Charles University

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Evolution of sex determination in skinks and related lineages

Evoluce determinace pohlaví u scinků a příbuzných linií

Doctoral thesis

Supervisor: Michail Rovatsos, PhD

Prague, 2021

DECLARATION OF ORIGINALITY / PROHLÁŠENÍ O ORIGINALITĚ

I declare that this thesis has not been submitted for the purpose of obtaining the same or another academic degree earlier or at another institution. My involvement in the research presented in this thesis is expressed through the authorship order of the included publications and explained in detail in the "Outline of publications" section of the dissertation. All literature sources I used when writing this thesis have been properly cited.

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Prague, June 2021 / Praha, červen 2021,

Alexander Kostmann

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Publications

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Kostmann A, Kratochvíl L, Rovatsos M. 2021 Poorly differentiated XX/XY sex chromosomes are widely shared across skink radiation. *Proc. R. Soc. B.* 288, 20202139. (doi:10.1098/rspb.2020.2139)

Chapter 2

Kostmann A, Kratochvíl L, Rovatsos M. 2021 First report of sex chromosomes in plated lizards (Squamata: Gerrhosauridae). *Sex. Dev.*, in press (doi:10.1159/000513764)

Chapter 3

Kostmann A, Augstenová B, Frynta D, Kratochvíl L, Rovatsos M. 2021 Cytogenetically elusive sex chromosomes in scincoidean lizards. *Int. J. Mol. Sci.* (submitted)

Chapter 4

Kostmann A, Kratochvíl L, Rovatsos M. 2021 ZZ/ZW sex chromosomes in the Madagascar girdled lizard, *Zonosaurus madagascariensis* (Squamata: Gerrhosauridae). (manuscript)

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Abstract

Scincoidean lizards, i.e. cordylids, gerrhosaurids, skinks and xantusiids, are known for their remarkable ecological and morphological variability. It was hypothesized that, at least in skinks, sex determining systems are highly variable as well. In the other three families, evidence for presence or absence of sex chromosomes has been scarce, with two species of night lizards with ZZ/ZW sex chromosomes being the exception. In this thesis, conventional and molecular cytogenetic methods, including C-banding, fluorescence *in situ* hybridization (FISH) with probes for telomeric motifs and rDNA loci and comparative genomic hybridization (CGH) were used to identify cytogenetically distinguishable sex chromosomes. Although most studied species showed no sex-specific differences by cytogenetic examination, some did. *Tracheloptychus petersi* has accumulations of rDNA loci on a pair of macrochromosomes and a pair of microchromosomes in males, while again on a pair of macrochromosomes and a single microchromosome in females. This distribution suggests a ZZ/ZW system in this species, which is the first report of sex chromosomes in any gerrhosaurid lizard. In *Zonosaurus madagascariensis*, CGH was able to identify the W chromosome in females, which is the second report of sex chromosomes in this family. Cytogenetic examinations in scincid lizards led to the identification of cytogenetically distinguishable XX/XY sex chromosomes in *Tiliqua scincoides* and confirmed sex-associated nucleolus organizer region (NOR) polymorphism in *Scincus scincus*. Subsequently, a genome coverage analysis in *S. scincus* led to the identification of several X-linked genes, which in turn uncovered homologous and poorly differentiated XX/XY sex chromosomes across all 13 tested scincid species. These results indicate that homologous XX/XY sex chromosomes are shared across nearly the whole scincid phylogeny, covering around 1700 species representing 15% of reptile species diversity. The age of the XX/XY sex determination system of skinks is estimated to be at least 85 million years, approaching the age of highly differentiated ZZ/ZW sex chromosomes of birds and advanced snakes. Scincid sex chromosomes demonstrate that even poorly differentiated sex chromosomes can be evolutionary stable in the long-term.

