UNIVERZITA KARLOVA

PŘÍRODOVĚDECKÁ FAKULTA

Studijní program: Ekologie

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Rozplétání složité evoluční historie pohlavních chromozomů hadů

Disentangling the complex evolutionary history of sex chromosomes in snakes

Disertační práce

Vedoucí práce: Michail Rovatsos, PhD

Praha, 2025

ACKNOWLEDGMENTS

Scientific work is usually the result of a joint effort among many colleagues and collaborators, and this thesis is no exception. It would not have been possible without the unwavering support of my supervisor, Michail Rovatsos, who provided me not only with his knowledge, experience, ideas, and funding but also with his friendship, for which I am deeply grateful.

I would also like to thank Bára Augstenová, who has been my partner in crime since the beginning of my studies and taught me most of the laboratory procedures.

The next person to thank is my previous supervisor, Lukáš Kratochvíl, who first introduced me to the topic of sex chromosome evolution and has always been willing to discuss fascinating reptile or evolutionary facts, sharing his passion.

The lab would not function the same without the contributions of Jana Thomayerová, who kindly prepares all the necessary materials, even when I forget to ask in time.

I would like to thank Honza Červenka for his help with animal handling and care.

For the sample donations, I would like to thank Daniel Frynta, Panagiotis Kornilios, Jan Hříbal, and the great people at Prague Zoo, Plzeň Zoo, and Jihlava Zoo.

Over the years, many amazing scientists have passed through our lab. Among them, special thanks go to Sofi, Lollo, Alex, Greg, Ele, and Nuria, as well as to those still around: Alona, Maruška, Dima, Lukáš, Bára, Tomáš, and Roel. I thank Pavel, Nataša, Míša, and Eleanna for sharing the ups and downs of a PhD student's life with me. I am also grateful for the moral support provided by Richard.

Finally, many thanks go to my family, especially my amazing wife Eliška, for her tremendous support, love, and continuing interest in my work.

This thesis was partially funded by the Charles University Grant Agency (GAUK 358522).

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 the publications" section of the dissertation. All literature sources I used in the writing of this thesis have been properly cited.

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Prague, January 2025 / Praha, leden 2025

Tomáš Pšenička

STATEMENTS OF CONTRIBUTION / PROHLÁŠENÍ O PŘÍSPĚVKU

I declare that my research effort for this doctoral thesis was conducted through international collaboration with scientists from several countries. My personal contribution to the experimental design, data collection, analyses and preparation of manuscripts for each chapter is explained in detail in the section “Outline of the publications”.

Prohlašuji, že mé výzkumné úsilí pro tuto disertační práci probíhalo v rámci mezinárodní spolupráce s vědci z několika zemí. Můj osobní podíl na návrhu experimentů, sběru dat, analýzách a přípravě rukopisů jednotlivých kapitol je podrobně vysvětlen níže v části „Přehled publikací“.

Prague, January 2025 / Praha, leden 2025

Tomáš Pšenička

As supervisor of the PhD thesis and to the best of my knowledge, I confirm the contribution of Mgr. Tomáš Pšenička to the chapters of this thesis is accurately explained in the section “Outline of the publications”.

Jako vedoucí disertační práce a podle svého nejlepšího vědomí potvrzuji, že přínos Mgr. Tomáše Pšeničky ke kapitolám této práce je přesně vysvětlen v části „Přehled publikací“.

Prague, January 2025 / Praha, leden 2025

Michail Rovatsos

TABLE OF CONTENTS

ABSTRAKT

Pohlavní chromozomy mohou být dynamickou, rychle se vyvíjející částí genomu, která podléhá jiným evolučním tlakům než autozomy. V rámci obrovské diverzity eukaryot se vyvinuly mnohokrát nezávisle a představují tak případ ohromující konvergence na genomové úrovni. Jedna z nejvlivnějších teorií o evoluci pohlavních chromozomů byla inspirována pohlavními chromozomy hadů, dobře známé a druhově bohaté skupiny šupinatých plazů. Díky cytogenetickým studiím provedeným před několika desetiletími bylo všeobecně přijímáno, že všichni hadi sdílejí homologní a evolučně stabilní pohlavní chromozomy ZZ/ZW, které jsou diferencované v druhově bohaté skupině Caenophidia a jen málo diferencované v ostatních liniích, kde nebyly zjištěny. Nedávné studie však prokázaly, že evoluce pohlavních chromozomů u hadů je komplikovanější, a to díky objevu dvou nehomologních systémů pohlavních chromozomů XX/XY u hadů mimo skupinu Caenophidia. Tato práce je zaměřena na odhalení systémů pohlavních chromozomů u hadích linií, u nichž přímé důkazy o nich doposud chyběly. Kromě toho si klade za cíl prozkoumat obsah satelitní DNA v genomech příslušníků čeledi Erycidae. Kombinace cytogenetických, molekulárních a bioinformatických metod byla použita k odhalení i málo diferencovaných pohlavních chromozomů a k určení motivů satelitní DNA a její topologie. U čeledí Erycidae, Sanziniidae, Tropidophiidae a Typhlopidae bylo objeveno několik dosud neznámých systémů pohlavních chromozomů a obsah genů pohlavních chromozomů byl použit k testování homologie pohlavních chromozomů mezi nestudovanými druhy hadů, což výrazně rozšířilo dosavadní znalosti. Tyto výsledky představují další důkaz, že evoluce pohlavních chromozomů u hadů je mnohem variabilnější, než se dříve předpokládalo, což značně kontrastuje s původním názorem. Navíc jsme prokázali množství zajímavých rysů evoluce pohlavních chromozomů u hadů, která musela zahrnovat například obměny pohlavních chromozomů, změny v heterogamii, nezávislou koopci stejných genomových oblastí pro pohlavní chromozomy nebo nerovnoměrnou míru diferenciace pohlavních chromozomů mezi jednotlivými liniemi. To vše dělá z hadů vynikající modelovou skupinu pro budoucí studie.

ABSTRACT

Sex chromosomes can be a dynamic, fast-evolving part of the genome and one that follows slightly different evolutionary pathways than the autosomes. They have evolved independently many times in the great diversity of eukaryotes, representing a case of incredible convergence on the genomic level. One of the most influential theories about sex chromosome evolution was inspired by sex chromosomes of snakes, a well-recognized and species-rich group of squamate reptiles. Due to the cytogenetic studies conducted several decades ago, it was widely accepted that all snakes share homologous and evolutionary stable ZZ/ZW sex chromosomes that are differentiated in the diverse group of caenophidian snakes and only poorly differentiated in the other lineages, where they were not detected. Recent advances have, however, proven the sex chromosome evolution in snakes more complex with the discovery of two non-homologous XX/XY sex chromosome systems in non-caenophidian snakes. This thesis is focused on uncovering sex chromosome systems in snake lineages where direct evidence for them was missing. In addition, it aimed to explore the satellite DNA content in the genomes of members of the family Erycidae. A combination of cytogenetic, molecular and bioinformatic methods was used to detect even poorly differentiated sex chromosomes and to determine satellite DNA motifs and their topology. Several previously unknown sex chromosome systems were discovered in the families Erycidae, Sanziniidae, Tropidophiidae and Typhlopidae and their sex chromosome gene content was used to test homology of sex chromosomes among unstudied snake species, greatly increasing the current knowledge. These results provide further evidence that sex chromosome evolution in snakes is much more variable than was previously thought, greatly contrasting the original view. Additionally, we demonstrated a plethora of interesting features of the sex chromosome evolution in snakes, which must have involved for example sex chromosome turnovers, changes in heterogamety, independent co-option of the same genomic regions for sex chromosomes or unequal rates of sex chromosome differentiation between lineages. All of these make snakes an excellent model group for future studies.

