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Bc. Tomáš Charvát

Evolution of sex chromosomes and karyotypes in boas and pythons

Evoluce pohlavních chromozomů a karyotypů u hroznýšů a krajt

Diploma thesis

Supervisor: Michail Rovatsos, PhD

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DECLARATION OF ORIGINALITY

I declare that this thesis is entirely the result of my own work and it has not been submitted for the purpose of obtaining the same or another academic degree earlier or at another institution. All literature sources I used when writing this thesis have been properly cited.

PROHLÁŠENÍ O ORIGINALITĚ

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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– TABLE OF CONTENTS –

– ABSTRACT –

Snakes (Serpentes) are a group of squamate reptiles (Squamata) that represents more than one third of the total reptile species diversity. Snake karyotype is generally conserved with the most common chromosome number of 36 (16 macro- and 20 microchromosomes) in diploid state. It is believed that this karyotype was also present in the common ancestor of all snakes. The majority of snake species belong to the group Caenophidia and share homologous ZW sex chromosomes. Snakes from the groups "Scolecophidia" and "Henophidia" have mostly poorly differentiated, homomorphic sex chromosomes, which made them impossible to distinguish from the autosomes in the past. These snakes were for many years assumed to have ZW sex chromosomes as well. However, recent studies demonstrated not only ZW but also two non-homologous XY sex chromosome systems in non-caenophidian snakes and thus the sex determination systems in snakes are much more variable than previously thought. In this thesis, eight species of henophidian snakes (representatives from the genera *Eryx*, *Cylidrophis*, *Python* and *Tropidophis*) and one caenophidian species (*Ophiophagus hannah*) were examined using conventional and molecular cytogenetic methods. However, sex chromosomes were not detected in the henophidian species, only in *Ophiophagus hannah*, the single studied caenophidian species. *Ophiophagus hannah* has highly heteromorphic ZW sex chromosomes with extensive accumulation of interstitial telomeric repeats (ITRs) and constitutive heterochromatin on its W chromosome. ITRs were also observed on one autosomal pair in *Ophiophagus hannah* and on three chromosome pairs in *Eryx muelleri*, one of the tested henophidian species, despite the shared chromosome number of 2n = 34 with the rest of the *Eryx* species. These results correspond with the growing evidence that ITRs might be more common in snakes even with their generally conserved karyotypes. Although the total chromosome number is shared in Erycinae and may be an apomorphy of this subfamily, differences in chromosome morphology and heterochromatin locations were found between some of its species.

Key words:

boas, evolution, FISH, heterochromatin, karyotypes, pythons, rDNA, sex chromosomes, telomeres

– ABSTRAKT –

Hadi (Serpentes) jsou skupinou šupinatých plazů (Squamata), která představuje více než třetinu druhové diverzity plazů. Karyotyp hadů je poměrně konzervativní a nejčastěji má v diploidním stavu 36 chromozomů (16 makro- a 20 mikrochromozomů). Předpokládá se, že toto uspořádání bylo přítomno už u společného předka všech hadů. Majoritní většina hadích druhů patří do skupiny Caenophidia a sdílí homologní ZW pohlavní chromozomy. Hadi ze skupin "Scolecophidia" a "Henophidia" mají převážně nerozlišené, homomorfní pohlavní chromozomy, což v minulosti znemožnilo jejich podrobnější studium. Přítomnost ZW pohlavních chromozomů byla u těchto hadů po desetiletí tedy pouze předpokládána. Nové studie doložily existenci nejen ZW, ale i dvou nehomologních systémů s XY pohlavními chromozomy mimo skupinu Caenophidia. Dokázaly, že systémy určení pohlaví jsou u hadů variabilnější, než se dříve předpokládalo. V této práci bylo studováno osm druhů hadů ze skupiny "Henophidia" (zástupci rodů *Eryx*, *Cylidrophis*, *Python* a *Tropidophis*) a jeden druh ze skupiny Caenophidia (*Ophiophagus hannah*) a to za použití konvenčních i molekulárních cytogenetických metod. Pohlavní chromozomy však nebyly nalezeny u žádného ze studovaných druhů skupiny "Henophidia", nýbrž pouze u *Ophiophagus hannah* ze skupiny Caenophidia. Tento druh má výrazně heteromorfní pohlavní chromozomy, přičemž W chromozom vykazuje rozsáhlou akumulaci intersticiálních telomerických repetic (ITR) a konstitutivního heterochromatinu. ITR byly nalezeny i na jednom páru autozomů u *Ophiophagus hannah* a na třech chromozomových párech u *Eryx muelleri* ze skupiny "Henophidia", a to i přestože je celkový počet chromozomů (2n = 34) u všech zástupců rodu *Eryx* stejný. Ačkoliv mají hadi obecně konzervativní karyotypy, tyto výsledky společně s mnohými jinými podporují hypotézu, že ITR u nich mohou být častější, než se dříve předpokládalo. Stejný počet chromozomů zástupců Erycinae by mohl být apomorfií této skupiny, avšak mezi některými druhy této podčeledi byly zjištěny rozdíly v morfologii chromozomů a distribuci heterochromatinu.

Klíčová slova:
evoluce, FISH, heterochromatin, hroznýši, karyotypy, krajty, pohlavní chromozomy, rDNA, telomery

– INTRODUCTION –

Diversity and phylogeny of snakes

Snakes (Serpentes) are iconic reptiles with a characteristic legless body plan. Their appearance and behavior are inducing fear as well as amazement in people for centuries. Snakes are considered a monophylum and are part of the most diverse group of reptiles, the order Squamata. Out of more than 10850 extant squamate species, roughly 3880 species are snakes (Uetz *et al.* 2020). With such a high species richness, it is not surprising that snakes possess a wide variety of phenotypes ranging from small insectivorous burrowing species to land-dwelling giants and from slim arboreal species to aquatic and even fully pelagic species. These features enabled them to inhabit many niches on all continents except for Antarctica.

Another well-known fact about many snake species is their ability to produce complex and, in some cases, deadly venoms. Especially snakes from the families Viperidae and Elapidae mastered this ability and some of them belong to the most venomous animals on the planet (Kocholaty *et al.* 1971). The lesser known fact is that not only snakes but also some of their other reptile relatives are able to produce venom. This inspired taxonomists to create the group Toxicofera, which includes snakes and their two sister groups Anguimorpha and Iguania (Vidal & Hedges 2005). These groups include for example monitors, beaded lizards and anguids (Anguimorpha), and iguanas, agamids and chameleons (Iguania).

Based on morphological data, the first ophidians (Pan-Serpentes) originated in the Jurassic after the splitting from other toxicoferan reptiles with the oldest known fossils dating back to roughly 170 million years ago (Caldwell *et al.* 2015; Hsiang *et al.* 2015). Molecular data estimate that the divergence of the extant snake lineages is at least 40 million years younger (Zheng & Wiens 2016; Harrington & Reeder 2017).

Currently, there are recognized 25 snake families (Fig. 1). Phylogenetic relationships between some of them are still poorly supported and the taxonomic position of some species is still under debate (Pyron *et al.* 2013; Pyron & Wallach 2014; Zheng & Wiens 2016). Snake families are traditionally divided into three groups: “Scolecophidia” (606 species), “Henophidia” (222 species) and “Caenophidia” (3148

species) (Uetz *et al.* 2020). The most species-rich and the only monophyletic group is Caenophidia. With almost 3150 species, it includes some of the most recognizable snakes such as vipers, rattlesnakes, colubrids and cobras. Even though "Henophidia" does not have so many species, they include almost half of all snake families (Uetz *et al.* 2020). Let's mention for example pythons and boas, the largest and heaviest squamates currently in existence. In contrast, representatives of the group "Scolecophidia" are usually small burrowing snakes living underground. It is hardly surprising that they are not given so much attention by the public. They feed on insects, mainly their larvae and are generally uniform in appearance. Both "Henophidia" and "Scolecophidia" were proven paraphyletic by molecular taxonomy (Pyron *et al.* 2013; Pyron & Wallach 2014; Zheng & Wiens 2016). However, these terms are still being used for historical reasons, and as the phylogeny of these groups is still not fully resolved (but see Reynolds *et al.* 2014; Miralles *et al.* 2018) and new terminology is yet to be suggested.

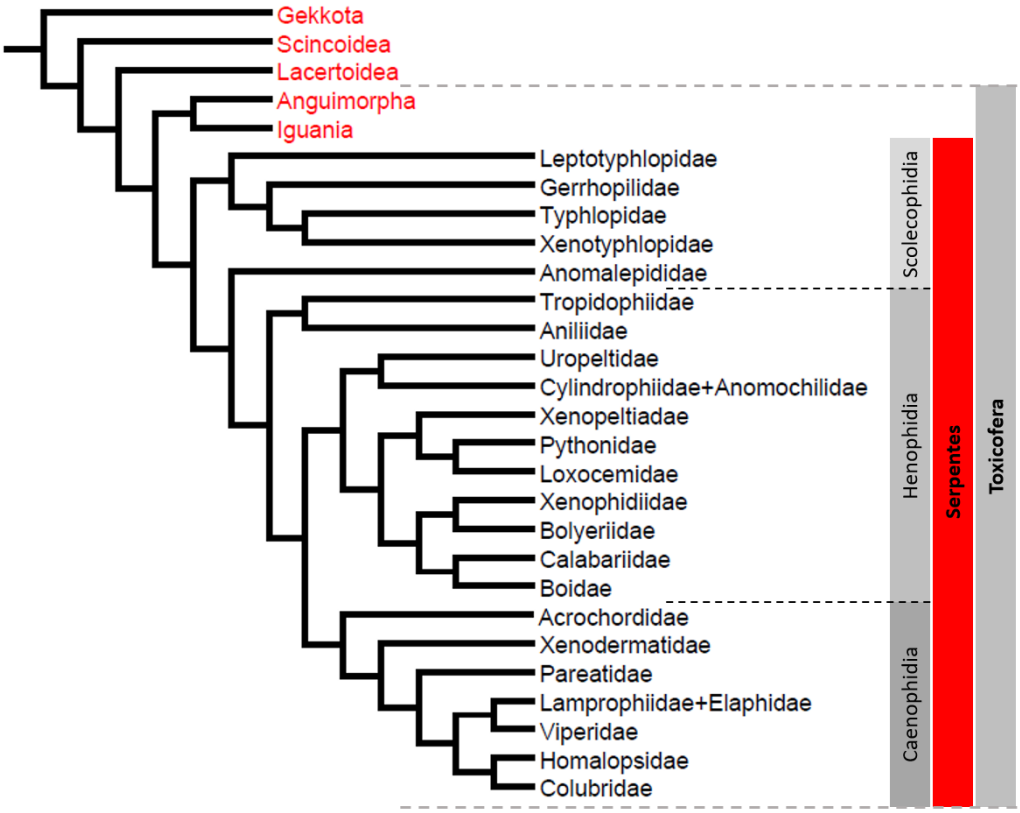


Fig. 1: Phylogeny of snake families and other squamate lineages. Data extracted from Zheng & Wiens (2016).

Evolution of snake karyotypes

Chromosomes in reptile karyotypes, birds included, can be divided into two major categories according to their size: microchromosomes and macrochromosomes. This organization is thought to reflect the ancestral state, although microchromosomes are missing in some reptile species or even lineages (Cohen & Gans 1970; Beçak & Beçak 1969; Srikulnath *et al.* 2015). This is most likely due to their fusions with macrochromosomes or other microchromosomes, but the karyotype evolution may vary in each lineage. In the past, microchromosomes were often overlooked and were believed to be an unimportant part of the reptile genome. However, latter studies declared that microchromosomes are essential in many species, as some proved to be gene-rich, and in some cases are even carrying the sex determining locus and thus have the role of sex chromosomes (Smith *et al.* 2000; Ezaz *et al.* 2005; 2006; Badenhorst *et al.* 2013; Matsubara *et al.* 2014).

The diploid chromosome number can be variable in snakes and it ranges between 2n = 24 and 2n = 52, possibly up to 2n = 56 (Beçak & Beçak 1969; Becak *et al.* 1990; Olmo & Signorino 2005). However, the most common karyotype found in the majority of snake species has 36 chromosomes out of which are 16 macrochromosomes and 20 microchromosomes (Olmo & Signorino 2005; Oguiura *et al.* 2009). It is feasible that this karyotype was already present in the common ancestor of all snakes. There are no visible trends for reduction or increase of total chromosome number through the snake phylogeny. Yet, the most diverse snake subfamily Dipsadinae (Colubridae, Caenophidia) shows striking variability in chromosome numbers, relative to other snake lineages, even at the generic level (Oguiura *et al.* 2009). Both above-mentioned extremes belong to this group: *Hydrodynastes gigas* with 24 chromosomes and *Sordellina punctata* with 52 chromosomes. It is plausible that chromosomal rearrangements and subsequent changes in chromosome numbers contributed to the species diversification in this subfamily.

