [CrossRef] [PubMed]
47. Endow, S.A. Polytenization of the ribosomal genes on the X and Y chromosomes of Drosophila melanogaster. *Genetics* **1982**, *100*, 375–385. [CrossRef]
48. Oguiura, N.; Ferrarezzi, H.; Batistic, R.F. Cytogenetics and molecular data in snakes: A phylogenetic approach. *Cytogenet. Genome Res.* **2009**, *127*, 128–142. [CrossRef] [PubMed]
49. Rovatsos, M.; Altmanová, M.; Johnson Pokorná, M.; Velenský, P.; Sanchez Baca, A.; Kratochvíl, L. Evolution of karyotypes in chameleons. *Genes* **2017**, *8*, 382. [CrossRef] [PubMed]
50. Gorman, G. The Chromosomes of the Reptilia, a Cytotaxonomic Interpretation. In *Cytotaxonomy and Vertebrate Evolution*; Chiarelli, A.B., Capanna, E., Eds.; Academic Press: Cambridge, MA, USA, 1973.
51. Gao, J.; Li, Q.; Wang, Z.; Zhou, Y.; Martelli, P.; Li, F.; Xiong, Z.; Wang, J.; Yang, H.; Zhang, G. Sequencing, de novo assembling, and annotating the genome of the endangered Chinese crocodile lizard Shinisaurus crocodilurus. *GigaScience* **2017**, *6*, gix041. [CrossRef]
52. Nanda, I.; Schrama, D.; Feichtinger, W.; Haaf, T.; Schartl, M.; Schmid, M. Distribution of telomeric $(TTAGGG)_n$ sequences in avian chromosomes. *Chromosoma* **2002**, *111*, 215–227. [CrossRef]
53. Mazzoleni, S.; Augstenová, B.; Clemente, L.; Auer, M.; Fritz, U.; Praschag, P.; Protiva, T.; Velenský, P.; Kratochvíl, L.; Rovatsos, M. Sex is determined by XX/XY sex chromosomes in Australasian side-necked turtles (Testudines: Chelidae). *Sci. Rep.* **2020**, *10*, 4276. [CrossRef]
54. Clemente, L.; Mazzoleni, S.; Pensabene Bellavia, E.; Augstenová, B.; Auer, M.; Praschag, P.; Protiva, T.; Velenský, P.; Wagner, P.; Fritz, U.; et al. Interstitial telomeric repeats are rare in turtles. *Genes* **2020**, *11*, 657. [CrossRef]
55. Rovatsos, M.; Pokorná, M.J.; Kratochvíl, L. Differentiation of sex chromosomes and karyotype characterisation in the dragon snake *Xenodermus javanicus* (Squamata: Xenodermatidae). *Cytogenet. Genome Res.* **2015**, *147*, 48–54. [CrossRef]
56. Burt, D.W. Origin and evolution of avian microchromosomes. *Cytogenet. Genome Res.* **2002**, *96*, 97–112. [CrossRef]
57. International Chicken Genome Sequencing Consortium. Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. *Nature* **2004**, *432*, 695–716. [CrossRef]
58. Backström, N.; Forstmeier, W.; Schielzeth, H.; Mellenius, H.; Nam, K.; Bolund, E.; Webster, M.T.; Öst, T.; Schneider, M.; Kempenaers, B.; et al. The recombination landscape of the zebra finch *Taeniopygia guttata* genome. *Genome Res.* **2010**, *20*, 485–495. [CrossRef]
59. Melek, M.; Shippen, D.E. Chromosome healing: Spontaneous and programmed de novo telomere formation by telomerase. *Bioessays* **1996**, *18*, 301–308. [CrossRef]
60. Shay, J.R.; Wright, W.E. Telomeres and telomerase: Three decades of progress. *Nat. Rev. Genet.* **2019**, *20*, 299–309. [CrossRef] [PubMed]