INTRODUCTION

From sex to sexes and sex determination

Sexual reproduction is one of the most fundamental traits of eukaryotic organisms. It is believed to be already present in their last common ancestor at least 1.6 - 1.8 billion years ago, partially because the molecular machinery of meiosis is largely homologous among all eukaryotic lineages (Betts *et al.* 2018; Hofstatter and Lahr 2019). As the complexity of some eukaryotes increased over time and they became multicellular (Valentine 1978; Knoll 2011; Niklas and Newman 2013), their cells differentiated, divided labor and specialized to better perform their designated functions (Bonner 1998; Kaiser 2001). For gametes, usually haploid cells mediating sexual reproduction by their fusion, the two most important tasks are to ensure fertilization and subsequently the survival of the developing zygote. This eventually led in some lineages to the rise of two distinct gamete types, each optimized to one of these tasks and dimorphic in size, a state called anisogamy. Larger gametes (ova) carry more nutrients and energy reserves, ensuring higher chances of embryonic survival, but their number is limited by the high production costs to the individual, which would lower the overall chances of fertilization. Combating this disadvantage of large gametes, small gametes (sperm), inexpensive to the individual, are produced in large quantities, some of them further becoming motile, increasing the chances of fertilization but not contributing resources to the embryo (Parker *et al.* 1972). This arrangement must be highly advantageous as anisogamy is believed to have evolved independently several times in eukaryotes (reviewed in Lehtonen and Parker 2014). Almost inevitably, the two sexes, male and female, arise as a direct consequence of anisogamy (Lehtonen *et al.* 2016), further enabling improvement in evolutionary roles dictated by the gametes. This specialization peaks in gonochorism or sequential hermaphroditism, sexual systems, where at a given time, an individual is of only one sex. While sequential hermaphrodites are usually born as one sex and can change sex during their postnatal life, such change is typically not possible in gonochoristic species, where once the sex is determined, it tends to remain stable for the rest of the life (Warner 1975; Gamble and Zarkower 2012).

With the emergence of gonochorism, the need to determine which sex an individual should assume emerges as well. Here is where we will abandon the immense variability of Eukaryota and constrict ourselves to a relatively small subgroup of gonochoristic lobe-finned fishes, that also evolved several extraembryonic membranes to protect their embryos while they are developing on dry land, amniotes at first, and ultimately, snakes.

Sex determination in amniotes

Sex determination is a mechanism for deciding which sex is adopted by the developing embryo (Gamble and Zarkower 2012). It acts on the bipotential gonad that later differentiates either to the testis or the ovary (e.g. Morrish and Sinclair 2002; MacLaughlin and Donahoe 2004). There are two different types of sex determination in amniote vertebrates (Bachtrog *et al.* 2014). One of them is environmental sex determination (ESD), where the intensity of an external environmental factor provides the initial signal to the cells of the bipotential gonad to trigger the developmental pathway towards the formation of testis or ovary, which will subsequently determine the sex of the individual (Charnov and Bull 1977). Temperature is the most prominent environmental factor with an undisputed effect on sex determination in amniotes (Bull 1980; Korpelainen 1990), although more factors, such as humidity, are hypothesized (Lolavar and Wyneken 2020).

Temperature-dependent sex determination

Temperature-dependent sex determination (TSD) was once thought to be quite a common mechanism, at least in reptiles. Based on word of mouth from animal breeders and small sample sizes, it was believed to be present in most of the major reptilian lineages (reviewed in Valenzuela and Lance 2004). Recent advances have however disproven many of these reports leaving us with verified TSD in all crocodilians, most turtles, the tuatara (*Sphenodon punctatus*) and, in squamates, only in several species of geckos and dragon lizards (Lang and Andrews 1994; Valenzuela and Lance 2004; Iannucci *et al.* 2019; Rovatsos *et al.* 2019a; Sidhom *et al.* 2020; Straková *et al.* 2024; Peš *et al.* 2024).

The best-studied TSD system to date is the one of the Red-eared slider (*Trachemys scripta*). Briefly, it works on the principle of concentration of calcium cations, increasing with rising temperature, in the cells of the bipotential gonad. This triggers methylation of the *Kdm6b* gene, an epigenetic regulator important for the testis development gene cascade, resulting in female phenotype (Weber *et al.* 2020). Moreover, it was shown that sex determination in this species can be influenced also by the number of primordial germ cells present in the bipotential gonad (Tezak *et al.* 2023). Interestingly, the differentiated splicing patterns of the genes *Kdm6b* and *Jarid2* were shown to have an important role also in the regulation of TSD in the Bearded dragon (*Pogona vitticeps*) and the American alligator (*Alligator mississippiensis*) (Yatsu *et al.* 2016; Whiteley *et al.* 2022). The presence of seemingly homologous molecular mechanisms of TSD in distantly related species might indicate its shared ancestry, which is consistent with the hypothesis about TSD as the ancestral sex determination system of amniotes (Pokorná and Kratochvíl 2016).

In general, TSD is, by definition, somewhat prone to biased primary sex ratios. This might pose a serious threat to some species, especially in the face of ongoing global warming (Jensen *et al.* 2018). Luckily, most amniote species do not depend on environmental factors to determine their sex, they leave it to their genes.

Genotypic sex determination

As the name suggests, genotypic sex determination (GSD) is based on sex-specific genomes, represented by existing genetic differences between males and females, located on sex chromosomes (Bull 1980; Gamble and Zarkower 2012). Here, it is the expression of a gene, or in some cases even several genes (Kocher *et al.* 2024), that gives the initial signal to the cells of the bipotential gonad to trigger further development as one sex and/or suppress the other. Although the determination itself happens at this relatively advanced stage of embryonic development, the sex of an individual is technically pre-destined already at the moment of zygote formation by the combination of its sex chromosomes - usually XX for female and XY for male in male heterogamety systems and ZZ for male and ZW for female in female heterogamety systems (Bachtrog *et al.* 2014).