Past chromosomal rearrangements can be detected by the presence of interstitial telomeric repeats (ITRs). Telomeres normally provide protective caps at the terminal parts of each chromosome. In the case of vertebrates, they consist of telosome or shelterin protein complex and $(TTAGGG)_n$ repeats (Blackbum 1991; Liu *et al.* 2004). Telomeres prevent the ends of chromosomes from losing genetic material during replication and from unintentional recombination or fusion (O'sullivan & Karlseder 2010). Although it

was proposed, that in some cases, ITRs might just be the products of high telomerase activity or transposon relocation, they are usually viewed as remnants of chromosomal rearrangements, such as chromosome fusions or inversions (Bolzán & Bianchi 2006; Rovatsos *et al.* 2015a). Just as telomeres themselves, they consist of the same repetitive hexamer motif. ITRs were observed in a few species of snakes but the data coverage is still poor. On autosomes, they are often located around the pericentromeric region but also in other, interstitial parts of the chromosome (Viana *et al.* 2016; Augstenová *et al.* 2019). Their distribution on sex chromosomes is variable between species and may differ greatly between Z and W (Rovatsos *et al.* 2015b; Augstenová *et al.* 2018a; Singchat *et al.* 2019).

Evolution of sex determination in snakes

The sex of a reptile can be determined either by environmental factors affecting the embryo during prenatal development or by inherited sex determining locus (Bull 1980). Snakes are reptiles with genotypic sex determination (GSD). This means, that the sex of an individual is determined at the zygote formation. It is so by the presence/absence or dose of inherited master sex determining gene or genes. Therefore, there is a genetic difference between males and females of species with GSD. In contrast, an animal with environmental sex determination (ESD) starts its development with the potential to become either male or female. The sex is determined by the intensity of an environmental factor, in reptiles usually temperature, during a sensitive period in prenatal development (Janzen & Paukstis 1991).

The presence of GSD in a species can be proved for example by equal sex ratio of the offspring across different incubation temperatures or by the presence of sex chromosomes. There is not a single known snake species with ESD, but a good portion of the total snake diversity remains unstudied. This holds especially for scolecophidian snakes, because they are not often bred in captivity.

It is believed that GSD evolves from ESD or another GSD when a pair of autosomes acquires a sex determining locus (Ohno 1967; Pokorná & Kratochvíl 2016). The mechanism then works on the principle of heterozygosity. When it is the male, who is heterozygous for the sex determining locus, we speak about male heterogamety with

XX and XY sex chromosome combinations. Correspondingly, there is also female heterogamety with ZZ or ZW sex chromosomes. Recombination between these newly established sex chromosomes or their parts might be suppressed, which leads to their eventual divergence. In such a case, heteromorphic sex chromosomes evolve (Ohno 1967). These usually differ in size and morphology. The non-pairing chromosome often loses most of its original genes and accumulates heterochromatin, mostly different kinds of repetitive elements. This process is called degeneration (Bachtrog 2006).

When one of the sex chromosomes fuses with an autosome or when one sex chromosome splits, multiple neo-sex chromosomes evolve (Toder *et al.* 1997; Rovatsos *et al*. 2016). Six such cases are known in the caenophidian family Elapidae and each of them is thought to have an independent origin (Pokorná *et al.* 2014). Let's mention for example *Bungarus caeruleus* with $Z_1Z_1Z_2Z_2/Z_1Z_2W$ or *Enhydrina schistosa* with ZZ/ZW_1W_2 sex chromosomes (Singh *et al.* 1970; Singh 1972b).

Among all snakes, sex chromosomes and sex determination systems are best known in the group Caenophidia. Multiple species from all Caenophidian families were proved to have homologous heteromorphic ZW sex chromosomes (Rovatsos *et al.* 2015c). The level of degeneration of W chromosomes, and the difference in size and morphology between Z and W sex chromosomes as well as the amount and nature of accumulated repetitive elements, vary across the snake phylogeny (Beçak & Beçak 1969; Singh 1972a; Vicoso *et al*. 2013; Augstenová *et al.* 2018a; Singchat *et al.* 2019). Many microsatellite motifs are accumulating on the caenophidian Ws (Matsubara *et al.* 2016). Some of the most common and well-studied are the *Bkm* repeats, rDNA repeats and already mentioned ITRs. Ribosomal DNA (rDNA) are sequences coding for ribosomal RNA and have thus an important role in ribosome biogenesis. rDNA sequences form large tandem repeats that amplify extensively when located on the caenophidian W (O'Meally *et al.* 2010). Although both ITRs and rDNA loci accumulate also on autosomes, their potentially unequal distribution on sex chromosomes can help characterize or even uncover cryptic sex chromosomes. *Bkm* repeats are enriched in $(GATA)_n$ and $(GACA)_n$ motifs and their distribution is often species-specific. They are found in all so far tested species of caenophidian snakes, with the exception of the family Acrochordidae (Singh *et al.* 1980; Rovatsos *et al.* 2015b; Matsubara *et al.* 2016; Augstenová *et al.* 2018a). As this family is sister to all other caenophidian snakes, it was

proposed that *Bkm* repeats started accumulating on the W chromosome of caenophidian snakes after the splitting of the family Acrochordidae from other caenophidian lineages (Rovatsos *et al.* 2018a). However, it is possible that these repeats were lost from the W in this family.

So far, studied snakes outside Caenophidia have homomorphic sex chromosomes, except for two species (Mengden & Stock 1980; Augstenová *et al.* 2018b; Matsubara *et al.* 2019). This means that they are identical in size, morphology and content - with the exception of the sex determining region. It was shown in a fish species that the sex determining region can be as small as a single nucleotide polymorphism (Kamiya *et al.* 2012). Homomorphic sex chromosomes retained the ability to recombine across most of their length which prevents degeneration (Ohno 1967; Charlesworth *et al.* 2005). On the other hand, degeneration of the W enables in Caenophidia the "fast Z" effect (Vicoso *et al.* 2013). Because of this phenomenon, genes on the Z chromosome evolve faster than genes on autosomes and it may be one of the reasons, why caenophidian snakes are so diversified in contrast to the other snake lineages (Rovatsos *et al.* 2015c).

For a long time, it was widely accepted, that not only caehophidian, but all snakes share homologous ZZ/ZW sex determination (e.g. Beçak & Beçak 1969; Matsubara *et al.* 2006; Booth *et al.* 2011; Vicoso *et al.* 2013). Before the era of molecular genetics and advanced cytogenetics, scientists were restricted to use conventional cytogenetic methods for sex chromosome research. These methods are only capable of uncovering heteromorphic sex chromosomes as they rely on different morphology of the chromosomes or unequal distribution of heterochromatin. Thus, it is not possible to distinguish homomorphic sex chromosomes using these techniques. Because of this, many henophidian and scolecophidian snakes were just assumed to have ZW sex chromosomes, that were thought to represent the ancestral or at least the more primitive state of sex chromosome evolution.

This view was however challenged many times by the results of facultative parthenogenesis in some pythons and boas. The offspring of such events were "hemiclonal" females, which is not consistent with female heterogamety (Groot *et al.* 2003; Booth *et al.* 2011, Kinney *et al.* 2013; Shibata *et al.* 2017). Finally, in 2017 a study using RAD sequencing proved the existence of male heterogamety in a python (*Python bivittatus*) and two species of boas (*Boa constrictor, B. imperator*) (Gamble *et al.* 2017).

Interestingly, these two systems proved to be non-homologous. Instead, python XY sex chromosomes are surprisingly homologous to caenophidian ZW. It remains unclear, whether this homology is due to shared ancestral sex chromosome system or independent co-option of this chromosome pair for the role of sex chromosomes, possibly due to yet unknown predispositions.

There are only two other species of non-caenophidian snakes with known sex chromosomes. One of them is *Acrantophis* sp. cf. *dumerili* from the family Boidae with heteromorphic ZW sex chromosomes (Mengden & Stock 1980). Heteromorphic nature of its sex chromosomes seems to be fairly young as it is not shared with its sister species and is probably an outcome of pericentromeric inversion (Augstenová *et al.* 2018b). Recently, heteromorphic ZW chromosomes were identified in the scolecophidian snake *Myriopholis macrorhyncha*, a species from the family Leptotyphlopidae (Matsubara *et al.* 2019). It is also suggested that these sex chromosomes might not be homologous to the ones of Caenophidia and *Python bivittatus*.

– AIMS OF THE STUDY –

For a better understanding of the karyotype evolution and sex determination systems in snakes, we decided to study mostly species from the group "Henophidia". Recent studies proved that sex determination systems in this group are much more variable than previously thought (Gamble *et al.* 2017; Augstenová *et al.* 2018b). Yet, a good portion of the total species diversity remains cytologically unstudied. Snakes from this group are often kept as pets or display animals at zoos, which makes them more accessible for research, relative to scolecophidian snakes.

As the main lineage for this thesis, we selected the subfamily Erycinae (sand boas) from the family Boidae. There are 12 species in this subfamily, out of which 5 were used for this study. This group of snakes was selected, as it is poorly studied and is phylogenetically nested between species of boas with XY (*Boa constrictor, B. imperator*) and ZW (*Acrantophis* sp. cf. *dumerili*) sex chromosomes. Sand boas are burrowing snakes living mostly in the loose substrate in arid areas. All sand boa species display remarkable sexual dimorphism in size with females being up to ten times larger than males. There are two species of sand boas that are believed to re-evolved oviparity and might be an example of breaking the Dollo's law (Lynch & Wagner 2010). Additional three henophidian and one caenophidian species were added to the thesis. These are *Cylindrophis ruffus* (Cylindrophiidae), *Tropidophis melanurus* (Tropidophiidae), *Python regius* (Pythonidae) and *Ophiophagus hannah* (Elapidae), respectively. XY sex chromosomes are highly expected in *P. regius* due to the observed pattern of inheritance of a sex-linked phenotypic trait. This pattern is best explained by male heterogamety, yet direct evidence is missing (Mallery Jr. *et al.* 2016).

The first main goal of this thesis was to explore the karyotype evolution and the distribution of repetitive elements in selected species of snakes. Despite the apparent karyotype conservatism in snakes, the examination of repetitive elements distribution might uncover cryptic chromosomal rearrangements and help to better understand the karyotype evolution in snakes.

The second main goal was to explore the presence of differentiated sex chromosomes by conventional and molecular cytogenetic methods. All selected species represent an interesting position in the snake phylogeny and discovery of sex

chromosomes in any of them would greatly improve current knowledge about snake sex determination systems.

– MATERIALS & METHODS –

The following methods were selected to characterize cytogenetically and to uncover sex chromosomes and sex determination systems in previously unstudied species of snakes. Animals were provided for this thesis from private breeders and collaborators, but also from our collection. Fresh blood was taken from the tail vein, which is one of the less invasive methods of blood taking in reptiles. The blood was then used for DNA isolation and to obtain chromosome suspensions.

Staining with Giemsa solution is commonly used in cytogenetics to visualize the chromosomes. Giemsa stained metaphases are used for karyotype reconstruction and in case of heteromorphic sex chromosomes, also for the description of sex determination. To add further characterization for the karyotype, C-banding is used to visualize the constitutive heterochromatin, which often accumulates near centromeric regions and on degenerated sex chromosomes. Unequal distribution of heterochromatin in both sexes might help uncover sex chromosomes, that are otherwise similar in size and morphology.

Fluorescence *in situ* hybridization was used to localize the distribution of telomeric repeats and rDNA loci. Both were shown to amplify on reptile sex chromosomes (O'Meally *et al*. 2010; Literman *et al.* 2014; Augstenová *et al.* 2018a; Lee *et al.* 2019; Singchat *et al.* 2019; Mazzoleni *et al.* 2020). Distribution of telomeric repeats might also uncover potential past chromosomal rearrangements.

Sequences of mitochondrial genes cytochrome b (CYTB) and cytochrome c oxidase subunit I (COI) were obtained from each individual. These sequences serve not only for species verification but also for a specimen-specific barcode. As cryptic species are common in reptiles, this method will ensure that the following results will remain valid even in case of future species splitting.

Blood taking

Blood was taken from selected individuals from the tail vein using U-100 1ml insulin syringes with approximately 10 µl of heparin. The amount of taken blood varied between individuals and was dependent on the size of each animal. The list of examined snakes per species are presented at Table 1.

