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Evoluce určení pohlaví a karyotypů u ještěrkovitých a příbuzných linií (Lacertoidea)

Evolution of sex determination and karyotypes in lacertid lizards and related lineages (Lacertoidea)

Doctoral thesis

Supervisor: Michail Rovatsos, PhD

Prague, 2024

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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.

Prague, August 2024 / Praha, srpen 2024

Grzegorz Tomasz Suwała

Preface & Acknowledgments

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Finally, I dedicate this work to my children: Kornelia, Gustaw, Tymoteusz, and Hugo. May you too find joy and beauty in the exploration of the natural world.

Abstract (English)

Lacertoidea, a clade of squamate reptiles, includes several families, with Lacertidae being the most species-rich and karyologically most explored. This widely diversified group is known for presence of all-acrocentric karyotypes and ZZ/ZW sex chromosomes. Despite this, the origins and evolutionary rates of their chromosomes have been previously unexplored. Additionally, the variability in their W sex chromosomes and reports of environmental sex determination (ESD) led some authors to believe in multiple independent origins of their sex chromosomes. This thesis addresses these gaps by applying bioinformatic, cytogenetic, and molecular approaches. We collected data on karyotypes and phylogenetic relationships of lacertoidean species to estimate the evolutionary rates of chromosome numbers between their clades via maximum likelihood models. Our analysis revealed that while the evolution of chromosome number is highly dynamic in Teiidae, Gymnophthalmidae, and Amphisbaenia, it is considerably decreased in Lacertidae. Further examination of chromosome synteny in lacertids and their outgroups indicated that two opposing processes - fusions of ancestral microchromosomes and fissions of homologs of bi-armed macrochromosomes - likely led to the origin of the derived and stable lacertid karyotype. Using fluorescence *in situ* hybridization (FISH) and C-banding, we examined the lacertid W chromosome across 15 species from ten genera, revealing species-specific accumulations of 22 microsatellite and telomeric motifs that do not follow the phylogenetic pattern. This suggests that the repetitive sequences within degenerated sex chromosomes are among the most evolutionarily dynamic regions of the genome. Finally, we examined the homology and the age of lacertid Z chromosomes across 45 species, including 26 genera, confirming homologous differentiated ZZ/ZW sex chromosomes dating back approximately 85 million years. This evidence challenges previous reports of transitions to ESD in lacertids, emphasising the stability of their sex determination system. Lacertid chromosomes demonstrate that derived all-acrocentric karyotypes and differentiated sex chromosomes can be extremely stable over a long evolutionary time.

Abstrakt (Česky)

Lacertoidea je skupina šupinatých plazů, která zahrnuje několik čeledí, z nichž druhově nejbohatší je čeleď Lacertidae (ještěrkovití). Ta je také nejvíce karyologicky prozkoumaná. Tato široce diverzifikovaná skupina je známá karyotypy s výhradně akrocentrickými chromosomy a ZZ/ZW pohlavními chromosomy. Navzdory tomu byly původ a evoluční rychlosti změn jejich chromosomů dosud neprozkoumány. Navíc variabilita ve vlastnostech W pohlavních chromosomů ještěrek a zprávy o environmentálním určení pohlaví (ESD) vedly některé autory k přesvědčení, že u ještěrek došlo k vícenásobným nezávislým vznikům pohlavních chromosomů. Tato práce se zabývá mezerami ve znalostech o genetice skupiny Lacertoidea pomocí bioinformatických, cytogenetických a molekulárních přístupů. Shromáždili jsme data o karyotypech a fylogenetických vztazích druhů skupiny, abychom odhadli evoluční rychlosti počtů chromosomů mezi jednotlivými jejich liniemi pomocí modelů maximální věrohodnosti. Naše analýza odhalila, že zatímco evoluce počtu chromozomů je vysoce dynamická u skupin Teiidae, Gymnophthalmidae a Amphisbaenia, u čeledi Lacertidae je značně snížena. Další zkoumání syntenie chromozomů ukázalo, že pravděpodobně dva protichůdné procesy vedly ke vzniku odvozeného a značně stabilního karyotypu ještěrek: fúze ancestrálních mikrochromosomů a rozpad homologů dvouramenných makrochromosomů. Pomocí fluorescenční *in situ* hybridizace (FISH) a C-pruhování jsme zkoumali W chromosom lacertidů u 15 druhů z deseti rodů ještěrek. Odhalili jsme druhově specifické akumulace 22 mikrosatelitních a telomerických motivů, které nesledují fylogenetický vzorec. To naznačuje, že repetitivní sekvence degenerovaných pohlavních chromosomů patří mezi evolučně nejdynamičtější oblasti genomu. Nakonec jsme zkoumali homologii a stáří Z chromosomů ještěrek napříč 45 druhy 26 rodů, a potvrdili jsme u všech homologické diferencované ZZ/ZW pohlavní chromosomy staré přibližně 85 milionů let. Zpochybňujeme předchozí tvrzení o přechodech k ESD v rámci ještěrek. Chromosomy ječtěrek ukazují, že odvozené karyotypy s výhradně akrocentrickými chromosomy a diferencované pohlavní chromosomy mohou být extrémně stabilní po dlouhé evoluční období.

