Chapter 1

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Article

Unequal rates of chromosome number evolution among lacertoidean lizards

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Abstract

Organisms vary dramatically in chromosome number, morphology, and evolutionary stability, yet this variability is poorly understood. We collected data on chromosome number and morphology in lacertoidean lizards and compared their available whole chromosome genomes to understand processes responsible for the karyotype dynamics in this group. We documented that karyotype evolution is highly dynamic in teiids, gymnophthalmids and amphisbaenas but that the rate of evolution of chromosome number is drastically (5-fold) lower in lacertids. The derived and highly stable lacertid karyotypes surprisingly evolved by two opposite mechanisms acting on chromosomes of different sizes: repeated fusions of the ancestral microchromosomes but fissions of homologs of the bi-armed macrochromosomes of many other squamates. Interestingly, the similar ancestral all-acrocentric karyotype of geckos probably evolved by analogous processes. The stability of the lacertid karyotype is associated not only with a low rate of centric fusions (although few exceptions can be found), but also with a notable lack of intrachromosomal rearrangements (pericentric inversions or centromere repositioning) leading to metacentric chromosomes. We suggest that a derived nature of lacertid centromeres could explain the peculiar stasis of their karyotypes.

Keywords: evolution; karyotype; chromosome; centromere; genome; synteny

Introduction

The organisation of the nuclear genome into chromosomes is a fundamental feature of eukaryotic organisms. The variability in chromosome number (CN), size and morphology is enormous, ranging from a single pair to several hundred of chromosomes (Imai et al. 2002; Ruiz-Herrera et al. 2012). Despite the lack of standardised definitions of these categories, karyotypes may contain different combinations of large chromosomes (macrochromosomes) and small chromosomes (microchromosomes), with the centromere at the terminal (acrocentric chromosomes) or the innermost position (metacentric chromosomes). It is clear that the current diversity in karyotypes is a manifestation of the underlying genomic dynamics including changes in ploidy level and chromosomal rearrangements, processes in which DNA breaks are followed by incorrect repair. (Mayrose & Lysak, 2021). However, despite decades of research, it is not known why organisms differ to such an extent in numbers and shapes of chromosomes and dynamics of their changes. The variability is particularly intriguing given that extended synteny – conservation of gene content and order in blocks – has existed in some lineages for over 700 million years (Putnam et al. 2008).

Among chromosome rearrangements, duplication, deletion, inversion, and/or translocation of chromosome segments can lead to changes in the chromosome arm length, centromere position, and altered linkage. Translocations involving recombination of non-homologous chromosomes may result in changes in CN (Mayrose & Lysak, 2021). Three main types of such translocations have traditionally been distinguished: centric fissions, centric fusions, and tandem fusions. The centric fission involves centromere breakage or miss-division during the segregation of a biarmed chromosome, yielding two acrocentric chromosomes. To remain stable, centromeric parts on both of these chromosomes must retain the ability to form kinetochores (Mayrose & Lysak, 2021). The centric fusion is a reverse process, joining the long arms of two acrocentric chromosomes through

their centromeric ends or short arms. Both centromeres in the resulting biarmed chromosome must either function as a single entity or one must be eliminated (Mayrose & Lysak, 2021). Tandem fusions are defined by the retention of the head-to-tail collinearity of two or more ancestral chromosomes, coupled with the loss of one of the centromeres (Yang et al. 1997).

It is evident that each fixed chromosome fusion and fission is associated with changes in the number of centromeres within the genome. Centromeres are essential chromosomal loci critical for genome stability. They facilitate the kinetochore assembly, which ensures accurate spindle attachment and segregation during cell divisions. Centromeres generally share the presence of the centromeric histone H3 variant (cenH3), yet centromeric DNA is highly variable in sequences and size among organisms (De Rop et al. 2012). Centromeres (both their sequence and cenH3) are considered to be among the most rapidly evolving parts of eukaryotic genomes (Malik & Henikoff, 2002). This variability might be attributed to centromere (meiotic) drive, a phenomenon where chromosomes with certain centromeres are preferentially segregated during oogenesis due to their stronger centromere-kinetochore interactions (Dudka & Lampson, 2022). This process should have a profound effect on karyotype evolution. For example, it is hypothesised to be responsible for the bimodality in chromosome numbers across mammals, where particular species tend to have either mostly metacentric or acrocentric chromosomes (de Villena & Sapienza, 2001).

Squamate reptiles, i.e. lizards and snakes, provide a rich area for the study of chromosome evolution. This group shows great diversity in CN and its morphology, in the presence of microchromosomes and sex chromosomes (Deakin & Ezaz, 2019; Olmo, 2008). The most obvious change in chromosome number occurs through the acquisition of an additional set of chromosomes - polyploidization - which, although common in plants, is rarely fixed in animals, particularly in amniotes (Otto & Whitton, 2000). Nevertheless, triploid and even tetraploid hybrids were reported

in several obligatory parthenogenetic squamate lineages (Lowe et al. 1970; Pellegrino et al. 2004; Darevsky & Danielyan, 1968). However, diploidy is the typical karyological state of squamates, with CN ranging from 2n = 16 in *Gonatodes tanieae* (Gekkonidae) up to 2n = 62 in *Notobrachia ablephara* (Gymnophthalmidae) and *Rieppeleon brevicaudatus* (Chamaeleonidae) (Pellegrino et al. 1999; Rovatsos et al. 2017; Schmid et al. 1994).

Squamate karyotypes can be broadly divided into two major groups. The first type - asymmetrical karyotypes (by some authors also described as "bimodal") - with a few large, often metacentric macrochromosomes and many dot-like microchromosomes, was predominantly reported in iguanian and scincoidean lizards and in snakes (Mezzasalma et al. 2021). Such a karyotype was probably ancestral in squamates (Waters et al. 2021). The second type - symmetrical karyotypes (also referred to as "unimodal"), characterised by an array of acrocentric chromosomes gradually decreasing in size (White, 1948), is typical mainly for geckos and lacertid lizards but also occur, for example, within agamids, anoles, chameleons, snakes, amphisbaenas, teiids and gymnophthalmids (Gorman & Shochat, 1972; Webster et al. 1972; Rovatsos et al. 2017(chamaeleon); Serafim et al. 2007; Huang et al. 1967; Falcione & Hernando, 2010; Gorman, 1970; Cole et al. 1995; Santos et al. 2007; Pellegrino et al. 1999; Pellegrino et al. 2004; Yonenaga-Yassuda et al. 2005). The symmetrical karyotypes are assumed to be derived, and it was hypothesised that they evolved via centric fissions of macrochromosomes leading to the disappearance of this category (Webster et al. 1972; Serafim et al. 2007).

Both symmetrical and asymmetrical karyotypes, along with forms that do not fit precisely into either category, have been described within the lacertoidean lizards, including nine or ten families (Lacertidae, Alopoglossidae - sometimes treated as a subfamily of Gymnophthalmidae, and Teiidae, and amphisbaenians now categorised into six families: Rhineuridae, Bipedidae,

Cadeidae, Blanidae, Trogonophidae and Amphisbaenidae) (Zheng & Wiens, 2015; Goicoechea et al. 2016). Certain clades have been reported to exhibit high variability in both chromosome shape and number (Pellegrino et al. 2001; Rojo-Orons, 2015; Laguna et al. 2010a; Carvalho et al. 2015), while little variation has been described in others (e.g., Gorman, 1969; Olmo et al. 1986; Rojo-Orons, 2015). This suggests that evolutionary rates in CN might not be homogenous among lacertoidean lineages. Furthermore, recent advances, including the publication of seven lacertoidean chromosome-level genome assemblies, have provided an unprecedented opportunity to compare chromosome homology within this group and to uncover processes leading to the evolution of the derived karyotypes.

Using available cytogenetic data on lacertoidean chromosomes, we conducted a phylogenetic analysis to investigate potential differences in the rates of chromosome number evolution among their clades. In addition, we explored the mechanisms underlying the potentially derived lacertid karyotypes using the newly available annotated genome data of squamates. This multidisciplinary approach aimed to shed light on the evolutionary dynamics of lacertoidean chromosomes and their implications for squamate genome evolution as a whole. We were particularly interested in whether there are any evolutionary trends in CN that are associated with karyotype stability, what are the characteristics of stable versus evolutionarily labile karyotypes, and which chromosomal rearrangements are involved in the evolutionary dynamics. For example, the meiotic drive hypothesis of karyotype evolution predicts that karyotypes with maximum or minimum possible numbers of chromosomes (centromeres) should be particularly stable (de Villena & Sapienza, 2001). According to this hypothesis, chromosome evolution should follow a trend with a limit given splits or fusions of all stable syntenic chromosome blocks.

Materials and Methods

Evolutionary rates of chromosome number changes

In total, we accumulated data on karyotypes in 249 lacertoidean species. Out of them, 196 species were included in the time-calibrated tree proposed by Zheng & Wiens (2015) and were thus used for the phylogenetic analysis. The families Lacertidae and Teiidae were slightly better represented (each with both karyotype and phylogenetic data in 23% of species) compared to Gymnophthalmidae and Amphisbaenia (13%). Nevertheless, species with available data were spread evenly across the tree, covering the major lineages.

Subsequently, alternative maximum likelihood models were used to estimate the chromosome number evolutionary rates. The single-rate model assumed a uniform chromosome number evolution rate for the entire superfamily (σ^2). The four-rate model adopted independent rates in each of the four monophyletic groups (Lacertidae, Gymnophthalmidae, Teiidae, and Amphisbaenia). The four variants (one per group) of the two-rate model assumed a different rate of the given group versus all other groups combined (i.e. Lacertidae vs all other groups, Gymnophthalmidae vs all other groups, Teiidae vs all other groups, and Amphisbaenia vs all other groups). Each of these multi-rate models was then compared to the single-rate model using a likelihood ratio test, each with a p-value calculated based on the χ^2 distribution. The computation was performed in brownie.lite function in the phytools R package v2.3-0 (Revell, 2012). We then used the Akaike information criterion to determine the best model, based on the highest likelihood and minimum number of parameters.

Synteny analysis and the origin of the lacertid karyotypes

We compared the available genomes of six lacertids and expanded our analysis to incorporate an assembly of another lacertoid, tegu (*Salvator merianae*), a member of the Teiidae family, as well as genomes of seven additional squamates. This broader approach resulted in a synteny map containing a web of homologies among 14 representative squamate species spanning Gekkota, Scincoidea, Lacertoidea and Toxicofera (Table 1; Fig. 2).

The identification of the synteny blocks between the genomes of the 14 squamate species was performed at the genes level using orthogroups computed by Orthofinder v2.5.4 (Emms & Kelly, 2019) and GENESPACE v1.2.3 (Lovell et al. 2022) R library. The amino acid sequences for *Eremias argus* and *Cryptoblepharus egeriae* were not available, hence we predicted them utilising AUGUSTUS v 3.3.2 (Stanke et al. 2008) with the *ab initio* mode and chicken (*Gallus gallus*) as a reference species. Additionally, we evaluated the completeness of genomic assemblies and predictions using BUSCO v4.1.4 (Benchmarking Universal Single-Copy Orthologs) (Manni et al. 2021) search against eukaryotic datasets (Fig, S1).

Results

Evolutionary rates in chromosome number changes

The model incorporating different evolutionary rates of chromosome number changes for lacertids compared to other lacertoidean lineages outperformed the model predicting a single rate for all lacertoids and other multi-rate models (Table 2). It was selected based on its low log-likelihood and minimum number of parameters expressed in the Akaike information criterion (AIC) estimator. Moreover, this model fits data better than other models compared to the single rate model, as indicated by the lowest P-value for a likelihood ratio test against the $\chi 2$ distribution. The

family Lacertidae revealed to exhibit an approximately five-times slower rate of chromosome number evolution ($\sigma^2_L = 88$) than the other lacertoidean lineages ($\sigma^2_{nL} = 442$) (Fig. 1).

Synteny analysis and the origin of the stable lacertid karyotypes

All six lacertid species with available chromosome-level genome assemblies have a strong synteny of their chromosomes (Fig. 2). The only exception is the sixth chromosome pair of *Zootoca vivipara*, where the terminal part corresponds to a microchromosome of the other species, which is a clear apomorphy of this lineage. The conserved synteny within the lacertids corresponds to their low evolutionary rates in chromosome numbers (Fig. 1).

Tegu exhibited remarkable chromosome-wide homology with scincoid and toxicoferan species. We observed that the five largest (macro-)chromosomes of the tegu are 1:1 homologous to those in the brown anole and cliff lizard, while three of these (macro-)chromosomes show 1:1 homology in the skink and snakes. Tegu's sixth and seventh chromosomes aligned to a single (macro-)chromosome homologous among the aforementioned species, suggesting putative fission in the tegu's ancestor. The 12 smallest (micro-)chromosomes of the tegu were present in all other species as syntenic blocks, either as independent (micro-)chromosomes or fused to other chromosomes. Interestingly, the colocalization of these syntenic blocks was unique to each lineage, implying that these are instances of independent fusions while the tegu represents the ancestral state.

Thus, tegu and cliff lizard chromosomes are highly conserved - despite these species sharing their last common ancestor at the divergence of Episquamata (= Lacertoidea + Toxicofera) and Scincoidea 196.9 million years ago (Zheng & Wiens, 2015) - and, therefore, most likely resemble the ancestral state for the entire Unidentata group (Squamata excluding Dibamidae and

Gekkota). In comparison, lacertid chromosomes appear to be products of fissions of large ancestral (macro-)chromosomes and fusions of the small ancestral (micro-)chromosomes.

Discussion

Based on our current understanding of squamate phylogeny and the synteny, we propose that the ancestral karyotype of Lacertoidea comprised 2n=36-38 chromosomes distinguishable to macro- and microchromosomes, resembling the state found in several extant species of the subfamily Tupinambinae, such as tegu (Gorman, 1970; Santos et al. 2008; Carvalho et al. 2015; da Silva et al. 2020; Fig. 1; Table S1). It corresponds to the large macrochromosomes of other Unidentata, namely toxicoferan and scincoidean species, Fig. 2) and 12 microchromosomes found in other reptiles (Waters et al. 2020; Pinto et al. 2023).

Compared to this putative ancestral state, the karyotypes of the family Lacertidae are clearly derived. We hypothesise that the ancestral karyotype of most lacertids (the subfamily Lacertinae encompassing 96% lacertid species, i.e. all except Gallotinae) contained 2n=38 acrocentric chromosomes, gradually decreasing in size and this configuration remains prevalent within the group. The small subfamily Gallotinae with 2n=40 acrocentric chromosomes may either have an intermediate state with one less fusion, or an autapomorphy evolved by a fission. Therefore, the derived karyotype in most lacertids emerged approximately 65-154 million years ago, at least since the split within Lacertinae, possibly even since their divergence from Amphisbaenia (Zheng & Wiens, 2015).

The derived lacertid karyotype likely originated through two opposing mechanisms: fissions of ancestral macrochromosomes and fusions involving ancestral microchromosomes (Fig. 2). This observation is intriguing as it indicates that two opposite mechanisms acted on different

size categories of the ancestral chromosomes. The tendency of microchromosomes to either remain conserved or to fuse with each other in a lineage-specific fashion was noted earlier (Pinto et al. 2023; Srikulnath et al. 2021; Waters et al. 2021). The tendency for the fusions could be primarily attributed to their colocalisation and more frequent interactions compared to the macrochromosomes in the 3D structure of the interphase nucleus (Perry et al. 2021). However, this suggestion does not explain the strong conservation of microchromosomes in many lineages (Waters et al. 2020; Pinto et al. 2023).

The largest chromosomes of tegu are biarmed (da Silva et al. 2020) and aligned to the derived lacertid chromosomes (Fig. 2), which are all acrocentric (Gorman, 1969; Vujošević & Blagojević, 1999; Odierna et al. 2004; Lisachov et al. 2020). This suggests that they have undergone centric fissions. The multiple fissions of the ancestral macrochromosomes during the formation of the lacertid karyotype might have happened independently of the microchromosome fusions. We can speculate that centromeric (meiotic) drive might explain the series of centric fissions. Chmátal et al. (2014) observed such biased binding in acrocentric chromosomes compared to their biarmed homologs in lab mice. de Villena & Sapienza (2001) and Blackmon et al. (2019) proposed that meiotic drive might act as a stabilising mechanism in all-acrocentric and all-biarmed karyotypes. They also observed that such karyotypes are associated with a lower CN evolutionary rate in mammals. The polarity reversal of the meiotic drive was proposed to lead to a reversed trend, favouring centric fusions instead of fissions (or the other way) (de Villena & Sapienza, 2001). It is remarkable that among squamates, a clear trend to lower chromosome numbers (fusions) was reported in chameleons (Mezzasalma et al. 2023), while lacertids, at least in macrochromosomes, tended to go through multiple fissions, while other lacertoideans seems not to follow any notable trend (Fig. 1).