Table 1: List of species used in current study and the number of tested individuals

Species	Family	Sex	
		♂	♀
Cylidrophis ruffus	Cylindrophiidae	0	1
Eryx colubrinus	Boidae	2	1
Eryx conicus	Boidae	1	1
Eryx miliaris	Boidae	1	1
Eryx muelleri	Boidae	1	1
Eryx tataricus	Boidae	1	1
Python regius	Pythonidae	2	2
Tropidophis melanurus	Tropidophiidae	0	1
Ophiophagus hannah	Elapidae	1	1

DNA isolation

DNA was isolated using DNeasy Blood and Tissue Kit (Qiagen). 3 drops of fresh blood, 180 µl of sterile PBS, 20 µl of proteinase K and 4 µl of RNase A (100 mg/ml) was added into 1.5 ml Eppendorf tube. The tubes were vortexed and incubated at room temperature for 2 min. Then, 200 µl of AL buffer was added, the tubes were vortexed again and incubated at 56 °C for 1 hour. Sequentially, 200 µl of 96% ethanol was added and the tubes were vortexed. The liquid part of the sample was transferred to DNeasy Mini spin column and centrifuged at 8000 rpm for 2 min. The tube was removed, and the column was transferred to a clean tube. 500 µl of AW1 buffer was added and the samples were centrifuged at 8000 rpm for 1 min. The tube was removed, and the column was transferred to a clean tube. 500 µl of AW2 buffer was added and the tubes were centrifuged at 13300 rpm for 3 min. The tube was removed, the column was transferred to a clean tube and centrifuged at 8000 rpm for 1 min. The tube was removed, the column was transferred to a clean 1.5 ml Eppendorf tube, 60 µl of AE buffer was added and the tubes were centrifuged at 8000 rpm for 1 min. The concentration of the DNA of the

sample was measured using the Thermo Scientific NanoDrop 1000 Spectrophotometer. The DNA samples were stored at -20 °C for future use.

Species verification

For species identification, sequences of two mitochondrial genes, cytochrome b and cytochrome c oxidase subunit I, were used. Master mix for one sample was prepared as: 18.5 µl of sterile milliQ H_2O, 2.5 µl of PCR buffer, 1.25 µl of $MgCl_2$, 1 µl of deoxynucleotides (dATP, dCTP, dGTP, dTTP), 0.25 µl of Taq polymerase and 0.5 µl of both forward and reverse primer (Table 1). 0.7 µl of DNA was used for each such prepared mix.

The PCR started with 3 min long denaturation phase at 94 °C, followed by 35 cycles of 40 seconds of denaturation at 94 °C, 40 seconds of annealing at 47 °C and 1 minute of extension at 72 °C. The extension step in the last cycle was prolonged to 5 min, followed by cooling to 4 °C. The results of the PCR were verified by electrophoresis using 1% agarose gel and sent for sequencing to Macrogen in South Korea.

Table 2: Primers used for species verification

Gene	Primer name	Primer sequence from 5' to 3'	Direction	Reference
COI	REPCOI R	ACTTCTGGRTGKCCAAARAATCA	reverse	Nagy *et al.* 2012
	REPCOI F	TNTTMTCAACNAACCACAAAGA	forward	Nagy *et al.* 2012
CYTB	H16064	CTTTGGTTTACAAGAACAATGCTTTA	reverse	Burbrink *et al.* 2000
	L14919	AACCACCGTTGTTATTCAACT	forward	de Queiroz *et al.* 2002

Cell cultures and chromosome harvesting

Leukocyte cultivation from fresh whole blood samples was used to obtain chromosome suspensions. The medium as well as the cell cultures were prepared in sterile laminar flow-box. For 100 ml of the final medium, was used 90 ml of D-MEM medium (Sigma-Aldrich; M2154) and 10 ml of fetal bovine serum (Baria; S 0125). To initiate mitosis of the leukocytes, 3 ml of phytohemagglutinin M (GIBCO; 10576-015)

was added to the mix, alongside with 1 ml of penicillin/streptomycin (Sigma-Aldrich; A5955), 1 ml of L- glutamine (Sigma-Aldrich; K1377) and 1 ml of lipopolysaccharide (Sigma-Aldrich; L4005). The medium was then evenly distributed into 15 ml falcon tubes to have approximately 5 ml in each one. After that, 100–300 µl of fresh blood was added to each tube. The cell cultures were left to incubate for one week at 30 °C without CO_2 supplementation.

After one week, 35 µl of colcemid solution (Roche; 10295892001) was added to the cultures to stop mitosis. The samples were left to incubate for additional 3.5 hours but were mixed after the first 2 hours of incubation. After 3.5 hours, the tubes were centrifuged at 1200 rpm at room temperature for 10 min. The supernatant was then removed using a glass pipette and 5 ml of pre-wormed 0.075M KCl were added to each tube. The samples were left at 37 °C for 30 min. After that, fresh fixation solution was prepared as 3:1 methanol:acetic acid and 500 µl was added to each tube. Here, the first fixation round started. The tubes were centrifuged at 1200 rpm at 4 °C for 10 min. The supernatant was then removed using glass pipette, 5 ml of fixation solution was added, and the samples were left at 4 °C for 20 min. Additional two fixation rounds were performed. After the third round of fixation, the tubes were centrifuged at 1200 rpm at 4 °C for 10 min, the supernatant was removed, and up to 500 µl of fixation solution was added. The volume of added fixation solution was adjusted according to the amount of chromosomal material. Such prepared chromosome suspensions were stored at -20 °C for future use.

Karyotype reconstruction

Microscope slides were cleaned in 96% ethanol. Once dry, two drops of chromosomal material were dropped to each slide. After they dried, the slides were stained in 3% Giemsa in phosphate buffer (4.5 g KH_2PO_4 + 4.7 g Na_2HPO_4 in 1 l of distilled water, pH = 6.8) solution for 15 min. The slides were then washed in distilled water and left to dry in a vertical position.

The pictures of Giemsa-stained metaphases were taken on Zeiss AxioImager Z2 with automatic slide scanning system Metafer-MSearch (MetaSystems). Karyograms were reconstructed using the Ikaros karyotyping system (MetaSystems).

C-banding

Constitutive heterochromatin was visualized using standard C-banding protocol by Sumner (1972). Microscope slides were cleaned in 96% ethanol. Once dry, two drops of chromosomal material were dropped to each slide. The slides were aged overnight at 37 °C or 1 hour at 60 °C, then treated with 0.2N HCl at room temperature for 30 min and washed in distilled water. Subsequently, the slides were treated with 5% $Ba(OH)_2$ solution at 45 °C for 5–18 min (Table 2), depending on the species, and washed in distilled water again. One-hour long incubation in 2xSSC (saline-sodium citrate) at 60 °C followed and then quick wash in distilled water. The slides were left to dry in a vertical position. Fluoroshield with DAPI (Vector Laboratories) was added to each slide and covered with a cover slide.

Table 3: $Ba(OH)_2$ treatment time for each tested species

Species	$Ba(OH)_2$ treatment time
Eryx colubrinus	5 min
Eryx conicus	15 min
Eryx miliaris	8 min
Eryx muelleri	18 min
Eryx tataricus	10 min
Cylindrophis ruffus	5 min
Ophiophagus hannah	5 min
Python regius	8 min

Fluorescence *in situ* hybridization (FISH)

For rDNA probe preparation, plasmid (pDmr.a 51#1) with an 11.5-kb insert encoding the 18S and 28S ribosomal units of Drosophila melanogaster (Endow 1982) was used to obtain replicated rDNA loci. The DNA was isolated and only samples with concentration 44 ng/µl or higher were used for the probe preparation. The probe is cut and labelled with dUTP-biotin using the nick translation kit ABBOTT (07J00-001). For this task, a deoxynucleotide mix was prepared as: 50 µl of dCTP, 50 µl of dGTP, 50 µl of dATP, 25 µl of dTTP and 7,5 µl of dUTP-biotin. DNA samples with higher concentration than 44 ng/µl were diluted accordingly. Master mix for one reaction was prepared as: 23 µl of sterile milliQ H_2O, 1 µg of DNA, 12 µl of deoxynucleotide mix, 5 µl of nick translation buffer and 10 µl of nick translation enzyme. As the nick translation

enzyme is thermo-sensitive, it was needed to work partially on ice to prevent degradation. The mix was then incubated in a cycler at 15 °C for 3-5 hours to obtain 200-300 bp long fragments. The reaction was stopped by increasing temperature to 70 °C for 10 minutes. The size of the probe was verified using electrophoresis 1% agarose gel.

The probe for telomeric repeats was prepared using polymerase chain reaction (PCR). Primers $(TTAGGG)_5$ and $(CCCTAA)_5$ were used for the reaction without any DNA template as in Ijdo *et al.* (1991). During the reaction, the probe was labelled with dUTP-biotin. Master mix for the PCR was prepared as: 5 µl of PCR buffer (Bioline), 2.5 µl of MgCl2 (50 mM), 1 µl of deoxynucleotides (dATP, dCTP, dGTP; 10mM each), 0.7 µl of dTTP (10mM), 1 µl of dUTP-biotin (1mM) and 1 µl of BioTaq DNA polymerase (5 U/µl, Bioline) and 0.4 µl of each primer (5 pmol/µl). Sterile milliQ H_2O was added up to 50 µl.

The PCR started with 5 min of the denaturation phase at 94 °C, followed by 10 cycles of 1-minute denaturation at 94 °C, 30 sec of annealing at 55 °C and 30 sec of extension at 72 °C. Additional 35 cycles where annealing temperature was raised to 60 °C followed. The extension step in the last cycle was prolonged to 5 min, followed by cooling to 4 °C. The results of the PCR were verified by electrophoresis using 1% agarose gel.

Both probes were precipitated overnight at -20 °C using 5 µl of salmon sperm, 5 µl of sodium acetate (3M) and 150 µl of 96 % cold ethanol (-20 °C) per 45 µl of PRC product/nick translation product. Next day the probes were centrifuged at 4 °C at 13 000 rpm for 30 min. The supernatant was removed and 500 µl of cold 75 % ethanol (-20 °C) was added to each tube. The tubes were then centrifuged at 4 °C at 13 000 rpm for 2 min. The supernatant was removed, and the pellet was left to dry at 37 °C. Dried pellet was dissolved in a hybridization buffer (50% formamide in 2×SSC) and stored at -20°C for future use.

Microscope slides were cleaned in 96% ethanol. Once dry, two drops of chromosomal material were dropped to each slide. The slides were aged overnight at 37 °C or 1 hour at 60 °C and then washed in 2xSSC for 5 min. The chromosomal material was then treated with RNAse (100 mg/ml) for 1 hour at 37 °C and washed 3 times in 2xSSC for 5 min. After that, 0.01% pepsin solution was added and the slides were

incubated for 10 min at 37 °C, then washed 3 times in PBS (phosphate-buffered saline) for 5 min. The slides were incubated for 10 min at room temperature in 1% formaldehyde solution (2.7 ml 37% formaldehyde, 250 µl $MgCl_2$ 1M, filled up to 100 ml with PBS), washed 3 times in PBS for 5 min and dehydrated using ethanol series of 70%, 85% and 96% ethanol (5 min in each) and left to dry in a vertical position. The chromosomal material was denaturated in 70% formamide solution (100 ml prepared as: 70 ml of formamide, 10 ml of 20xSSC and 20 ml of ddH_2O) for 4 min at 72 °C and washed in 2xSSC for 1 min. The slides were then dehydrated using ethanol series once again and left to dry. The previously prepared probes were preheated to 37°C, then denaturated at 73 °C for 6 min and quickly cooled at -20 °C for 10 min. 22 µl of the probe was added per each slide (per 2 drops of chromosomal material). The slides were covered with a cover slide and left to hybridize overnight.