Abstrakt

Ještěři skupiny Scincoidea, tj. kruhochvosti (Cordylidae), ještěrkovci (Gerrhosauridae), scinci (Scincidae) a xantusie (Xantusiidae), mají pozoruhodnou ekologickou a morfologickou variabilitou. Předpokládalo se, že přinejmenším u skinků jsou systémy určení pohlaví také velmi variabilní. U ostatních tří čeledí byly důkazy pro přítomnost nebo nepřítomnost pohlavních chromozomů vzácné, přičemž výjimkou byly dva druhy xantusií s pohlavními chromozomy typu ZZ/ZW. V této práci byly k identifikaci cytogeneticky odlišitelných pohlavních chromosomů použity konvenční a molekulární cytogenetické metody včetně C-pruhování, fluorescenční *in situ* hybridizace (FISH) se sondami pro telomerické motivy a rDNA lokusy a komparativní genomová hybridizace. Většina studovaných druhů nevykazovala pohlavně-specifické rozdíly zjistitelné cytogenetickým vyšetřením. Nicméně jsme prokázali akumulaci lokusů rDNA na páru makrochromosomů a páru mikrochromosomů u samců, zatímco opět na páru makrochromosomů a jediném mikrochromosomu u samic druhu *Tracheloptychus petersi*. Pohlavně-specifická distribuce rDNA lokusů naznačuje, že tento druh má ZZ/ZW pohlavní chromosomy, což představuje první doklad o pohlavních chromosomech u zástupce čeledi Gerrhosauridae. U dalšího zástupce této čeledi, konkrétně u druhu *Zonosaurus madagascariensis* dokázala CGH identifikovat samičí chromosom W, což dále podporuje první nález. Cytogenetické vyšetření u scinků vedlo k identifikaci cytogeneticky odlišitelných pohlavních chromozomů XX/XY u druhu *Tiliqua scincoides* a potvrzení pohlavně vázaného polymorfismu v rDNA lokusech u druhu *Scincus scincus*. Následně analýza pokrytí genomu při sekvenování u *S. scincus* vedla k identifikaci několika genů specifických pro chromosom X, což dále vedlo k odhalení homologických, málo diferencovaných pohlavních chromosomů XX/XY u všech 13 testovaných druhů scinků. Tyto výsledky naznačují, že homologické pohlavní chromosomy XX/XY jsou sdíleny přes linii scinků pokrývají přibližně 1700 druhů, tj. zhruba 15% druhové rozmanitosti všech neptačích plazů. Stáří XX/XY chromosomů scinků jsme odhadli na nejméně 85 milionů let, což se blíží stáří vysoce diferencovaných ZZ/ZW pohlavních chromozomů ptáků a hadů skupiny Caenophidia. Analýza pohlavních chromosomů scinků ukazuje, že i málo diferencované pohlavní chromosomy mohou být dlouhodobě evolučně stabilní.

Outline of publications

This thesis consists of four publications, focusing on conventional and molecular cytogenetics to identify sex chromosomes and whole-genome sequencing and quantitative polymerase chain reaction (qPCR) to test their homology across the large scincoidean diversity. Two of the mentioned publications are already published in reputable journals, one has been submitted to a journal recently and the last one is still in preparation.

In the first publication (**Chapter 1**), two members of Scincidae, *Scincus scincus* and *Tropidophorus baconi*, were cytogenetically examined. Caputo et al. (1994) reported two active Nucleolus Organizer Regions (NORs) per female metaphase, while only a single active NORs in males. First, we confirmed this observation by fluorescence *in situ* hybridization (FISH) with the probe for rDNA loci. Additional cytogenetic experiments, including comparative genomic hybridization (CGH), as well as all cytogenetic experiments in *Tropidophorus baconi*, did not uncover any sex-specific differences which could be attributed to sex chromosomes. With these now confirmed XX/XY sex chromosomes in *S. scincus*, a genome coverage analysis was performed to identify its X-linked genes. An accumulation of these genes are linked to *Podarcis muralis* chromosome 10 (PMU10), their homologues are linked to chicken chromosome 1 (GGA1). Among amniotes, this PMU10/GGA1 syntenic block was only known to be a part of sex chromosomes in the distantly related geckos of the genus *Coleonyx* (Rovatsos et al. 2019, Pensabene et al. 2020), which supports the hypothesis that skink sex chromosomes evolved independently from other amniote sex chromosomes. Testing the X-specificity of these genes in 13 species of skinks, covering a phylogenetic spectrum of nearly 1700 species, led to the conclusion that these poorly differentiated sex chromosomes are highly conserved across the skink radiation. Although these sex chromosomes are poorly differentiated, and only have a small X-specific region (as was also shown recently in another skink, *Eulamprus heatwolei*; Cornejo-Páramo et al. 2020), they are at least 85 million years old and therefore of similar age as the highly differentiated ZZ/ZW sex chromosomes of birds and advanced snakes (Zhou et al. 2014, Rovatsos et al. 2015).

The second publication (**Chapter 2**) is a conventional and molecular cytogenetic examination of the Malagasy Peters' keeled plated lizard *Tracheloptychus petersi*. Several species of gerrhosaurid lizards have been examined in the past, at least by conventional cytogenetics, but sex-specific differences, and therefore sex chromosomes, have never been identified in any of them. The sex chromosomes of *T. petersi* are homomorphic and even CGH failed to identify them. The only cytogenetically observable difference is the accumulation of rDNA loci; they are present on a pair of macrochromosomes in both sexes, and additionally, on two microchromosomes in males, but only a single microchromosome in females. This female-specific heterozygosity suggests a putative ZZ/ZW system and is the first report of sex chromosomes in plated lizards (Gerrhosauridae).

The third publication (**Chapter 3**) employs conventional and molecular cytogenetics on a wider phylogenetic scale of scincoidean lizards, including six species of skinks, two cordylids and one gerrhosaurid. Diploid chromosome numbers are rather conserved among these species, but chromosome morphology, presence of interstitial telomeric sequences and distribution of rDNA loci vary significantly. Although we confirmed XX/XY sex chromosomes in all studied skink species (except *Acontias percivali*) by qPCR test, sex-specific differences by cytogenetic methods were only identified in *Tiliqua scincoides*, where rDNA loci were only present on the X chromosome.