Introduction

1. What are Lacertoidea?

The superfamily Lacertoidea comprises ten recognised families: Lacertidae, Rhineuridae, Bipedidae, Cadeidae, Blanidae, Trogonophidae, Amphisbaenidae, Teiidae, Gymnophthalmidae, and Alopoglossidae (Uetz and Hošek, 2024).

The lacertid lizards (**Lacertidae**) include 375 species categorised into 42 genera (Figure 2, Uetz and Hošek, 2024) distributed across Europe, Asia, Africa, and adjacent islands (Figure 1A). They are divided into two subfamilies: Gallotinae and the more diverse Lacertinae (Uetz & Hošek, 2024). The small subfamily Gallotinae includes 14 species found in the West Mediterranean and the Canary Islands. The more speciose Lacertinae includes two tribes: Eremiadini, primarily found in Africa and the Middle East, and Lacertini, spread across Europe, Asia, and parts of North Africa. Lacertids inhabit an extensive range of environments, from rainforests and grasslands to deserts, with a single species - the viviparous lizard (*Zootoca vivipara*) - thriving in the cold plains of the Arctic tundra. All species are diurnal insectivores, and most are terrestrial, but some specialise in more diversified lifestyles. *Meroles anchietae* consumes seeds on a daily basis. Strongly saxicolous *Australolacerta* and parthenogenetic *Darevskia* flourish in rocky areas. *Takydromus* spend most of their time off the ground in dense grasses. The arboreal prehensile tail of *Gastropholis* helps it to move through treetops, while the flat-bodied *Holaspis* can glide between high trees (Vitt & Caldwell, 2014).

The worm lizards (**Amphisbaenia**) are the closest lacertid relatives of lacertids and together, they form the Lacertibaenia group. Amphisbaenia monophylum includes 20 genera with 203 species across six families: Rhineuridae, Bipedidae, Cadeidae, Blanidae, Trogonophidae, and Amphisbaenidae (Figure 2, Uetz and Hošek, 2024). Distributed mainly in Africa and South

America (Figure 1B-G), amphisbaenians are exclusively burrowing creatures with an annulated snake-like appearance. All are limbless except for the three species in Bipedidae, with large mole-like forearms. Challenging sample collection associated with their secretive lifestyle makes them possibly one of the least-studied groups of squamates, with many aspects of their biology still largely unknown (Vitt & Caldwell, 2014).

Sister to Lacertibaenia is Teiioidea; it includes the families Gymnophthalmidae, Allopoglossidae and Teiidae. The teiid lizards (**Teiidae**), also known as macroteiids, include 173 species divided into two subfamilies: Tupinambinae, found in Latin America, and Teiinae, found in both Americas (Figure 2, Figure 1H). The Tupinambinae subfamily includes 16 species in five genera (Uetz and Hošek, 2024). They are mostly large lizards with diverse habits and habitats. Tegus (*Tupinambis* and *Salvator*) are opportunistic omnivores that adapted to various environments, from rainforests and grasslands to semiarid regions. Semiaquatic caiman lizards (*Dracaena*) and crocodile tegu (*Crocodilurus*) are excellent swimmers, while the false monitors (*Callopistes*) are the saurovorous predators of the arid coastal areas. The Teiinae subfamily includes 157 species in 13 genera (Uetz and Hošek, 2024). Although widespread from Canada to Argentina, these lizards share a remarkably similar morphology. They are characterised by an elongated, streamlined body, long hindlimbs and a long whip-like tail. Most of the species are active insectivores and prefer terrestrial habitats. *Dicrodon guttulatum* and two *Cnemidophorus* species are herbivorous. Some *Aspidoscelis* and *Cnemidophorus* hybrids reproduce via obligatory parthenogenesis. Due to their similar morphology and habitats, these lizards are often compared to lacertids as the Old and New World ecological equivalents (Vitt & Caldwell, 2014).