For a long time, there were only two known primary sex-determining genes in amniotes, each determining sex by a different mechanism. *Sry*, the sex determiner of eutherian mammals, is located on the Y chromosome and acts as a dominant locus, i.e. its presence in the genome triggers the male developmental pathway (Koopman *et al.* 1990; Sinclair *et al.* 1990). In contrast, *Dmrt1* is located on the Z chromosome of birds. Since both sexes carry Z chromosomes, it is believed that the presence/absence of this gene does not offer sufficient explanation for sex determination. Instead, *Dmrt1* works on the principle of gene dose, when only two copies per cell (on two Z chromosomes) are capable of triggering the male phenotype (Smith *et al.* 2009). Additionally, recent studies have shown the anti-Müllerian hormone gene (*Amh*) as a promising candidate for primary sex-determining gene in monotreme mammals (Deakin 2017). It has two alleles in the genome, X- and Y-specific, both expressed in the bipotential gonad during the embryonic gonadal differentiation. The authors speculate that co-expression of both alleles is required to trigger the testis development, while the ovary will only develop in the absence of the Y-specific allele (Shearwin-Whyatt *et al.* 2024). The true mechanism, however, is yet to be verified.

Sex chromosomes evolved likely independently more than 40 times in non-avian reptiles, greatly contrasting the conserved systems of mammals and birds (Pokorná and Kratochvíl 2009, 2016; Gamble *et al.* 2015; Thépot 2021). No primary sex-determining genes have been truly verified in this group, although some candidates have been proposed. Drawing inspiration from other vertebrate taxa, the focus in a search for primary sex-determining genes is often directed towards genes generally involved in the sex determination pathway. These so-called "usual suspects" (including their paralogs) were shown to be the primary sex-determining genes in independently evolved systems (Herpin and Schartl 2015). Among them is for example the already mentioned *Dmrt1*, which determines the sex in birds, the African clawed frog (*Xenopus laevis*), some Medaka species (*Oryzias latipes, O. curvinotus*) or the Chinese tongue sole (*Cynoglossus semilaevis*). Similar can be said for example about *Sox3*, the ancestor of mammalian *Sry*, *Amh* or its type II receptor (*Amhr2*) (e.g. Nanda *et al.* 2002; Matsuda *et al.* 2003; Yoshimoto *et al.* 2008; Takehana *et al.* 2014; Cui *et al.* 2017; Song *et al.* 2021; Hattori *et al.* 2022).

Among the proposed primary sex-determining genes in reptiles is for example *Dmrt1* as sex determiner of the turtles of the genus *Staurotypus* or the gecko *Gekko hokouensis*. *Sox3*

could be the sex determiner of the gecko *Christinus marmoratus,* while *Amh* is the candidate for anguimorphan reptiles – Varanids and Beaded lizards and *Foxl2* has been recently proposed as a candidate for sex determination in the Chinese crocodile lizard *Shinisaurus crocodilurus* (Kawai *et al.* 2009; Kawagoshi *et al.* 2014; Deakin 2016; Rovatsos *et al.* 2019c; Pinto *et al.* 2024).

Sex chromosomes and their evolution

The history of sex chromosome research dates all the way back to 1891, when Hermann Henking noticed a mysterious non-pairing chromosome during his studies of spermatocyte divisions of *Pyrrhocoris* firebugs (X0 sex determination system). He named this chromosome "Element X", not yet knowing its true purpose or why it behaves differently from the rest of the chromosomes (Henking 1891). Other researchers took interest in his work and started uncovering similar, now called accessory, chromosomes in additional taxa. It was Clarence McClung in 1902, who first proposed the idea of the accessory chromosomes having the sex-determining function (McClung 1902). Following his research, while discovering the first Y chromosome in *Tenebrio* mealworms, Nettie Stevens further developed and popularized this view (Stevens 1905). The idea of sex chromosomes evolving from autosomes was published over a decade later by Hermann Muller (Muller 1918). Fast forward to 1967, Susumu Ohno formulated the theory of gradual sex chromosome evolution and laid a foundation for many future studies (Ohno 1967), including this thesis.

According to this now classical paradigm, sex chromosomes evolve when a pair of autosomes acquires the sex-determining locus. Subsequent accumulation of sexually antagonistic alleles near the sex-determining locus promotes recombination suppression. In the absence of recombination between the whole chromosomes or their parts, the non-recombining chromosome (or its part) degenerates, i.e. often loses functional genes and accumulates repetitive elements which ultimately leads to heterochromatinization. In contrast, its counterpart, either X or Z chromosome, depending on the system, stays more conserved as it can still recombine in the homogametic sex (XX females or ZZ males). This further promotes sex chromosomes to differentiate from one another not only sequentially, but also morphologically, leading to heteromorphic sex chromosomes (Fisher 1931; Ohno 1967; Rice

1987; Charlesworth 1991; 2005), a state so familiar from many species and one that originally caught the attention of Stevens over a century ago. And even though it is today known, that this model is greatly oversimplified, the role of sexually antagonistic alleles as drivers of recombination suppression is questionable (Ironside 2010; Perrin 2021), and the fact that sex chromosomes do not need to differentiate even over large evolutionary timescales (Kostmann *et al.* 2021a; Kuhl *et al.* 2021), and more (reviewed in Kratochvíl *et al.* 2021a), the original premise remains relevant and continues to be at the base of most of the recent, more complex, models (Abbott *et al.* 2017; Vicoso *et al.* 2019; Furman *et al.* 2020; Kratochvíl *et al.* 2021a). However, these models agree that sex chromosome evolution does not need to follow the originally proposed one-way trajectory. In fact, it has been demonstrated many times that the sex chromosome pair can be replaced by a different one, in a process called sex chromosome turnover, especially if the original pair was only poorly differentiated (Abbott *et al.* 2017; Vicoso *et al.* 2019). This can be achieved by the emergence of a new, more dominant, primary sex-determining gene on a different chromosome pair or by translocation of the already existing sex-determining gene to a different chromosome pair. In contrast, well-differentiated sex chromosomes were shown to be quite stable in many lineages and were even hypothesized to act as evolutionary traps (Pokorná and Kratochvíl 2009; but see Nielsen *et al.* 2019a; Rovatsos *et al.* 2019b; Pinto *et al.* 2024). Turnover of such differentiated sex chromosomes would be likely connected with the eventual complete loss of one of the original sex chromosomes with its beneficial genes and alleles, which would lower fitness. It might be also the reason why we observe many more transitions from TSD to GSD than in the opposite direction. On the other hand, the non-recombining parts of Y or W sex chromosomes were shown to accumulate not only repetitive elements but also deleterious mutations under the effect of Muller's ratchet (Muller *et al.* 1964). It is hard to imagine that losing highly degenerated sex chromosomes would have a negative impact on fitness, at least in some cases (Blaser *et al.* 2013). It was hypothesized that the accumulation of repetitive elements and deleterious mutations is the actual driver of the gradual loss of recombination between sex chromosomes, shielding the X or Z chromosomes from the negative effects (Jay *et al.* 2022).