Next day, the slides were incubated in 2xSSC for 2 min to remove the cover slide. They were then washed 3 times in 50% formamide solution (100 ml prepared as: 50 ml of formamide, 10 ml of 20xSSC and 40 ml of ddH_2O) at 37°C for 5 min to remove the excess probe. The slides were then washed twice in 2xSSC and once in 4T (1 l prepared as: 200 ml of 20xSSC, 800 ml of ddH_2O a 500 µl of Tween-20; Sigma) for 5 min. After that, 200 µl of 4B solution (5% blocking agent (Roche) in 4xSSC) was added to the slide and the slides were left to incubate at 37°C for 45 min. In the next step, 200 µl of 4B containing 0.2 µl of avidin-FICH (Vector Laboratories) was added to each slide and the slides were left to incubate at 37 °C for 30 min. The slides were then washed 3 times in 4T for 5 min. The fluorescence signal was then amplified with 200 µl of 4B with 2 µl of biotinylated anti-avidin (Vector Laboratories) per slide and the slides were left to incubate at 37 °C for 30 min and washed 3 times in 4T for 5 min. These steps using 4B with avidin-FICH or anti-avidin to amplify the signal were repeated one more time with one additional avidin-FICH application. The slides were then washed twice in 4T and once in PBS for 5 min, dehydrated using the ethanol series and left to dry in a vertical position. Fluoroshield with DAPI (Vector Laboratories) was added to each slide and covered with a cover slide.

Microscopy analysis

Provis AX70 (Olympus) fluorescence microscope with DP30BW digital camera (Olympus) was used to analyze the results of C-banding and FISH. All the images were acquired in black and white and later processed in DP Manager imaging software (Olympus).

– RESULTS –

Species verification

Obtained sequences of two mitochondrial genes (CYTB, COI) from each tested animal were compared with available sequences in the Genbank database using the Basic local alignment search tool – "BLAST" (Altschul *et al.* 1990) (Table 4).

Table 4: Table of the most similar sequences found in Genbank. Only sequences with percent identity higher than 90% were included. In the case sequences from both studied individuals of the same species were aligned to the same available sequence, percent identity of both is depicted.

Studied species	N. of studied individuals	Gene	The most similar sequence description	Percent identity	Accession
Cylidrophis ruffus	1	CYTB	*Cylindrophis ruffus* mitochondrial DNA, complete genome	**100%**	AB179619.1
		COI	*Cylindrophis ruffus* mitochondrial DNA, complete genome	**100%**	AB179619.1
Eryx colubrinus	2	CYTB	*Eryx colubrinus colubrinus* cytochrome b (cytb) gene, mitochondrial gene encoding mitochondrial protein, partial cds	**99.06%; 97.43%**	U69811.1
		COI	-	-	-
Eryx conicus	2	CYTB	*Eryx conicus* cytochrome b (cytb) gene, mitochondrial gene encoding mitochondrial protein, partial cds	**99.40%; 99.29%**	U69817.1
		COI	-	-	-
Eryx miliaris	2	CYTB	*Eryx tataricus* voucher UMFS 11688 cytochrome b (cytb) gene, partial cds; mitochondrial	**99.43%; 98.69%**	KF811117.1
		COI	*Eryx tataricus* mitochondrion, complete genome	**99.09%; 99.09%**	MN646174.1
Eryx muelleri	2	CYTB	*Eryx muelleri* voucher UMFS 11723 cytochrome b (cytb) gene, partial cds; mitochondrial	**99.85%; 99.85%**	KF811116.1
		COI	-	-	-
Eryx tataricus	2	CYTB	*Eryx tataricus* mitochondrion, complete genome	**99.55%; 99.43%**	MK780743.1
		COI	*Eryx tataricus* mitochondrion, complete genome	**99.67%; 99.85%**	MN646174.1
Python regius	2	CYTB	*Python regius* cytochrome b (cytb) gene, mitochondrial gene encoding mitochondrial protein, partial cds	**99.31%**	U69856.1
			Python regius mitochondrial DNA, complete genome	**99.62%**	AB177878.1
		COI	*Python regius* haplotype hap5 cytochrome c oxidase subunit 1 (cox1) gene, partial cds; mitochondrial	**100%**	MN295678.1
			Python regius haplotype hap3 cytochrome c oxidase subunit 1 (cox1) gene, partial cds; mitochondrial	**100%**	MN295676.1
Tropidophis melanurus	1	CYTB	*Tropidophis canus* curtus voucher Tcurt1 cytochrome b (cytb) gene, partial cds; mitochondrial	**93.17%**	KF811123.1
		COI	*Tropidophis melanurus* voucher USNM:Herp:515936 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	**98.78%**	MH274736.1
Ophiophagus hannah	2	CYTB	*Ophiophagus hannah* mitochondrion, complete genome	**99.91%; 98.36%**	EU921899.1
		COI	*Ophiophagus hannah* voucher PUCZM/X/SL819 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	**99.04%; 99.20%**	MH107864.1

Karyotype reconstruction and C-banding

Cylindrophis ruffus

The karyotype of *Cylindrophis ruffus* was reconstructed based on one female individual. It has 36 chromosomes in the diploid state, out of which 16 macrochromosomes and 20 microchromosomes (Fig. 2a). The first, third and fourth chromosome pairs are metacentric, the second is submetacentric, the pairs 5–8 are acrocentric. Morphology of microchromosomes is not distinguishable. Heteromorphic sex chromosomes are not present.

A constitutive heterochromatin was detected in the centromeric region of all chromosomes and the terminal region of the q-arm of the second chromosome pair (Fig. 2b).

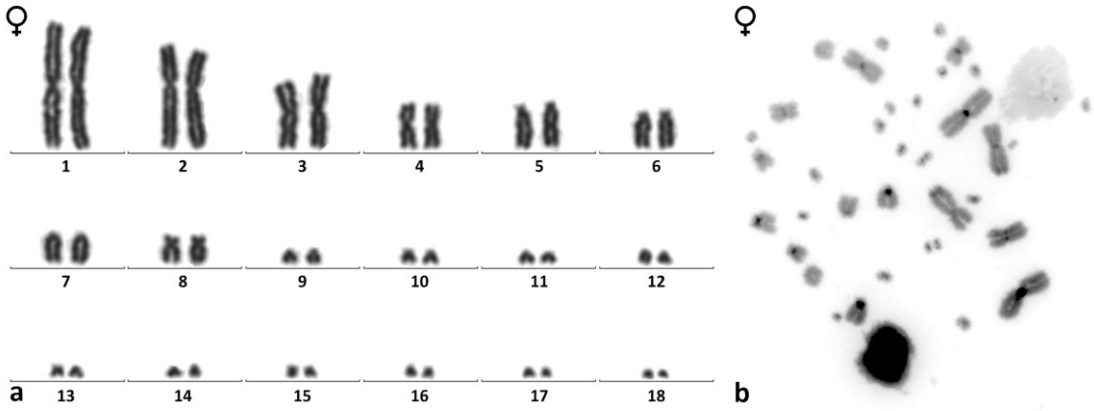


Fig. 2: Karyogram (a) and C-banding (b) of *Cylindrophis ruffus.* Sex is indicated.

Eryx colubrinus

The karyotype of *Eryx colubrinus* consists of 34 chromosomes in the diploid state, out of which 20 are macrochromosomes and 14 microchromosomes (Fig. 3a,c). The first, third and fourth chromosome pairs are metacentric, the second and ninth submetacentric and the remaining macrochromosome pairs are acrocentric. It is not possible to determine the morphology of the microchromosomes. Heteromorphic sex chromosomes are not present.

The heterochromatin accumulates in the pericentromeric region on the q arms of the pairs 4, 5, 6 and 8. The pairs 9 and 10 are heavily heterochromatinized and five pairs of microchromosomes show heterochromatin accumulations as well. Only one chromosome from the seventh pair was observed with a heterochromatic block in the telomeric region of the q arm. This state was however observed in both sexes (Fig. 3b,d).

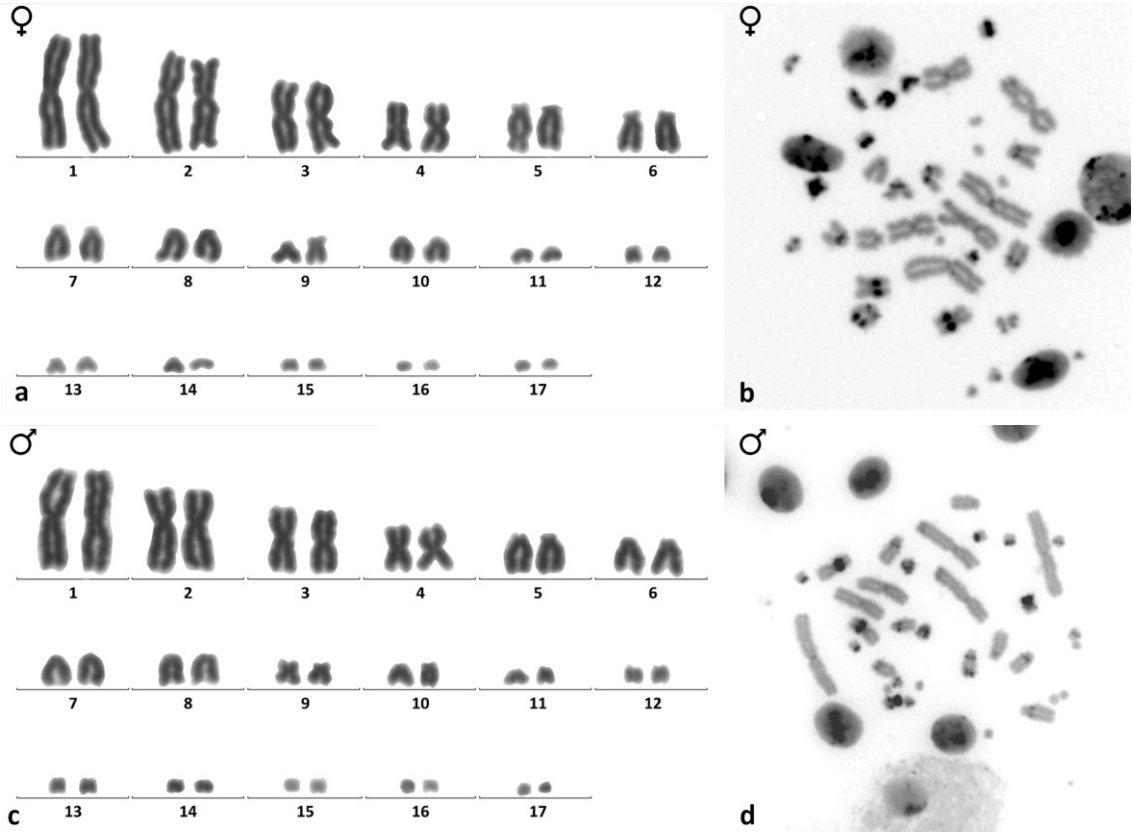


Fig. 3: Karyogram (a,c) and C-banding (b,d) of *Eryx colubrinus*. Sex is indicated.

Eryx conicus

The karyotype of *Eryx conicus* was constructed for the first time in 1972 by Singh. In agreement with the original study, we found that this species has 34 chromosomes in diploid state, out of which 16 are macrochromosomes and 18 microchromosomes (Fig. 4a,c). The first, third and fourth chromosome pairs are metacentric, the second is submetacentric and the remaining macrochromosomes have centromeres in their terminal parts. Morphology of the microchromosome pairs is not distinguishable. Heteromorphic sex chromosomes are not present.

The heterochromatin was detected in the centromeric region of all macrochromosomes. In addition, the second chromosomal pair has a heterochromatic block in the terminal region of the q arm (Fig. 4b,d).