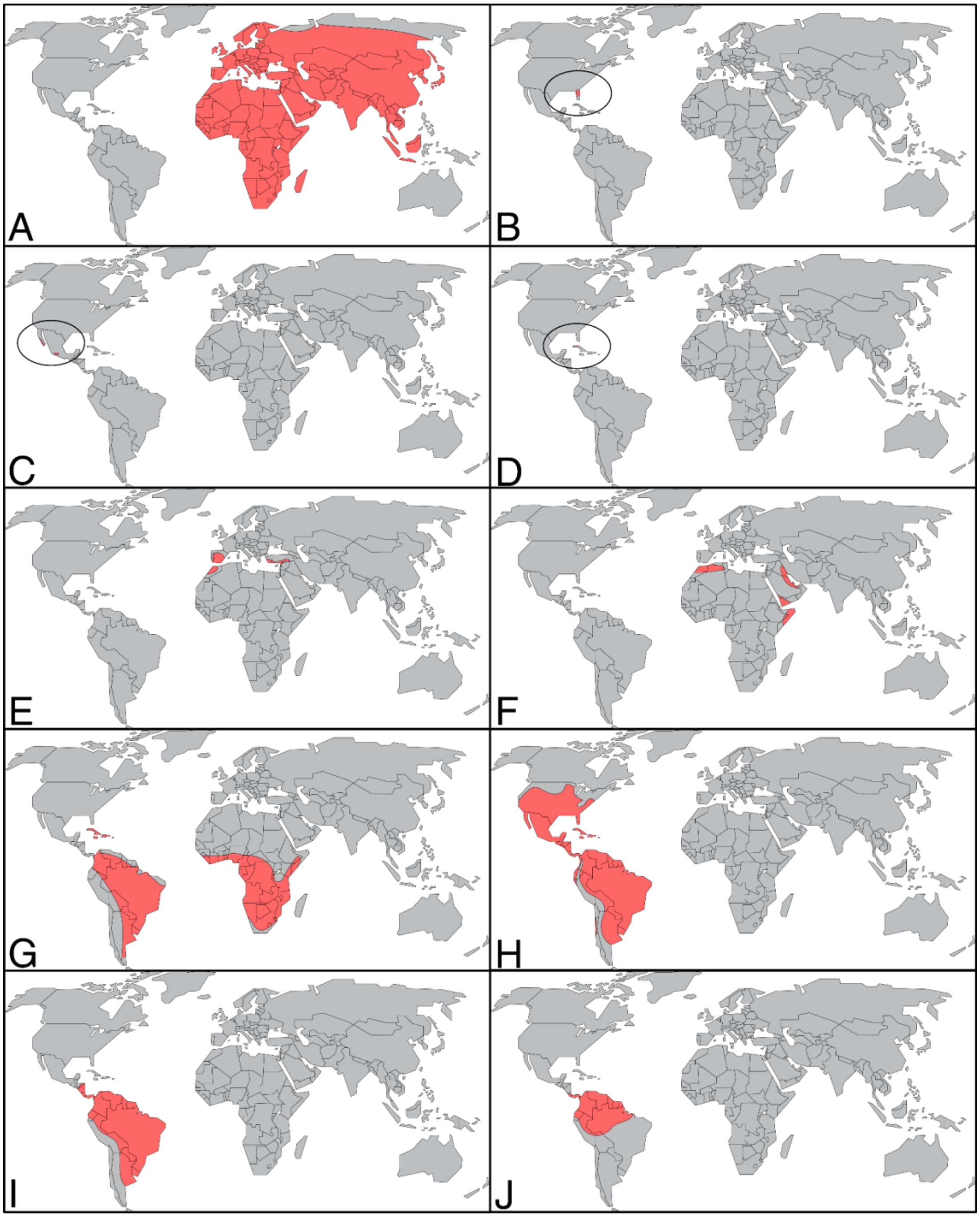


Figure 1. Geographical distribution of Lacertoidea: **A.** Lacertidae, **B.** Rhineuridae, **C.** Bipedidae, **D.** Cadeidae, **E.** Blanidae, **F.** Trogonophidae, **G.** Amphisbaenidae, **H.** Teiidae, **I.** Gymnophthalmidae and **J.** Alopoglossidae. The maps were created based on simplified species' distribution by Uetz and Hošek (2024).