The last publication (**Chapter 4**) is a cytogenetic examination of the Madagascar girdled lizard *Zonosaurus madagascariensis*. In this gerrhosaurid lizard, all conventional and molecular cytogenetic methods failed to show sex-specific differences, apart from CGH. CGH revealed a strong female-specific signal in one of the larger microchromosomes in females, which suggests homomorphic ZZ/ZW sex chromosomes. If these sex chromosomes are homologous to the ZZ/ZW sex chromosomes of *T. petersi* remains to be uncovered.

T**able 1:** Overview of contribution for each chapter.

	contribution					
chapter	funding	experimental design	experimental procedures	figure/table preparation	data analysis	manuscript preparation
1	✓		✓	✓	✓	✓
2	✓	✓	✓	✓	✓	✓
3	✓		✓	✓	✓	✓
4	✓	✓	✓	✓	✓	✓

STATEMENTS OF CONTRIBUTION / PROHLÁŠENÍ O PŘÍSPĚVKU

I declare that my research effort for this dissertation was conducted in collaboration with scientists international. My personal contribution to the conception, data collection, analysis and manuscript preparation for each of the following chapters is accurately explained above.

Prohlašuji, že moje výzkumné úsilí ústící v tuto disertační práci bylo vedeno v mezinárodní spolupráci. Můj osobní příspěvek ke koncepci, sběru dat, analýze a rukopisné přípravě pro každou z kapitol byl přesně vysvětlen výše.

Prague, June 2021 / Praha, červen 2021,

Alexander Kostmann

As supervisor of the PhD thesis and to the best of my knowledge, I confirm that the contribution of MSc. Alexander Kostmann to the chapters and appendices of this thesis is accurately explained above.

Jako vedoucí disertační práce a podle mého nejlepšího vědomí potvrzuji, že příspěvek Alexandera Kostmanna ke kapitolám a dodatkům této práce byl přesně vysvětlen výše.

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Michail Rovatsos

Introduction

There are two major modes of sex determination in amniote vertebrates: genotypic sex determination (GSD) and environmental sex determination (ESD). GSD, where sex is determined by sex chromosomes, is the sole system in birds and mammals (Shetty et al. 1999, Graves 2006). ESD, where environmental factors, most commonly temperature, determine gonadal development is common in turtles, all crocodilians and many lizards (Bull 1983, Lang and Andrews 1994, Janzen & Krenz 2004).

In GSD, we differentiate between several sex chromosome systems. XX/XY sex chromosomes, where males are the heterogametic sex and carry one X and the male-specific Y, while females carry two X chromosomes, is present in mammals, but also occurs in many other lineages (Graves 2006, Carvalho 2002). In eutherian mammals, the gene called *Sry* (sex-determining region Y) located on the Y chromosome is responsible for the initiation of the male developmental pathway (Sinclair et al. 1990, McLaren 1991). Although they share the same (but obviously non-homologous) sex chromosome system, the mechanism of sex determination in *Drosophila melanogaster* is completely different: its Y is not involved in determining sex, but the ratio between X chromosomes and autosomes (Bridges 1921, Bridges 1925). Even within mammals, sex determination differs significantly: monotremes, which diverged early from mammalian evolution, have multiple sex chromosomes (5X5Y in platypus and 5X4Y in echidna), which are not homologous to eutherian XY and miss the *Sry* gene (Grützner et al. 2004, Rens et al. 2004, Rens et al. 2007). On the contrary, the gene *Amh* (Anti-Müllerian hormone) is the likely sex-determining locus in monotremes (Cortez et al. 2014). Sex determination in few rodents, including members of the genera *Ellobius* and *Tokudaia*, varies from other eutherian mammals. Some species in these genera lost the Y chromosome, with it the *Sry* gene, leaving them with X0/X0, while others even acquired isomorphic XX sex chromosomes in males and females (Castro-Sierra & Wolf 1967, Just et al. 1995, Soullier et al. 1998, Matveevsky et al. 2017).