Despite the outstanding dominance of the all-acrocentric karyotypes, biarmed chromosomes have evolved at least five times independently in lacertids, namely in the genera *Darevskia, Zootoca, Timon, Iberolacerta* and *Parvilacerta*. All known cases point towards centric fusions as the responsible mechanism. Interestingly, two of the five cases involve the presence of a single biarmed heterechromosome evolved under special inheritance patterns potentially able to overcome general operation of the meiotic drive: one evolved in the "clonal" parthenogenetic hybrid *Darevskia unisexualis,* and the second concerns a W chromosome-autosome fusion within *Zootoca vivipara* (Spangenberg et al. 2021; Kupriyanova & Melashchenko, 2011). However, the other cases concern autosomes. The species of the genus *Timon* are characterised by a single biarmed chromosome pair (Giovannotti et al. 2018; Odierna et al. 1990; Rykena & Nettmann, 1986; Suwala et. al., 2020) documented to be homologous to two ancestral acrocentric chromosomes (Naveira et al. 2023). The most extreme changes were observed in species of the genera *Iberolacerta* and *Parvilacerta*. Some of their species are even characterised by the majority of chromosomes being biarmed with CN as low as 2n=24 (Odierna et al. 1996; Olmo et al. 2001). Such a sudden burst of centric fusions fits the hypothesis of the polarity switch of centromeric drive. However, the exact mechanism leading to the preference for fused or split chromosomes in the centromeric drive remains unclear.

Intrachromosomal rearrangements - pericentric inversions and centromere repositioning - are additional mechanisms that can generate biarmed chromosomes. They have been documented as the primary mechanism in geckos, another lineage with a putative ancestral all-acrocentric karyotype (Pensabene et al. 2024; Pokorná et al. 2015), and in monitor lizards, another lineage known for conserved chromosome numbers (Iannucci et al. 2019). We document that gekkotan and lacertid karyotypes evolved likely convergently by repeated fusions of the ancestral

microchromosomes, however, that the biarmed macrochromosomes of most other squamates underwent repeated Robertsonian fissions to form the ancestral all-acrocentric karyotypes of these diversified lineages (Fig. 2). Remarkably, in contrast to geckos, there is not a single case of this type of rearrangement in lacertids. This further suggests that lacertid centromeres might have unique properties making them resistant not only to chromosome fusions, but also to intrachromosomal rearrangements.

Given the resistance of lacertid centromeres to fusions or repositioning, we speculate they play a key role in stabilising lacertid karyotypes. Future research should focus on investigating the epigenetic makeup of lacertid centromeres, their repetitive content, and the existing variants of associated histones and kinetochore proteins. We suggest implementing long-read sequencing technologies (Marx, 2023) to uncover the composition of lacertid centromeres, combined with comparative mapping between their biarmed and acrocentric counterparts. Another approach could exploit the strong tolerance of lacertid species to interspecies hybridisation (up to 19% genetic distance between parental species) (Jančúchová-Lásková et al. 2015; Rykena, 2002). A back-cross between a species with a derived karyotype and a closely related species with an ancestral karyotype could be attempted - for example, a cross between *Iberolacerta aranica* and *Iberolacerta galani* or between *Parvilacerta* and *Atlantolacerta*. If successful, F1 hybrids could be used to compare the centromeres of parental chromosomes and their inheritance.

In summary, we documented a dynamic karyotype evolution in teiids, gymnophthalmids, and amphisbaenas, contrasted by its dramatic decrease in lacertids. The derived lacertid karyotypes evolved surprisingly through two opposing mechanisms operating on chromosomes of different sizes: repeated fusions of the ancestral microchromosomes and fissions of the homologs of biarmed macrochromosomes common in many squamates. Interestingly, the gekkotan ancestral karyotype

likely evolved by analogous processes. The stability of the karyotype in lacertids is not only connected with low rate of centric fusions (although exceptions can be found in this respect), but also by a notable lack of pericentric inversions or centromere reposition leading to metacentric chromosomes. We suggest that the potentially derived nature of lacertid centromeres could explain the peculiar stasis in their karyotypes.

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Data Accessibility: Data are provided in the Supplementary material.

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References

Blackmon, H., Justison, J., Mayrose, I., & Goldberg, E. E. (2019). Meiotic drive shapes rates of karyotype evolution in mammals. *Evolution, 73*, 511–523. https://doi.org/10.1111/evo.13682

Carvalho, N. D. M., Arias, F. J., Da Silva, F. A., Schneider, C. H., & Gross, M. C. (2015). Cytogenetic analyses of five Amazon lizard species of the subfamilies Teiinae and

Tupinambinae and review of karyotyped diversity in the family Teiidae. *Comparative Cytogenetics, 9*, 625–644. https://doi.org/10.3897/CompCytogen.v9i4.5371

Chmátal, L., Gabriel, S. I., Mitsainas, G. P., Martínez-Vargas, J., Ventura, J., Searle, J. B., Schultz, R. M., & Lampson, M. A. (2014). Centromere strength provides the cell biological basis for meiotic drive and karyotype evolution in mice. *Current Biology: CB, 24*, 2295–2300. https://doi.org/10.1016/j.cub.2014.08.017

Da Silva, M. J., De Araújo Vieira, A. P., Galvão Cipriano, F. M., Dos Santos Cândido, M. R., De Oliveira, E. H. C., Gimenez Pinheiro, T., & Da Silva, E. L. (2020). The karyotype of *Salvator merianae* (Squamata, Teiidae): Analyses by classical and molecular cytogenetic techniques. *Cytogenetic and Genome Research, 160*, 94–99. https://doi.org/10.1159/000506140

Darevsky, I. S., & Danielyan, F. D. (1968). Diploid and triploid progeny arising from natural mating of parthenogenetic *Lacerta armeniaca* and *L. unisexualis* with bisexual *L. saxicola valentini*. *Journal of Herpetology, 2*, 65–69. https://doi.org/10.2307/1563104

De Rop, V., Padeganeh, A., & Maddox, P. S. (2012). CENP-A: The key player behind centromere identity, propagation, and kinetochore assembly. *Chromosoma, 121*, 527–538. https://doi.org/10.1007/s00412-012-0386-5

De Villena, F. P.-M., & Sapienza, C. (2001). Female meiosis drives karyotypic evolution in mammals. *Genetics, 159*, 1179–1189. https://doi.org/10.1093/genetics/159.3.1179

Deakin, J. E., & Ezaz, T. (2019). Understanding the evolution of reptile chromosomes through applications of combined cytogenetics and genomics approaches. *Cytogenetic and Genome Research, 157*, 7–20. https://doi.org/10.1159/000495974

Dudka, D., & Lampson, M. A. (2022). Centromere drive: Model systems and experimental progress. *Chromosome Research: An International Journal on the Molecular,*

Supramolecular and Evolutionary Aspects of Chromosome Biology, 30, 187–203. https://doi.org/10.1007/s10577-022-09696-3

Falcione, C., & Hernando, A. (2010). A new karyotypic formula for the genus *Amphisbaena* (Squamata: Amphisbaenidae). *Phyllomedusa: Journal of Herpetology, 9*, 75. https://doi.org/10.11606/issn.2316-9079.v9i1p75-80

Giovannotti, M., Nisi Cerioni, P., Rojo, V., Olmo, E., Slimani, T., Splendiani, A., & Caputo Barucchi, V. (2018). Characterization of a satellite DNA in the genera *Lacerta* and *Timon* (Reptilia, Lacertidae) and its role in the differentiation of the W chromosome. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution, 330*, 83–95. https://doi.org/10.1002/jez.b.22790

Goicoechea, N., Frost, D. R., De la Riva, I., Pellegrino, K. C. M., Sites Jr, J., Rodrigues, M. T., & Padial, J. M. (2016). Molecular systematics of teioid lizards (Teioidea/Gymnophthalmoidea: Squamata) based on the analysis of 48 loci under tree-alignment and similarity-alignment. *Cladistics, 32*, 624–671. https://doi.org/10.1111/cla.12150

Gorman, G. C. (1969). New chromosome data for 12 species of lacertid lizards. *Journal of Herpetology, 3*, 49. https://doi.org/10.2307/1563223

Gorman, G. C. (1970). Chromosomes and the systematics of the family Teiidae (Sauria, Reptilia). *Copeia, 1970*, 230. https://doi.org/10.2307/1441645

Gorman, G. C., & Shochat, D. (1972). A taxonomic interpretation of chromosomal and electrophoretic data on the agamid lizards of Israel with notes on some East African species. *Herpetologica, 28*, 106–112.

Huang, C. C., Clark, H. F., & Gans, C. (1967). Karyological studies on fifteen forms of amphisbaenians (Amphisbaenia-Reptilia). *Chromosoma, 22*, 1–15. https://doi.org/10.1007/BF00291283

Iannucci, A., Altmanová, M., Ciofi, C., Ferguson-Smith, M., Milan, M., Pereira, J. C., Pether, J., Rehák, I., Rovatsos, M., Stanyon, R., Velenský, P., Ráb, P., Kratochvíl, L., & Johnson Pokorná, M. (2019). Conserved sex chromosomes and karyotype evolution in monitor lizards (Varanidae). *Heredity, 123*, 215–227. https://doi.org/10.1038/s41437-018-0179-6

Imai, H. T., Satta, Y., Wada, M., & Takahata, N. (2002). Estimation of the highest chromosome number of eukaryotes based on the minimum interaction theory. *Journal of Theoretical Biology, 217*, 61–74. https://doi.org/10.1006/jtbi.2002.3016

Jančúchová-Lásková, J., Landová, E., & Frynta, D. (2015). Are genetically distinct lizard species able to hybridize? A review. *Current Zoology, 61*, 155–180. https://doi.org/10.1093/czoolo/61.1.155

Kupriyanova, L., & Melashchenko, O. (2011). The common Eurasian lizard *Zootoca vivipara* (Jacquin, 1787) from Russia: Sex chromosomes, subspeciation, and colonization. *Russian Journal of Herpetology, 18*, 99-104. (Corrected full name)

Laguna, M. M., Amaro, R. C., Mott, T., Yonenaga-Yassuda, Y., & Rodrigues, M. T. (2010). Karyological study of *Amphisbaena ridleyi* (Squamata, Amphisbaenidae), an endemic species of the Archipelago of Fernando de Noronha, Pernambuco, Brazil. *Genetics and Molecular Biology, 33*, 57–61. https://doi.org/10.1590/S1415-47572010005000009

Laguna, M. M., Rodrigues, M. T., Dos Santos, R. M. L., Yonenaga-Yassuda, Y., Ávila-Pires, T. C. S., Hoogmoed, M. S., & Pellegrino, K. C. M. (2010). Karyotypes of a cryptic diploid form of the unisexual *Leposoma percarinatum* (Squamata, Gymnophthalmidae) and the bisexual

Leposoma ferreirai from the lower Rio Negro, Amazonian Brazil. *Journal of Herpetology, 44*, 153–157. https://doi.org/10.1670/08-250.1

Lisachov, A. P., Giovannotti, M., Pereira, J. C., Andreyushkova, D. A., Romanenko, S. A., Ferguson-Smith, M. A., Borodin, P. M., & Trifonov, V. A. (2020). Chromosome painting does not support a sex chromosome turnover in *Lacerta agilis* Linnaeus, 1758. *Cytogenetic and Genome Research, 160*, 134–140. https://doi.org/10.1159/000506321

Lovell, J. T., Sreedasyam, A., Schranz, M. E., Wilson, M., Carlson, J. W., Harkess, A., Emms, D., Goodstein, D. M., & Schmutz, J. (2022). GENESPACE tracks regions of interest and gene copy number variation across multiple genomes. *eLife, 11*, e78526. https://doi.org/10.7554/eLife.78526

Lowe, C. H., Wright, J. W., Cole, C. J., & Bezy, R. L. (1970). Chromosomes and evolution of the species groups of *Cnemidophorus* (Reptilia: Teiidae). *Systematic Zoology, 19*, 128. https://doi.org/10.2307/2412450

Malik, H. S., & Henikoff, S. (2002). Conflict begets complexity: The evolution of centromeres. *Current Opinion in Genetics & Development, 12*, 711–718. https://doi.org/10.1016/S0959-437X(02)00351-9

Marx, V. (2023). Method of the year: Long-read sequencing. *Nature Methods, 20*, 6–11. https://doi.org/10.1038/s41592-022-01730-w

Mayrose, I., & Lysak, M. A. (2021). The evolution of chromosome numbers: Mechanistic models and experimental approaches. *Genome Biology and Evolution, 13*, evaa220. https://doi.org/10.1093/gbe/evaa220

Mezzasalma, M., Guarino, F. M., & Odierna, G. (2021). Lizards as model organisms of sex chromosome evolution: What we really know from a systematic distribution of available data? *Genes, 12*, Article 9. https://doi.org/10.3390/genes12091341

Mezzasalma, M., Streicher, J. W., Guarino, F. M., Jones, M. E. H., Loader, S. P., Odierna, G., & Cooper, N. (2023). Microchromosome fusions underpin convergent evolution of chameleon karyotypes. *Evolution, 77*, 1930–1944. https://doi.org/10.1093/evolut/qpad097

Naveira, H., Rojo, V., Gómez-Seoane, I., Ferguson-Smith, M. A., Pereira, J. C., & Martínez-Lage, A. (2023). Chromosome evolution in a genus that deviates from the standard karyotype formula of Lacertidae. 16 May 2023, PREPRINT (Version 1) available at Research Square [https://doi.org/10.21203/rs.3.rs-2917305/v1] https://doi.org/10.21203/rs.3.rs-2917305/v1

Odierna, Aprea, G., Capriglione, T., Caputo, V., & Olmo, E. (1996). The karyology of the Iberian rock lizards. *Herpetologica*, 542-550.

Odierna, G., Aprea, G. E. N. N. A. R. O., Capriglione, T., & Puky, M. I. K. L. Ó. S. (2004). Chromosomal evidence for the double origin of viviparity in the European common lizard, *Lacerta* (*Zootoca*) *vivipara*. *Herpetological Journal, 14*, 157-160.

Odierna, G., Olmo, E., Capriglione, T., & Caputo, V. (1990). Karyological differences between *Lacerta lepida* and *Lacerta pater*. *Journal of Herpetology, 24*, 97. https://doi.org/10.2307/1564300

Olmo, E. (2008). Trends in the evolution of reptilian chromosomes. *Integrative and Comparative Biology, 48*, 486–493. https://doi.org/10.1093/icb/icn049

Olmo, E., Odierna, G., & Cobror, O. (1986). C-band variability and phylogeny of Lacertidae. *Genetica, 71*, 63–74. https://doi.org/10.1007/BF00123234

Otto, S. P., & Whitton, J. (2000). Polyploid incidence and evolution. *Annual Review of Genetics, 34*, 401–437. https://doi.org/10.1146/annurev.genet.34.1.401

Pellegrino, K. (2001). A molecular perspective on the evolution of microteiid lizards (Squamata, Gymnophthalmidae), and a new classification for the family. *Biological Journal of the Linnean Society, 74*, 315–338. https://doi.org/10.1006/bijl.2001.0580

Pellegrino, K. C. M., Rodrigues, M. T., & Yonenaga-Yassuda, Y. (1999). Chromosomal polymorphisms due to supernumerary chromosomes and pericentric inversions in the eyelidless microteiid lizard *Nothobachia ablephara* (Squamata, Gymnophthalmidae). *Chromosome Research, 7*, 247–254. https://doi.org/10.1023/A:1009218628942

Pellegrino, K. C. M., Rodrigues, M. T., & Yonenaga-Yassuda, Y. (2004). Chromosomal evolution in the Brazilian lizards of genus *Leposoma* (Squamata, Gymnophthalmidae) from Amazon and Atlantic rain forests: Banding patterns and FISH of telomeric sequences. *Hereditas, 131*, 15–21. https://doi.org/10.1111/j.1601-5223.1999.00015.x

Pensabene, E., Augstenová, B., Kratochvíl, L., & Rovatsos, M. (2024). Differentiated sex chromosomes, karyotype evolution, and spontaneous triploidy in carphodactylid geckos. *Journal of Heredity, 115*, 262–276. https://doi.org/10.1093/jhered/esae010

Perry, B. W., Schield, D. R., Adams, R. H., & Castoe, T. A. (2021). Microchromosomes exhibit distinct features of vertebrate chromosome structure and function with underappreciated ramifications for genome evolution. *Molecular Biology and Evolution, 38*, 904–910. https://doi.org/10.1093/molbev/msaa253

Pinto, B. J., Gamble, T., Smith, C. H., & Wilson, M. A. (2023). A lizard is never late: Squamate genomics as a recent catalyst for understanding sex chromosome and microchromosome evolution. *Journal of Heredity, 114*, 445–458. https://doi.org/10.1093/jhered/esad023

Putnam, N. H., Butts, T., Ferrier, D. E. K., Furlong, R. F., Hellsten, U., Kawashima, T., Robinson-Rechavi, M., Shoguchi, E., Terry, A., Yu, J.-K., Benito-Gutiérrez, E., Dubchak, I., Garcia-Fernàndez, J., Gibson-Brown, J. J., Grigoriev, I. V., Horton, A. C., De Jong, P. J., Jurka, J., Kapitonov, V. V., … Rokhsar, D. S. (2008). The amphioxus genome and the evolution of the chordate karyotype. *Nature, 453*, 1064–1071. https://doi.org/10.1038/nature06967

Rovatsos, M., Altmanová, M., Johnson Pokorná, M., Velenský, P., Sánchez Baca, A., & Kratochvíl, L. (2017). Evolution of karyotypes in chameleons. *Genes, 8*, 382. https://doi.org/10.3390/genes8120382

Ruiz-Herrera, A., Farré, M., & Robinson, T. J. (2012). Molecular cytogenetic and genomic insights into chromosomal evolution. *Heredity, 108*, 28–36. https://doi.org/10.1038/hdy.2011.102

Rykena, S. (2001). Experimental hybridization in green lizards (*Lacerta* s. str.), a tool to study species boundaries. *Mertensiella, 13*, 78-88.