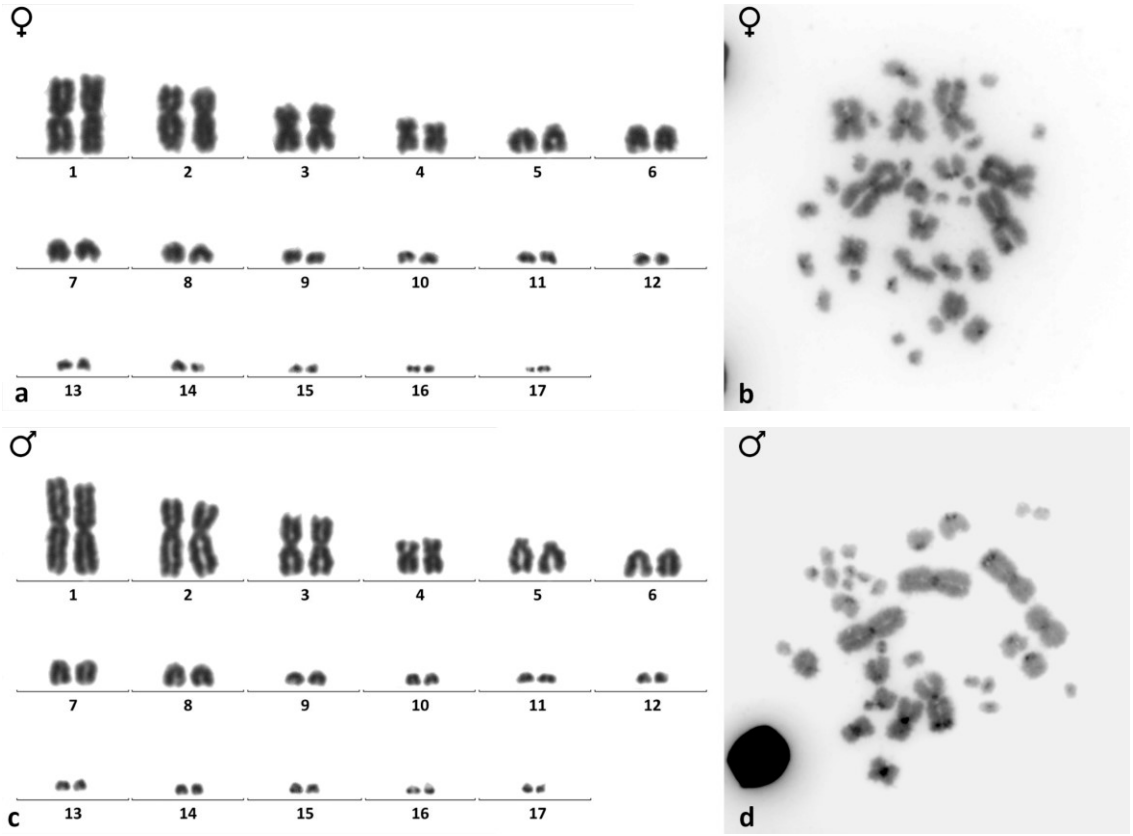


Fig. 4: Karyogram (a,c) and C-banding (b,d) of *Eryx conicus.* **S**ex is indicated.

Eryx miliaris

Eryx miliaris has the diploid number of chromosomes 34, out of which 16 are macrochromosomes and 18 microchromosomes (Fig. 5a,c). The first, third and fourth chromosome pairs are metacentric, the second is submetacentric and the remaining macrochromosome pairs are acrocentric. Because of their size, it is not possible to determine the morphology of the microchromosomes. Heteromorphic sex chromosomes are not present.

The heterochromatin signal was detected in the pericentromeric region of the second and eight chromosomal pair and in the terminal region of the q arm of the second chromosome pair. Only one chromosome from the fourth pair was observed with a large heterochromatic block in the pericentromeric region of the q arm. As in the case of *Eryx colubrinus*, this state was observed in both sexes (Fig 5b,d).

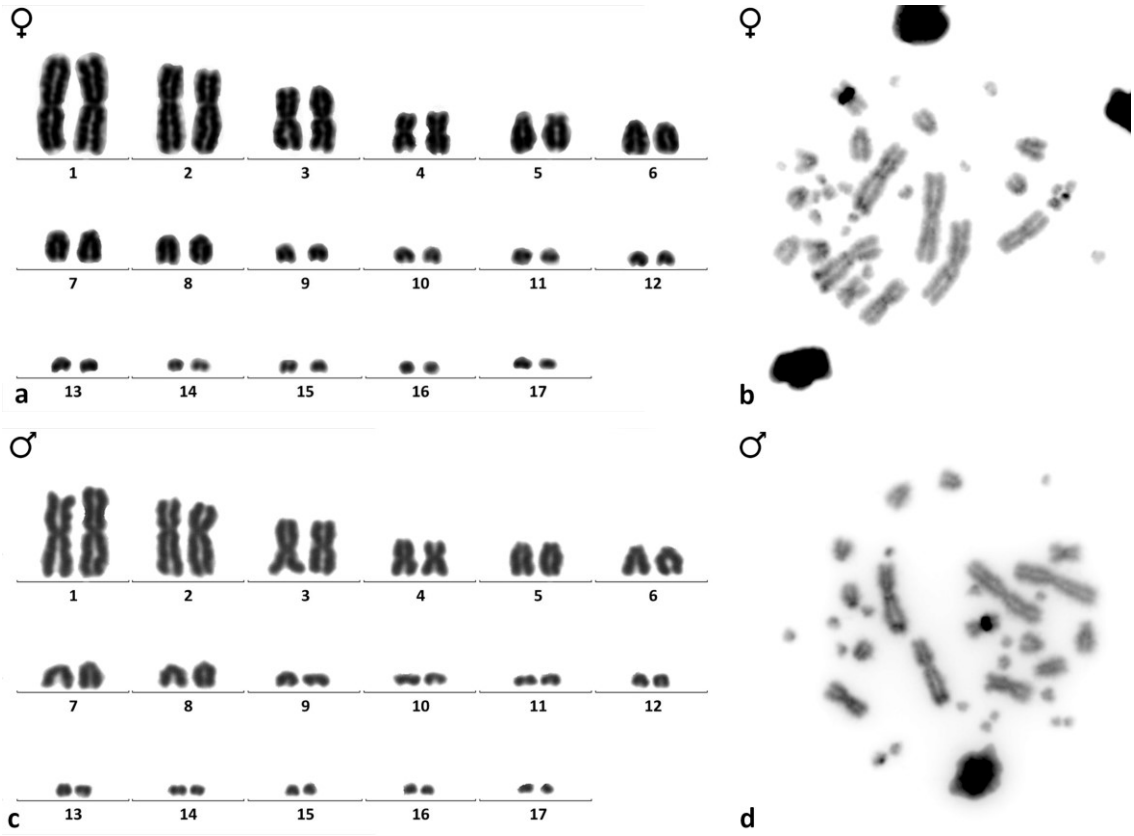


Fig. 5: Karyogram (a,c) and C-banding (b,d) of *Eryx miliaris.* Sex is indicated.

Eryx muelleri

The karyotype of *Eryx muelleri* has 36 chromosomes in the diploid state with 16 macro- and 18 microchromosomes (Fig. 6a,c). The first, third and fourth chromosome pairs are metacentric, the second is submetacentric and the remaining macrochromosome pairs are acrocentric. It is not possible to determine the morphology of the microchromosomes. Heteromorphic sex chromosomes are not present.

The heterochromatin was detected in the centromeric region on all macrochromosomes. The second chromosome pair has a heterochromatic block in the terminal region of the q arm (Fig. 6b,d).

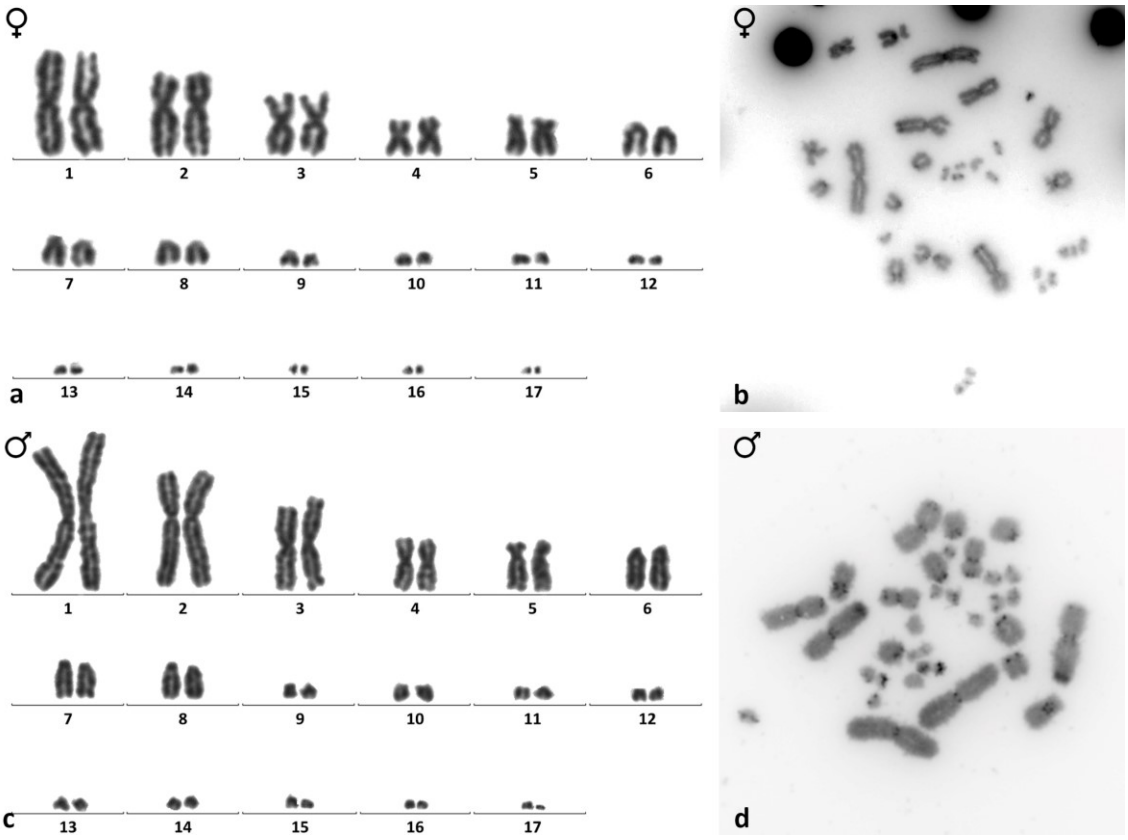


Fig. 6: Karyogram (a,c) and C-banding (b,d) of *Eryx muelleri.* Sex is indicated.

Eryx tataricus

The diploid chromosome number of *Eryx tataricus* is 34, out of which 16 are macrochromosomes and 18 microchromosomes (Fig. 7a,c). The first, third and fourth chromosome pairs are metacentric, the second is submetacentric, the pairs 5–8 are acrocentric. Morphology of microchromosomes is not resolved. Heteromorphic sex chromosomes are not present.

The heterochromatin accumulates in a small amount on all macrochromosomes in the pericentromeric region and the terminal region of the q arm of the second chromosome pair (Fig. 7b,d).

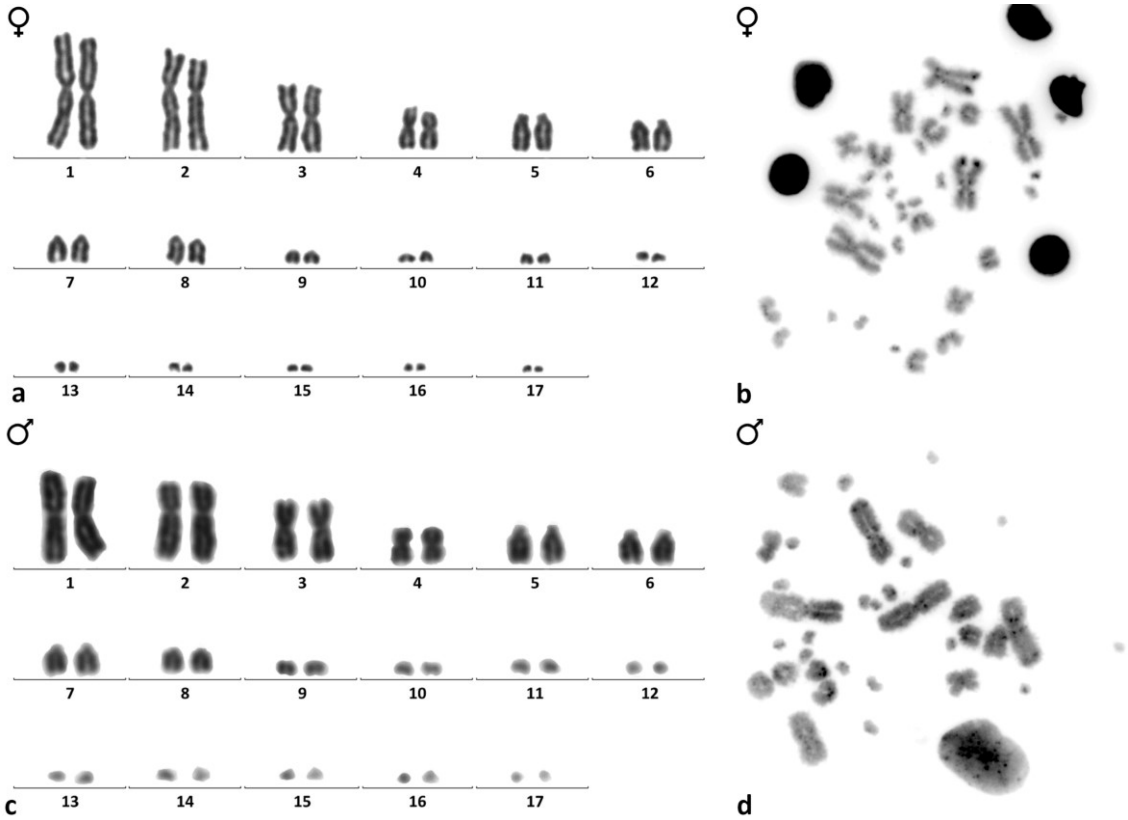


Fig. 7: Karyogram (a,c) and C-banding (b,d) of *Eryx tataricus.* Sex is indicated.