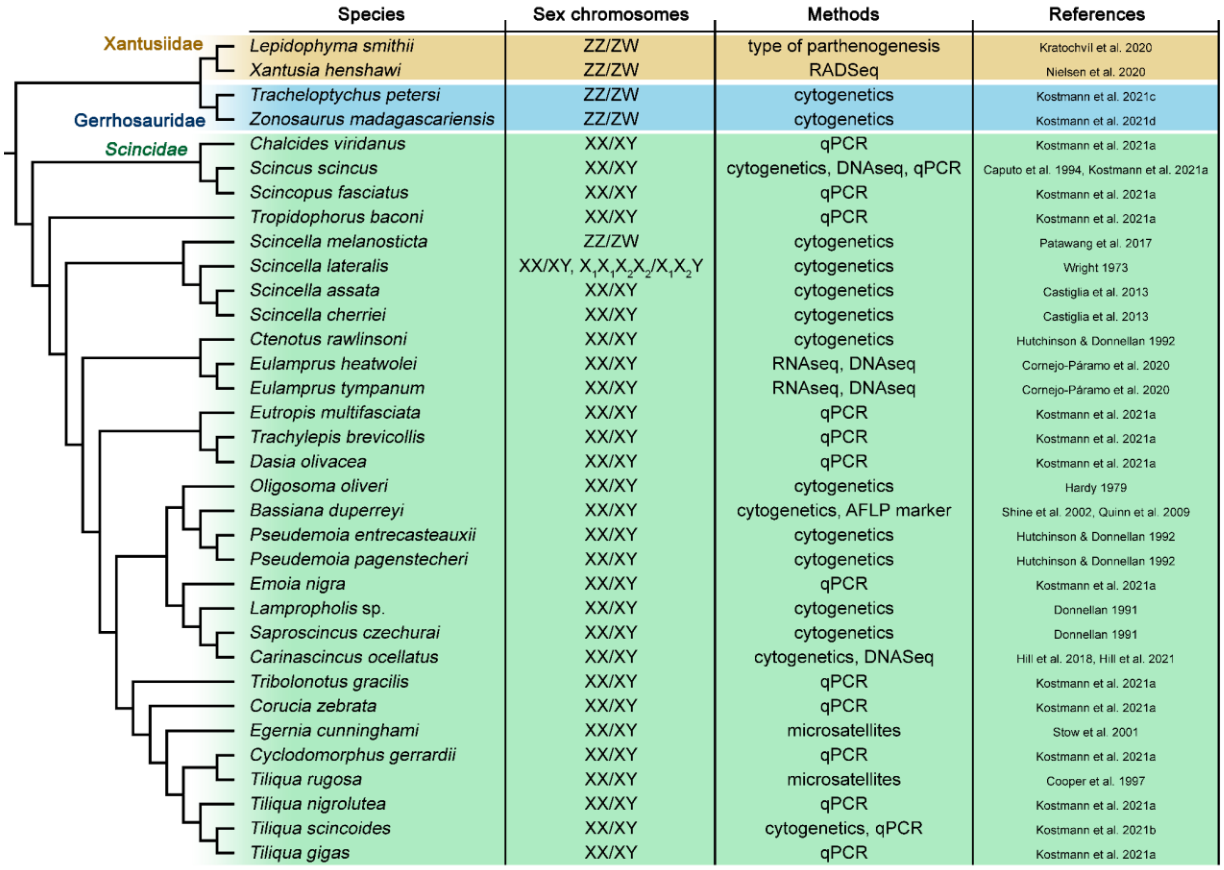
Female heterogamety, where females possess a Z and a female-specific W chromosome, while males carry two Z chromosomes, is the predominant sex determination system in several lineages, including birds, caenophidian snakes, trionychid turtles and butterflies (Traut & Rhadjens 1973, Shetty et al. 1999, Rovatsos et al. 2015, Rovatsos et al. 2017). One could hypothesize that the female-specific W may carry an ovary determinant, but it was shown that at least in birds, the dosage of the Z-linked *Dmrt1* (doublesex and mab-3 related transcription factor 1) is the trigger in sex determination and two doses are required for male development (Smith et al. 2009). In caddisflies (Trichoptera) and basal families of butterflies (Lepidoptera), an ancestral ZZ/Z0 sex chromosome system is prevalent, while the W chromosome only occurs in more derived lepidopteran families (Marec and Novák 1998, Marec et al. 2010, Sahara et al. 2012). Contrary to the Z counting mechanism of birds, the silk moth, *Bombyx mori*, has a W chromosome with strong female-determining effect. In polyploids, the embryo enters the female pathway if the W chromosome is present, no matter how many Z chromosomes are present (Tazima 1964, Fujii & Shimada 2007). While different XX/XY and ZZ/ZW systems are well documented in a plethora of species, there is another, lesser-known system: UV sex chromosomes. This system occurs in some algae and bryophytes, where the diploid stage is always heterogametic (UV), while the haploid stage is either female, and carries a U, or is male, and carries a V chromosome (Bachtrog et al. 2011).