Rykena, S., & Nettmann, H. K. (1986). The karyotype of *Lacerta princeps kurdistanica* and its meaning in phylogeny. *Studies in Herpetology*, 193-196.

Schmid, M., Felchtinger, W., Nanda, I., Schakowski, R., Garcia, R. V., Puppo, J. M., & Badillo, A. F. (1994). An extraordinarily low diploid chromosome number in the reptile *Gonatodes taniae* (Squamata, Gekkonidae). *Journal of Heredity, 85*, 255–260. https://doi.org/10.1093/oxfordjournals.jhered.a111452

Serafim, H., Peccinini-Seale, D. M., & Batistic, R. F. (2007). Estudo cariotípico de duas espécies brasileiras do gênero *Micrurus* (Ophidia: Elapidae). *Biota Neotropica, 7*, 75–80. https://doi.org/10.1590/S1676-06032007000100010

Spangenberg, V., Arakelyan, M., Galoyan, E., Martirosyan, I., Bogomazova, A., Martynova, E., de Bello Cioffi, M., Liehr, T., Al-Rikabi, A., Osipov, F., Petrosyan, V., & Kolomiets, O.

(2021). Meiotic synapsis of homeologous chromosomes and mismatch repair protein detection in the parthenogenetic rock lizard *Darevskia unisexualis*. *Molecular Reproduction and Development, 88*, 119–127. https://doi.org/10.1002/mrd.23450

Srikulnath, K., Ahmad, S. F., Singchat, W., & Panthum, T. (2021). Why do some vertebrates have microchromosomes? *Cells, 10*, 2182. https://doi.org/10.3390/cells10092182

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*, 531. https://doi.org/10.3390/genes11050531

Waters, P. D., Patel, H. R., Ruiz-Herrera, A., Álvarez-González, L., Lister, N. C., Simakov, O., Ezaz, T., Kaur, P., Frere, C., Grützner, F., Georges, A., & Graves, J. A. M. (2021). Microchromosomes are building blocks of bird, reptile, and mammal chromosomes. *Proceedings of the National Academy of Sciences, 118*, e2112494118. https://doi.org/10.1073/pnas.2112494118

Webster, T. P., Hall, W. P., & Williams, E. E. (1972). Fission in the evolution of a lizard karyotype. *Science, 177*, 611–613. https://doi.org/10.1126/science.177.4049.611

Yang, F., O'brien, P. C. M., Wienberg, J., & Ferguson-Smith, M. A. (1997). A reappraisal of the tandem fusion theory of karyotype evolution in the Indian muntjac using chromosome painting. *Chromosome Research, 5*, 109-117.

Yonenaga-Yassuda, Y., Rodrigues, M. T., & Pellegrino, K. C. M. (2005). Chromosomal banding patterns in the eyelidless microteiid lizard radiation: The X1X1X2X2:X1X2Y sex chromosome system in *Calyptommatus* and the karyotypes of *Psilophthalmus* and *Tretioscincus* (Squamata, Gymnophthalmidae). *Genetics and Molecular Biology, 28*, 700–709. https://doi.org/10.1590/S1415-47572005000500009

Table 1. Species used to construct the synteny map of alignments

short_id	family	species	Common name	Source/ Gene Bank accession	Notes
emac	Eublepharidae (Gekkota)	*Eublepharis macularius*	Common Leopard Gecko	GCA_028583425.1	
sphtow	Sphaerodactylidae (Gekkota)	*Sphaerodactylus townsendi*	Puerto Rican Sandy Geckolet	GCF_021028975.2	
hemcap	Cordylidae (Scincoidea)	*Hemicordylus capensis*	Cape Cliff Lizard	GCF_027244095.1	
cryege	Scincidae (Scincoidea)	*Cryptoblepharus egeriae*	Christmas Island Blue-tailed Shinning-skink	GCA_030015325.1	genes were predicted using Augustus with chicken as a model
tegu	Teiidae (Lacertoidea)	*Salvator merianae*	Argentine Black and White Tegu	GCA_003586115.2	Scaffold-level assembly
eragus	Lacertidae (Lacertoidea)	*Eremias argus*	Mongolia Racerunner	https://figshare.com/articles/dataset/Chromosome-level_genome_assembly_and_population_genomics_of_Mongolian_racerunner_Eremias_argus_provide_insights_into_high-altitude_adaptation_in_lizards/21098470	genes were predicted using Augustus with chicken as a model
podmur	Lacertidae (Lacertoidea)	*Podarcis muralis*	Common Wall Lizard	GCF_004329235.1	
podraf	Lacertidae (Lacertoidea)	*Podarcis raffonei*	Aeolian Wall Lizard	GCF_027172205.1	
podlia	Lacertidae (Lacertoidea)	*Podarcis lilfordi*	Lilford's Wall Lizard	GCA_947686815.1	
lacagi	Lacertidae (Lacertoidea)	*Lacerta agilis*	Sand Lizard	GCF_009819535.1	
zviv	Lacertidae (Lacertoidea)	*Zootoca vivipara*	Common Lizard	GCF_963506605.1	
anosag	Anolidae (Iguania)	*Anolis sagrei ordinatus*	Brown anole	GCF_025583915.1	Scaffold-level assembly
najana	Elapidae (Serpentes)	*Naja naja*	Common cobra	GCA_009733165.1	
ahapra	Colubridae (Serpentes)	*Ahaetulla prasina*	Gunther's whip snake	GCA_028640845.1	

Table 2. Comparison of fitted models for the rate of chromosome number evolution. The best model is determined based on ΔAIC and highlighted in bold.

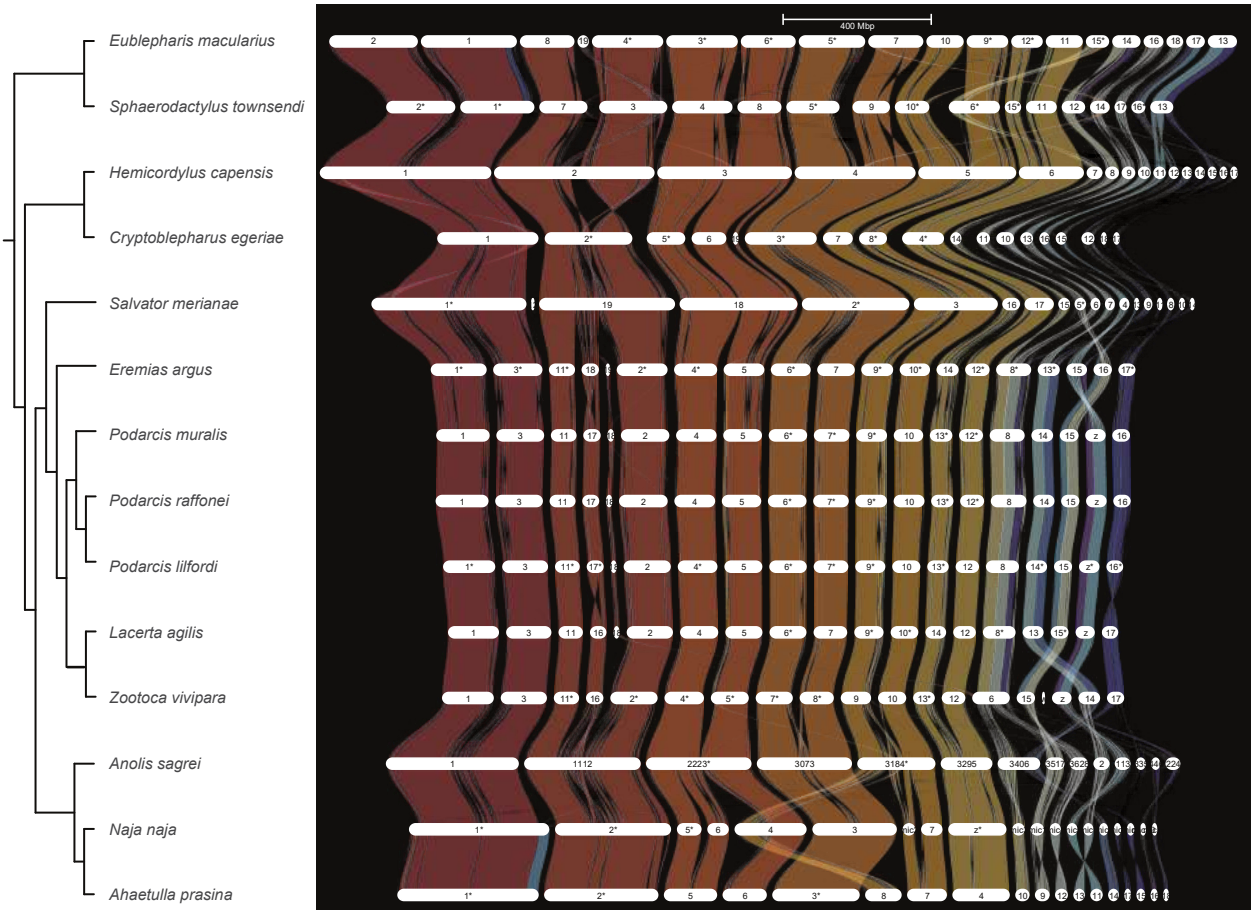
Model description	log-likelihood	p-value	AIC	ΔAIC
Two-rate: Lacertidae-vs-other groups	**-509.698**	**1.04E-13**	**1023.395**	**0**
Four-rate	-508.194	1.36E-12	1024.387	0.992
Two-rate: Gymnophthalmidae-vs-other groups	-530.534	2.25E-4	1065.068	41.673
Two-rate: Teiidae-vs-other groups	-532.948	3.04E-3	1069.896	46.501
Single-rate	-537.339	—	1076.678	53.283
Two-rate: Amphisbaenia-vs-other groups	-537.241	6.58E-1	1078.482	55.087

Model description	Parameters	log-likelihood	p-value	AIC	ΔAIC
Lacertidae vs all other groups	**2**	**-509.698**	**1.04E-13**	**1023.395**	**0**
Individual rates per each group	4	-508.194	1.36E-12	1024.387	0.992
Gymnophthalmidae vs all other groups	2	-530.534	2.25E-4	1065.068	41.673
Teiidae vs all other groups	2	-532.948	3.04E-3	1069.896	46.501
Single-rate	1	-537.339	—	1076.678	53.283
Amphisbaenia vs all other groups	2	-537.241	6.58E-1	1078.482	55.087

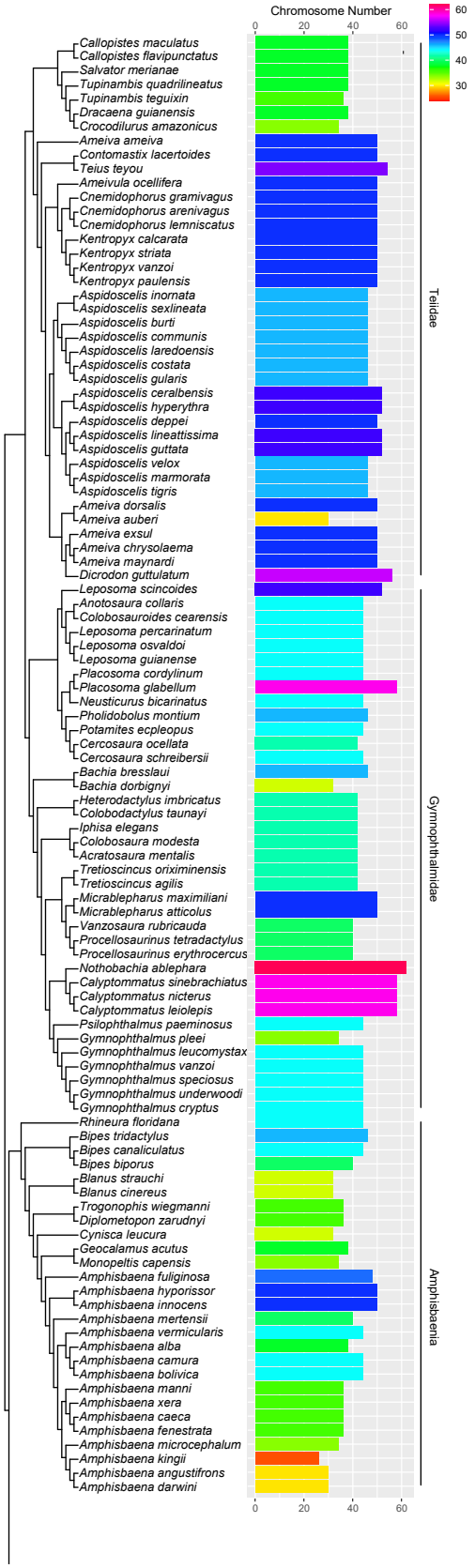
Figure legends

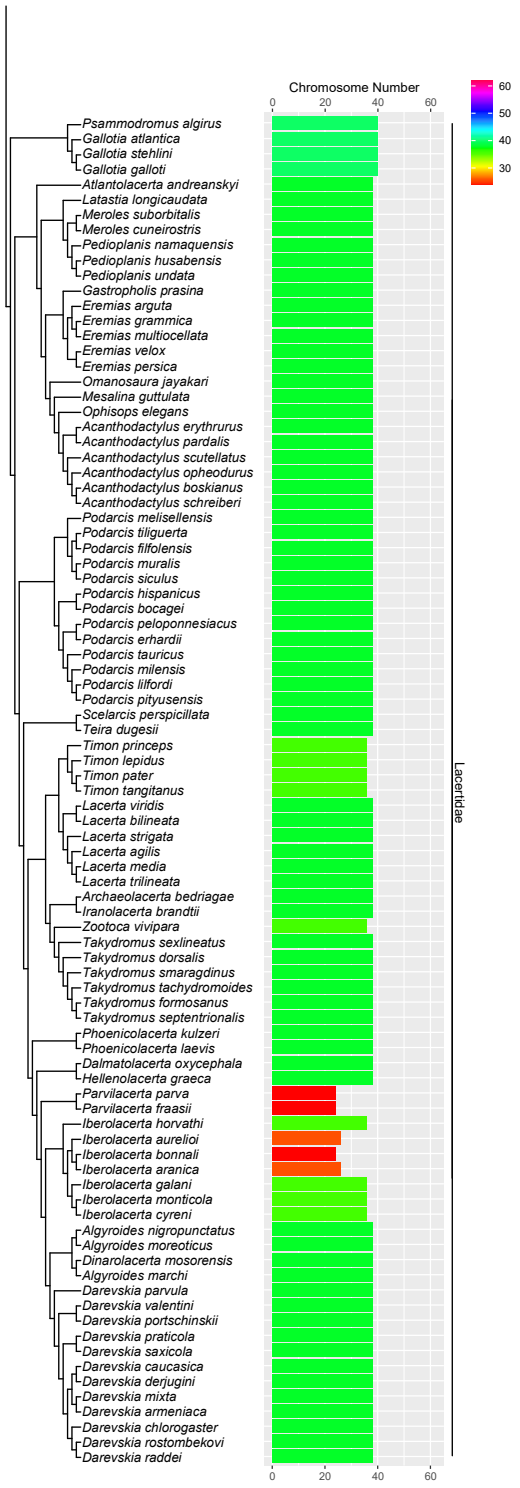
Figure 1. Phylogenetic reconstruction of chromosome diploid numbers under the maximum parsimony based on the phylogenetic hypothesis of Lacertoidea by Zheng & Wiens (2015).

Figure 2. Chromosomal synteny comparison between the 14 squamate species: 2 geckos, 2 scincoids, 1 teiid, 6 lacertids, an iguanid, and 2 snakes. The colors of bands linking the syntenic blocks are based on *Hemicordylus capensis* chromosomes. Chromosomes marked with an asterisk have been inverted to align with the rest of their homologs.