Python regius

The karyotype of *Python regius* has 36 chromosomes in the diploid state with 16 macro- and 20 microchromosomes (Fig. 8a,c). The first, third and fourth chromosome pair is metacentric, the second is submetacentric, the pairs 5–8 are acrocentric. It is not possible to determine the morphology of the microchromosomes. Heteromorphic sex chromosomes are not present.

The heterochromatin accumulates in the centromeric region of all chromosomes in this species (Fig. 8b,d).

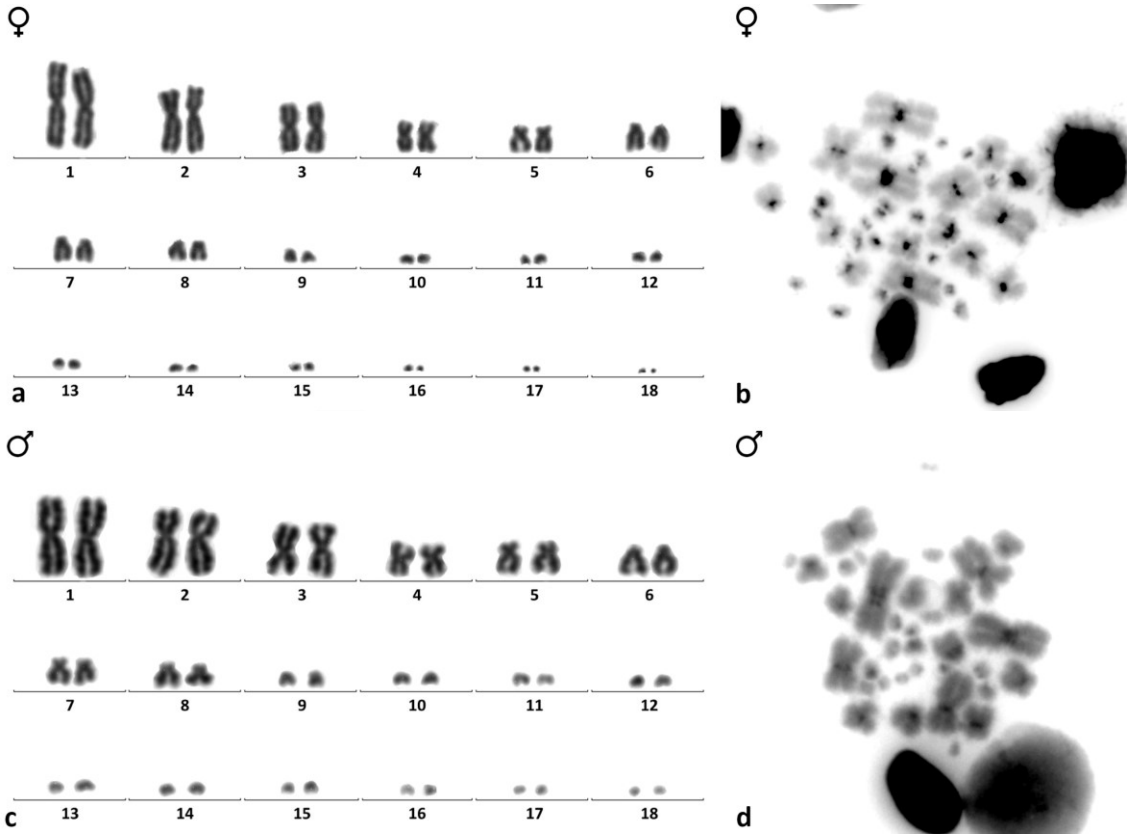


Fig. 8: Karyogram (a,c) and C-banding (b,d) of *Python regius.* Sex is indicated.

Tropidophis melanurus

For the karyotype reconstruction of *Tropidophis melanurus*, only one female individual was examined. It has 34 chromosomes in the diploid state out of which 22 are macrochromosomes and 12 microchromosomes (Fig. 9). The first three chromosome pairs are submetacentric and the remaining macrochromosome pairs are acrocentric. Morphology of microchromosomes was not identified.

C-banding did not reveal any visible heterochromatin patterns in this species.

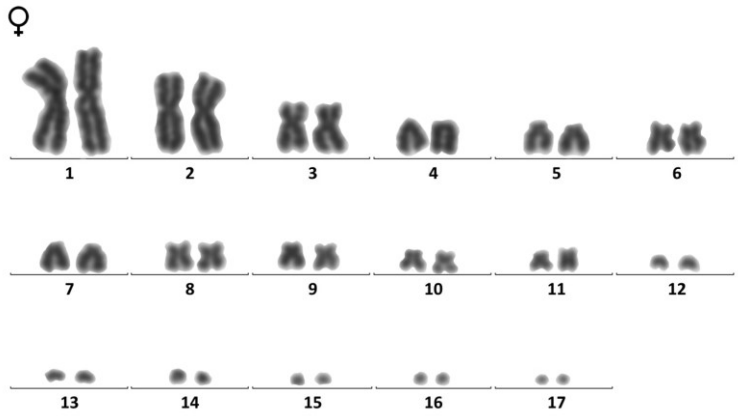


Fig. 9: Karyogram of *Tropidophis melanurus* Sex is indicated.

Ophiophagus hannah

The karyotype of the king cobra was reconstructed for the first time in 1982 in the doctoral thesis by Mengden (1982) using only male specimens. It was reported that king cobras have 36 chromosomes in the diploid state with 18 macro- and 18 microchromosomes. While our data support this statement for males, the karyotype of the studied female has 37 chromosomes with 18 macro- and 19 microchromosomes (Fig. 10a,c). The ZW sex chromosomes are the fourth chromosome pair.

The first and third chromosome pair is metacentric, the second as well as the Z chromosome are submetacentric. The W chromosome is much larger than the Z and it is telocentric. The remaining macrochromosomes are acrocentric. Morphology of microchromosomes remains unresolved.

Heterochromatin signal was detected in the centromeric region of all macrochromosomes. The W chromosome is completely heterochromatic (Fig. 10b,d).

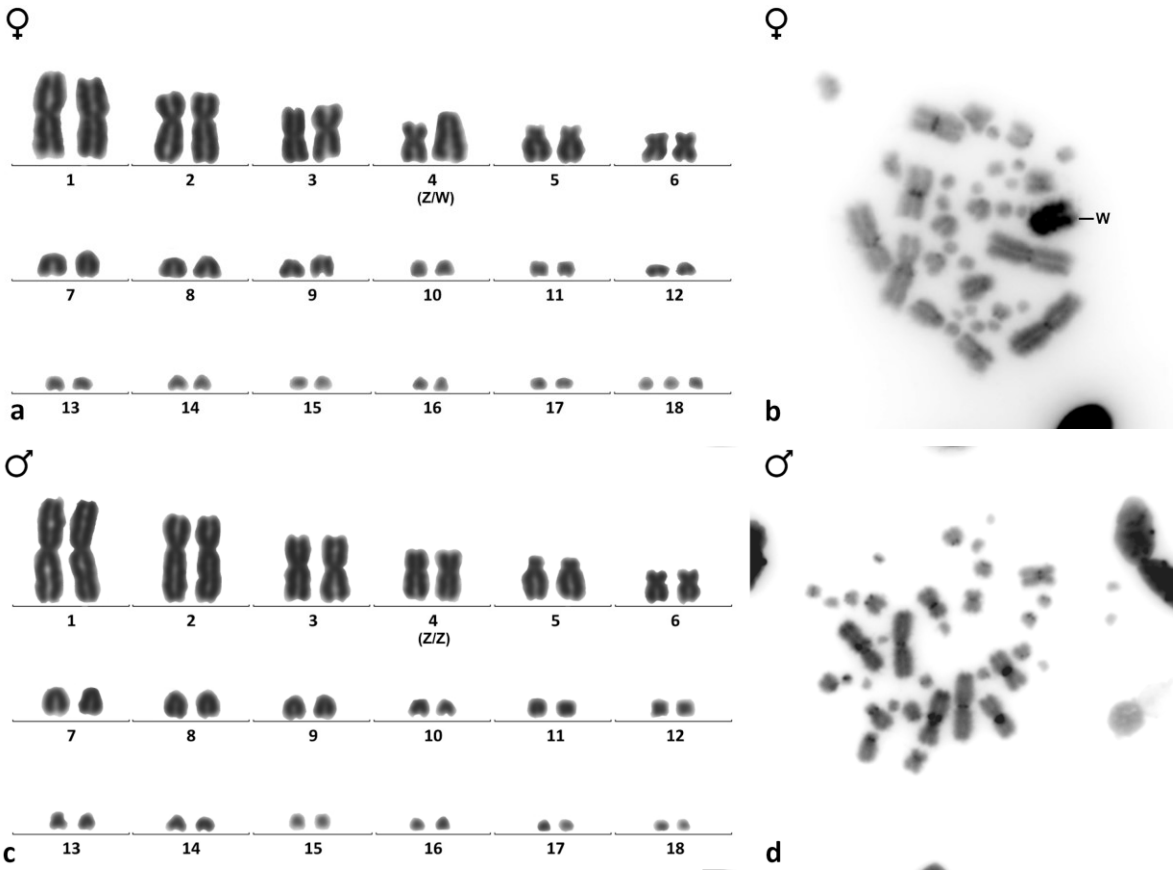


Fig. 10: Karyogram (a,c) and C-banding (b,d) of *Ophiophagus hannah.* Sex is indicated.

Fluorescence *in situ* hybridization with telomeric probe

FISH with the telomeric probe was performed to determine the $(TTAGGG)_n$ motif distribution in each species. In the case of *Eryx colubrinus*, *E. conicus*, *E. miliaris*, *E. tataricus*, *Cylindrophis ruffus*, *Python regius* and *Tropidophis melanurus* the signal was detected only in the telomeric regions of the chromosomes (Fig. 11). These species thus have no interstitial telomeric repeats in their karyotypes. In contrast, ITRs were detected in *Eryx muelleri* and *Ophiophagus hannah* (Fig. 12). In the case of *E. muelleri*, ITRs are located in the centromeric regions of the first three pairs of macrochromosomes. No sex-specific amplification was observed in this species. *Ophiophagus hannah* has ITRs in the pericentromeric region of both arms of the second macrochromosome pair. The signal from the q arm of the chromosomes is much stronger than on the p arm. The W chromosome shows extensive accumulation of ITRs in three different locations spread through the whole length of the chromosome.

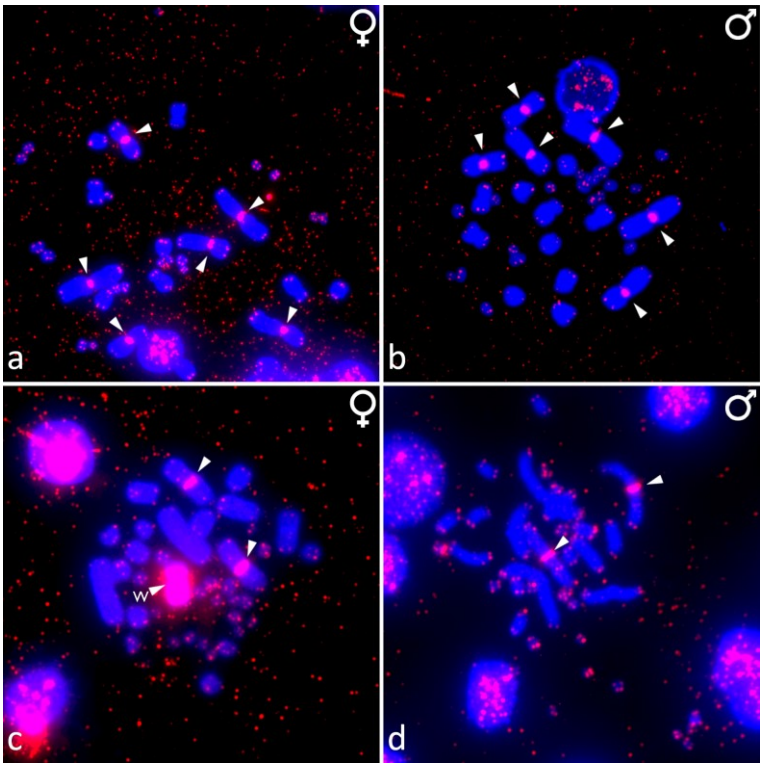


Fig. 12: FISH with telomeric probe of *Eryx muelleri* (a,b), *Ophiophagus hannah* (c,d). Sex is indicated.