Sex chromosomes evolve from autosomes by acquiring sex determining genes and subsequent recombination suppression, initially around these genes (Muller 1914, Ohno 1967). Charlesworth et al. (2005) hypothesized that sex chromosomes at an early state are homomorphic, but gradual gene loss and accumulation of repetitive elements in the non-recombining region leads to molecularly more differentiated and heteromorphic sex chromosomes. Theoretical predictions about the differentiation of W and Y have been controversial. Faster differentiation of the Y was predicted because of stronger sexual selection in males, male mutation bias and smaller effective population size (Bachtrog et al. 2011). More frequent fixation of inversions in Z and W chromosomes, which reduces recombination, is assumed to be the main reason why the W chromosome should differentiate faster (Sardell & Kirkpatrick 2020). Ongoing degeneration of Y (or W), and therefore gene loss, leads to differences in copy numbers of genes on X (or Z). To cope with these differences, many taxa evolved dosage compensation, ranging from epigenetic silencing of one entire X chromosome in females in eutherian mammals and partial silencing of the Z chromosome in male birds to two-fold upregulation of X-linked genes in male anoles, a compensation mechanism well-known from fruit fly (Graves 2016, Marin et al. 2017). Highly differentiated sex chromosomes and their mechanisms to achieve dosage balance between sexes makes turn-overs of sex chromosomes, and even more so, the loss of sex chromosomes, unlikely, which led to the hypothesis that these sex chromosomes act as an evolutionary trap (Pokorná & Kratochvíl 2009). Highly differentiated and evolutionary stable sex chromosomes are well-known in eutherian mammals and birds (Cortez et al. 2014, Zhou et al. 2014), while sex chromosomes in many lineages of amphibians and teleost fishes are mostly homomorphic and prone to turn-overs (Devlin & Nagahama 2002, Miura 2017). In non-avian reptiles, the picture is less clear: highly differentiated, evolutionary stable sex chromosomes are known in some lineages (e.g., ZZ/ZW in caenophidian snakes, see Rovatsos et al. 2015), while in others, turn-overs are frequent, different sex determining systems exist simultaneously and differentiated sex chromosomes even disappear (e.g., geckos, Gamble 2010, Rovatsos et al. 2019). One of the most species-rich and ecologically diverse groups of lizards has been highly neglected in this regard: the scincoidean lizards.

The lizard clade Scincoidea consists of four families: Cordylidae, Gerrhosauridae, Scincidae and Xantusiidae. The girdled lizards (Cordylidae) contain 70 species (Uetz et al. 2020), inhabiting Sub-Saharan Africa. They are divided in two subfamilies, the monogeneric Platysaurinae, and the more diverse Cordylinae (Pyron et al. 2013). Members of the genus *Platysaurus* are highly flattened and lightly armored, an adaptation to their retreat sites, which are narrow rock crevices (Stanley et al. 2011). The phylogenetic classification within the subfamily Cordylinae was subject to many changes in the recent past, and the non-monophyletic genus *Cordylus* was split up into several small genera (Frost et al. 2001, Stanley et al. 2011, Pyron et al. 2013). The members of the subfamily Cordylinae are ecologically and morphologically diverse, ranging from the nearly limbless, snake-like *Chaemaesaura* to large, heavily armored lizards of the genus *Smaug*. The plated lizards (Gerrhosauridae) contain 37 species, split among two subfamilies: Gerrhosaurinae, inhabiting Sub-Saharan Africa, and Zonosaurinae, inhabiting Madagascar and nearby islands. In Gerrhosaurinae, five genera are recognized: *Broadleysaurus*, *Cordylosaurus*, *Gerrhosaurus*, *Matobosaurus* and *Tetradactylus* (Bates et al. 2013). In Zonosaurinae, two genera are traditionally recognized: *Tracheloptychus* and *Zonosaurus*, but a fast initial radiation with up to five distinct groups has been hypothesized by Recknagel et al. (2013). Skinks (Scincidae) are not only by far the most species-rich family among Scincoidea, but with around 1700 species they account for 15% of reptile species diversity. Thanks to their ecological and morphological diversity, they are nearly cosmopolitan and are able to occupy a wide range of habitats. There are terrestrial, subterranean, arboreal and semiaquatic species, and traits like limblessness, viviparity and nocturnality evolved several times independently within skinks (Vitt & Caldwell 2014). According to Pyron et al. (2013) only three subfamilies are recognized: basal legless Acontinae, Scincinae and Lygosominae. The night lizards (Xantusiidae) are a small family of secretive lizards with 36 species (Uetz et al. 2020). They are split among three subfamilies: Cricosaurinae, with only one species, the Cuban night lizard *Cricosaura typica*, the North American Xantusiinae and the Central American Lepidophyminae (Pyron et al. 2013).