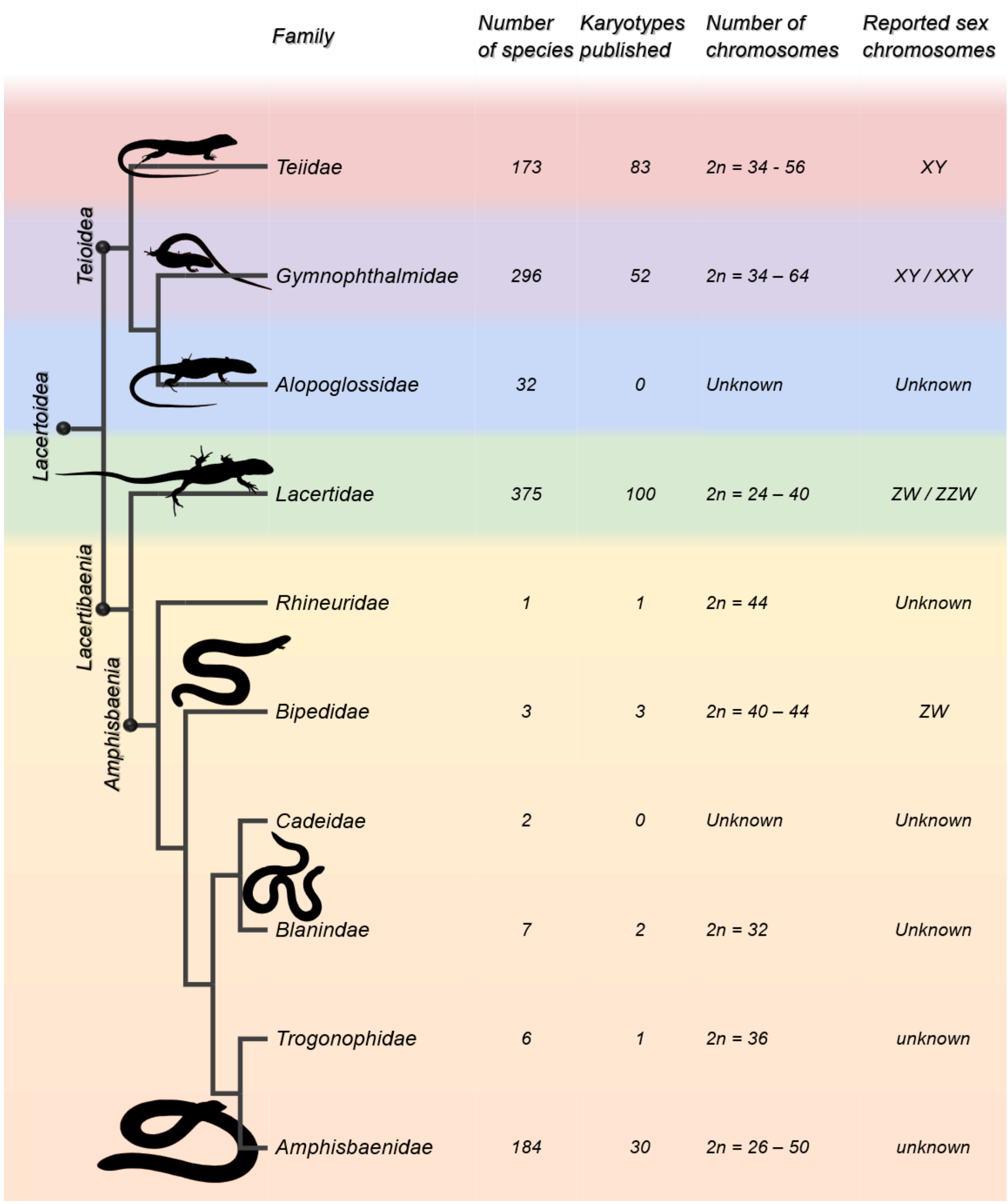
The spectacled lizards (**Gymnophthalmidae**), also known as microteiids, consist of 296 species in 56 genera, divided into five subfamilies: Bachiinae, Cercosaurinae, Ecpleopinae, Gymnophthalminae, and Rhachisaurinae (Figure 2, Uetz and Hošek, 2024). These neotropical lizards are distributed across South and Central America, from rainforests to high Andes, savannas, and semiarid areas (Figure 1I). They are a family of small but ecologically diverse lizards. Most species are diurnal and terrestrial. Yet, *Potamites* members adapted to a semiaquatic lifestyle, while *Bachia* and *Calyptommatus* are subterranean and semi-nocturnal, with their reduced limbs and elongated bodie*s* (Teixeira et al. 2013). Alopoglossid lizards (**Alopoglossidae**) include 32 species of a single genus *Alopoglossus*. It was formerly known as a subfamily within Gymnophthalmidae, but was recently proposed as an independent family. These diurnal, small lizards inhabit the leaf litter in Neotropical forests and fields from northern South America to Costa Rica (Figure 1J), blending into their environment with brownish colouration and cryptic behaviour (Goicoechea et al., 2016; Hernández Morales et al., 2020; Uetz and Hošek, 2024).

2. Karyotype evolution in Lacertoidea

Karyotypes of **lacertoidean** lizards exhibit significant diversity, ranging from 2n=24 in species like *Iberolacerta bonnali*, *Parvilacerta fraasii*, and *Parvilacerta parva*, to 2n=62 in *Nothobachia ablephara* (Odierna et al., 1995, 1996; Pellegrino et al., 1999). This superfamily includes species with unimodal, bimodal, and mixed karyotypes dispersed throughout the phylogeny. Bimodal karyotypes, featuring a few large biarmed macrochromosomes and numerous microchromosomes, are found in species like tegu (*Salvator merianae*) and are proposed as the ancestral state of the group (**Chapter 1**). Unimodal karyotypes, composed of a gradient of medium-sized acrocentric chromosomes, are common in Lacertidae and Teiinae and are likely derived.

Mixed karyotypes, with various ratios of biarmed, acrocentric, and microchromosomes, are prevalent within the superfamily (Carvalho et al., 2015; Laguna et al. 2010; Laguna 2011). Evolutionary rates of chromosome number changes are highly variable among lacertoideans, with lacertid lizards displaying a five times lower rate than other lineages (**Chapter 1**).

Lacertids are the most karyologically studied family within Lacertoidea, accounting for nearly half of the studied species in this clade (i.e. 100 out of 250 species, see Figure 2). Their historical significance is highlighted by the fact that their chromosomes, specifically those of *Lacerta agilis*, were the first reptilian chromosomes ever described (Tellyesniczky, 1897). Lacertid karyotypes are remarkably conserved, with most species exhibiting 36 acrocentric chromosomes that gradually decrease in size, along with 2 microchromosomes (2n = 38) (Figure 3, Gorman, 1973; Olmo et al., 1993; **Chapter 1**). Only a few variations have been identified, including decreased chromosome numbers and the presence of biarmed chromosomes in genera such as *Darevskia, Timon, Parvilacerta, Iberolacerta,* and *Zootoca*. These variations likely result from centric fusions. Notably, Iberolacerta and Zootoca also lack a pair of microchromosomes, which is exceptional in Lacertoidea (Spangenberg et al., 2021; Kupriyanova & Melashchenko, 2011; Giovannotti et al., 2018; Odierna et al., 1990; Rykena & Nettmann, 1986; Suwala et al., 2020; Odierna et al., 1996; Olmo et al., 2001). An elevated diploid number (2n=40) was observed only in Gallotinae members, potentially representing the sole instance of chromosomal fission within the group (Capula et al., 1982; Lopez-Jurado et al., 1986; Suwala et al., 2020). Additionally, no evidence of pericentric inversions or centromere repositioning has been reported in Lacertidae, highlighting the peculiar nature of their conserved chromosome structure (**Chapter 1**).