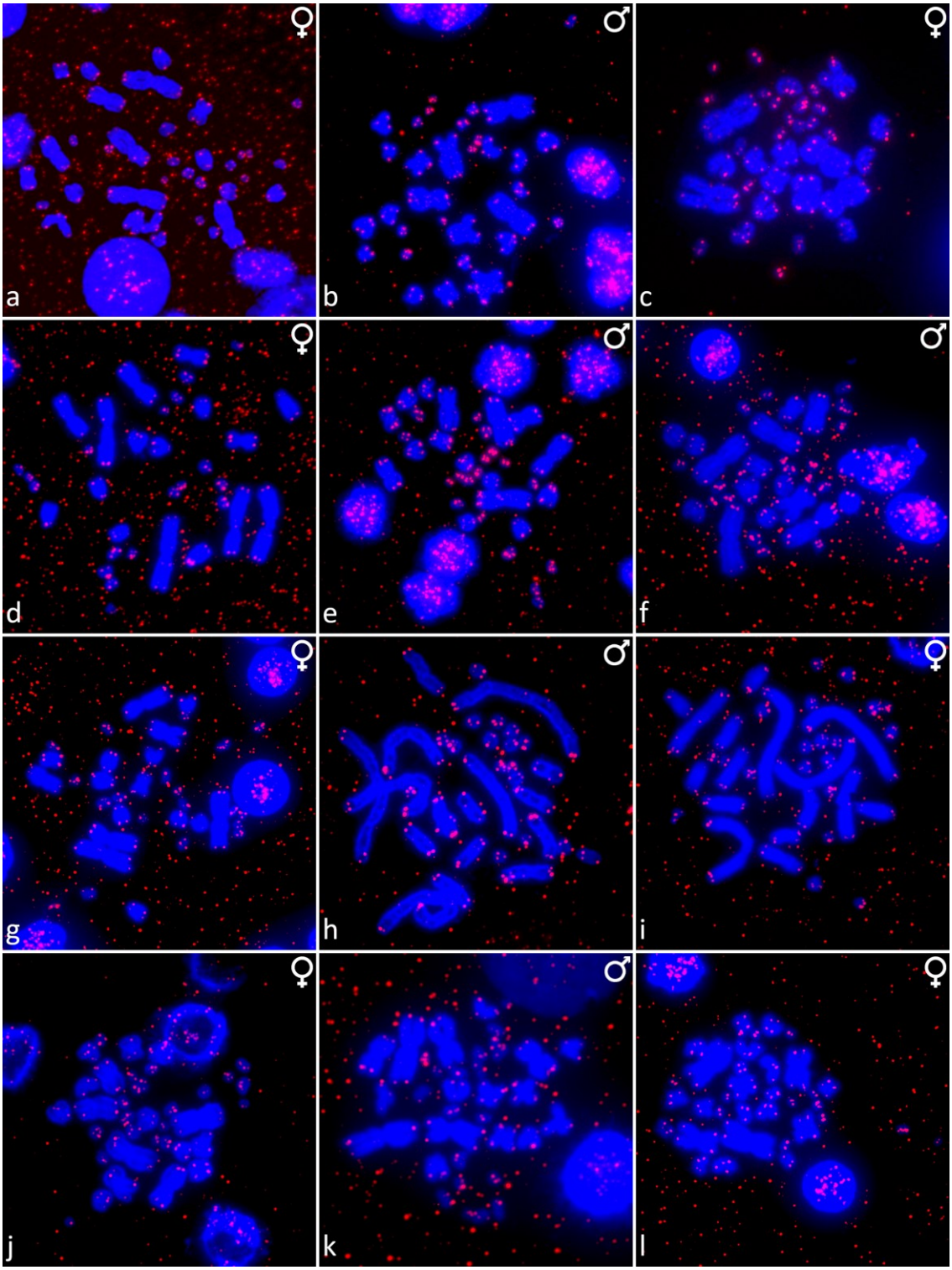


Fig. 11: FISH with telomeric probe of *Eryx colubrinus* (a,b), *E. conicus* (c,f), *E. miliaris* (d,e), *E. tataricus* (g,h), *Cylindrophis ruffus* (i), *Python regius* (j,k) and *Tropidophis melanurus* (l). Sex is indicated.

Fluorescence *in situ* hybridization with rDNA probe

The distribution of ribosomal RNA gene repeats was explored using FISH with an rDNA probe in all species included in this thesis, except for *Python regius* due to the limited amount of chromosomal material obtained from this species. We discovered that rDNA loci are located at one microchromosome pair in *Eryx colubrinus*, *E. conicus*, *E. miliaris* (Fig. 13), *E. tataricus, E. muelleri*, *Ophiophagus hannah* and *Cylindrophis ruffus* (Fig. 14). In *Tropidophis melanurus* rDNA repeats accumulate on the 10th macrochromosome pair (Fig. 14h). No sex-specific differences in the distribution of rDNA loci were observed in any of the studied species.

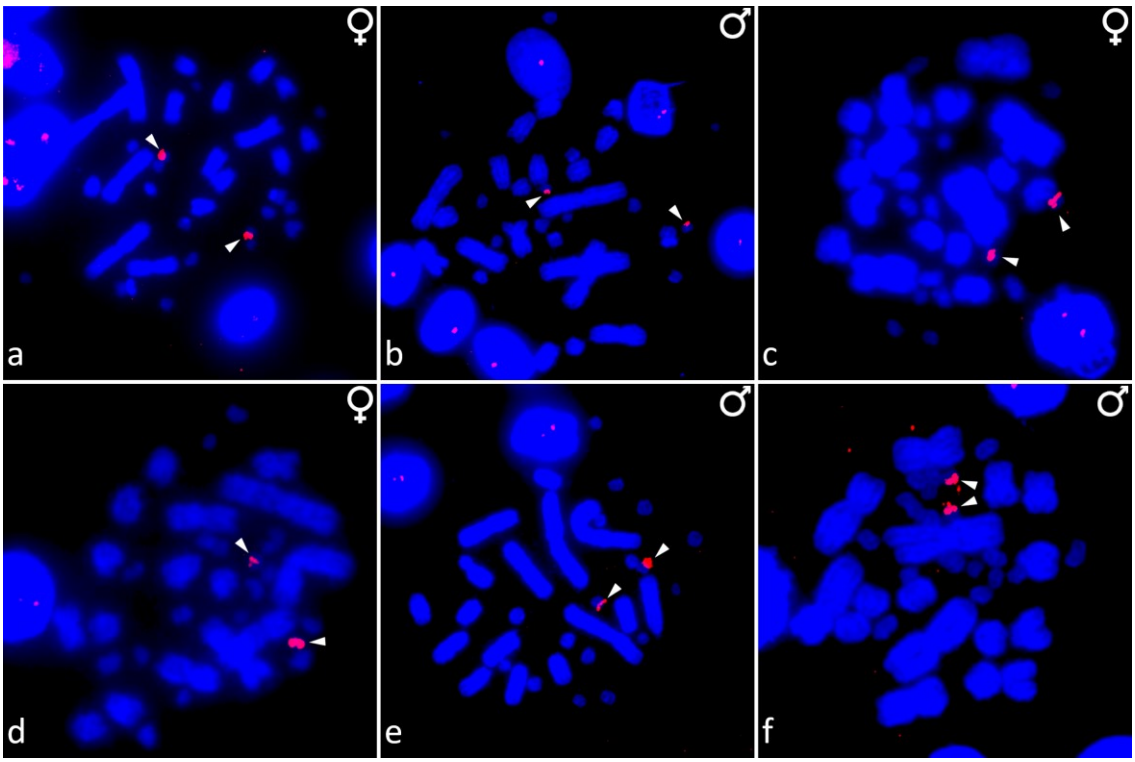


Fig. 13: FISH with rDNA probe of *Eryx colubrinus* (a,b), *E. conicus* (c,f) and *E. miliaris* (d,e). Sex is indicated.

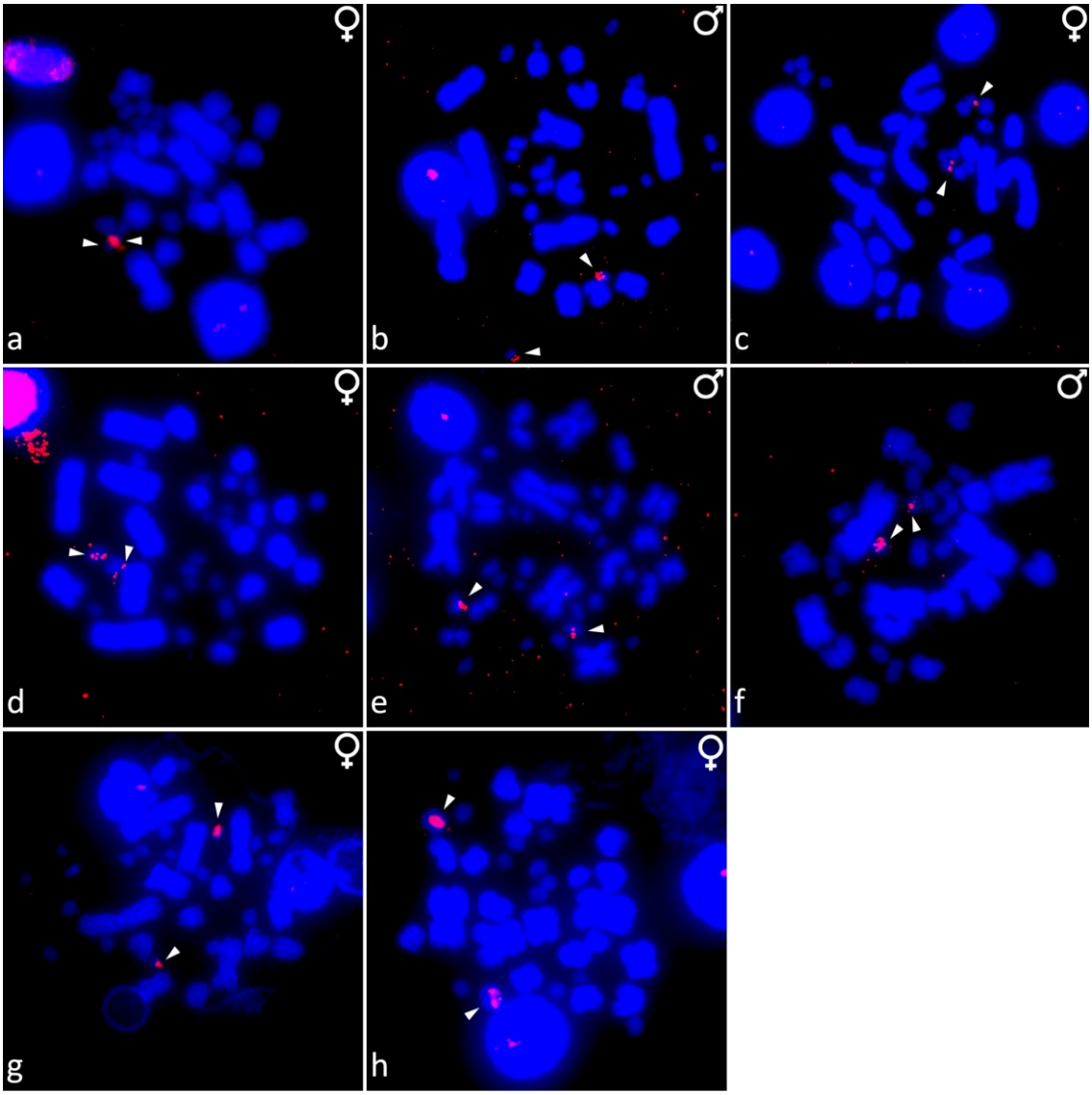


Fig. 14: FISH with rDNA probe of *Eryx muelleri* (a,b), *E. tataricus* (c,f), *Ophiophagus hannah* (d,e), *Cylindrophis ruffus* (g) and *Tropidophis melanurus* (h). Sex is indicated.