Although scincoidean lizards are such a species-rich, ecologically and morphologically diverse group, their karyotype is rather conservative. In Cordylidae and Gerrhosauridae, a diploid chromosome number of 2n = 34 is prevalent (Odierna et al. 2002). Scincid diploid chromosome numbers show higher variation, ranging from 2n = 22 to 2n = 36, categorized in macro- and microchromosomes (Olmo & Signorino 2005, Giovannotti et al. 2009). In the subfamily Acontinae, the genera *Acontias*, *Typhlosaurus* and *Acontophiobs* have a diploid chromosome number of 2n = 22 (Gordon et al. 1989). In Scincinae, some genera show low variation, e.g. *Chalcides* and *Sphenops* share 2n = 28 chromosomes (Caputo 1993), while diploid chromosome numbers in *Eumeces* and *Scincus* vary from 2n = 26 to 2n = 36 (Deweese & Wright 1970, Branch 1980, Caputo 1994). Lygosomine skinks have mostly diploid chromosome numbers of 2n = 30 to 2n = 32 (King 1973), but exceptions exist, e.g., *Apterygodon vittatus*, with 2n = 28 (Ota et al. 1996). Among xantusiid lizards, *Cricosaura typica* has the lowest diploid chromosome number with 2n = 24 (Hass & Hedges 1992), while in other species, it ranges from 2n = 30 to 2n = 34 (Bezy 1972).

Many careful cytogenetic studies on scincoidean lizards were done in the past, but sex chromosomes were rarely identified (Figure 1). Heteromorphic XX/XY sex chromosomes have been detected in *Cyclodina oliveri* (Hardy 1979), *Saproscincus czechurai*, *Lampropholis* sp. C (Donnellan 1991), *Ctenotus rawlinosoni*, *Pseudemoia entrecasteauxii*, *P. pagenstecheri* (Hutchinson & Donnellan 1992), *Scincella assata* and *S. cherriei* (Castiglia et al. 2013). A special case is *Scincella lateralis*, where XX/XY sex chromosomes were identified in one population, while another population has a $X_1X_1X_2X_2/X_1X_2Y$ multiple sex chromosome system (Wright 1973, Hedin et al. 1990). The only report of ZZ/ZW sex chromosomes in skinks was based on size difference between the putative Z and W in *Scincella melanosticta* (Patawang et al. 2017). In *Bassiana duperreyi*, size difference of X and Y, later confirmed by C-banding, FISH with a probe for telomeric repeats and CGH was reported (Shine et al. 2002, Matsubara et al. 2015). Additionally, Quinn et al. (2009) identified Y-specific markers by screening amplified fragment length polymorphism markers from pooled monosex templates. Dissanayake et al. (2020) applied *in silico* whole genome subtraction to identify these markers in *B. duperreyi*. Y-specific markers have been identified by a similar subtraction method, but from transcriptome data in *Eulamprus heatwolei*, supplemented by genome coverage analysis to find X-specific markers (Cornejo-Páramo et al. 2020). In xantusiid lizards, ZZ/ZW sex chromosomes have been identified in *Xantusia henshawi* by restriction site-associated DNA sequencing (Nielsen et al. 2020), and female heterogamety was also implied in *Lepidophyma smithii*, which produces both sexes via facultative parthenogenesis (Kratochvíl et al. 2020). Previous research has found no signs of sex chromosomes in the families Gerrhosauridae and Cordylidae.



Species	Sex chromosomes	Methods	References
Lepidophyma smithii	ZZ/ZW	type of parthenogenesis	Kratochvil et al. 2020
Xantusia henshawi	ZZ/ZW	RADSeq	Nielsen et al. 2020
Tracheloptychus petersi	ZZ/ZW	cytogenetics	Kostmann et al. 2021c
Zonosaurus madagascariensis	ZZ/ZW	cytogenetics	Kostmann et al. 2021d
Chalcides viridanus	XX/XY	qPCR	Kostmann et al. 2021a
Scincus scincus	XX/XY	cytogenetics, DNAseq, qPCR	Caputo et al. 1994, Kostmann et al. 2021a
Scincopus fasciatus	XX/XY	qPCR	Kostmann et al. 2021a
Tropidophorus baconi	XX/XY	qPCR	Kostmann et al. 2021a
Scincella melanosticta	ZZ/ZW	cytogenetics	Patawang et al. 2017
Scincella lateralis	XX/XY, $X_1X_1X_2X_2$/X_1X_2Y	cytogenetics	Wright 1973
Scincella assata	XX/XY	cytogenetics	Castiglia et al. 2013
Scincella cherriei	XX/XY	cytogenetics	Castiglia et al. 2013
Ctenotus rawlinsoni	XX/XY	cytogenetics	Hutchinson & Donnellan 1992
Eulamprus heatwolei	XX/XY	RNAseq, DNAseq	Cornejo-Páramo et al. 2020
Eulamprus tympanum	XX/XY	RNAseq, DNAseq	Cornejo-Páramo et al. 2020
Eutropis multifasciata	XX/XY	qPCR	Kostmann et al. 2021a
Trachylepis brevicollis	XX/XY	qPCR	Kostmann et al. 2021a
Dasia olivacea	XX/XY	qPCR	Kostmann et al. 2021a
Oligosoma oliveri	XX/XY	cytogenetics	Hardy 1979
Bassiana duperreyi	XX/XY	cytogenetics, AFLP marker	Shine et al. 2002, Quinn et al. 2009
Pseudemoia entrecasteauxii	XX/XY	cytogenetics	Hutchinson & Donnellan 1992
Pseudemoia pagenstecheri	XX/XY	cytogenetics	Hutchinson & Donnellan 1992
Emoia nigra	XX/XY	qPCR	Kostmann et al. 2021a
Lampropholis sp.	XX/XY	cytogenetics	Donnellan 1991
Saproscincus czechurai	XX/XY	cytogenetics	Donnellan 1991
Carinascincus ocellatus	XX/XY	cytogenetics, DNASeq	Hill et al. 2018, Hill et al. 2021
Tribolonotus gracilis	XX/XY	qPCR	Kostmann et al. 2021a
Corucia zebrata	XX/XY	qPCR	Kostmann et al. 2021a
Egernia cunninghami	XX/XY	microsatellites	Stow et al. 2001
Cyclodomorphus gerrardii	XX/XY	qPCR	Kostmann et al. 2021a
Tiliqua rugosa	XX/XY	microsatellites	Cooper et al. 1997
Tiliqua nigrolutea	XX/XY	qPCR	Kostmann et al. 2021a
Tiliqua scincoides	XX/XY	cytogenetics, qPCR	Kostmann et al. 2021b
Tiliqua gigas	XX/XY	qPCR	Kostmann et al. 2021a