Although recombination suppression is, at various degrees, almost ubiquitous, even among independently evolved sex chromosome systems based on different genomic regions (but see Kamiya *et al.* 2012), non-adaptive explanations for it have been proposed as well

(Jeffries *et al.* 2021). Some of them rely on the pre-existing unequal distribution of recombination throughout different parts of the genome or heterochiasmy (even achiasmy), a state where sexes differ in their recombination rates (e.g. Satomura *et al.* 2019).

Recombination suppression is often connected to chromosomal rearrangements such as inversions, deletions, translocations or fusions, which can distort local sequential homology needed for successful crossover (Charlesworth *et al.* 2005; Kirkpatrick 2010). These rearrangements can be then either fixed by genetic drift, if neutral, or selected for, if advantageous. In some cases, we can observe differently differentiated areas on the Y and W chromosomes. These evolutionary strata correspond to separate events of recombination suppression between sex chromosomes with the sex-determining gene located in the oldest one (Lahn and Page 1999; Handley *et al.* 2004).

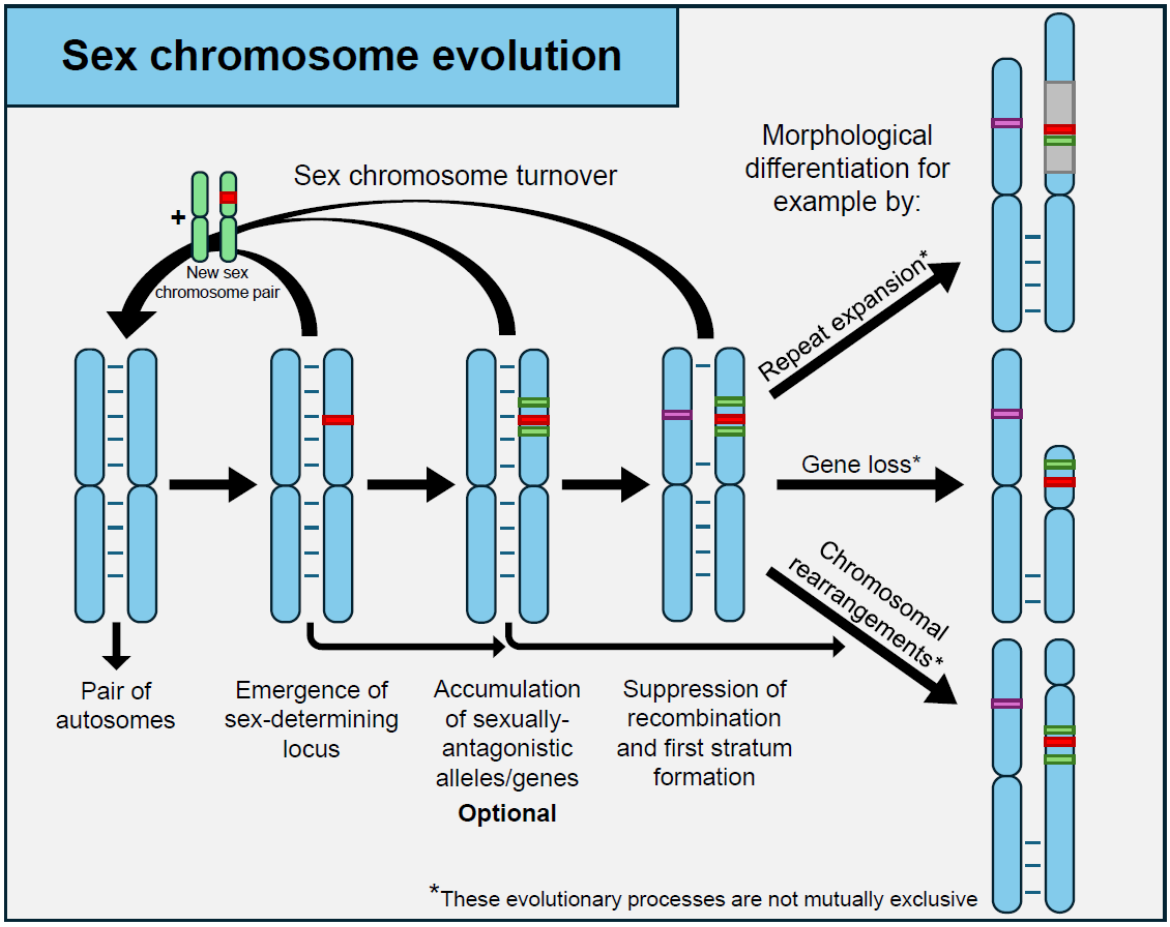


Figure 1: Sex chromosome evolution. Simplified from Kratochvíl *et al.* (2021a).

Despite all these circumstances, even the usually non-recombining sex chromosomes may obtain more genetic material, either by duplication of already existing genes, translocation or by occasional recombination in sex-reverted individuals (Ross *et al.* 2005; Perrin 2009; Soh *et al.* 2014). Furthermore, sex chromosomes may fuse with autosomes, creating so-called neo-sex chromosomes (Steinemann and Steinemann 1998). If the autosome pair fuses with both sex chromosomes, the newly added region can act as a new pseudoautosomal region, a region on sex chromosomes that retains recombination, or can rapidly degenerate (Sigeman *et al.* 2024). If only one autosome fuses with one sex chromosome, while the other autosome from the same pair does not, multiple neo-sex chromosomes might arise (Pokorná *et al.* 2014). All these systems are a popular research subject as they provide a unique opportunity to study the dynamics of recombination suppression, strata formation and degeneration (Sigeman *et al.* 2021, 2024; Jayaprasad *et al.* 2024).

So far, most of the attention has focused on the non-pairing sex chromosomes, Y and W. However, also X and Z chromosomes exhibit interesting evolutionary dynamics, different from both autosomes and also the Y or W chromosomes. For example, it was shown that X and Z chromosomes evolve faster than autosomes if their counterparts, Y and W, are differentiated and degenerated enough (Charlesworth *et al.* 1987). This can be attributed to both genetic drift and selection. The effective population size for X or Z chromosomes is that of 3:4 of autosomes and as such, more prone to the effects of genetic drift. At the same time, genes and alleles on X and Z sex chromosomes are hemizygous in the heterogametic sex, and thus directly exposed to selection (Vicoso and Charlesworth 2006, 2009). Furthermore, this unequal copy number of X-/Z-specific genes between sexes may cause unbalanced expression, which leads in several systems to the evolution of gene dose regulatory mechanisms. Gene dosage compensation is a mechanism by which the expression of hemizygous X-/Z-specific genes is elevated to the ancestral expression level, to autosomes, and as such equal between sexes (Charlesworth 1996; Mank 2009). On the other hand, dosage balance is the mechanism by which the expression of hemizygous X-/Z-specific genes is equalized between sexes but not matched to the ancestral expression level. Gene dose regulatory mechanisms can act on whole chromosomes or just on specific dosage-sensitive genes or can be absent altogether in some systems (Gu and Walters 2017).