61. Bolzán, A.D. Interstitial telomeric sequences in vertebrate chromosomes: Origin, function, instability and evolution. *Mutat. Res.* **2017**, *773*, 51–65. [CrossRef] [PubMed]
62. Bolzán, A.D.; Bianchi, M.S. Telomeres, interstitial telomeric repeat sequences, and chromosomal aberrations. *Mutat. Res.* **2006**, *612*, 189–214. [CrossRef] [PubMed]
63. Ruiz-Herrera, A.; Nergadze, S.G.; Santagostino, M.; Giulotto, E. Telomeric repeats far from the ends: Mechanisms of origin and role in evolution. *Cytogenet. Genome Res.* **2008**, *122*, 219–228. [CrossRef] [PubMed]
64. Birchler, J.A.; Presting, G.G. Retrotransposon insertion targeting: A mechanism for homogenization of centromere sequences on nonhomologous chromosomes. *Genes. Dev.* **2012**, *26*, 638–640. [CrossRef] [PubMed]
65. Rovatsos, M.T.; Marchal, J.A.; Romero-Fernández, I.; Fernández, F.J.; Giagia-Athanosopoulou, E.B.; Sánchez, A. Rapid, independent, and extensive amplification of telomeric repeats in pericen-tromeric regions in karyotypes of arvicoline rodents. *Chromosome Res.* **2011**, *19*, 869–882. [CrossRef] [PubMed]

66. Rovatsos, M.; Marchal, J.A.; Giagia-Athanasopoulou, E.; Sánchez, A. Molecular composition of heterochromatin and its contribution to chromosome variation in *Microtus thomasi/Microtus atticus* species complex. *Genes* **2021**, *12*, 807. [CrossRef] [PubMed]
67. Augstenová, B.; Mazzoleni, S.; Kostmann, A.; Altmanová, M.; Frynta, D.; Kratochvíl, L.; Rovatsos, M. Cytogenetic analysis did not reveal differentiated sex chromosomes in ten species of boas and pythons (Reptilia: Serpentes). *Genes* **2019**, *10*, 934. [CrossRef] [PubMed]
68. Gvoždík, V.; Benkovský, N.; Crottini, A.; Bellati, A.; Moravec, J.; Romano, A.; Sacchi, R.; Jandzik, D. An ancient lineage of slow worms, genus *Anguis* (Squamata: Anguidae), survived in the Italian Peninsula. *Mol. Phylogenet. Evol.* **2013**, *69*, 1077–1092. [CrossRef] [PubMed]
69. Lin, L.-H.; Wiens, J.J. Comparing macroecological patterns across continents: Evolution of climatic niche breadth in varanid lizards. *Ecography* **2017**, *40*, 960–970. [CrossRef]
70. De Oca, A.N.M.; Barley, A.J.; Meza-Lázaro, R.N.; García-Vázquez, U.O.; Zamora-Abrego, J.G.; Thomson, R.C.; Leaché, A.D. Phylogenomics and species delimitation in the knob-scaled lizards of the genus *Xenosaurus* (Squamata: Xenosauridae) using ddRADseq data reveal a substantial underestimation of diversity. *Mol. Phylogenet. Evol.* **2017**, *106*, 241–253. [CrossRef]
71. Brennan, I.G.; Lemmon, A.R.; Lemmon, E.M.; Portik, D.M.; Weijola, V.; Welton, L.; Donnellan, S.C.; Keogh, J.S. Phylogenomics of monitor lizards and the role of competition in dictating body size disparity. *Syst. Biol.* **2021**, *70*, 120–132. [CrossRef]
72. Porter, C.A.; Hamilton, M.J.; Sites, J.W., Jr.; Baker, R.J. Location of ribosomal DNA in chromosomes of squamate reptiles: Systematic and evolutionary implications. *Herpetologica* **1991**, *47*, 271–280.