– DISCUSSION –

We cytogenetically examined nine snake species out of which only *Eryx conicus*, *Cylindrophis ruffus* and *Ophiophagus hannah* had previously known karyotypes. All tested sand boas have 34 chromosomes in the diploid state. However, while *E. colubrinus* has 20 macrochromosomes and 14 microchromosomes, the rest of the tested *Eryx* species as well as two previously studied sand boa species have 16 macrochromosomes and 18 microchromosomes (Singh *et al.* 1968; Olmo & Signorino 2005). It is feasible that two pairs of former microchromosomes increased in size in *E. colubrinus*, possibly by amplification of repetitive elements, as both chromosome pairs show strong constitutive heterochromatin accumulation. Alternatively, a small chromosomal region might have been translocated from a macrochromosome to a microchromosome. Nevertheless, both alternative scenarios are just a speculation as none of the repetitive elements used in the thesis proved to accumulate on these chromosomes. Comparative BAC-FISH, chromosome painting or whole genome sequencing at the chromosome level could resolve the mechanism leading to this difference in chromosome morphology. Despite the slight differences between chromosome morphology in *E. colubrinus* and the rest of the sand boa species, all representatives of the subfamily Erycinae share chromosome number of 2n = 34, while their closest relatives have mostly the common number of 2n = 36 (Fig. 15). This chromosome number might thus be an apomorphy of sand boas, however it might be also the result of convergent evolution as the tendency to decrease chromosome number was also observed in the subfamily Sanziniinae (Boidae) (Mengden & Stock 1980). This however cannot be interpreted as a general trend for Boidae, as the genus *Corallus* shows exceptional increase in chromosome numbers, unparalleled by any other henophidian lineage (Olmo & Signorino 2005; Viana *et al.* 2016).

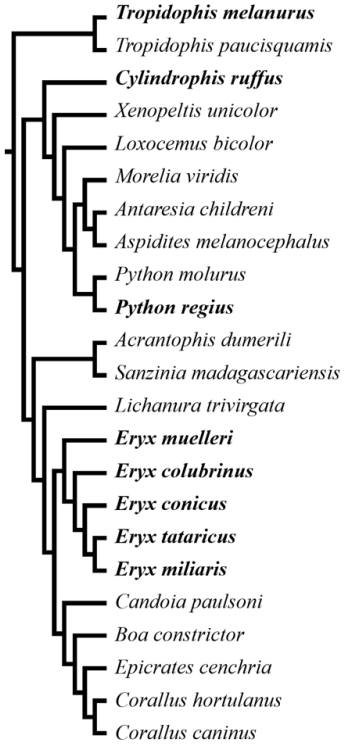
Because cryptic species as well as species complexes are rather common in reptiles (e.g. Donnellan *et al.* 1993; Harris & Sa-Sousa 2002; Oliver *et al.* 2009; Gvoždík *et al.* 2010), the species status of all tested snakes was verified using sequences of two mitochondrial genes: cytochrome b and cytochrome c oxidase subunit I. Interestingly, the BLAST nucleotide search revealed that the sequences from tested *Eryx miliaris* specimens are slightly more similar to the available sequences of *E. tataricus* than of *E. miliaris*. The difference between the available sequences of both species is however

small (<5%) and some authors claim that these two forms are just a single species (Reynolds *et al.* 2014). We found differences between these species in C-banding patterns, although with unequal distribution of heterochromatin in *E. miliaris*. This might be just a result of hybridization of animals from two different populations with different heterochromatin patterns. Unequal distribution of constitutive heterochromatin forming a heterochromatin heteromorphism was observed not only in *Eryx miliaris* but also in *E. colubrinus.* As this state was observed in both sexes, discovered heteromorphism is likely not linked to sex chromosomes. Beside the already mentioned hybrid nature of such heteromorphism, mechanism of how such polymorphism might be conserved in a population was described on European newts from the genus *Triturus*. The first chromosome pair of *T. carnifex* and *T. marmoratus* is heteromorphic in size but also in heterochromatin patterns. Embryos of both species with homomorphic chromosomes of the first pair fail to develop and only individuals with observed heteromorphism survive (Sims *et al.* 1984). Heterochromatin heteromorphism was described in many species in the past and was proposed to be the cause of some diseases in humans (Heneen *et* al. 1980; Freitas *et al.* 1982; Haaf & Schmid 1987; Bressa *et al.* 2008; Ferreira *et al.* 2019).

Beside the presence of heterochromatin blocks, C-banding also uncovered interesting variability in chromosomal resistance to $Ba(OH)_2$ treatment among tested species. Even closely related species, such as *Eryx colubrinus* and *E. muelleri* differ greatly in the treatment times needed to visualize the constitutive heterochromatin (Table 1).

The diploid karyotype with 34 chromosomes (22 macro- and 12 microchromosomes) was revealed also in *Tropidophis melanurus* (Fig. 9). This result is interesting as the only other species from this family with known karyotype (*Tropidophis paucisquamis*) was reported to have 26 chromosomes (Oguiura *et al.* 2009). Such diversity is unusual in henophidian snakes and more species from this family will have to be tested to better understand the karyotype evolution in this lineage. C-banding did not reveal any heterochromatic blocks in *Tropidophis melanurus* even when long treatment times were used. Similar scenario was observed also in other henophidian snakes (Augstenová *et al*. 2019). Karyotype of *Cylindrophis ruffu*s has 36 chromosomes (16 macro- and 20 microchromosomes). The same chromosome number has also

Python regius, which is not surprising as all other previously studied representatives of the *Python* genus share the same karyotype (Singh *et al.* 1968; Olmo & Signorino 2005).



Family	Chromosome number	Reference
Tropidophiidae	**34 (22+12)**	**this thesis**
Tropidophiidae	26	Batistic *et al.* (2002) in Oguiura *et al.* (2009)
Cylindrophiidae	**36 (16+20)**	**this thesis**
Xenopeltidae	36 (16+20)	Cole & Dowling (1970) in Olmo & Signorino (2005)
Loxocemidae	36	Fischman *et al.* (1972) in Olmo & Signorino (2005)
Pythonidae	36 (16+20)	Mengden (1982)
Pythonidae	36 (16+20)	Mengden (1982)
Pythonidae	36 (16+20)	Mengden (1982)
Pythonidae	36 (16+20)	Singh *et al.* (1968)
Pythonidae	**36 (16+20)**	**this thesis**
Boidae	34 (16+18)	Mengden & Stock (1980)
Boidae	34 (18+16)	Mengden & Stock (1980)
Boidae	36 (16+20)	Augstenová *et al.* (2019)
Boidae	**34 (16+18)**	**this thesis**
Boidae	**34 (20+14)**	**this thesis**
Boidae	**34 (16+18)**	**this thesis**
Boidae	**34 (16+18)**	**this thesis**
Boidae	**34 (16+18)**	**this thesis**
Boidae	36 (16+20)	Augstenová *et al.* (2019)
Boidae	36 (16+20)	Beçak & Beçak (1972)
Boidae	36 (16+20)	Augstenová *et al.* (2019)
Boidae	40 (20+20)	Beçak (1965) in Olmo & Signorino (2005)
Boidae	44 (24+20)	Viana *et al.* (2016)

Fig. 15: Evolution of chromosome numbers in henophidian snakes. Phylogenetic relationships inferred from Reynolds *et al.* (2014).

The karyotype of the studied female king cobra (*Ophiophagus hannah*) has 37 chromosomes (18 macro- and 19 microchromosomes) in diploid state, while the karyotype of studied male has 36 chromosomes (18 macro- and 18 microchromosomes). There are several possible explanations for this state. As chromosome aberrations were described in snakes, it is possible that the odd number of microchromosomes might be due to aneuploidy of one of the microchromosome pairs (Beçak & Beçak 2003). Snakes seem to be quite tolerant to polyploidy or even functional "aneuploidy" evolved via degeneration of the W, as they show no signs of global dosage compensation (Wynn *et al.* 1987; Vicoso *et al.* 2013; Rovatsos *et al.* 2018b). Although less likely, observed aneuploidy can be even a technical artifact and could have been induced artificially in the

cell culture. It is also feasible that the extra microchromosome is a B-chromosome. B-chromosomes are "*additional dispensable chromosomes that are present in some individuals from some populations in some species, which have probably arisen from the A chromosomes but follow their own evolutionary pathway*" (Camacho & Parker in Beukeboom 1994). They were found in hundreds of species from numerous plant and animal lineages, including reptiles (reviewed in Camacho 2005). We also cannot rule out the potential role of hybridization. Even though the studied female comes from captive breeding, her parents were wild caught individuals of unknown origin. It is possible that wild populations of this species differ in chromosome number and their F_1 hybrids show an intermediate chromosome number (Badr & Badr 1970). The other possible explanation is that the extra microchromosome in the female karyotype could be a W_2 chromosome. King cobras belong to the family Elapidae, which includes all known snake species with multiple sex chromosomes. For example, the already mentioned representative of this family, *Enhydrina schistosa*, has two W chromosomes, moreover the W_2 is a microchromosome (Singh 1972b). FISH with a telomeric probe uncovered extensive amplification of ITRs on the W of the studied female. ITRs can accumulate on the W due to the lack of recombination. Furthermore, female-specific hormone-induced increase in telomerase activity, as demonstrated in the Siamese cobra (*Naja kaouthia*), could have strengthened the effect and further promote ITR amplification on the W (Singchat *et al.* 2019). Chromosomal regions that are rich in ITRs were shown to be more unstable and prone to breakage and subsequent chromosomal rearrangements (Bolzán & Bianchi 2006). It is thus plausible that the putative W_2 chromosome originated by fission of the original W. More female individuals of this species will have to be tested in the future and/or Comparative genomic hybridization (CGH) experiments will have to be performed to uncover the true nature of the extra microchromosome.

ITRs were found also in the pericentromeric region of the second chromosome pair in *Ophiophagus hannah* and in the centromeric region of the first three chromosome pairs in *Eryx muelleri* but not in the other sand boas. Because the morphology of these chromosomes is relatively conserved among snakes, and both *O. hannah* and *E. muelleri* are not an exception, it is unlikely that the observed ITRs are an outcome of interchromosomal rearrangements in these species (Beçak & Beçak 1969; Mengden 1982; Mezzasalma *et al.* 2016; Augstenová *et al.* 2019). The more feasible explanation might be the intrachromosomal rearrangements, likely inversions or retrotranspositions.

It was not long ago postulated that ITRs accumulate on chromosomes of squamate reptiles more than previously expected, despite their generally conserved karyotype (Rovatsos *et al.* 2015a). This seems to be true, as the presence of ITRs was also proved in many recently studied snakes (Viana *et al.* 2016; Augstenová *et al.* 2019; Matsubara *et al.* 2019).

rDNA loci are located at one microchromosome pair in all tested species, except for *Tropidophis melanurus*. This arrangement seems to be quite common in so far studied species of henophidian snakes, although rDNA loci were also shown to accumulate even on multiple chromosome pairs (Augstenová *et al.* 2019). In *T. melanurus,* rDNA loci are located on one macrochromosome pair. Sex specific accumulation of rDNA or ITRs was not observed in any henophidian species. Because heteromorphic sex chromosomes are absent in all tested henophidian snakes, we can assume, that these species have poorly differentiated homomorphic sex chromosomes. Nevertheless, we cannot rule out that some henophidian snakes might also show TSD, although such sex determination system has not been yet reported in snakes.

– CONCLUSIONS –

This thesis is a cytogenetic study of eight henophidian and one caenophidian snake species, with conventional and molecular cytogenetic methods. To the best of our knowledge, karyotypes are presented for the first time for six snake species, namely *Eryx colubrinus*, *E. miliaris*, *E. tataricus*, *E. muelleri*, *Python regius* and *Tropidophis melanurus*. We conclude that all so far tested sand boa species, with the exception of *Eryx colubrinus*, share diploid karyotype number of 34 (16 macro- and 18 microchromosomes) and chromosome morphology, despite the presence of ITRs on first three chromosome pairs in *E. muelleri*. Two former microchromosome pairs probably increased in size in the evolution of *E. colubrinus* karyotype, as it is otherwise shared with the rest of the sand boas. Interesting finding of 34 chromosomes in diploid state in *Tropidophis melanurus* suggests higher variability in chromosome numbers in the family Tropidophiidae. Sex chromosomes were not observed in any of the studied species of henophidian snakes by cytogenetic methods, even if XY sex chromosomes were expected in *Python regius*, based on the results from previous studies.

On the contrary, heteromorphic sex chromosomes were observed in the single studied representative of the group Caenophidia, the king cobra. The W is larger than Z, completely heterochromatic and shows extensive amplification of interstitial telomeric repeats. The karyotype of the studied female has 37 chromosomes in the diploid state, while the karyotype of the studied male has 36. Nevertheless, it requires further study to explore if this difference reflects hybridization, spontaneous aneuploidy, presence of B chromosomes or multiple sex chromosomes.

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