As mentioned, sex chromosomes evolved many times. Furthermore, it has been shown that certain genomic regions often emerge as sex chromosomes in unrelated lineages of amniotes (Kratochvíl *et al.* 2021b). For example, the genomic region homologous to the sex chromosomes of therian mammals plays the role of sex chromosomes also in the chameleons of the genus *Furcifer*, some geckos of the genus *Paroedura* and lacertid lizards (Rovatsos *et al.* 2016, 2019b, 2024). Similarly, the genomic region homologous to the sex chromosomes of birds is also known to have emerged as sex chromosomes in three distinct lineages of reptiles (Kawai *et al.* 2008; Kawagoshi *et al.* 2014; Nielsen *et al.* 2019b). Although similar homologies were sometimes hypothesized to be the result of fissions of the amniote ancestral "proto-sex chromosome", a more plausible explanation involves independent co-option, either due to limited options or favourable pre-existing predispositions of the autosomal pair (Marshall Graves and Peichel 2010; Ezaz *et al.* 2017; Singchat *et al.* 2018, 2020; Kratochvíl *et al.* 2021b).

In summary, sex chromosomes represent a dynamic and fast-evolving part of the genome that greatly impacts their bearers. There is an immense variability between systems - in their genetic background, how or why they suppress recombination, differentiate or how they cope with expression imbalance. However, sex chromosome evolution tends to follow similar pathways even in these variable and independently evolved systems, resulting in the most remarkable case of genomic convergence in eukaryotes.

Snakes - phylogenetic overview

Snakes (Serpentes) are an iconic group of squamate reptiles (Squamata), notable to many. They were praised, feared and hated characters throughout the cultural history of our species. Today, snakes are desired research subjects due to their unique morphology, physiology and diverse ecology (Brischoux and Shine 2011; Simoes *et al.* 2015; Leal and Cohn 2018; Silva *et al.* 2018). Especially prominent is the research regarding the evolution of their venom (Koh et. al 2006; Tasoulis and Isbister 2017; Casewell *et al.* 2020).

Snakes are an extremely successful vertebrate group that with more than 4000 species comprise roughly one-third of the total diversity of non-avian reptiles (Uetz *et al.* 2024). Their origin dates to the Jurassic period and they since then colonized all the continents, except Antarctica (Caldwell *et al.* 2015; Uetz *et al.* 2024). Although major phylogenetic relationships

are mostly solved within snakes, a big debate continues about the family/subfamily status of some clades. Despite this fact, snakes can be divided into approximately 30 families (Pyron *et al.* 2013, 2014; Zheng and Wiens 2016; Das *et al.* 2023). More than 80% of all snake species belong to the group Caenophidia. Some of the most recognizable snake families belong to this group, among others for example Viperidae, a family including vipers and rattlesnakes, Elapidae, including cobras and sea snakes, or Colubridae, a species-rich family including rat snakes. The remaining 20% of snake species were historically divided into two other groups, Henophidia and Scolecophidia. Since both of them were proven paraphyletic and new terminology is currently missing, we will refer to them as non-caenophidian snakes. These include, for example, blind snakes, small, mostly fossorial species specializing to eating insects, predominantly termites and ants, or in stark contrast, boas and pythons, currently the biggest and heaviest squamates. And even though non-caenophidian snakes represent only one-fifth of the snake species diversity, more than half of all snake families belong to this group (Pyron *et al.* 2013; 2014; Zheng and Wiens 2016; Das *et al.* 2023; Uetz *et al.* 2024).

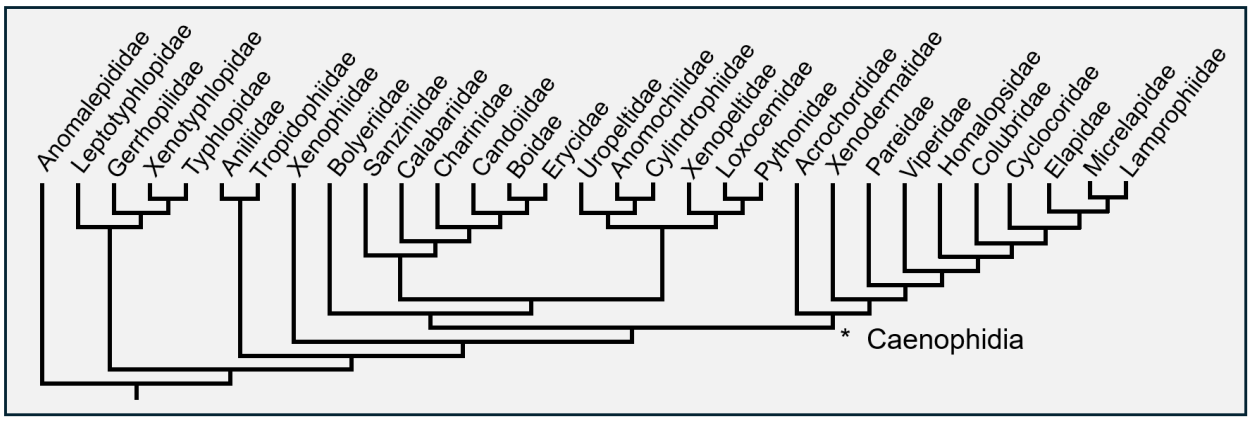


Figure 2: Phylogenetic relationships among snake families according to Pyron *et al.* (2013) and Das *et al.* (2023).

Sex chromosome evolution in snakes

In contrast to other reptilian groups, the effect of temperature on sex determination was never observed in any snake species, although temperature-dependent sex-specific mortality of developing embryos was (Burger and Zappalorti 1988; Valenzuela and Lance 2004). It is

therefore believed that all snakes rely on GSD and have sex chromosomes. The ZZ/ZW sex chromosomes of caenophidian snakes have been studied for more than 60 years. They consist of a large pair of macrochromosomes, they are well differentiated in sequence level and often heteromorphic. This made them easily recognizable using conventional and later also molecular cytogenetic methods, as the W chromosome was shown to be heterochromatic and tends to accumulate many kinds of repetitive elements (Beçak *et al.* 1962, 1964; Singh *et al.* 1968, 1972a, 1980; Beçak and Beçak 1969; Mengden and Stock 1980; Augstenová *et al.* 2018b). For example, the well-known and among other vertebrates widespread Bkm repeats, consisting of GATA and GACA motifs, were first identified on the W chromosome of Banded krait (*Bungarus fasciatus*), from where they got their name (Singh *et al.* 1976, 1980). The association of Bkm repeats with sex chromosomes of among vertebrates led to speculations of its role in sex chromosome differentiation or even sex determination itself, which was later dismissed (Jones and Singh 1981; Arnemann *et al.* 1986; Matsukuma *et al.* 1987; Subramanian *et al.* 2003).

As differentiated sex chromosomes in many other systems, caenophidian sex chromosomes were shown to be evolutionary stable, at least 69 MY old and shared among all studied members (Rovatsos *et al.* 2015; Kumar *et al.* 2017). No sex chromosome turnovers were detected among caenophidian snakes, although multiple sex chromosomes are known in several species of the family Elapidae, emerging probably as a result of sex chromosome-autosome fusions or sex chromosome fissions (Singh *et al.* 1970, 1972b).