Family	Number of species	Karyotypes published	Number of chromosomes	Reported sex chromosomes
Teiidae	173	83	2n = 34 - 56	XY
Gymnophthalmidae	296	52	2n = 34 – 64	XY / XXY
Alopoglossidae	32	0	Unknown	Unknown
Lacertidae	375	100	2n = 24 – 40	ZW / ZZW
Rhineuridae	1	1	2n = 44	Unknown
Bipedidae	3	3	2n = 40 – 44	ZW
Cadeidae	2	0	Unknown	Unknown
Blanindae	7	2	2n = 32	Unknown
Trogonophidae	6	1	2n = 36	unknown
Amphisbaenidae	184	30	2n = 26 – 50	unknown

Figure 2. Phylogenetic overview of the lacertoidean families with respective species richness, number of species karyotyped, chromosome number ranges and reported sex chromosomes. Phylogenetic reconstruction and species richness according to Uetz and Hošek (2024). The number of karyotypes, CN range and sex chromosomes reported are based on the data from Table S1 from **Chapter 1**.

Among **teiids**, the Tupinambinae subfamily members exhibit similar bimodal karyotypes, with diploid numbers ranging from 2n=34 to 2n=38, characterised by a few large biarmed macrochromosomes and numerous microchromosomes (Figure 3A, Carvalho et al., 2015). In contrast, Teiinae karyotypes are more diverse, with diploid numbers ranging from 2n=30 in *Ameiva auberi* to 2n=56 in *Dicrodon guttulatum* (Figure 2, Lowe et al., 1970; Gorman, 1970). Most Teiinae karyotypes feature a gradient of acrocentric chromosomes but differ from lacertids by having numerous microchromosomes and often at least one biarmed pair (Carvalho et al., 2015). Gorman (1970) proposed an ancestral unimodal karyotype of 2n=50 acrocentric chromosomes for Teiinae and 2n=36 with 12 biarmed macrochromosomes and 24 microchromosomes for Tupinambinae. Robertsonian rearrangements were hypothesised as the key mechanism driving the divergence of these karyotypes (Olmo, 1986). Da Silva et al. (2020) suggested that the Tupinambinae karyotype is derived, based on the accumulation of interstitial telomeric repeats in the pericentromeric regions of tegu's biarmed chromosomes. However, as revealed in **Chapter 1**, conserved chromosome-wide syntenies shared with other squamates indicate that the tegu karyotype likely resembles the ancestral state, suggesting that the acrocentric chromosomes of Teiinae are derived. Unlike lacertids, pericentric inversions are frequently reported in Teiinae, especially in the genus *Aspidoscelis*, and are thought to be responsible for transitions between acrocentric and biarmed chromosomes (Gorman, 1970; Peccinini-Seale, 1981, 1989). Nucleolar organiser regions (NORs) have been identified on differently sized and sometimes multiple chromosomes in Teiinae members, while in all tested Tupinambinae species, NORs are located on the pair 2 macrochromosome, supporting the more conserved nature of Tupinambinae karyotypes (Carvalho et al., 2015).

Gymnophthalmids are largely unstudied cytogenetically, with only 49 out of 296 species karyotyped (Figure 2.). Their karyotypes are highly diverse, with intrageneric chromosome variability even among closely related species, such as within the genera *Gymnophthalmus, Bachia, Leposoma,* and *Placosoma* (Yonenaga-Yassuda et al., 1995; Pellegrino, 1998; Pellegrino et al., 1999b; Pellegrino et al., 2001). Chromosome numbers in gymnophthalmids range from 2n=32 in *Bachia dorbignyi* to 2n=64 in *Nothobachia ablephara* (Figure 2, Pellegrino et al., 1999b, 2001). Chromosome fusions, pericentric inversions, heterochromatin amplification or deletion, and shifts in nucleolar organiser region distribution are proposed to have accompanied speciation events in this family (Yonenaga-Yassuda et al., 2005). Diploid and triploid parthenogenetic hybrids were identified in the genera *Gymnophthalmus* and *Leposoma* (Yonenaga-Yassuda et al.,1995; Laguna et al., 2010). None of the 32 **Allopoglossidae** members have been karyotyped to date.