73. Stults, D.M.; Killen, M.W.; Pierce, H.H.; Pierce, A.J. Genomic architecture and inheritance of human ribosomal RNA gene clusters. *Genome Res.* **2008**, *18*, 13–18. [CrossRef]
74. Altmanová, M.; Rovatsos, M.; Kratochvíl, L.; Johnson Pokorná, M. Minute Y chromosomes and karyotype evolution in Madagascan iguanas (Squamata: Iguania: Opluridae). *Biol. J. Linn. Soc.* **2016**, *118*, 618–633. [CrossRef]
75. Sochorová, J.; Garcia, S.; Gálvez, F.; Symonová, R.; Kovařík, A. Evolutionary trends in animal ribosomal DNA loci: Introduction to a new online database. *Chromosoma* **2017**, *127*, 141–150. [CrossRef]
76. Mazzoleni, S.; Rovatsos, M.; Schillaci, O.; Dumas, F. Evolutionary insight on localization of 18S, 28S rDNA genes on homologous chromosomes in Primates genomes. *Comp. Cytogenet.* **2018**, *12*, 27–40. [CrossRef]
77. Micolino, R.; Cristiano, M.P.; Travenzoli, N.M.; Lopes, D.M.; Cardoso, D.C. Chromosomal dynamics in space and time: Evolutionary history of *Mycetophylax* ants across past climatic changes in the Brazilian Atlantic coast. *Sci. Rep.* **2019**, *9*, 18800. [CrossRef] [PubMed]
78. Degrandi, T.M.; Gunski, R.J.; Garnero, A.D.V.; Oliveira, E.H.C.; Kretschmer, R.; Souza, M.S. Barcellos, S.A.; Hass, I. The distribution of 45S rDNA sites in bird chromosomes suggests multiple evolutionary histories. *Genet. Mol. Biol.* **2020**, *43*, e20180331. [CrossRef]
79. Literman, R.; Badenhorst, D.; Valenzuela, N. qPCR-based molecular sexing by copy number variation in r RNA genes and its utility for sex identification in soft-shell turtles. *Methods Ecol. Evol.* **2014**, *5*, 872–880. [CrossRef]
80. Literman, R.; Radhakrishnan, S.; Tamplin, J.; Burke, R.; Dresser, C.; Valenzuela, N. Development of sexing primers in *Glyptemys insculpta* and *Apalone spinifera* turtles uncovers an XX/XY sex-determining system in the critically-endangered bog turtle *Glyptemys muhlenbergii*. *Conserv. Genet. Resour.* **2017**, *9*, 651–658. [CrossRef]
81. Rovatsos, M.; Praschag, P.; Fritz, U.; Kratochvíl, L. Stable Cretaceous sex chromosomes enable molecular sexing in softshell turtles (Testudines: Trionychidae). *Sci. Rep.* **2017**, *7*, 42150. [CrossRef] [PubMed]
82. Kostmann, A.; Kratochvíl, L.; Rovatsos, M. First report of sex chromosomes in plated lizards (Squamata: Gerrhosauridae). *Sex. Dev.* **2021**, 1–6. [CrossRef]
83. Zurita, F.; Sánchez, A.; Burgos, M.; Jiménez, R.; de la Guardia, R.D. Interchromosomal, intercellular and interindividual variability of NORs studied with silver staining and *in situ* hybridization. *Heredity* **1997**, *78*, 229–234. [CrossRef]
84. Zurita, F.; Jimenez, R.; Burgos, M.; de La Guardia, R.D. Sequential silver staining and *in situ* hybridization reveal a direct association between rDNA levels and the expression of homologous nucleolar organizing regions: A hypothesis for NOR structure and function. *J. Cell Sci.* **1998**, *111*, 1433–1439. [CrossRef]
85. Nirchio, M.; Oliveira, C.; Ferreira, I.A.; Granado, A.; Ron, E. Extensive polymorphism and chromosomal characteristics of ribosomal DNA in the characid fish *Triportheus venezuelensis* (Characiformes, Characidae). *Genet. Mol. Biol.* **2007**, *30*, 25–30. [CrossRef]