Until recently, not much was known about the sex chromosomes of non-caenophidian snakes. Heteromorphic ZZ/ZW sex chromosomes were found only in *Acrantophis* sp. cf. *dumerili* from the family Sanziniidae but not in any other studied species (Beçak *et al.* 1962, 1964; Mengden and Stock 1980; Augstenová *et al.* 2018a, 2019). This led to the assumption that all snakes have homologous ZZ/ZW sex chromosomes, only in non-caenophidian snakes they are poorly differentiated and as such, not detectable by cytogenetic methods. This view became widely accepted by the scientific community even despite the absence of direct evidence (e.g. Ray-Chaudhuri *et al.* 1971; Bull 1980; Jones and Singh 1985; Marshall Graves and Shetty 2001; Ezaz at al. 2006; Matsubara *et al.* 2006, 2016b; Pokorná and Kratochvíl 2009; Vicoso *et al.* 2013). Snakes have become a model group in shaping the understanding of sex chromosomes and their evolution. It was the notable differences between the sex chromosomes

of caenophidian and non-caenophidian snakes and variable sex chromosome morphology in Caenophidia that inspired Susumu Ohno to formulate his influential theory about gradual, step-by-step differentiation of sex chromosomes (Ohno 1967).

Years later, the reported results of facultative parthenogenesis in several non-caenophidian species seemed inconsistent with the presence of ZZ/ZW sex chromosomes as exclusively female, mostly homozygous, offspring were produced (Booth *et al.* 2011a,b, 2014; Kinney *et al.* 2013; Shibata *et al.* 2017; Seixas et. al. 2020, Bailey *et al.* 2024). Together with the observed pattern of inheritance of a color mutation in the Ball python (*Python regius*), these results strongly indicated the presence of XX/XY sex chromosomes (Mallery Jr. and Carrillo 2014). This was later verified by a study of Gamble *et al.* (2017) which uncovered, using RAD-seq-based methodology, two non-homologous XX/XY sex chromosome systems in *Boa constrictor* and *B. imperator* (Boidae) and in *Python bivittatus* (Pythonidae). Interestingly, the XX/XY sex chromosomes of *P. bivittatus* were demonstrated to share partial homology of genes with the ZZ/ZW sex chromosomes of caenophidian snakes. Sex chromosome evolution in snakes thus proved much more complex than was previously believed, with a series of presumed sex chromosome turnovers. The knowledge about sex determination systems in non-caenophidian snakes was however still constricted to only four species from three families. This thesis expanded it to 17 species and seven families.

Satellite DNA in snakes

Satellite DNA (satDNA) is formed by tandem repetitions of the same DNA motif and was historically famously labeled “junk DNA” as it typically does not code for proteins (Ohno 1972; Charlesworth *et al.* 1994). The increasing number of studies demonstrating the importance of satDNA in for example chromosome structure regulation or kinetochore formation have, however, proven the previous description wrong (Henikoff *et al.* 2001; Martienssen 2003; Bolzán and Bianchi 2006; Jagannathan *et al.* 2018).

After the discovery of Bkm repeats, the focus of many studies had shifted to other taxa and the satDNA sequences of snakes remained generally poorly studied. Only a handful of publications have enabled us to peek into their evolutionary dynamics. One of the first targets was of course the W chromosome of caenophidian snakes, which harbours a plethora of

mostly, but not only, microsatellite motifs, sometimes with species-specific topology suggesting a dynamic evolution of these sequences (Singh *et al.* 1976; Matsubara *et al.* 2016a; Augstenová *et al.* 2018b; Rovatsos *et al.* 2018; Viana *et al.* 2019). In contrast, some identified satellites, not necessarily constricted only to sex chromosomes, have been shown to be sequentially remarkably stable among distantly related snake lineages (Lisachov *et al.* 2023). An extreme case of recorded satellite conservatism in snakes is the PBI-DdeI satellite, which was originally found to be present in the centromeric region of all chromosomes of *Python bivittatus* (Matsubara *et al.* 2015). A later study of Thongchum *et al.* (2019) verified the presence of this satellite in 15 additional snake species from 10 snake families, both caenophidian and non-caenophidian. Moreover, it demonstrated extensive amplification of this satellite on the W chromosome of *Naja kaouthia* (Elapidae) and suggested it could have played a role in W chromosome differentiation and heterochromatinization.

AIMS OF THE THESIS

This thesis aimed to explore the variability of sex chromosome systems in snakes, especially in non-caenophidian lineages, which were only poorly studied in the past. This aim is highly relevant to our general knowledge about sex chromosome evolution, not only because of the historical importance of snakes in the field but also because the recent advances uncovered unexpected variability in their sex chromosome systems, implicating sex chromosome turnovers, changes in heterogamety and more. I obtained samples from snake species from several phylogenetically informative caenophidian and non-caenophidian families and used them to:

Chapter 1: Cytogenetically uncover putative heteromorphic sex chromosomes, if they are present.

Chapter 2: Explore sex chromosome gene content and homology with emphasis on systems with homomorphic or poorly differentiated sex chromosomes using bioinformatic and molecular approaches.

Chapter 3: Explore the dynamics of satDNA in the genome of the sand boas of the genus *Eryx* by bioinformatic and cytogenetic methods.

MATERIALS AND METHODS

This thesis represents the combination of standard and molecular cytogenetic methods as well as bioinformatic approaches to uncover both differentiated and poorly differentiated sex chromosomes and to analyze satellitome of selected species. All approaches start with obtaining blood samples from phylogenetically informative species, ideally from several individuals of both sexes to overcome individual-specific differences. The blood is taken from the tail vein. The whole procedure is only minimally invasive on the animal, taking less than one minute. Blood samples are then used for cell cultures, which are later harvested to obtain chromosomal material, or for DNA isolation, mostly for sequencing and further bioinformatic analyses. I summarize briefly the methodology of each chapter.

Chapter 1

The chromosomal material was used for cytogenetic analysis. Karyograms were reconstructed for previously unstudied species with the expectation to uncover putative heteromorphic sex chromosomes. To visualize the topology of constitutive heterochromatin, which often accumulates on Y or W sex chromosomes, standard C-banding methodology was used (Sumner 1972). In addition, fluorescence *in situ* hybridization (FISH) was used in order to uncover the distribution of rDNA loci and telomeric repeats. These repetitive sequences were shown to accumulate on sex chromosomes in several reptilian lineages and have helped to uncover homomorphic but differentiated sex chromosomes in the past (Lee *et al.* 2019; Kostmann *et al.* 2021b). In addition, telomeric repeats might help to uncover cryptic chromosomal rearrangements if they are present in the interstitial parts of the chromosomes. Lastly, comparative genomic hybridization between sexes was performed in order to visualize the sex-specific region, if it is differentiated and large enough.