Chromosomes scaled by physical position


Chromosome Number
0
20
40
60
60
50
40
30
Callopistes maculatus
Callopistes flavipunctatus
Salvator merianae
Tupinambis quadrilineatus
Tupinambis teguixin
Dracaena guianensis
Crocodilurus amazonicus
Ameiva ameiva
Contomastix lacertoides
Teius teyou
Ameivula ocellifera
Cnemidophorus gramivagus
Cnemidophorus arenivagus
Cnemidophorus lemniscatus
Kentropyx calcarata
Kentropyx striata
Kentropyx vanzoi
Kentropyx paulensis
Aspidoscelis inornata
Aspidoscelis sexlineata
Aspidoscelis burti
Aspidoscelis communis
Aspidoscelis laredoensis
Aspidoscelis costata
Aspidoscelis gularis
Aspidoscelis ceralbensis
Aspidoscelis hyperythra
Aspidoscelis deppei
Aspidoscelis lineattissima
Aspidoscelis guttata
Aspidoscelis velox
Aspidoscelis marmorata
Aspidoscelis tigris
Ameiva dorsalis
Ameiva auberi
Ameiva exsul
Ameiva chrysolaema
Ameiva maynardi
Dicrodon guttulatum
Leposoma scincoides
Anotosaura collaris
Colobosauroides cearensis
Leposoma percarinatum
Leposoma osvaldoi
Leposoma guianense
Placosoma cordylinum
Placosoma glabellum
Neusticurus bicarinatus
Pholidobolus montium
Potamites ecpleopus
Cercosaura ocellata
Cercosaura schreibersii
Bachia bresslaui
Bachia dorbignyi
Heterodactylus imbricatus
Colobodactylus taunayi
Iphisa elegans
Colobosaura modesta
Acratosaura mentalis
Tretioscincus oriximinensis
Tretioscincus agilis
Micrablepharus maximiliani
Micrablepharus atticolus
Vanzosaura rubricauda
Procellosaurinus tetradactylus
Procellosaurinus erythrocercus
Nothobachia ablephara
Calyptommatus sinebrachiatus
Calyptommatus nicterus
Calyptommatus leiolepis
Psilophthalmus paeminosus
Gymnophthalmus pleei
Gymnophthalmus leucomystax
Gymnophthalmus vanzoi
Gymnophthalmus speciosus
Gymnophthalmus underwoodi
Gymnophthalmus cryptus
Rhineura floridana
Bipes tridactylus
Bipes canaliculatus
Bipes biporus
Blanus strauchi
Blanus cinereus
Trogonophis wiegmanni
Diplometopon zarudnyi
Cynisca leucura
Geocalamus acutus
Monopeltis capensis
Amphisbaena fuliginosa
Amphisbaena hyporissor
Amphisbaena innocens
Amphisbaena mertensii
Amphisbaena vermicularis
Amphisbaena alba
Amphisbaena camura
Amphisbaena bolivica
Amphisbaena manni
Amphisbaena xera
Amphisbaena caeca
Amphisbaena fenestrata
Amphisbaena microcephalum
Amphisbaena kingii
Amphisbaena angustifrons
Amphisbaena darwini
Teiidae
Gymnophthalmidae
Amphisbaenia
0
20
40
60


Chromosome Number
0
20
40
60
60
50
40
30
Psammodromus algirus
Gallotia atlantica
Gallotia stehlini
Gallotia galloti
Atlantolacerta andreanskyi
Latastia longicaudata
Meroles suborbitalis
Meroles cuneirostris
Pedioplanis namaquensis
Pedioplanis husabensis
Pedioplanis undata
Gastropholis prasina
Eremias arguta
Eremias grammica
Eremias multiocellata
Eremias velox
Eremias persica
Omanosaura jayakari
Mesalina guttulata
Ophisops elegans
Acanthodactylus erythrurus
Acanthodactylus pardalis
Acanthodactylus scutellatus
Acanthodactylus opheodurus
Acanthodactylus boskianus
Acanthodactylus schreiberi
Podarcis melisellensis
Podarcis tiliguerta
Podarcis filfolensis
Podarcis muralis
Podarcis siculus
Podarcis hispanicus
Podarcis bocagei
Podarcis peloponnesiacus
Podarcis erhardii
Podarcis tauricus
Podarcis milensis
Podarcis lilfordi
Podarcis pityusensis
Scelarcis perspicillata
Teira dugesii
Timon princeps
Timon lepidus
Timon pater
Timon tangitanus
Lacerta viridis
Lacerta bilineata
Lacerta strigata
Lacerta agilis
Lacerta media
Lacerta trilineata
Archaeolacerta bedriagae
Iranolacerta brandtii
Zootoca vivipara
Takydromus sexlineatus
Takydromus dorsalis
Takydromus smaragdinus
Takydromus tachydromoides
Takydromus formosanus
Takydromus septentrionalis
Phoenicolacerta kulzeri
Phoenicolacerta laevis
Dalmatolacerta oxycephala
Hellenolacerta graeca
Parvilacerta parva
Parvilacerta fraasii
Iberolacerta horvathi
Iberolacerta aurelioi
Iberolacerta bonnali
Iberolacerta aranica
Iberolacerta galani
Iberolacerta monticola
Iberolacerta cyreni
Algyroides nigropunctatus
Algyroides moreoticus
Dinarolacerta mosorensis
Algyroides marchi
Darevskia parvula
Darevskia valentini
Darevskia portschinskii
Darevskia praticola
Darevskia saxicola
Darevskia caucasica
Darevskia derjugini
Darevskia mixta
Darevskia armeniaca
Darevskia chlorogaster
Darevskia rostombekovi
Darevskia raddei
Lacertidae
0
20
40
60

Supplementary material:

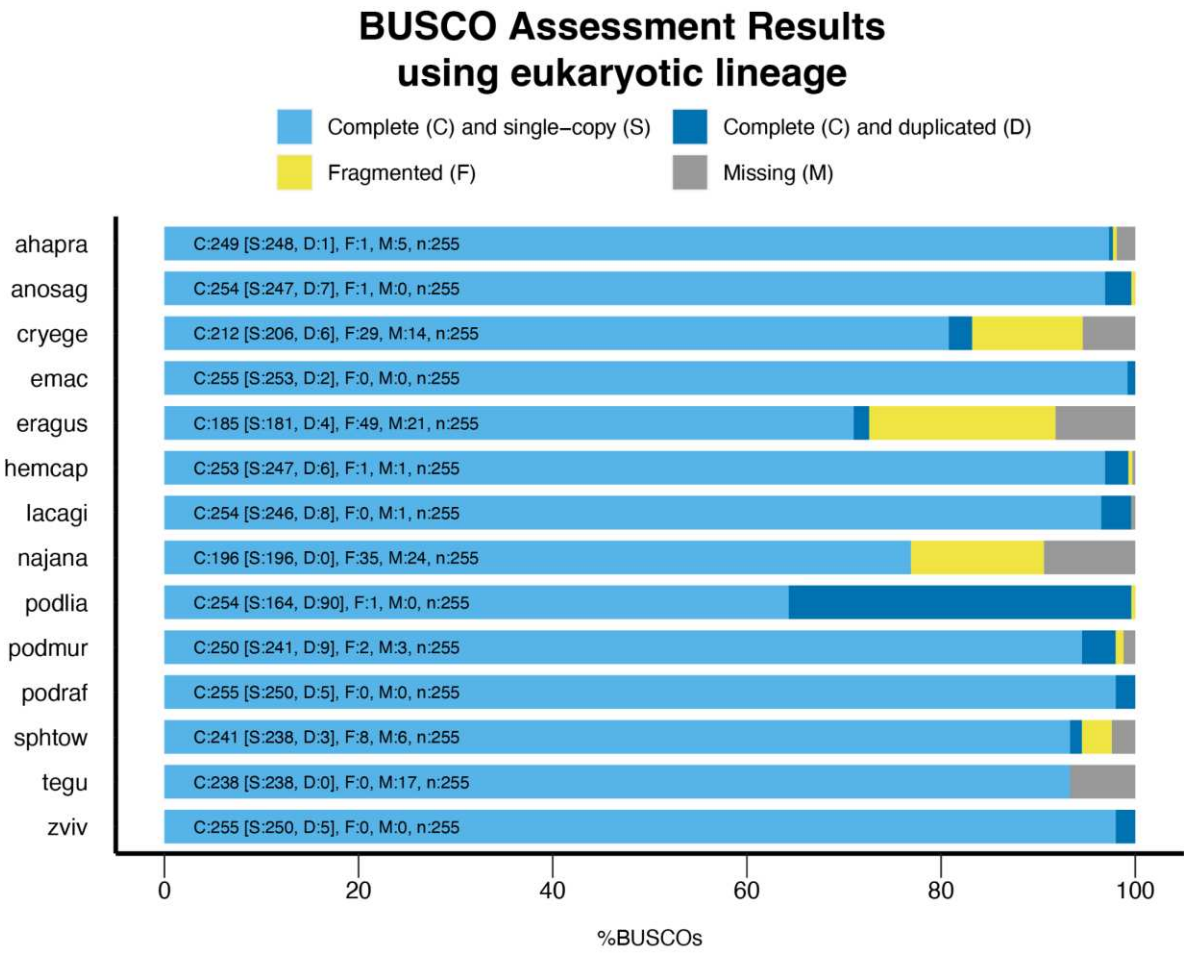


Figure S1. Completeness of the datasets used for the syntenic blocks inference estimated using BUSCO scores against eukaryotic lineage. For species abbreviations see Table 1.

Table S1. Data on chromosome numbers in lacertoidean lizards.

Family	Species (sensu)	2n	Reference
Teiidae	*Callopistes maculatus*	38	Gorman (1970)
Teiidae	*Callopistes flavipunctatus*	38	Gorman (1970)
Teiidae	*Salvator merianae*	38	da Silva et al. (2020)
Teiidae	*Tupinambis quadrilineatus*	38	Santos et al. (2008)
Teiidae	*Tupinambis teguixin*	38	Santos et al. (2008)
Teiidae	*Dracaena guianensis*	38	Gorman (1970)
Teiidae	*Crocodilurus amazonicus*	34	Santos et al. (2008)
Teiidae	*Ameiva ameiva*	50	Santos et al. (2007)
Teiidae	*Contomastix lacertoides*	50	Veronese et al. (2003)
Teiidae	*Teius teyou*	54	Gorman (1970)
Teiidae	*Ameivula ocellifera*	50	Santos et al. (2007)
Teiidae	*Cnemidophorus gramivagus*	50	Peccinini-Seale & Almeida (1986)
Teiidae	*Cnemidophorus arenivagus*	50	Markezich et al. (1997)
Teiidae	*Cnemidophorus lemniscatus*	50	Gorman (1970)
Teiidae	*Kentropyx calcarata*	50	Cole et al. (1995)
Teiidae	*Kentropyx striata*	50	Cole et al. (1995)
Teiidae	*Kentropyx vanzoi*	50	Santos et al. (2007)
Teiidae	*Kentropyx paulensis*	50	Santos et al. (2007)
Teiidae	*Ameiva dorsalis*	50	Gorman (1970)
Teiidae	*Ameiva auberi*	30	Porte et al. (1989)
Teiidae	*Dicrodon guttulatum*	56	Gorman (1970)
Teiidae	*Ameiva exsul*	50	Gorman (1970)
Teiidae	*Ameiva maynardi*	50	Gorman (1970)
Teiidae	*Ameiva chrysolaema*	50	Gorman (1970)
Teiidae	*Aspidoscelis inornata*	46	Lowe et al. (1970)
Teiidae	*Aspidoscelis sexlineata*	46	Lowe et al. (1970)
Teiidae	*Aspidoscelis burti*	46	Lowe et al. (1970)
Teiidae	*Aspidoscelis communis*	46	Lowe et al. (1970)
Teiidae	*Aspidoscelis laredoensis*	46	Robinson (1973)
Teiidae	*Aspidoscelis costata*	46	Lowe et al. (1970)
Teiidae	*Aspidoscelis gularis*	46	Lowe et al. (1970)
Teiidae	*Aspidoscelis ceralbensis*	52	Robinson (1973)
Teiidae	*Aspidoscelis hyperythra*	52	Lowe et al. (1970)
Teiidae	*Aspidoscelis deppei*	50	Lowe et al. (1970)
Teiidae	*Aspidoscelis lineattissima*	52	Lowe et al. (1970)
Teiidae	*Aspidoscelis guttata*	52	Lowe et al. (1970)
Teiidae	*Aspidoscelis velox*	46	Lowe et al. (1970)
Teiidae	*Aspidoscelis marmorata*	46	Porte et al. (1989)
Teiidae	*Aspidoscelis tigris*	46	Lowe et al. (1970)
Gymnophthalmidae	*Leposoma scincoides*	52	Pellegrino et al. (2004)
Gymnophthalmidae	*Anotosaura collaris*	44	Rodrigues et al. (2013)
Gymnophthalmidae	*Colobosauroides cearensis*	44	Pellegrino et al. (2001)

Gymnophthalmidae	*Leposoma percarinatum*	44	Laguna et al. (2010b)
Gymnophthalmidae	*Leposoma osvaldoi*	44	Pellegrino et al. (2004)
Gymnophthalmidae	*Leposoma guianense*	44	Pellegrino et al. (2004)
Gymnophthalmidae	*Neusticurus bicarinatus*	44	Pellegrino & Yonenaga-Yassuda (1998)
Gymnophthalmidae	*Placosoma cordylinum*	44	Pellegrino & Yonenaga-Yassuda (1998)
Gymnophthalmidae	*Placosoma glabellum*	58	Pellegrino & Yonenaga-Yassuda (1998)
Gymnophthalmidae	*Pholidobolus montium*	46	Gorman (1970)
Gymnophthalmidae	*Potamites ecpleopus*	44	Sherbrooke & Cole, 1972
Gymnophthalmidae	*Cercosaura ocellata*	42	Pellegrino & Yonenaga-Yassuda (1998)
Gymnophthalmidae	*Cercosaura schreibersii*	44	Pellegrino & Yonenaga-Yassuda (1998)
Gymnophthalmidae	*Bachia bresslaui*	46	Pellegrino & Yonenaga-Yassuda (1998)
Gymnophthalmidae	*Bachia dorbignyi*	32	Pellegrino & Yonenaga-Yassuda (1998)
Gymnophthalmidae	*Heterodactylus imbricatus*	42	Laguna (2011)
Gymnophthalmidae	*Colobodactylus taunayi*	42	Laguna (2011)
Gymnophthalmidae	*Iphisa elegans*	42	Pellegrino et al. (2001)
Gymnophthalmidae	*Colobosaura modesta*	42	Pellegrino et al. (2001)
Gymnophthalmidae	*Acratosaura mentalis*	42	Laguna (2011)
Gymnophthalmidae	*Tretioscincus oriximinensis*	42	Yonenaga-Yassuda et al. 2005
Gymnophthalmidae	*Tretioscincus agilis*	42	Yonenaga-Yassuda et al. 2005
Gymnophthalmidae	*Micrablepharus maximiliani*	50	Yonenaga-Yassuda & Rodrigues (1999)
Gymnophthalmidae	*Micrablepharus atticolus*	50	Yonenaga-Yassuda & Rodrigues (1999)
Gymnophthalmidae	*Vanzosaura rubricauda*	40	Yonenaga-Yassuda et al. 1996
Gymnophthalmidae	*Procellosaurinus tetradactylus*	40	Yonenaga-Yassuda et al. 1996
Gymnophthalmidae	*Procellosaurinus erythrocercus*	40	Yonenaga-Yassuda et al.,1996
Gymnophthalmidae	*Nothobachia ablephara*	62	Laguna (2011)
Gymnophthalmidae	*Calyptommatus sinebrachiatus*	58	Yonenaga-Yassuda et al. (2005)
Gymnophthalmidae	*Calyptommatus nicterus*	58	Yonenaga-Yassuda et al. (2005)
Gymnophthalmidae	*Calyptommatus leiolepis*	58	Yonenaga-Yassuda et al. (2005)
Gymnophthalmidae	*Psilophthalmus paeminosus*	44	Yonenaga-Yassuda et al. (2005)
Gymnophthalmidae	*Gymnophthalmus pleei*	34	Cole et al. (1993)
Gymnophthalmidae	*Gymnophthalmus leucomystax*	44	Yonenaga-Yassuda et al. (1995)
Gymnophthalmidae	*Gymnophthalmus vanzoi*	44	Yonenaga-Yassuda et al. (1995)
Gymnophthalmidae	*Gymnophthalmus speciosus*	44	Cole et al. (1993)
Gymnophthalmidae	*Gymnophthalmus underwoodi*	44	Yonenaga-Yassuda et al. (1995)
Gymnophthalmidae	*Gymnophthalmus cryptus*	44	Cole et al. (1993)
Rhineuridae	*Rhineura floridana*	44	Huang et al. (1967)
Bipedidae	*Bipes tridactylus*	46	Cole and Gans (1987)
Bipedidae	*Bipes canaliculatus*	44	Macgregor & Klosterman (1979)
Bipedidae	*Bipes biporus*	40	Cole and Gans (1987)
Blanidae	*Blanus strauchi*	32	Huang et al. (1967)
Blanidae	*Blanus cinereus*	32	Huang et al. (1967)
Trogonophidae	*Trogonophis wiegmanni*	36	Huang et al. (1967)
Trogonophidae	*Diplometopon zarudnyi*	36	Branch (1980)
Amphisbaenidae	*Cynisca leucura*	30	Huang & Gans (1971)
Amphisbaenidae	*Geocalamus acutus*	38	Huang & Gans (1971)