Amphisbaenians' karyology is also largely understudied, with karyotypes known only in 35 species, and the most recent report was published over a decade ago (Laguna et al., 2010). Their chromosomes exhibit considerable diversity, with diploid numbers ranging from 2n=26 in *Amphisbaena dubia* and *A. kingi* to 2n=50 in *A. leberi* and *A. innocens* (Figure 2, Huang & Gans, 1971; Beçak et al., 1971a, 1972; Cole & Gans, 1987). Despite this variability, macrochromosomes and microchromosomes are clearly distinguishable in all studied species. Amphisbaenians vary in their biarmed-to-acrocentric chromosome ratios, with higher diploid numbers associated with more acrocentric chromosomes. Therefore, Robertsonian translocations of macrochromosomes are hypothesised to be the underlying mechanism (Cole & Gans, 1987; Hernando, 2005; Huang et al., 1967; Huang & Gans, 1971; Olmo, 1986). Nucleolar organiser regions (NORs) have been identified in four species, typically located on the subterminal regions of a single chromosomal pair, except in *Amphisbaena heterozonata*, which has multiple NORs.

Figure 3. A representative bimodal karyotype of **A.** *Salvator merianea*, and a representative all-acrocentric lacertid karyotype of **B.** *Gastropholis prasina* (female), based on karyotypes published by: da Silva et al. (2020) (A) and Chapter 2 (B).

3. Sex chromosomes and repetitive content

Sex chromosomes are an important aspect of organisms' karyology. In Lacertoidea, two types of genotypic sex determination (GSD) systems have been identified: female heterogamety and male heterogamety, each including species with multiple sex chromosomes. In GSD, sex is determined by a sex-specific genotype (Leonard 2018).

Female heterogamety involves ZZ/ZW or $Z_1Z_1Z_2Z_2/Z_1Z_2W$ sex chromosomes, found in all 73 tested lacertids and a single amphisbaenid species, *Bipes tridactylus* (Figure 2, **Chapter 3**, Cole

& Gans 1987). In contrast, male heterogamety involves XX/XY or $X_1X_1X_2X_2/X_1X_2Y$ sex chromosomes, observed in two teiids: *Aspidoscelis tigris* and *Glaucomastix littoralis,* and eight gymnophthalmids: *Bachia dorbignyi, Calyptommatus leiolepis, C. nicterus, C. sinebrachiatus, Gymnophthalmus pleei, Micrablepharus atticolus, M. maximiliani,* and *Nothobachia ablephara* (Figure 2, Lowe et al. 1970; Peccinini-Seale et al. 2004; Pellegrino et al. 1999; Yonenaga-Yassuda & Rodrigues 1999; Cole et al. 1990; Pellegrino & Yonenaga-Yassuda 1998; Yonenaga-Yassuda et al. 2005).

In female heterogamety, females carry the W heterochromosome, while males carry the Y heterochromosome in male heterogamety. The differentiation of heterochromosomes varies among species. Sometimes, they are much smaller, and often, they are heterochromatic. However, in some cases, the W/Y chromosomes are homomorphic and cytogenetically indistinguishable from their Z/X counterparts, as reported in Skinks (Kostmann et al. 2021).

Sex chromosomes evolve from autosomes when a sex-determining locus is acquired, leading later to suppressed recombination around this locus (Ohno 1967). This suppression is often achieved through inversions (Kirkpatrick et al. 2010). The sex chromosomes of two gymnophthalmid species might exemplify this differentiation stage. In both species, the Y chromosome is telocentric and the same size as its X counterpart, while the X chromosome is either metacentric (*Nothobachia ablephara*) or submetacentric (*Gymnophthalmus pleei*). Recombination suppression often results in the accumulation of repetitive elements, gene degradation, and heterochromatization, making sex heterochromosomes smaller and distinct from autosomes over time (Bachtrog et al. 2011). This degeneration, with the Y chromosome much smaller than the X chromosome, can be observed in a teiid (*Glaucomastix littoralis*) and two other gymnophthalmids (*Micrablepharus atticolus* and *M. maximiliani*) (Yonenaga-Yassuda & Rodrigues 1999; Peccinini-

Seale et al. 2004). In lacertids, the W chromosome size varies between species. It can be much smaller than the Z chromosome - in the microchromosome range - as in *Lacerta media* or *Timon lepidus*. In some cases, it is the same size as the Z chromosomes, as in *T. tangitanus*. However, in all investigated lacertids, the W chromosome is heterochromatic (Odierna et al. 1993, **Chapter 2**).

Environmental sex determination (ESD), an alternative to GSD, lacks sex-specific differences in genomes. Environmental conditions determine sex during egg incubation, most commonly by temperature (Charnov & Bull 1977; Head et al. 1987; Mrosovsky & Pieau 1991; Bull 1983; Lang & Andrews 1994; Janzen & Krenz 2004). Many authors consider ESD to be the ancestral state in reptiles, and GSD to be derived, a notion well-supported by the phylogenetic distribution patterns of sex determination systems (Pokorná & Kratochvíl 2009; Johnson Pokorná & Kratochvíl 2016, Kratochvil et al. 2020). Highly differentiated sex chromosomes (like those in mammals and birds) are thought to be evolutionarily stable, making transitions from GSD to ESD unlikely (Pokorná & Kratochvíl 2009). However, cases of environment-dependent sex reversal in GSD species such as *Pogona vitticeps* suggest that such transitions might be possible (Holleley et al. 2015; Ehl et al. 2017). ESD was previously reported in two lacertid species: *Podarcis pityusensis* (Eichenberger 1981) and *Eremias multiocellata* (Zhang et al. 2010; Tang et al. 2012; Wang et al. 2015), interpretations we challenge in **Chapter 3**. No reports of ESD in other lacertoidean lizards have been published to date. Thus, most teiids, gymnophthalmids, and amphisbaenians may possess GSD with homomorphic sex chromosomes.