Chapter 2

In this chapter, we aimed to uncover sex chromosomes and their gene content by comparative gene coverage analysis. It is based on the fact that the sexes differ in the gene dose of their X-/Z- specific genes in contrast to their autosomal or pseudoautosomal genes. For example, XX female must have twice as many copies of X-specific genes than a XY male, while they should

not differ in the copy number of their autosomal genes. Since Illumina sequencing is relatively unbiased, it should produce twice as many reads of X-specific genes for females than males. The same logic works for ZZ/ZW systems, only vice versa. After high-coverage sequencing of one male and one female per species, filtering, trimming and mapping those reads to a reference list of exons, we calculated the male-to-female gene coverage ratio for each species, normalized to the average coverage. Once we had a list of candidate X-/Z-specific genes, we designed qPCR primers for their exons. These primers were later used not only to verify the results of the comparative analysis on multiple individuals of the same species, but they also allowed us to test the homology of the newly uncovered sex chromosome system across related species.

Chapter 3

We sequenced the genome of two sand boa species, *Eryx colubrinus* and *E. miliaris* (Erycidae) in Illumina platform, and the high-throughput sequencing data were used for repetitive element analysis by RepeatExplorer. After filtering duplicates, FISH probes for each obtained satDNA motif were constructed and their topology was examined in the previously obtained chromosomal material. In addition, the newly assembled genome of *E. miliaris* allowed us to compare the results of FISH experiments with bioinformatic analysis of topology of the same satellites.

PUBLICATIONS OVERVIEW

Chapter 1:

Charvát T, Augstenová B, Frynta D, Kratochvíl L, Rovatsos M. Cytogenetic analysis of the members of the snake genera *Cylindrophis*, *Eryx*, *Python*, and *Tropidophis*. *Genes* **2022**:13(7):1185. https://doi.org/10.3390/genes13071185

Chapter 2:

Pšenička T, Augstenová B, Frynta D, Kornilios P, Kratochvíl L, Rovatsos M. Sex chromosome turnovers and stability in snakes. *Mol Biol Evol.* **2024**:msae255. https://doi.org/10.1093/molbev/msae255

Chapter 3:

Pšenička T, Rovatsos M. Satellitome analysis of the Old World sand boas - ***manuscript***

OUTLINE OF THE PUBLICATIONS

This thesis consists of three original investigations (two published and one unpublished), each representing a separate chapter.

Chapter 1 is a cytogenetic analysis of seven non-caenophidian snake species from genera *Cylindrophis*, *Eryx*, *Python*, and *Tropidophis*. Although the main goal of this study was to uncover heteromorphic sex chromosomes, it was not fulfilled even though a similar methodology was successfully used in the past for the same objective in other reptilian lineages. Studied species thus must possess not only homomorphic but also poorly differentiated sex chromosomes, which are not detectable by the selected cytogenetic methods. Nonetheless, we assembled karyograms, and explored the topology of constitutive heterochromatin, rDNA loci and telomeric repeats, elements that are commonly cytogenetically studied and important for karyotype evolution reconstructions. For example, despite the conserved chromosome number of 2n=34 in the genus *Eryx*, only the Saharan sand boa (*Eryx muelleri*) possesses interstitial telomeric repeats, suggesting cryptic chromosomal rearrangements. Interestingly, we uncovered heterochromatin heteromorphism, not linked to sex, in two other species of sand boas, *E. colubrinus* and *E. miliaris*. The heterochromatin topology in *E. colubrinus* was generally interesting, as it had big heterochromatin blocks on several chromosomal pairs, and as it was not shared with closely related species, suggesting rapid repetitive DNA expansion even on autosomes, a state unusual in snakes.

After the results of chapter 1 were obtained, it was clear that cytogenetic approaches are not efficient at uncovering sex chromosome systems in non-caenophidian snakes. Hence, in chapter 2, we used the comparative gene coverage analysis in *Acrantophis* sp. cf. *dumerili* (Sanziniidae), *Eryx colubrinus* (Erycidae), *Tropidophis melanurus* (Tropidophiidae) and *Xerotyphlops vermicularis* (Typhlopidae). We verified the old report about ZZ/ZW sex chromosomes in *A.* sp. cf. *dumerili* and uncovered previously unknown XX/XY sex chromosome systems in the remaining three species. The subsequent test of homology proved the poorly differentiated sex chromosomes of *A.* sp. cf. *dumerili* are homologous not only to the sex chromosomes of other members of family Sanziniiidae, but also Calabariidae and caenophidian snakes. Interestingly, the distantly related *X. vermicularis* and *E. colubrinus* share homologous sex chromosomes, although at quite different states of differentiation. We

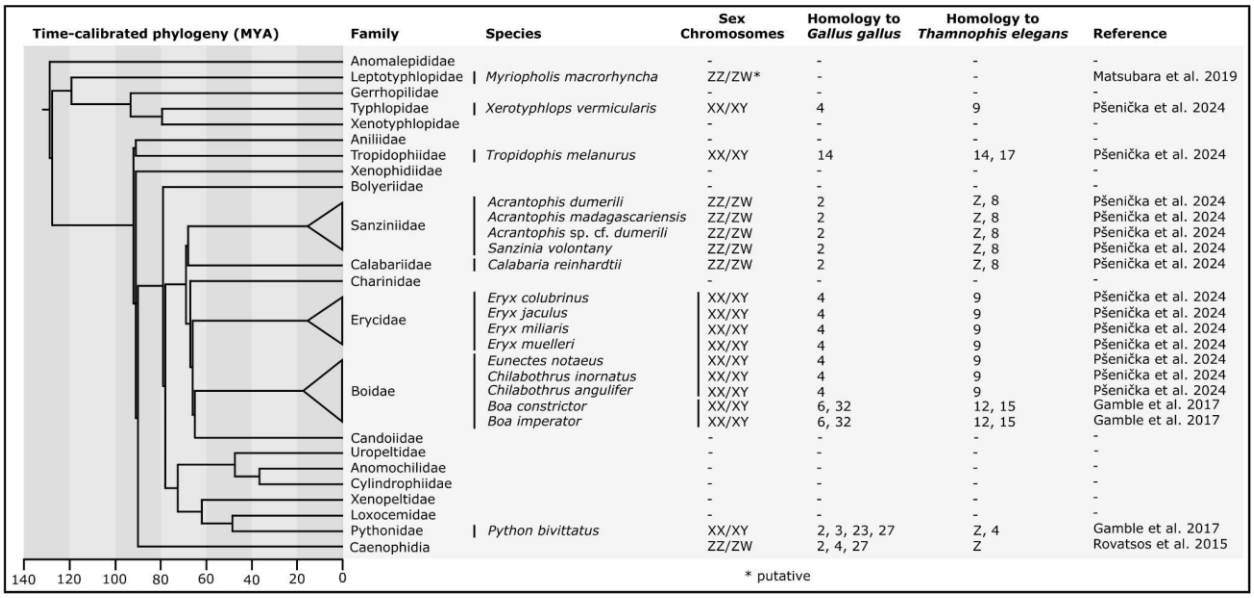
found these sex chromosomes also in the other species from the family Erycidae and some members of Boidae. The XX/XY sex chromosomes of *T. melanurus* did not prove homologous to any other studied snake species, although closely related species were not studied due to their rarity, and might be specific to this lineage. Since the sex chromosome homology between Sanziniidae, Calabariidae and Caenophidia, and Boidae, Erycidae and Typhlopidae, respectively, is phylogenetically conflicting, it can only be explained by independent co-option of the same genomic region for the role of sex chromosomes in some of these lineages. Moreover, these systems with homologous sex chromosomes differ dramatically in the level of differentiation, despite the fact that at least some of them are of similar age.