Amphisbaenidae	*Monopeltis capensis*	34	Huang et al. (1967)
Amphisbaenidae	*Amphisbaena fuliginosa*	48	Huang et al. (1967)
Amphisbaenidae	*Amphisbaena hyporissor*	50	Cole and Gans (1987)
Amphisbaenidae	*Amphisbaena innocens*	50	Huang & Gans (1971)
Amphisbaenidae	*Amphisbaena mertensii*	40	Hernando & Alvarez (2005)
Amphisbaenidae	*Amphisbaena vermicularis*	44	Beçak et al. (1973)
Amphisbaenidae	*Amphisbaena alba*	38	Beçak et al. (1971)
Amphisbaenidae	*Amphisbaena camura*	44	Huang et al. (1967)
Amphisbaenidae	*Amphisbaena bolivica*	44	Falcione & Hernando (2010)
Amphisbaenidae	*Amphisbaena manni*	36	Huang & Gans (1971)
Amphisbaenidae	*Amphisbaena xera*	36	Huang et al. (1967)
Amphisbaenidae	*Amphisbaena caeca*	36	Huang et al. (1967)
Amphisbaenidae	*Amphisbaena fenestrata*	36	Huang & Gans (1971)
Amphisbaenidae	*Amphisbaena microcephalum*	34	Huang et al. (1967)
Amphisbaenidae	*Amphisbaena kingii*	26	Huang & Gans (1971)
Amphisbaenidae	*Amphisbaena angustifrons*	30	Huang et al. (1967)
Amphisbaenidae	*Amphisbaena darwini*	30	Hernando & Alvarez (2005)
Lacertidae	*Psammodromus algirus*	40	Capula et al. (1982)
Lacertidae	*Gallotia atlantica*	40	LopezJurado et al. (1986)
Lacertidae	*Gallotia stehlini*	40	LopezJurado et al. (1986)
Lacertidae	*Gallotia galloti*	40	Suwala et al. (2020)
Lacertidae	*Atlantolacerta andreanskyi*	38	Giovannotti et al. (2020)
Lacertidae	*Latastia longicaudata*	38	Suwala et al. (2020)
Lacertidae	*Meroles suborbitalis*	38	Odierna et al. (1990)
Lacertidae	*Meroles cuneirostris*	38	Olmo et al. (1987)
Lacertidae	*Pedioplanis namaquensis*	38	Odierna et al. (1990)
Lacertidae	*Pedioplanis husabensis*	38	Odierna et al. (1990)
Lacertidae	*Pedioplanis undata*	38	Odierna et al. (1990)
Lacertidae	*Gastropholis prasina*	38	Suwala et al. (2020)
Lacertidae	*Eremias arguta*	38	Ivanov & Fedorova (1973)
Lacertidae	*Eremias grammica*	38	Kupriyanova (1986)
Lacertidae	*Eremias multiocellata*	38	Eremchenko et al. (1992)
Lacertidae	*Eremias velox*	38	Lisachov et al. (2019)
Lacertidae	*Eremias persica*	38	Ivanov et al. (1973)
Lacertidae	*Omanosaura jayakari*	38	Fritz et al. (1991)
Lacertidae	*Mesalina guttulata*	38	Kupriyanova (1994)
Lacertidae	*Ophisops elegans*	38	Bhatnagar & Yoniss (1976)
Lacertidae	*Acanthodactylus erythrurus*	38	Olmo et al. (1987)
Lacertidae	*Acanthodactylus pardalis*	38	Gorman (1969)
Lacertidae	*Acanthodactylus scutellatus*	38	Gorman (1969)
Lacertidae	*Acanthodactylus opheodurus*	38	Branch (1980)
Lacertidae	*Acanthodactylus boskianus*	38	Gorman (1969)
Lacertidae	*Acanthodactylus schreiberi*	38	Gorman (1969)
Lacertidae	*Podarcis melisellensis*	38	Olmo et al. (1987)
Lacertidae	*Podarcis tiliguerta*	38	Olmo et al. (1987)

Lacertidae	*Podarcis filfolensis*	38	Capula et al. (1982)
Lacertidae	*Podarcis muralis*	38	Vujošević & Blagojević (1999)
Lacertidae	*Podarcis siculus*	38	Odierna et al. (1985)
Lacertidae	*Podarcis hispanicus*	38	Calera & Cano (1979)
Lacertidae	*Podarcis bocagei*	38	Cano (1984)
Lacertidae	*Podarcis peloponnesiacus*	38	Olmo et al. (1987)
Lacertidae	*Podarcis erhardii*	38	Stille et al. (1983)
Lacertidae	*Podarcis tauricus*	38	Orlova & Orlov (1969)
Lacertidae	*Podarcis milensis*	38	Stille et al. (1983)
Lacertidae	*Podarcis lilfordi*	38	Gorman (1969)
Lacertidae	*Podarcis pityusensis*	38	Capula et al. (1982)
Lacertidae	*Scelarcis perspicillata*	38	Cano (1984)
Lacertidae	*Teira dugesii*	38	De Smet (1981)
Lacertidae	*Archaeolacerta bedriagae*	38	Capula et al. (1982)
Lacertidae	*Iranolacerta brandti*	38	Olmo (2001)
Lacertidae	*Zootoca vivipara*	36	Odierna et al. (2004)
Lacertidae	*Takydromus sexlineatus*	38	Suwala et al. (2020)
Lacertidae	*Takydromus dorsalis*	38	Suwala et al. (2020)
Lacertidae	*Takydromus smaragdinus*	38	Nogusa (1953)
Lacertidae	*Takydromus tachydromoides*	38	Nogusa (1953)
Lacertidae	*Takydromus formosanus*	38	Nakamura (1935)
Lacertidae	*Takydromus septentrionalis*	38	Nakamura (1935)
Lacertidae	*Timon princeps*	36	Rykena & Nettmann (1986)
Lacertidae	*Timon lepidus*	36	Giovannotti et al. (2018)
Lacertidae	*Timon pater*	36	Odierna et al. (1990)
Lacertidae	*Timon tangitanus*	36	Suwala et al. (2020)
Lacertidae	*Lacerta viridis*	38	De Smet (1981)
Lacertidae	*Lacerta bilineata*	38	Giovannotti et al. (2018)
Lacertidae	*Lacerta strigata*	38	Giovannotti et al. (2018)
Lacertidae	*Lacerta agilis*	38	Lisachov et al. (2020)
Lacertidae	*Lacerta media*	38	Kupriyanova (1968)
Lacertidae	*Lacerta trilineata*	38	Giovannotti et al. (2018)
Lacertidae	*Phoenicolacerta kulzeri*	38	Odierna & Arribas (2005)
Lacertidae	*Phoenicolacerta laevis*	38	Odierna & Arribas (2005)
Lacertidae	*Dalmatolacerta oxycephala*	38	Gorman et al. (1970)
Lacertidae	*Hellenolacerta graeca*	38	Olmo et al. (1987)
Lacertidae	*Parvilacerta parva*	24	Odierna et al. (1995)
Lacertidae	*Parvilacerta fraasii*	24	Odierna et al. (1995)
Lacertidae	*Iberolacerta horvathi*	36	Capula et al. (1989)
Lacertidae	*Iberolacerta aurelioi*	26	Odierna et al. (1996)
Lacertidae	*Iberolacerta bonnali*	24	Odierna et al. (1996)
Lacertidae	*Iberolacerta aranica*	26	Odierna et al. (1996)
Lacertidae	*Iberolacerta galani*	36	Giovannotti et al. (2014)
Lacertidae	*Iberolacerta monticola*	36	Giovannotti et al. (2014)
Lacertidae	*Iberolacerta cyreni*	36	Odierna & Arribas (2004)

Lacertidae	*Algyroides nigropunctatus*	38	Olmo et al. (1990)
Lacertidae	*Algyroides moreoticus*	38	Olmo et al. (1990)
Lacertidae	*Dinarolacerta mosorensis*	38	Odierna & Arribas (2005)
Lacertidae	*Algyroides marchi*	38	Calera & Cano (1979)
Lacertidae	*Darevskia parvula*	38	Kupriyanova (1976)
Lacertidae	*Darevskia valentini*	38	Darevsky et al. (1961)
Lacertidae	*Darevskia portschinskii*	38	Darevsky & Kupriyanova (1982)
Lacertidae	*Darevskia praticola*	38	Orlova & Orlov (1969)
Lacertidae	*Darevskia saxicola*	38	Kupriyanova (1969)
Lacertidae	*Darevskia chlorogaster*	38	Orlova & Orlov (1969)
Lacertidae	*Darevskia rostombekovi*	38	Darevsky & Danyelian (1968)
Lacertidae	*Darevskia raddei*	38	Kupriyanova (1994)
Lacertidae	*Darevskia caucasica*	38	Kupriyanova (1990)
Lacertidae	*Darevskia derjugini*	38	Orlova & Orlov (1969)
Lacertidae	*Darevskia mixta*	38	Darevsky et al. (1961)
Lacertidae	*Darevskia armeniaca*	38	Kupriyanova (1969)

Supplementary materials References:

Bhatnagar, A. N., & Yoniss, Y. Th. (1976). A proof of female heterogamety in a lacertid lizard, *Ophisops elegans*. *Cytologia, 41*, 507–511. https://doi.org/10.1508/cytologia.41.507

Capula, M., Lapini, L., & Capanna, E. (1989). The karyotype of *Lacerta horvathi* (Reptilia, Sauria, Lacertidae). *Genetica, 79*, 11–16.

Cole, C. J., Dessauer, I. H. C., & Markezich, A. L. (1993). Missing link found: The second ancestor of *Gymnophthalmus underwoodi* (Squamata: Teiidae), a South American unisexual lizard of hybrid origin. *American Museum Novitates, 3055*.

Da Silva, M. J., De Araújo Vieira, A. P., Galvão Cipriano, F. M., Dos Santos Cândido, M. R., De Oliveira, E. H. C., Gimenez Pinheiro, T., & Da Silva, E. L. (2020). The karyotype of *Salvator merianae* (Squamata, Teiidae): Analyses by classical and molecular cytogenetic techniques. *Cytogenetic and Genome Research, 160*, 94–99. https://doi.org/10.1159/000506140

Darevsky, I. S., & Danielyan, F. D. (1968). Diploid and triploid progeny arising from natural mating of parthenogenetic *Lacerta armeniaca* and *L. unisexualis* with bisexual *L. saxicola valentini*. *Journal of Herpetology, 2*, 65–69. https://doi.org/10.2307/1563104

De Smet, W. H. O. (1981). Description of the orcein strained karyotypes of 36 lizard species (Lacertilia, Reptilia) belonging to the families Teiidae, Scincidae, Lacertidae, Crodylidae and Varanidae (Autarchoglossa). *Acta Zoologica Pathologica Antverpiensia, 73*, 73-118.

Falcione, C., & Hernando, A. (2010). A new karyotypic formula for the genus *Amphisbaena* (Squamata: Amphisbaenidae). *Phyllomedusa: Journal of Herpetology, 9*, 75.

Giovannotti, M., Nisi Cerioni, P., Rojo, V., Olmo, E., Slimani, T., Splendiani, A., & Caputo Barucchi, V. (2018). Characterization of a satellite DNA in the genera *Lacerta* and *Timon* (Reptilia, Lacertidae) and its role in the differentiation of the W chromosome. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution, 330*, 83–95. https://doi.org/10.1002/jez.b.22790

Giovannotti, M., Rojo, V., Nisi Cerioni, P., González-Tizón, A., Martínez-Lage, A., Splendiani, A., Naveira, H., Ruggeri, P., Arribas, Ó., Olmo, E., & Caputo Barucchi, V. (2014). Isolation and characterization of two satellite DNAs in some Iberian rock lizards (Squamata, Lacertidae). *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution, 322*, 13–26.

Giovannotti, M., S'Khifa, A., Nisi Cerioni, P., Splendiani, A., Slimani, T., Fioravanti, T., Olmo, E., & Caputo Barucchi, V. (2020). Isolation and characterization of two satellite DNAs in *Atlantolacerta andreanskyi* (Werner, 1929) (Reptilia, Lacertidae). *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution, 334*, 178–191.

Gorman, G. C. (1969). New chromosome data for 12 species of lacertid lizards. *Journal of Herpetology, 3*, 49. https://doi.org/10.2307/1563223

Gorman, G. C. (1970). Chromosomes and the systematics of the family Teiidae (Sauria, Reptilia). *Copeia, 1970*, 230.

Hernando, A., & Alvarez, B. (2005). Estudios cromosómicos en saurios y anfisbénidos del litoral fluvial argentino y área de influencia. Estado del conocimiento. *Miscelánea, 14*, 428.

Huang, C. C., Clark, H. F., & Gans, C. (1967). Karyological studies on fifteen forms of amphisbaenians (Amphisbaenia-Reptilia). *Chromosoma, 22*, 1–15.

Huang, C. C., & Gans, C. (1971). The chromosomes of 14 species of amphisbaenians (Amphisbaenia, Reptilia). *Cytogenetic and Genome Research, 10*, 10–22.

Laguna, M. M. (2011). Estudos cromossômicos e moleculares em espécies de lagartos microteídeos, com ênfase na tribo Ecpleopodini (Gymnophthalmidae, Squamata) [Mestrado em Biologia, Universidade de São Paulo]. (Thesis title, not a journal) https://doi.org/10.11606/D.41.2011.tde-23092011-154628

Lisachov, A. P., Galkina, S. A., Saifitdinova, A. F., Romanenko, S. A., Andreyushkova, D. A., Trifonov, V. A., & Borodin, P. M. (2019). Identification of sex chromosomes in *Eremias velox* (Lacertidae, Reptilia) using lampbrush chromosome analysis. *Comparative Cytogenetics, 13*, 17–28. https://doi.org/10.3897/CompCytogen.v13i2.34116

Lisachov, A. P., Giovannotti, M., Pereira, J. C., Andreyushkova, D. A., Romanenko, S. A., Ferguson-Smith, M. A., Borodin, P. M., & Trifonov, V. A. (2020). Chromosome painting does not support a sex chromosome turnover in *Lacerta agilis* Linnaeus, 1758. *Cytogenetic and Genome Research, 160*, 134–140. https://doi.org/10.1159/000506321

Lowe, C. H., Wright, J. W., Cole, C. J., & Bezy, R. L. (1970). Chromosomes and evolution of the species groups of *Cnemidophorus* (Reptilia: Teiidae). *Systematic Zoology, 19*, 128.

Macgregor, H., & Klosterman, L. (1979). Observations on the cytology of *Bipes* (Amphisbaenia) with special reference to its lampbrush chromosomes. *Chromosoma, 72*, 67–87. https://doi.org/10.1007/BF00286430

Nogusa, S. (1953). A study of the chromosomes of some lizards, with special remarks on the m-chromosomes. *Cytologia, 18*, 29–35. https://doi.org/10.1508/cytologia.18.29

Odierna, G., Aprea, G., Arribas, O. J., Capriglione, T., Caputo, V., & Olmo, E. (1996). The karyology of the Iberian rock lizards. *Herpetologica*, 542-550.

Odierna, G., Aprea, G., Capriglione, T., & Puky, M. (2004). Chromosomal evidence for the double origin of viviparity in the European common lizard, *Lacerta* (*Zootoca*) *vivipara*. *Herpetological Journal, 14*, 157-160.

Odierna, G., & Arribas, O. J. (2005). The karyology of *Lacerta mosorensis* Kolombatovic, 1886, and its bearing on phylogenetic relationships to other European mountain lizards. *Italian Journal of Zoology, 72*, 93–96. https://doi.org/10.1080/11250000509356658

Odierna, G., Olmo, E., Capriglione, T., & Caputo, V. (1990). Karyological differences between *Lacerta lepida* and *Lacerta pater*. *Journal of Herpetology, 24*, 97. https://doi.org/10.2307/1564300

Odierna, G., Olmo, E., Caputo, V., Capriglione, T., & Bosch, H. A. J. in den. (1995). Karyological affinity between *Lacerta fraasii* Lehrs, 1910 and *Lacerta parva* Boulenger, 1887. *Amphibia-Reptilia, 16*, 293–297. https://doi.org/10.1163/156853895X00091

Odierna, G., Olmo, E., & Cobror, O. (1985). C-band variability in some Lacertidae (Sauria, Reptilia). *Experientia, 41*, 944–946.