Figure 1: Phylogeny of scincoidean lizards with known sex chromosomes. Phylogenetic relationships follow Pyron et al. (2013).

Aims

The aim of this thesis was to explore the evolution of sex determination in scincoidean lizards While in some lineages, mainly gerrhosaurid and cordylid lizards, research on sex determination has been highly neglected, results were contradictory in skinks. There have been reports about XX/XY, ZZ/ZW and multiple sex chromosome systems in some species, while temperature dependent sex determination was reported in others. In this thesis, conventional (Giemsa staining, C-banding) and molecular cytogenetic (FISH with rDNA and telomeric probes, comparative genomic hybridization) methods, complemented by genomic data and quantitative PCR are used to identify new sex chromosomes in these lineages and test for homology of sex chromosomes among families. The thesis is split into four chapters, with slightly different aims:

Chapter 1: Identification of X-specific genes in *Scincus scincus* and test of homology of sex chromosomes across skink radiation and outgroups.

Chapter 2: Identification of sex chromosomes in the gerrhosaurid lizard *Tracheloptychus petersi* by cytogenetic methods.

Chapter 3: Comparative cytogenetic study to discover possible cytogenetic characteristics of sex chromosomes in several scincoidean species.

Chapter 4: Identification of cytogenetically distinguishable sex chromosomes in the gerrhosaurid lizard *Zonosaurus madagascariensis.*

Materials & methods

Blood and tissue samples of as many as possible phylogenetically informative species of the scincoidean megadiversity were gathered from the pet trade, private breeders and zoological gardens to explore the evolution of sex determination in this important lineage. Wherever possible, the least possible invasive method (blood sampling) was used to acquire these samples. The sex of sampled specimens was identified by external morphology, everting hemipenes by palpation and after development of our molecular sexing method in skinks, by qPCR.