The extensive accumulation of heterochromatin in the karyotype of *E. colubrinus*, discovered in chapter 1, together with the newly acquired knowledge about sex chromosome system of this species, obtained in chapter 2, inspired us to investigate its satellite content in comparison to its related species, *E. miliaris* (chapter 3). We identified 18 satellites in these two species, 11 in *E. colubrinus* and 7 in *E. miliaris*, some of them species-specific, some of them conserved and shared between the two species. The two most abundant satellite families of *E. colubrinus* represent more than 2.3% of its whole genome and are not shared with *E. miliaris* suggesting a de-novo emergence, rapid amplification and dispersal across the genome. One of them, EcolSat01-26, is responsible not only for the previously observed heterochromatin heteromorphism on chromosome 7 but also for size increase of one ancestrally microchromosome pair, where it forms large heterochromatin blocks.

I contributed to experimental design and procedures, table and figure preparations, data analysis and manuscript preparation of each of these three chapters. Additionally, I contributed to the financial support of these projects from the grant I was awarded by the Charles University Grant Agency (GAUK 358522). I am the first author of all three investigations, equally contributing in chapter 2 with Barbora Augstenová.

CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis significantly expanded our knowledge about sex chromosome evolution in snakes. We uncovered several previously unknown sex chromosome systems using comparative gene coverage analysis, a bioinformatic approach. We uncovered the sex chromosome gene content of four phylogenetically informative species, which enabled us to later design primers for qPCR and test the homology of sex chromosomes among other snake species. On the contrary, we were not able to uncover sex chromosomes in non-caenophidian snakes by cytogenetic methods. The snake species studied by cytogenetic methods possess homomorphic and poorly differentiated sex chromosomes. This agrees with previous studies conducted on other non-caenophidian species. The single known non-caenophidian species with heteromorphic sex chromosomes is *Acrantophis* sp cf. *dumerili*. However, we demonstrated that even its sex chromosomes, although heteromorphic, are poorly differentiated and are likely an outcome of a recent chromosomal rearrangement.



Family	Species	Sex Chromosomes	Homology to *Gallus gallus*	Homology to *Thamnophis elegans*	Reference
Anomalepididae		-	-	-	-
Leptotyphlopidae	*Myriopholis macrorhyncha*	ZZ/ZW*	-	-	Matsubara et al. 2019
Gerrhopilidae		-	-	-	-
Typhlopidae	*Xerotyphlops vermicularis*	XX/XY	4	9	Pšenička et al. 2024
Xenotyphlopidae		-	-	-	-
Aniliidae		-	-	-	-
Tropidophiidae	*Tropidophis melanurus*	XX/XY	14	14, 17	Pšenička et al. 2024
Xenophidiidae		-	-	-	-
Bolyeriidae		-	-	-	-
Sanziniidae	*Acrantophis dumerili*	ZZ/ZW	2	Z, 8	Pšenička et al. 2024
	Acrantophis madagascariensis	ZZ/ZW	2	Z, 8	Pšenička et al. 2024
	Acrantophis sp. cf. *dumerili*	ZZ/ZW	2	Z, 8	Pšenička et al. 2024
	Sanzinia volontany	ZZ/ZW	2	Z, 8	Pšenička et al. 2024
Calabariidae	*Calabaria reinhardtii*	ZZ/ZW	2	Z, 8	Pšenička et al. 2024
Charinidae		-	-	-	-
Erycidae	*Eryx colubrinus*	XX/XY	4	9	Pšenička et al. 2024
	Eryx jaculus	XX/XY	4	9	Pšenička et al. 2024
	Eryx miliaris	XX/XY	4	9	Pšenička et al. 2024
	Eryx muelleri	XX/XY	4	9	Pšenička et al. 2024
Boidae	*Eunectes notaeus*	XX/XY	4	9	Pšenička et al. 2024
	Chilabothrus inornatus	XX/XY	4	9	Pšenička et al. 2024
	Chilabothrus angulifer	XX/XY	4	9	Pšenička et al. 2024
	Boa constrictor	XX/XY	6, 32	12, 15	Gamble et al. 2017
	Boa imperator	XX/XY	6, 32	12, 15	Gamble et al. 2017
Candoiidae		-	-	-	-
Uropeltidae		-	-	-	-
Anomochilidae		-	-	-	-
Cylindrophiidae		-	-	-	-
Xenopeltidae		-	-	-	-
Loxocemidae		-	-	-	-
Pythonidae	*Python bivittatus*	XX/XY	2, 3, 23, 27	Z, 4	Gamble et al. 2017
Caenophidia		ZZ/ZW	2, 4, 27	Z	Rovatsos et al. 2015

* putative

Figure 3: Current knowledge about sex chromosome systems in non-caenophidian snakes. Simplified from chapter 2 (Pšenička *et al.* 2024)

Going further, genomic and bioinformatic approaches will be likely more efficient at discovering sex chromosomes in unstudied snake lineages. Not only are they more sensitive,

but they allow us to determine the sex chromosome gene content and subsequently also to uncover their putative homology. This knowledge, combined with the rising number of reptilian genome projects has the potential to uncover primary sex-determining genes. Not only would it benefit the general knowledge about sex determination in reptiles, information about sex-determining genes would allow us to establish what was the ancestral sex chromosome system in snakes and which of the observed phylogenetically conflicting sex chromosome homologies are thus due to independent co-option. In addition, a large portion of the non-caenophidian snake families were not yet studied, leaving the possibility of even more exciting discoveries. Largely unexplored are also the satellite sequences in snakes. We demonstrated interesting satDNA dynamics in two sand boa species, where we can observe cases of both conservatism and species-specific amplification and distribution.

Taken together, we demonstrated a big variability in sex chromosome evolution in snakes, where we can now recognize up to seven distinct systems. It seems that snakes originally possessed less differentiated sex chromosomes prone to turnovers with some genomic regions likely independently co-opted as sex chromosomes in more lineages. On the other hand, the sex chromosomes of caenophidian snakes became highly stable once their differentiation proceeded, creating a stark contrast especially to sex chromosomes of Sanziniidae and Calabariidae, which are based on the same genomic region, yet remained homomorphic and poorly differentiated for comparable evolutionary time. Snakes thus re-emerge as an ideal model system to study turnovers, co-option or unequal rates of differentiation of sex chromosomes, all in a single lineage.

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