Olmo, E. (1990). DNA and chromosome evolution in lacertid lizards. *Cytogenetic and Genome Research, 160*, 505–505. https://doi.org/10.1159/000511195

Peccinini-Seale, D., & De Almeida, T. M. B. (1986). Chromosomal variation, nucleolar organizers and constitutive heterochromatin in the genus *Ambiva* and *Cnemidophorus* (Sauria; Teiidae). *Caryologia, 39*, 227–237.

Pellegrino, K. (2001). A molecular perspective on the evolution of microteiid lizards (Squamata, Gymnophthalmidae), and a new classification for the family. *Biological Journal of the Linnean Society, 74*, 315–338. https://doi.org/10.1006/bijl.2001.0580

Pellegrino, K. C. M., Rodrigues, M. T., & Yonenaga-Yassuda, Y. (2004). Chromosomal evolution in the Brazilian lizards of genus *Leposoma* (Squamata, Gymnophthalmidae) from Amazon and Atlantic rain forests: Banding patterns and FISH of telomeric sequences. *Hereditas, 131*, 15–21. https://doi.org/10.1111/j.1601-5223.1999.00015.x

Rodrigues, M. T., Jr, M. T., Vechio, F. D., Amaro, R. C., NiSA, C., Guerrero, A. C., Damasceno, R., Roscito, J. G., Nunes, P. M. S., & Recoder, R. S. (2013). Rediscovery of the earless microteiid lizard *Anotosaura collaris* Amaral, 1933 (Squamata: Gymnophthalmidae): A redescription complemented by osteological, hemipenial, molecular, karyological, physiological and ecological data. *Zootaxa, 3731*, 345. https://doi.org/10.11646/zootaxa.3731.3.5

Rykena, S., & Nettmann, H. K. (1986). The karyotype of *Lacerta princeps kurdistanica* and its meaning in phylogeny. *Studies in Herpetology*, 193-196.

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*, 531. https://doi.org/10.3390/genes11050531

Veronese, L. B., Freitas, T. R. O. D., & Krause, L. (2003). Cytogenetic studies of four Brazilian species of teiid lizards (Squamata, Teiidae). *Caryologia, 56*, 107–114.

Yonenaga-Yassuda, Y., Rodrigues, M. T., & Pellegrino, K. C. M. (2005). Chromosomal banding patterns in the eyelid-less microteiid lizard radiation: The $X_1X_1X_2X_2$:X_1X_2Y sex chromosome system in *Calyptommatus* and the karyotypes of *Psilophthalmus* and *Tretioscincus* (Squamata, Gymnophthalmidae). *Genetics and Molecular Biology, 28*, 700–709.

Yonenaga-Yassuda, Y., Vanzolini, P. E., Rodrigues, M. T., & De Carvalho, C. M. (1995). Chromosome banding patterns in the unisexual microteiid *Gymnophthalmus underwoodi* and in two related sibling species (Gymnophthalmidae, Sauria). *Cytogenetic and Genome Research, 70*, 29–34.

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

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Article

Evolutionary Variability of W-Linked Repetitive Content in Lacertid Lizards

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Abstract: Lacertid lizards are a widely radiated group of squamate reptiles with long-term stable ZZ/ZW sex chromosomes. Despite their family-wide homology of Z-specific gene content, previous cytogenetic studies revealed significant variability in the size, morphology, and heterochromatin distribution of their W chromosome. However, there is little evidence about the accumulation and distribution of repetitive content on lacertid chromosomes, especially on their W chromosome. In order to expand our knowledge of the evolution of sex chromosome repetitive content, we examined the topology of telomeric and microsatellite motifs that tend to often accumulate on the sex chromosomes of reptiles in the karyotypes of 15 species of lacertids by fluorescence in situ hybridization (FISH). The topology of the above-mentioned motifs was compared to the pattern of heterochromatin distribution, as revealed by C-banding. Our results show that the topologies of the examined motifs on the W chromosome do not seem to follow a strong phylogenetic signal, indicating independent and species-specific accumulations. In addition, the degeneration of the W chromosome can also affect the Z chromosome and potentially also other parts of the genome. Our study provides solid evidence that the repetitive content of the degenerated sex chromosomes is one of the most evolutionary dynamic parts of the genome.

Keywords: C-banding; evolution; FISH; GATA; heterochromatin; karyotype; microsatellites; sex chromosomes; telomeres

1. Introduction

Sex chromosomes evolve from a pair of autosomes, after one of them acquires a sex-determining locus [1–3]. This locus is on the Y or W chromosome and thus is restricted to a single sex, which affects subsequent processes in the nearby, linked loci. The region around this sex-determining locus progressively stops recombining with their respective homologous regions on the X/Z counterpart, possibly due to inversions [4] or other mechanisms decreasing the frequency of recombination. Over time, the cessation of recombination triggers more structural changes, mainly on the Y and W chromosomes, including the accumulation of deleterious mutations, the degradation of the gene content, the accumulation of repetitive elements, and/or the heterochromatinization. The differentiation process of the X/Z and Y/W chromosomes differs significantly among independently evolved sex determination systems in traits such as the degree of recombination suppression, the heteromorphism of sex chromosomes, and the sharing of gene and repeat content between sex chromosomes.

Sex chromosomes evolved independently in numerous animal and plant lineages, probably mainly to ensure a stable sex ratio in populations and to contribute to the resolution of the conflict between sexes over traits expression via the accumulation of sexually antagonistic alleles [5]. The differentiation of sex chromosomes is a complex and only partially understood process connected to a balance between adaptive and potentially harmful processes. The loss of numerous functional genes from the Y/W chromosomes, the increased frequency of transposons and other repetitive elements in the genomes, heterochromatinization, and the changes in gene expression due to these processes should often have negative fitness effects on the organism. On the other hand, many organismal lineages were able to cope with these potentially detrimental effects associated with sex chromosome differentiation, and differentiated sex chromosomes seem even to act as an "evolutionary trap" [6] in the sense that once evolved, they appear to be very evolutionary stable in the long term. Differentiated sex chromosomes stabilize the sex determination system for dozens of millions of years, as was documented by molecular and cytogenetic evidence for example in anguimorphan lizards, birds, caenophidian snakes, iguanas, lacertids, geckos of the genus *Paroedura*, softshell turtles, and viviparous mammals [7–18]. Recent studies in viviparous mammals, birds, iguanas, anoles, monitor lizards, and caenophidian snakes revealed a striking dichotomy: the gene and repetitive content of their Y/W chromosomes differs significantly even between closely related species [19–30], despite the long-lasting stability of sex determination systems in these lineages and the extensive between-species homology of their X/Z-specific gene content [7,8,10,12,25]. The contradiction between the similarity of the gene content of the X/Z chromosomes in comparison to the variability of the gene and repetitive content of their Y/W counterparts still remains unresolved. Notably, heterochromatic and/or low-complexity genomic regions such as centromeres and differentiated Y/W sex chromosomes are insufficiently sequenced, assembled, and annotated with the current high-throughput sequencing methodologies and bioinformatic tools [31]. As a result, either the heterogametic sex is often excluded from genome sequencing projects, or the regions from the Y/W chromosomes are poorly assembled and annotated [31]. Therefore, we currently have limited knowledge if the between-species variability of the Y/W gene and repetitive content is exceptional, as research has been restricted among amniotes mainly to a few up to now studied lineages, or whether it is common during sex chromosome differentiation.

Simple repeats, such as mini- and microsatellites, are often overabundant on sex chromosomes [32,33]. Their function is largely unknown. It was speculated that they contribute to the cessation of recombination, formation of heterochromatin, changes in gene expression, or that different kinds of these repeats are accumulated on sex chromosomes randomly, largely reflecting historical contingency [32,34]. The important functional role of such sequences would predict that the pattern of the distribution of their accumulation should be relatively conserved across species of the same lineage. In this context, we selected the lizards of the family Lacertidae to explore the variability of the repetitive content of sex chromosomes between species across a wide phylogenetic scale in another model system. Previous cytogenetic studies demonstrated that all studied lacertids have highly differentiated ZZ/ZW sex chromosomes [34–44]. The majority of chromosomes in lacertids are acrocentric gradually decreasing in size. Therefore, the sex chromosomes cannot be identified by morphology; however, the W chromosome is heterochromatic, visible after C-banding in all studied species [34,36]. The size of the W chromosome varies among lacertid species from small to medium [34,36,45]. In addition, the W chromosome seems to be enriched in satellite motifs in *Acanthodactylus lineomaculatus*, *Eremias velox*, and several species from the genera *Lacerta* and *Timon* [38,40–44]. The Z chromosome is also acrocentric, small to medium in size in *E. velox* [46], and with more than 800 protein-coding genes in *Lacerta agilis* and *Podarcis muralis* [47,48]. Based on qPCR-based methodology applied to 45 species, it was recently revealed that the ZZ/ZW sex chromosomes are homologous across lacertids and that they were highly differentiated already in the common ancestor of the family living approximately 85 million years ago [16,49].

In the current study, we tested the presence of accumulations of selected microsatellite motifs that tend to accumulate on the sex chromosomes of vertebrates by fluorescence in situ hybridization

and compared their distribution on the W chromosome of 15 species of lacertids selected for their phylogenetic position. Our aim is to explore the evolutionary dynamics of the accumulation of microsatellite motifs and heterochromatin distribution on the sex chromosomes across the phylogenetic scale of lacertids and to expand our knowledge on the processes of sex chromosome differentiation.

2. Materials and Methods

2.1. Studied Material

We studied 30 individuals belonging to 15 species of lacertids: *Acanthodactylus schreiberi*, *Eremias arguta*, *Gallotia galloti*, *Gastropholis prasina*, *Lacerta bilineata*, *Lacerta media*, *Lacerta strigata*, *Lacerta trilineata*, *Latastia longicaudata*, *Phoenicolacerta troodica*, *Podarcis siculus*, *Takydromus dorsalis*, *Takydromus sexlineatus*, *Timon lepidus*, and *Timon tangitanus* (Table S1). Individuals from the species *A. schreiberi* and *Ph. troodica* were collected from the wild in Cyprus (permissions 02.15.007.003.001/04.05.002.005.006 issued from Department of Environment, Ministry of Agriculture, Republic of Cyprus), while individuals from 13 other species were obtained from the pet trade. Blood samples were collected from the vein located at the ventral side of tails with a heparinized syringe. The processing of the biological material was carried out under the supervision and with the approval of the Ethics Committee of the Faculty of Science, Charles University in Prague followed by the Ministry of Education, Youth and Sports of the Czech Republic (permissions No. 15251/2012-30, 35484/2015-14 and 8604/2019-7).

2.2. Chromosome Preparations and Staining

Mitotic metaphase chromosome spreads were prepared from whole blood cell cultures following the protocol described by Pokorná et al. [50]. Chromosomal preparations were stained with Giemsa and karyogram reconstruction was used to identify the diploid number (2n) and morphology of chromosomes. To visualize the accumulation of constitutive heterochromatin, we applied C-banding following the protocol of Sumner [51] with modifications described by Pokorná et al. [50]. Giemsa staining, fluorescence in situ hybridization with probe for telomeric or GATA motifs, and C-banding were applied sequentially in the same metaphase in order to unequivocally identify the sex chromosomes and compare the results among the methods.

2.3. Fluorescence In Situ Hybridization (FISH) with Probes for Telomeric and Microsatellite Motifs

The distribution of telomeric repeats in the karyotype was examined by fluorescence in situ hybridization with the pan-telomeric peptide nucleic acid (PNA) probe directly labeled with Cy3 fluorochrome (DAKO, Glostrup, Denmark), following the manufacturer's protocol with a slight modification of longer hybridization time for 1–2 h. Furthermore, we analyzed the pattern of accumulation of microsatellite repeats in lacertid sex chromosomes by FISH using probes for 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 $(TTTC)_8$. The probes were synthesized and 5'-end biotin-labeled by Macrogen (Macrogen, Seoul, South Korea). Microsatellite mapping was performed on metaphase spreads following the protocol used by Rovatsos et al. [52]. The microsatellite signal was amplified and detected using a system of avidin–fluorescein and anti-avidin antibodies (Vector Laboratories, Burlingame, CA, USA) [28,30,52]. Slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and the antifade medium Fluoroshield (Sigma-Aldrich, St. Louis, MO, USA) or Vectashield (Vector Laboratories, Burlingame, CA, USA). The FISH with telomeric probe and the $(GATA)_8$ probe were performed in all studied species (Table S1). Other probes were hybridized to only four species (*Gal. galloti*, *Gas. prasina*, *Lac. media* and *Ti. lepidus*) selected with respect to the phylogenetic and karyotype diversity of lacertids.

2.4. Microscopy and Image/Data Analyses

We studied at least 10 metaphases from each specimen per method. We used Ikaros karyotyping software (Metasystems, Altlussheim, Germany) to prepare karyograms from Giemsa-stained metaphases of each species. Images were captured using a Provis AX70 fluorescence microscope (Olympus, Tokyo, Japan) equipped with a DP30BW digital camera (Olympus, Tokyo, Japan) or using an Imager Z2 microscope (Zeiss, Oberkochen, Germany) equipped with a CoolCube 1 digital camera (Metasystems, Altlussheim, Germany). Photos of in situ hybridization experiments were superimposed with color and processed with DP Manager imaging software (Olympus, Tokyo, Japan) or an Isis Fluorescence Imaging System (Metasystems, Altlussheim, Germany).

3. Results

3.1. Karyotype Reconstruction and Heterochromatin Distribution

Karyotypes for the species *A. schreiberi, E. arguta, Gal. galloti, Lac. bilineata, Lac. media, Lac. strigata, Lac. trilineata, Lat. longicaudata, Ph. troodica, Po. siculus, Ta. sexlineatus*, and *Ti. lepidus* agreed with the previous reports [34,35,37,39,53–58]. To the best of our knowledge, the karyotypes of *Gas. prasina*, *Ta. dorsalis*, and *Ti. tangitanus* have not been published up to date. Both *Gas. prasina* and *Ta. dorsalis* possess the typical lacertid karyotypes with 2n = 38 chromosomes gradually decreasing in size, with all larger chromosomes acrocentric shared by other lacertids studied here, with the exception of the genera *Timon* and *Gallotia* (female karyotypes are presented in Figure 1, male karyotypes in Figure 2). The karyotype of *Ti. tangitanus* is composed of 2n = 36 chromosomes with the largest pair being metacentric as in the previously studied *Ti. lepidus* [34,35]. The notable difference between karyotypes of the two species from the genus *Timon* can be found only in females. Females of *Ti. lepidus*, but not *Ti. tangitanus*, have 3 microchromosomes that are notably smaller than the other chromosomes. C-banding revealed a strong accumulation of heterochromatin on the smallest macrochromosomes of females in *Ti. tangitanus* and the largest microchromosome of females in *Ti. lepidus*; thus, these chromosomes can be identified as the W chromosomes. *Gal. galloti* has an all-acrocentric karyotype with 2n = 40 chromosomes gradually decreasing in size as previously reported by Cano et al. [57]. In all studied species, the W chromosomes can be identified by C-banding; however, Z chromosomes are difficult to distinguish from autosomes (Figure 1). We were able to identify the Z chromosomes only in four species (both studied species from the genus *Timon* and both species from the genus *Takydromus*; Figures 1 and 2) thanks to their distinct pattern in the FISH experiments with microsatellite probes (Figure 3).

3.2. In Situ Hybridization with Telomeric and Microsatellite Repeat Probes

The expected terminal position of the signals with the telomeric probe was observed in all studied lacertids (Figure 3). The hybridization with the telomeric probe was not tested in *Ta. sexlineatus* due to the limited availability of chromosomal material. Centromeric or pericentromeric accumulations of telomeric-like sequences were detectable in acrocentric chromosomes in all tested species with the exceptions of *Ta. dorsalis* and *E. arguta* (Figure 3). Notably, in *Ti. lepidus* and *Ti. tangitanus*, the only metacentric chromosomes (the largest chromosomes in the complement), possess a weak signal in the centromeric region. In *Ta. tangitanus*, additional interstitial telomeric repeats (ITRs) are present in the arm of the metacentric chromosome (Figure 3j,p). The only other species exhibiting ITRs within the chromosomal arms is *Lac. media* (Figure 3m).