86. Miller, L.; Knowland, J. Reduction of ribosomal RNA synthesis and ribosomal RNA genes in a mutant of *Xenopus laevis* which organizes only a partial nucleolus: II. The number of ribosomal RNA genes in animals of different nucleolar types. *J. Mol. Biol.* **1970**, *53*, 329–338. [CrossRef]
87. Gibbons, J.G.; Branco, A.T.; Godinho, S.A.; Yu, S.; Lemos, B. Concerted copy number variation balances ribosomal DNA dosage in human and mouse genomes. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 2485–2490. [CrossRef]
88. Xu, B.; Li, H.; Perry, J.M.; Singh, V.P.; Unruh, J.; Yu, Z.; Zakari, M.; McDowell, W.; Li, L.; Gerton, J.L. Ribosomal DNA copy number loss and sequence variation in cancer. *PLoS Genet.* **2017**, *13*, e1006771. [CrossRef] [PubMed]
89. Kobayashi, T. How does genome instability affect lifespan? Roles of rDNA and telomeres. *Genes Cells* **2011**, *16*, 617–624. [CrossRef] [PubMed]
90. Rovatsos, M.; Augstenová, B.; Altmanová, M.; Sloboda, M.; Kodym, P.; Kratochvíl, L. Triploid colubrid snake provides insight into the mechanism of sex determination in advanced snakes. *Sex. Dev.* **2018**, *12*, 251–255. [CrossRef]
91. Tiersch, T.R.; Figiel, C.R. A triploid snake. *Copeia* **1991**, *1991*, 838–841. [CrossRef]

92. Peters, G. Die intragenerischen Gruppen und die Phylogenese der Schmetterlingsagamen (Agamidae: *Leiolepis). Zool. Jahrb. Syst.* **1971**, *98*, 11–130.
93. Moritz, C. Parthenogenesis in the endemic Australian lizard *Heteronotia binoei* (Gekkonidae). *Science* **1983**, *220*, 735–737. [CrossRef]
94. Moritz, C.; Case, T.J.; Bolger, D.T.; Donnellan, S. Genetic diversity and the history of pacific island house geckos (*Hemidactylus* and *Lepidodactylus*). *Biol. J. Linn. Soc.* **1993**, *48*, 113–133. [CrossRef]
95. Darevsky, I.S.; Kupriyanova, L.A.; Roshchin, V.V. A new all-female triploid species of gecko and karyological data on the bisexual *Hemidactylus frenatus* from Vietnam. *J. Herpetol.* **1984**, *18*, 277–284. [CrossRef]
96. Wynn, A.H.; Cole, C.J.; Gardner, A.L. Apparent triploidy in the unisexual Brahminy blind snake, *Ramphotyphlops braminus*. *Am. Mus. Novit.* **1987**, *2868*, 1–7.
97. Adams, M.; Foster, R.; Hutchinson, M.N.; Hutchinson, R.G.; Donnellan, S.C. The Australian scincid lizard Menetia greyii: A new instance of widespread vertebrate parthenogenesis. *Evolution* **2003**, *57*, 2619–2627. [CrossRef] [PubMed]
98. Lutes, A.A.; Baumann, D.P.; Neaves, W.B.; Baumann, P. Laboratory synthesis of an independently reproducing vertebrate species. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 9910–9915. [CrossRef] [PubMed]
99. Vergun, A.A.; Martirosyan, I.A.; Semyenova, S.K.; Omelchenko, A.V.; Petrosyan, V.G.; Lazebny, O.E.; Tokarskaya, O.N.; Korchagin, V.I.; Ryskov, A.P. Clonal diversity and clone formation in the parthenogenetic Caucasian rock lizard Darevskia dahli. *PLoS ONE* **2014**, *9*, e91674. [CrossRef]
100. Trifonov, V.A.; Paoletti, A.; Caputo Barucchi, V.; Kalinina, T.; O'Brien, P.C.M.; Ferguson-Smith, M.A.; Giovannotti, M. Comparative chromosome painting and NOR distribution suggest a complex hybrid origin of triploid *Lepidodactylus lugubris* (Gekkonidae). *PLoS ONE* **2015**, *10*, e0132380. [CrossRef]