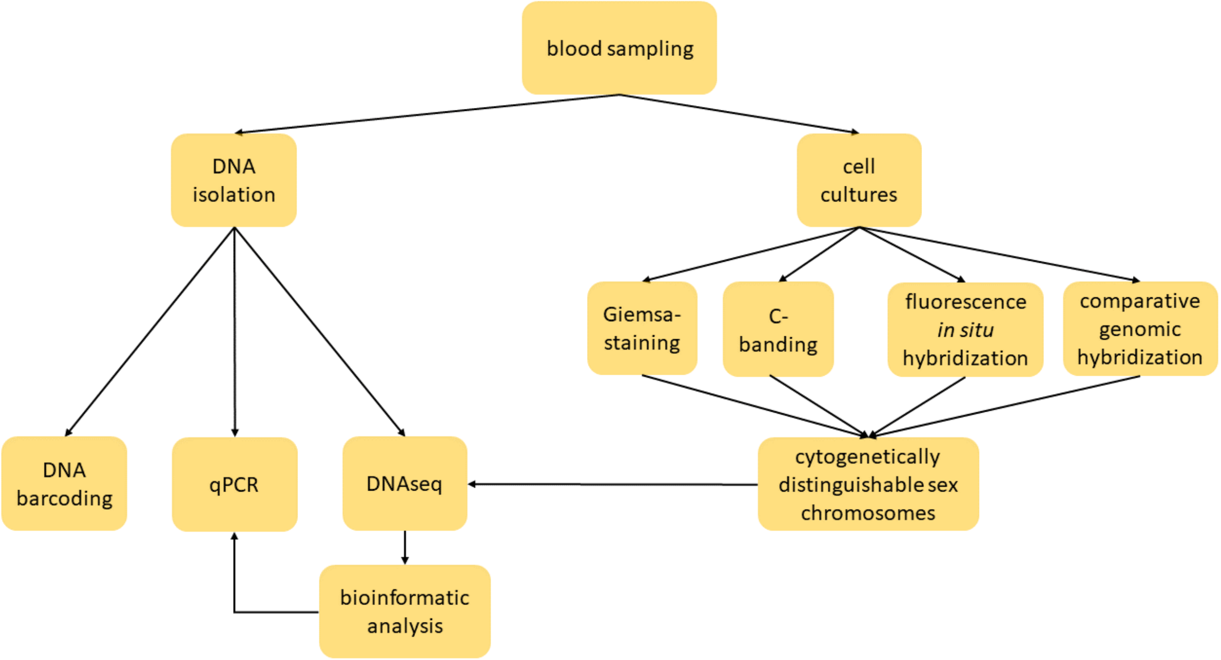


Figure 2: Depiction of the workflow used in this thesis.

Cells from whole blood were cultivated to acquire chromosome suspensions. These suspensions, dropped on microscope slides, are the basis of all cytogenetic methods, applied in this thesis. Giemsa-stained chromosome spreads were used to construct karyograms. This conventional cytogenetic method is used for karyogram preparation, in order to identify heteromorphic sex chromosomes by shape and size differences between X and Y or Z and W.

Differentiation and degeneration of sex chromosomes are often accompanied by accumulation of heterochromatin. The distribution of heterochromatin can be detected by C-banding and gives estimation on the presence of sex chromosomes. Other markers, which are often found accumulated on sex chromosomes, are the rDNA loci and telomeric sequences. Fluorescence *in situ* hybridization (FISH) is used to identify sex chromosomes which carry accumulations of these markers. Theoretically the best method in molecular cytogenetics to identify sex chromosomes is comparative genomic hybridization (CGH). Male and female DNA are differently labelled using a nick translation kit. Using the labelled DNA as probes in a similar treatment as in FISH allows detection of genomic regions differing between a male and a female in metaphase spreads, which might represent sex chromosomes (W or Y). In species, where sex chromosomes are confirmed by cytogenetics, an additional method, the genome coverage analysis, can be used. Under the assumption that X- (or Z-) specific genes have only half the copy numbers in the heterogametic sex, these differences should be proportional to the differences in coverage of the reads in DNA sequencing in Illumina platforms. Additionally, these genes, as long as they are single-copy, are hemizygous in the heterogametic sex and should therefore be free of single nucleotide polymorphisms (SNPs). Subsequently, quantitative polymerase chain reaction (qPCR) was used to test the homology of sex chromosomes across major lineages of scincoidean lizards.

Conclusions

Cytogenetically distinguishable sex chromosomes are rare among scincoidean lizards. The ZZ/ZW sex chromosomes of *Tracheloptychus petersi*, which are the first reported for any gerrhosaurid lizard, were identified by accumulations of rDNA loci, which are present on a pair of autosomes and on the Z chromosome. Other techniques failed to show sex-specific differences in these homomorphic sex chromosomes. In *Zonosaurus madagascariensis*, a closely related lizard, only CGH was able to identify their ZZ/ZW sex chromosomes. A comparative cytogenetic study on several scincoidean lizards found sex-specific differences only in *Tiliqua scincoides*, where accumulations of rDNA loci are found only on the X chromosome. These results suggest that scincoidean sex chromosomes are poorly differentiated. Genomic coverage analysis in *Scincus scincus*, which has cytogenetically distinguishable XX/XY sex chromosomes, again based on the sex-specific polymorphism in rDNA loci, identified a small X-specific region. These XX/XY sex chromosomes are homologous in all 13 studied skink species which represent most of the phylogenetic spectrum of the currently known 1700 species (around 15% of reptile species diversity). These poorly differentiated XX/XY sex chromosomes have been evolutionary stable for at least 85 million years, approaching the age of highly differentiated ZZ/ZW sex chromosomes of birds and advanced snakes.

Further research is needed to understand the evolution of sex chromosomes in scincoidean lizards. In Cordylidae, sex chromosomes have not been identified so far. Most studies, which applied cytogenetic methods to cordylid lizards, used exclusively conventional methods. Molecular cytogenetics might uncover otherwise hidden sex-specific differences. In the families Gerrhosauridae and Xantusiidae, sex chromosomes have been identified in some species. Genome coverage analysis to identify Z-linked genes and subsequent testing for homology in these families would give an estimation of the size of the Z-specific region, the level of differentiation in gene content, the age of these sex chromosomes and the putative homology between gerrhosaurid and xantusiid ZZ/ZW. Although homology of XX/XY sex chromosomes has been proven across a wide phylogenetic spectrum of skinks, some important taxa are still missing. Priority should be given to members of the small subfamily Acontinae, which is sister to other skink subfamilies, species where putative temperature-dependent sex determination was reported, and *Scincella melanosticta*, which is supposed to have ZZ/ZW sex chromosomes.

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