Aims of the study

This thesis investigates the evolutionary dynamics and structural variability of karyotypes and sex chromosomes in lacertid lizards in the context of other lacertoideans. Lacertids, a karyologically well-documented group of Old World lizards, with postulated stability of their all-acrocentric karyotypes and ZZ/ZW sex chromosomes. However, several gaps and contradictions exist within this pattern.

In this thesis, I seek to address these gaps via the following aims:

1. To test the rate of chromosome number evolution across the lacertoidean phylogeny, explore the syntenic relationships among their chromosomes, and assess the types of rearrangements that might have been involved in lacertid karyotype formation.

2. To explore the evolutionary dynamics behind lacertid W sex chromosome differentiation in a broad phylogenetic context of this group, focusing on the accumulation of microsatellite motifs and patterns of heterochromatization.

3. To investigate the stability of lacertid ZZ/ZW sex chromosomes, potential deviations from this pattern, and the potential age of their differentiation.

Publications overview

Chapter 1:

Suwala, G., Wisniewska, M., M., Kratochvíl, L., & Rovatsos, M. (2024). Unequal rates of chromosome number evolution among lacertoidean lizards. Manuscript under preparation.

Chapter 2:

Suwala, G., Altmanová, M., Mazzoleni, S., Karameta, E., Pafilis, P., Kratochvíl, L., & Rovatsos, M. (2020). Evolutionary variability of W-linked repetitive content in lacertid lizards. Genes, 11(5), 531. https://doi.org/10.3390/genes11050531

Chapter 3:

Rovatsos, M., Vukić, J., Mrugała, A., **Suwala, G.,** Lymberakis, P., & Kratochvíl, L. (2019). Little evidence for switches to environmental sex determination and turnover of sex chromosomes in lacertid lizards. Scientific Reports, 9(1), 7832. https://doi.org/10.1038/s41598-019-44192-5

Outline of the publications

This thesis integrates bioinformatic, cytogenetic, and molecular approaches to explore the fate of karyotypes and sex chromosomes in lacertid lizards. It consists of three chapters, each presenting original investigations. The first chapter explores the evolutionary rates and syntenic relationships of lacertoidean chromosomes through a bioinformatic analysis of available genomic and karyotype data. The next two chapters focus on sex chromosomes within the Lacertidae family. The second chapter explores the variability of the repetitive content on the lacertid W chromosome using molecular cytogenetic methods. The third chapter focuses on identifying genes on the lacertid Z chromosome and testing their homology across major lacertid genera by applying a combination of genomic data and quantitative PCR, challenging the reported transitions to environmental sex determination in lacertids.

Table 1. Overview of contribution for each chapter.

Chapter	Experimental design	Experimental procedures	Figure/table preparation	Manuscript preparation
1	✓	✓	✓	✓
2	-	✓	✓	✓
3	-	✓	-	-

Chapter 1 investigates the variability in evolutionary rates of chromosome numbers among lacertoidean lizards in the context of synteny relationships of their chromosomes. Although previous studies (e.g., Olmo et al. 1986; Gorman 1970) noted the similarities between lacertid karyotypes, these observations lacked statistical validation. Moreover, the uniform acrocentric

shape and similar size of lacertid chromosomes have historically impeded the investigation of their interspecies homologies through cytogenetic methods. In this chapter, we provide statistical evidence demonstrating that the rate of chromosome number evolution in lacertids is five times lower than in the other Lacertoidean lineages. Additionally, we establish that the chromosomal content in tested lacertid species is syntenic across both the Lacertini and Eremiadini clades, encompassing 96% of the species in the family. Our analysis revealed no interchromosomal rearrangements, except for a single microchromosome fusion in the viviparous lizard. Furthermore, based on the pattern of shared syntenies with the outgroups, we propose that the bimodal karyotype of tegu likely resembles the ancestral state of all lacertoideans. Consequently, we postulate that the lacertid karyotype, shared by most extant species, originated through centric fissions of macrochromosomes and fusions of microchromosomes. In this work, I contributed to the gathering of karyotypes and genomes, the data analyses, the preparation of figures and tables, and the preparation of the manuscript.