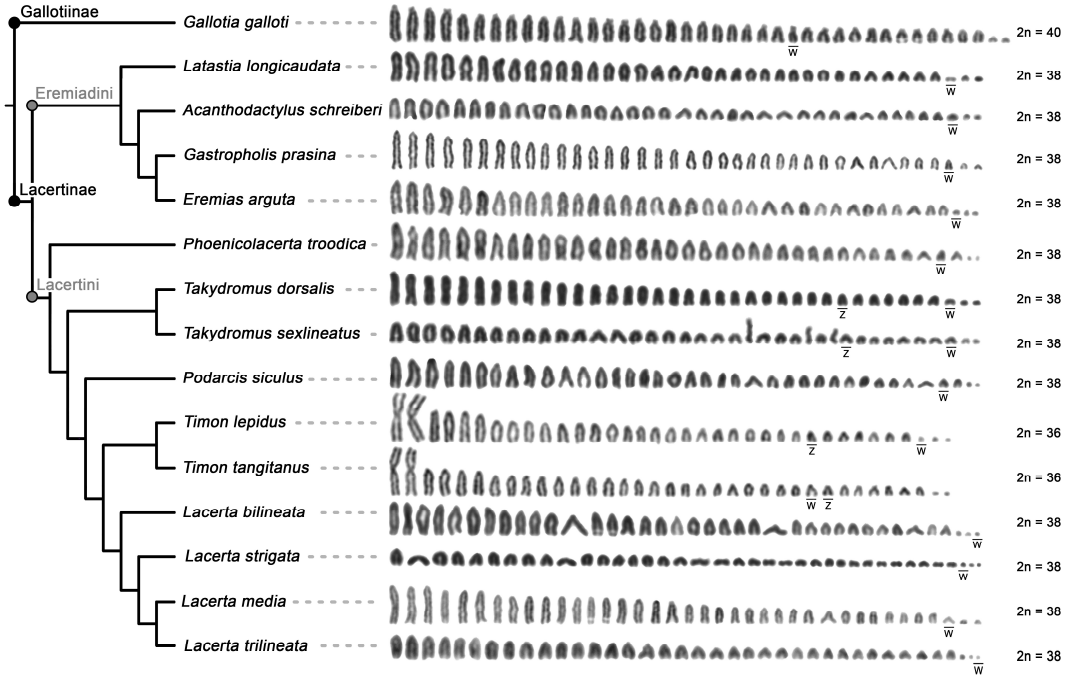

Figure 1. Phylogenetic relationships and Giemsa-stained karyotypes of females in the studied species of the family Lacertidae. The phylogenetic relationships follow Pyron et al. [59] (for an alternative topology see Garcia-Porta et al. [60]). The W chromosomes were identified by C-banding. The Z chromosomes in members of the genera *Timon* and *Takydromus* were detected by fluorescence in situ hybridization (FISH) with a $(GATA)_8$ probe.

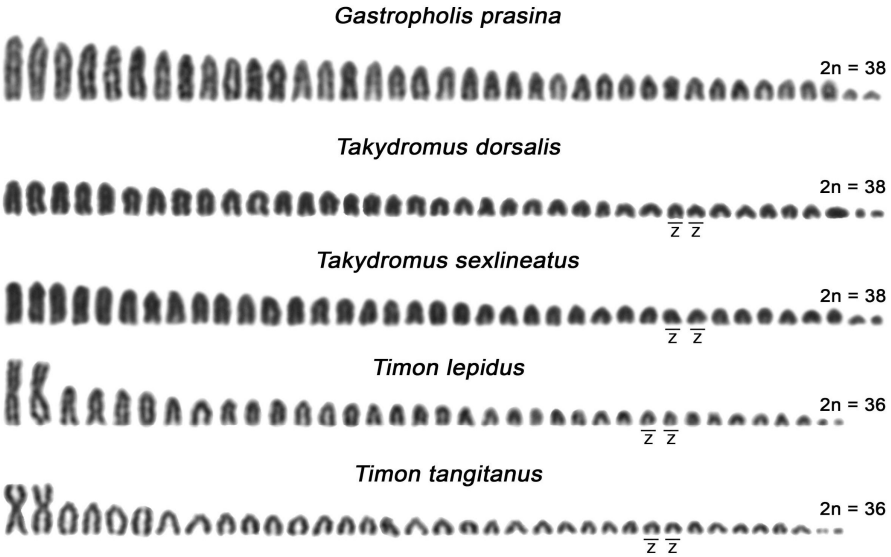

Figure 2. Male karyotypes of previously unstudied species and those with detectable Z chromosomes. The chromosomes were stained by Giemsa. The Z chromosomes of *Timon* and *Takydromus* were detected by FISH with the $(GATA)_8$ probe.

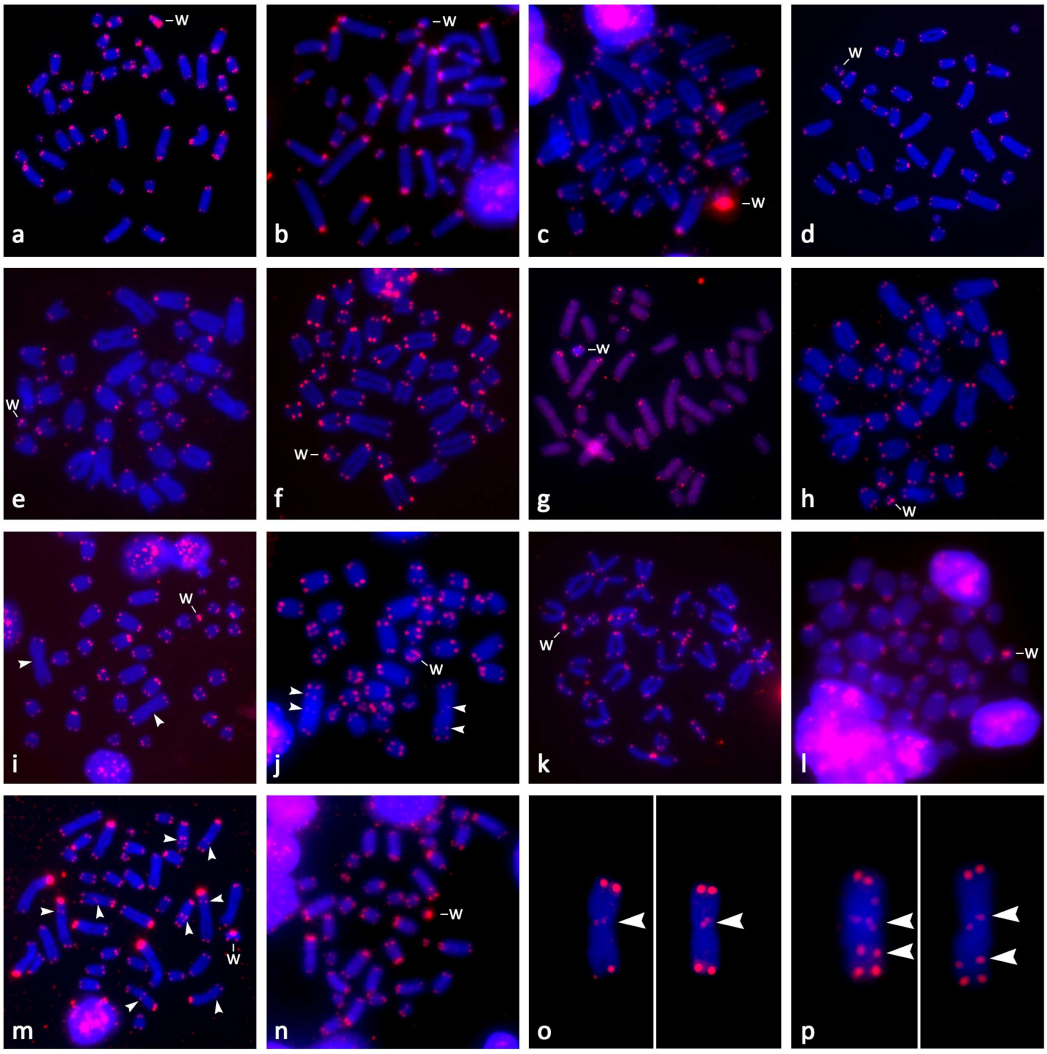

Figure 3. Mitotic metaphase chromosomes hybridized with the telomeric probe in females of (**a**) *Gallotia galloti*, (**b**) *Latastia longicaudata*, (**c**) *Acanthodactylus schreiberi*, (**d**) *Gastropholis prasina*, (**e**) *Eremias arguta*, (**f**) *Phoenicolacerta troodica*, (**g**) *Takydromus dorsalis*, (**h**) *Podarcis siculus*, (**i**) *Timon lepidus*, (**j**) *Timon tangitanus*, (**k**) *Lacerta bilineata*, (**l**) *Lacerta strigata*, (**m**) *Lacerta media*, and (**n**) *Lacerta trilineata*. In *Ti. lepidus* (**o**) and *Ti. tangitanus* (**p**), the largest, metacentric chromosomes were enlarged and the exposure was increased to show the weak signal near the centromere. An additional signal within a chromosome arm is present in *Ti. tangitanus*. Chromosomes were counterstained with DAPI, and the hybridization probes were detected with Cy3 (red). The W chromosomes are indicated; white arrows point at interstitial telomeric repeats.

Regarding accumulations of telomeric-like repeats on the W chromosomes (Figures 3 and 4), among the 15 studied species, *Gal. galloti* presents the most prominent accumulations distributed evenly throughout its W chromosome, excluding the strongly heterochromatic centromere. Other significant accumulations of telomeric-like motifs were found in the pericentromeric region in *A. schreiberi*. The W chromosomes of *Lac. bilineata*, *Lac. strigata*, *Lac. trilineata*, and *Ti. lepidus* had stronger accumulations of terminal telomeric repeats than their autosomes, while autosomes and the W chromosomes do not differ in this respect in *E. arguta*, *Gas. prasina*, *Lac. media*, *Lat. longicaudata*, *Ph. troodica*, *Po. siculus*, *Ta. dorsalis*, and *Ti. tangitanus* (Figures 3 and 4).

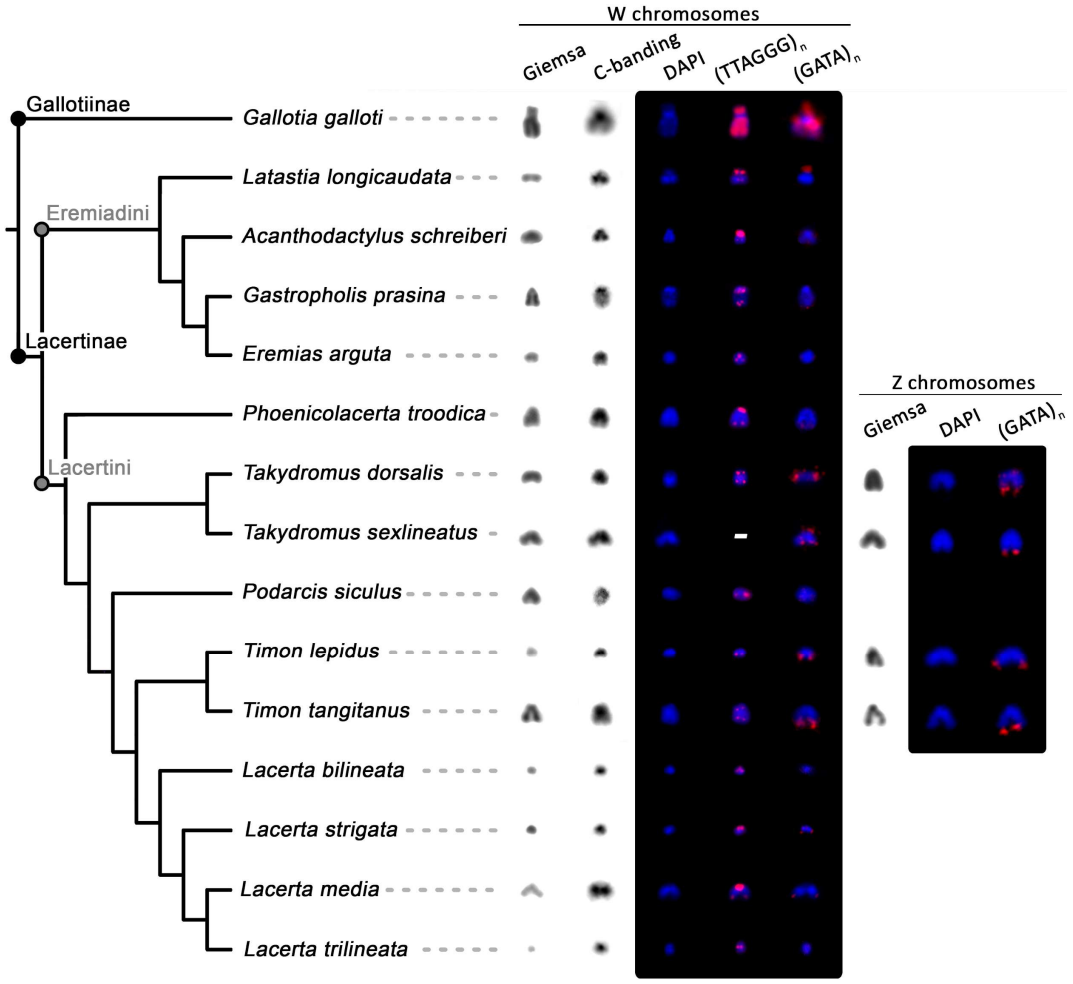

Figure 4. Comparison of morphology, heterochromatinization, and repetitive content (accumulations of telomeric-like repeats $(TTAGGG)_n$ and $(GATA)_n$ motifs) of the W chromosomes and identified Z chromosomes across the family Lacertidae. In *Ta. sexlineatus*, the telomeric probe was not tested due to the limited availability of chromosomal material. The phylogenetic tree is based on Pyron et al. [59] (for an alternative topology, see Garcia-Porta et al. [60]). Photos of C-banding are inverted. The chromosomes after FISH treatment were counterstained with DAPI (blue), and the probes were detected with fluorescein-avidin D (red).

The $(GATA)_8$ probe hybridized near the centromeric region of the W chromosome in *Lat. longicaudata* and *Gal. galloti*, while it hybridized to telomeric regions in *Lac. strigata*, *Ph. troodica*, *Ta. dorsalis*, *Ta. sexlineatus*, *Ti. lepidus*, and *Ti. tangitanus*. This probe showed no signal on the W chromosomes of *A. schreiberi*, *Gas. prasina*, *Lac. bilineata*, *Lac. media*, *Lac. trilineata* and *Po. siculus* (Figure 4). Additionally, in females of *Ta. dorsalis*, *Ta. sexlineatus*, *Ti. lepidus*, and *Ti. tangitanus*, the $(GATA)_8$ probe hybridized also in telomeric regions of an additional small chromosome. We hypothesized that it could be the Z chromosome and tested this hypothesis by the replication of the FISH with $(GATA)_8$ probe and C-banding in males of these species (Figure S1). The results supported the hypothesis that the chromosome bearing accumulation of the $(GATA)_8$ motif is the Z chromosome: as predicted, two chromosomes possess $(GATA)_8$ accumulations in males in all species with the exception of *Ta. sexlineatus*, where a pair of small chromosomes was strongly labeled in both sexes as well (Figure S1c,d). As revealed by C-banding, in contrast to the W chromosomes, the Z chromosomes did not show any accumulations of heterochromatin that would differentiate them from autosomes (results not shown). The only repetitive motif that hybridized to them was the $(GATA)_8$ probe (Figure S1). Out of the remaining 21 tested microsatellite probes, only the $(AAGG)_8$, $(GAC)_{10}$, and $(GA)_{15}$

motifs showed some degree of accumulation on the W chromosomes in the four species where all motifs were tested (Figure 5). The remaining motifs showed various levels of accumulation on the W chromosome across species, with *Gal. galloti* showing the most extensive accumulations in both the amount and variability of the tested motifs (Figure 5).