Chapter 2 focuses on the evolutionary dynamics of repetitive content on the W chromosome in lacertid lizards using molecular cytogenetics methods. We analysed 15 species across ten genera representing both subfamilies within Lacertidae: Gallotinae and Lacertinae, encompassing both tribes of the latter (Lacertini and Eremiadini). We employed fluorescence *in situ* hybridisation (FISH) with telomeric repeats and 22 microsatellite motifs $(A)_{30}$, $(C)_{30}$, $(CA)_{15}$, $(CG)_{15}$, $(GA)_{15}$, $(TA)_{15}$, $(CAA)_{10}$, $(CAC)_{10}$, $(CAG)_{10}$, $(CAT)_{10}$, $(CGG)_{10}$, $(GAA)_{10}$, $(GAC)_{10}$, $(GAG)_{10}$, $(TAA)_{10}$, $(TAC)_{10}$, $(AAGG)_{8}$, $(AATC)_{8}$, $(ACGC)_{8}$, $(GACA)_{8}$, $(GATA)_{8}$, and $(TTTC)_{8}$. Additionally, we used C-banding to compare heterochromatin distribution on the W chromosome. All studied W chromosomes were heterochromatic, yet the accumulation of microsatellite motifs was highly variable and did not align with phylogenetic relationships, suggesting species-specific patterns. This variability in W chromosome size and content contrasts with the overall conservation of karyotypes within lacertids, indicating higher evolutionary dynamics for the W chromosome than the Z chromosome. Notably, we observed a strong GATA signal on the Z chromosomes of *Timon* and *Takydromus* species, suggesting the potential crossover of these motifs from the degenerated W chromosome through the pseudoautosomal region. Additionally, here we described the karyotypes of *Gastropholis prasina* and *Timon tangitanus* for the first time. In this work, I contributed to the blood sample collection, the cytogenetic and molecular cytogenetic analyses, the preparation of the figures and tables, and the preparation of the manuscript.

In **Chapter 3**, we aimed to verify the homology and assess the age of the lacertid Z chromosomes in a wide phylogenetic spectrum. Although lacertids show long-term stability of their ZZ/ZW sex-determination system, transitions to ESD were reported in two species: *Podarcis pityusensis* and *Eremias multiocellata*. This led some authors to hypothesise that the differentiation of sex chromosomes occurred repeatedly and independently in Lacertidae (Eichenberger 1981; Zhang et al. 2010). Our analysis revealed that all examined lacertid species possess homologous differentiated ZZ/ZW sex chromosomes. These sex chromosomes are absent in the closely related amphisbaenians and teiid lizards. Suggesting that they date back to the Lacertidae common ancestor approximately 85 million years ago. In this work, I contributed to the molecular assays and analyses, table preparation and assisted manuscript preparation.

Statements of contribution / Prohlášení o příspěvku

I declare that my research for this dissertation was conducted in 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 above and in each published manuscript of this thesis.

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

Prague, August 2024 / Praha, srpen 2024,

Grzegorz Tomasz Suwała

As supervisor of the PhD thesis and to the best of my knowledge, I confirm the contribution of MSc. Grzegorz Suwala to the chapters of this thesis is accurately explained above.

Jako vedoucí disertační práce a podle mého nejlepšího vědomí potvrzuji, že příspěvek MSc. Grzegorz Suwala ke kapitolám a dodatkům v této práci byl přesně vysvětlen níže.

Prague, August 2024 / Praha, srpen 2024,

Michail Rovatsos

Conclusions and Future Perspectives

This thesis provides new insights into the evolution of chromosomes and sex determination in lacertid lizards. We demonstrated that the rate of chromosome number evolution in lacertids is five times lower than in the other lacertoidean lineages, with syntenic chromosomal content shared across the clades Lacertini and Eremiadini. This indicates that the conserved karyotype was already present in the ancestor of the majority of the lacertid species (Suwala et al. 2024 - Chapter 1). Additionally, our study of the lacertid W chromosomes revealed highly variable accumulations of microsatellite motifs in species-specific patterns, providing substantial evidence that the repetitive content on degenerated sex chromosomes represents one of the most dynamically evolving segments of the genome (Suwala et al. 2020 - Chapter 2). Finally, we confirmed that all examined lacertid species possess homologous differentiated ZZ/ZW sex chromosomes, dating back approximately 85 million years. This finding challenges previous reports of transitions to environmental sex determination in lacertids (Rovatsos et al. 2019 - Chapter 3).

Further research is needed to better understand the unique nature of lacertid chromosomes, including the reasons behind the exceptional stability of their karyotypes. This stability is likely related to the content of their centromeres, yet these regions are missing from their genomes. The repetitive content of eukaryotic centromeres is notoriously difficult to sequence, but it can be successfully assembled using long-read sequencing technologies, such as PacBio or Oxford Nanopore. The sequenced centromeric regions of lacertid chromosomes could be compared between species with conserved karyotypes and those with fusion chromosomes and might uncover otherwise hidden patterns.

Further research is required to comprehend the evolution of sex chromosomes in lacertoidean lizards. In the vast majority of teiid, gymnophthalmid, and amphisbaenian species, sex chromosomes have not been identified. Most studies that identified the sex chromosomes in their species applied exclusively conventional cytogenetic methods. Molecular cytogenetics, including the investigation of microsatellite motifs, may reveal sex-specific differences that are otherwise undetectable. Furthermore, genome coverage analysis of both sexes of a gymnophthalmid or a teiid species with known sex chromosomes could yield identification of their X-linked genes. These genes could then be tested across Teiioidea taxa revealing the age of these sex chromosomes and the potential homology between teiid and gymnophthalmid XX/XY chromosomes.

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