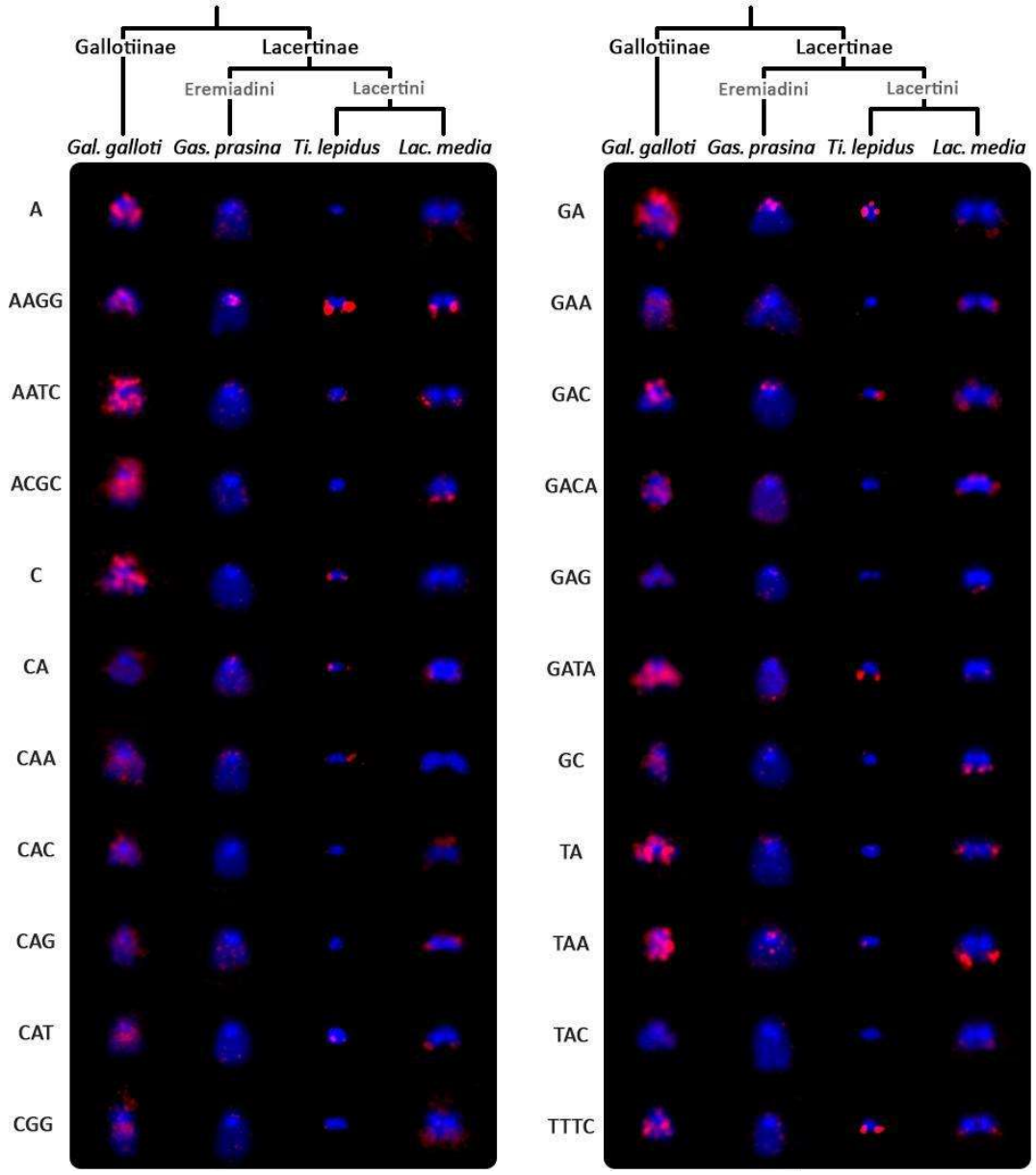

Figure 5. Comparison of the accumulation of 22 microsatellite motifs on the W chromosome of the lacertids: *Gallotia galloti*, *Gastropholis prasina*, *Timon lepidus*, and *Lacerta media*. The chromosomes were counterstained with DAPI (blue), the microsatellite probes were detected with fluorescein–avidin D (red).

4. Discussion

Evidence for ZZ/ZW sex chromosomes based on the copy-number variation of Z-linked genes and/or cytogenetics was available for 72 species of lacertids (recently reviewed by Rovatsos et al. [16]). We have added *Gas. prasina* and *Ta. dorsalis* to this long list covering over 20% of currently recognized lacertid lizards [44]. Nevertheless, the data on the sequence content of the W chromosome and the cytogenetic identification of the Z chromosomes in lacertid karyotypes are scarce. Our FISH

experiments with the $(GATA)_8$ probe (Figure 4 and Figure S1) revealed that the Z chromosome in four species of lacertids is a small acrocentric chromosome located between the 13th and 16th pair of the complement (Figure 2). Our findings are in accordance with recent cytogenetic evidence in *Eremias velox*, where the Z chromosome was identified as the 13th pair of the complement by the chromosome painting of a W-specific probe hybridized to the lampbrush sex bivalent [46]. Giovannotti et al. [42] based on FISH with a telomeric probe and the C-banding pattern of *Acanthodactylus lineomaculatus* chromosomes identified putative Z chromosomes among the 12th–13th pairs of the complement. In addition, we have estimated from the size of sequencing scaffolds from the genome projects of *Podarcis muralis* and *Lacerta agilis* that the Z chromosomes in these species should be in size between the 13th and 16th pair of the complement [47,48].

In the current study, we confirmed that the W chromosomes across lacertids are quite variable in size as was previously demonstrated for example in birds [61], monitor lizards [62], and snakes [63]. The W chromosomes are tiny in some lacertid species, but small to medium-sized in others (Figure 1). Observations based on the classical cytogenetic techniques led to the suggestion that the variability in the size of the W chromosomes in lacertids reflects the independent emergence of the ZZ/ZW sex chromosomes within this lineage [34]; however, molecular evidence for the homology of ZZ/ZW sex chromosomes across the family [16,64] disproved this hypothesis. As the size of the W chromosome is very different even in closely related lacertid species with otherwise very similar karyotypes (e.g., closely related *Ti. lepidus* and *Ti. tangitanus*, Figure 1), it seems that the size variability in lacertid W chromosomes cannot be attributed to chromosome fissions, fusions, or other significant interchromosomal rearrangements, but it is a result of repeated expansions and contractions of repeat content, as was suggested also for other lineages [26,30,65,66].

Matsubara et al. [40] and Giovannotti et al. [42] identified that the W chromosomes in *Lac. agilis* and in *A. lineomaculatus* are highly enriched in telomeric-like sequences. The accumulation of these sequences in the non-recombining part of the W can be expected, as they are also accumulated on independently evolved sex chromosomes in other squamate lineages [28,38,67]. However, only six out of the 15 here studied lacertid species have accumulations of telomeric and telomeric-like repeats on the W chromosome notably stronger than on autosomes or Z chromosomes (Figures 3 and 4). The strongest accumulation was found in the exceptionally large W chromosome in *Gal. galloti*. The larger accumulation of telomeric-like repeats on the W chromosomes can be considered as an apomorphy of the genus *Lacerta* (Figures 3 and 4), but otherwise, it is difficult to find any clear phylogenetic signal in the pattern. The situation resembles the analogous phylogenetic distribution in caenophidian snakes, where particular lineages exhibit a very diverse extent of the accumulation of telomeric-like repeats on W chromosomes [28]. As telomere shortening is related to aging and numbers of telomeric repeats are an important marker in aging research, we stress that the amount of telomeric-like repeats within chromosomes have to be taken into account during measurements of telomere length. Many techniques for the measurement of telomere size are not able to distinguish between terminal and interstitial positions. Therefore, telomeric-like repeats can give very biased results for the comparison of aging and heredity of telomere length when the variable amount on sex chromosomes is not taken into account [28]. Future studies of telomeres in lacertids have to keep in mind that W chromosomes are highly enriched with telomeric-like repeats in some but not all species of lacertids.

In the past, Banded krait minor satellite DNA repeats (Bkm) consisting of tandem arrays of 26 and 12 copies, respectively, of two tetranucleotides, GATA and GACA repeats, were isolated from caenophidian snakes. Bkm repeats were expected to occur on the heterochromatic sex chromosomes of amniotes, as it was assumed that they play an important role in the emergence of heterochromatin [68,69]. However, it was shown that GATA repeats are notably missing on the heterochromatic W in the lacertid *Eremias velox*, as well as in the heterochromatic regions of the sex chromosomes of several amniote lineages [38,67]. The present results support these findings. The GATA motif is accumulated on some, but not all heterochromatic W chromosomes in lacertids (Figure 4), and it does not co-localize with

GACA accumulations on the W across lacertids (Figure 5). Recently, it was shown that the emergence of the accumulations of Bkm repeats during the evolutionary history of caenophidian snakes was not correlated with the emergence of sex-linked heterochromatin, as heterochromatinization likely predeceased the accumulation of GATA-containing repeats on their W chromosomes [70].

The presence and accumulation of the other 21 tested microsatellite repeats varied greatly among four studied lacertid species (Figure 5) as well. *Gal. galloti*, a representative of the basal clade of lacertid phylogeny, had the most prominent accumulation and the biggest W chromosome. On the other hand, the W chromosome of *Gas. prasina* did not present significant accumulations of any of the tested motifs, despite the fact that it has a relatively large W chromosome, too. The variability in the repetitive content of the W chromosomes in lacertid lizards was recently presented also by Giovannotti et al. [43], who showed that the IMO-TaqI satellite DNA repeat accumulates on the W chromosomes of four species from the genus *Lacerta*, but not in *Ti. lepidus*. Future studies based on genomic approaches that enable catalogizing other repeat types in lacertids should test whether the variability in the repetitive content of the W chromosomes is restricted only to microsatellites, or whether the pattern revealed in them is general also for other repeats.

Several authors [38,67] compared repetitive content across independently evolved sex chromosomes and concluded that the identity of the accumulations at least of particular microsatellite sequences on the degenerated sex chromosomes reflect more likely historical contingency rather than a functional aspect of particular repeats. Later studies among reptiles, e.g., in monitor lizards [62,71] and in caenophidian snakes [28,72], documented that the repeat content of degenerated sex chromosomes is highly variable also within a lineage possessing homologous sex chromosomes [29,30,73]. Lacertids can be added to these groups as another example of the high evolutionary dynamics of repetitive content of degenerated chromosomes.

The highly dynamic content of W chromosomes across lacertids contrasts with their otherwise large conservation in karyotypes [45]. In fact, only three different chromosomal numbers occurred among the 15 here studied species (Figure 1), with the most common being the karyotype with 2n = 38 acrocentric chromosomes. The karyotype with 2n = 40 was found also in other members of the subfamily Gallotiinae, which is sister to all other lacertids. It occurs in all species from the genera *Gallotia* and *Psammodromus* [34,35,45,57,58] and might correspond to the ancestral karyotype of the subfamily. Among the studied lacertid species, metacentric chromosomes are present only in the genus *Timon*. The karyotype with 2n = 36 derived from the ancestral 2n = 38 via a Robertsonian fusion [39], and it seems to be a synapomorphy of this genus, as it is present also in *Ti. princeps* [74] and *Ti. pater* [75]. ITRs used to be considered as a marker of chromosomal rearrangements, although there are more mechanisms responsible for their emergence [76]. A previous study by Rojo et al. [39] did not detect any ITRs as a remnant of the fusion in the metacentric chromosome in *Ti. lepidus*. However, we noticed that there is a weak telomeric-like signal corresponding to ITRs in the assumed fusion point, i.e., in the centromeric region in both *Ti. lepidus* (Figure 3o) and *Ti. tangitanus* (Figure 3p). There are also additional ITRs in the chromosome arm of the same metacentric chromosome in *Ti. tangitanus* (Figure 3p). In addition to *Ti. lepidus* and *Ti. tangitanus*, ITRs within chromosomal arms were detected in *Lac. media* (Figure 3m). There was speculation that the origin of ITRs within chromosome arms, a relatively common trait in squamates, can be connected to intrachromosomal rearrangements (e.g., inversions) [73]. This hypothesis should be tested in lacertids when well-assembled genomes enabling the detailed detection of inversions will be available.

In contrast to the heterochromatic W chromosomes, the Z chromosomes were cytogenetically unequivocally distinguished from autosomes in only a few lacertid species [39,46,77] thanks to chromosome-specific hybridization probes or specific DNA methylation patterns. Thus, an interesting observation is that we were able to identify Z chromosomes in four species of two genera. In them, not only W, but also Z chromosomes possess notable accumulations of GATA repeats. With the exception of a pair of small chromosomes in *Ta. sexlineatus*, which can be an evolutionary novelty of this species, the accumulations of these motifs do not accumulate on autosomes, but rather only on

the sex chromosomes. We speculate that the Z chromosomes (and in *Ta. sexlineatus* maybe also a pair of autosomes) became "infected" by the repeats from the degenerated W chromosomes. Although largely different in sequences including gene content [64], the lacertid Z and W chromosomes make a bivalent during female meiosis and have pseudoautosomal regions [46]. We assume that a genomic region with GATA repeats was transferred from the W to the Z through recombination and in the case of *Ta. sexlineatus* to autosomes via translocation. These cases suggest that the degeneration process of the W/Y chromosomes might also affect the Z and X chromosomes and potentially also other parts of genomes, which is a phenomenon deserving further study.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4425/11/5/531/s1, Table S1. List of individuals per species and sex analyzed in this study. Figure S1. Mitotic metaphase chromosomes hybridized with the $(GATA)_8$ probe in (a) female and (b) male of *Takydromus dorsalis* (TADO), (c) female and (d) male of *Takydromus sexlineatus* (TASE), (e) female and (f) male of *Timon lepidus* (TILE), (g) female and (h) male of *Timon tangitanus* (TITA).

Author Contributions: G.S. and M.A. performed the cytogenetic analyses and prepared the figures; S.M., L.K. and M.R. contributed to the experimental part; P.P. and E.K. provided part of the material and useful consultations; L.K. and M.R. conceived and led the project; G.S., L.K. and M.R. drafted the first version of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: We declare no conflict of interest.

References

1. Ohno, S. *Sex Chromosomes and Sex-Linked Genes*; Monographs on Endocrinology; Springer: Berlin/Heidelberg, Germany, 1966; ISBN 978-3-642-88180-0.
2. Charlesworth, D.; Charlesworth, B.; Marais, G. Steps in the evolution of heteromorphic sex chromosomes. *Heredity* **2005**, *95*, 118–128. [CrossRef] [PubMed]
3. Vicoso, B. Molecular and evolutionary dynamics of animal sex-chromosome turnover. *Nat. Ecol. Evol.* **2019**, *3*, 1632–1641. [CrossRef] [PubMed]
4. Kirkpatrick, M. How and why chromosome inversions evolve. *PLoS Biol.* **2010**, *8*, e1000501. [CrossRef] [PubMed]
5. Bachtrog, D.; Kirkpatrick, M.; Mank, J.E.; McDaniel, S.F.; Pires, J.C.; Rice, W.; Valenzuela, N. Are all sex chromosomes created equal? *Trends Genet.* **2011**, *27*, 350–357. [CrossRef]
6. Pokorná, M.; Kratochvíl, L. Phylogeny of sex-determining mechanisms in squamate reptiles: Are sex chromosomes an evolutionary trap? *Zool. J. Linn. Soc.* **2009**, *156*, 168–183. [CrossRef]
7. Scherthan, H.; Cremer, T.; Arnason, U.; Weier, H.-U.; Lima-de-Faria, A.; Frönicke, L. Comparative chromosome painting discloses homologous segments in distantly related mammals. *Nat. Genet.* **1994**, *6*, 342–347. [CrossRef]
8. Ferguson-Smith, M.A.; Yang, F.; O'Brien, P.C.M. Comparative mapping using chromosome sorting and painting. *ILAR J.* **1998**, *39*, 68–76. [CrossRef] [PubMed]
9. Mank, J.E.; Ellegren, H. Parallel divergence and degradation of the avian W sex chromosome. *Trends Ecol. Evol.* **2007**, *22*, 389–391. [CrossRef]
10. Graphodatsky, A.S.; Trifonov, V.A.; Stanyon, R. The genome diversity and karyotype evolution of mammals. *Mol. Cytogenet.* **2011**, *4*, 22. [CrossRef] [PubMed]
11. Rovatsos, M.; Pokorná, M.; Altmanová, M.; Kratochvíl, L. Cretaceous park of sex determination: Sex chromosomes are conserved across iguanas. *Biol. Lett.* **2014**, *10*, 20131093. [CrossRef] [PubMed]
12. Rovatsos, M.; Vukić, J.; Lymberakis, P.; Kratochvíl, L. Evolutionary stability of sex chromosomes in snakes. *Proc. R. Soc. B* **2015**, *282*, 20151992. [CrossRef]
13. Rovatsos, M.; Praschag, P.; Fritz, U.; Kratochvíl, L. Stable Cretaceous sex chromosomes enable molecular sexing in softshell turtles (Testudines: Trionychidae). *Sci. Rep.* **2017**, *7*, 42150. [CrossRef]

14. Rovatsos, M.; Farkačová, K.; Altmanová, M.; Johnson Pokorná, M.; Kratochvíl, L. The rise and fall of differentiated sex chromosomes in geckos. *Mol. Ecol.* **2019**, *28*, 3042–3052. [CrossRef]
15. Rovatsos, M.; Rehák, I.; Velenský, P.; Kratochvíl, L. Shared ancient sex chromosomes in varanids, beaded lizards, and alligator lizards. *Mol. Biol. Evol.* **2019**, *36*, 1113–1120. [CrossRef] [PubMed]
16. Rovatsos, M.; Vukić, J.; Mrugała, A.; Suwala, G.; Lymberakis, P.; Kratochvíl, L. Little evidence for switches to environmental sex determination and turnover of sex chromosomes in lacertid lizards. *Sci. Rep.* **2019**, *9*, 7832. [CrossRef] [PubMed]
17. Altmanová, M.; Rovatsos, M.; Johnson Pokorná, M.; Veselý, M.; Wagner, F.; Kratochvíl, L. All iguana families with the exception of basilisks share sex chromosomes. *Zoology* **2018**, *126*, 98–102. [CrossRef]
18. Xu, L.; Auer, G.; Peona, V.; Suh, A.; Deng, Y.; Feng, S.; Zhang, G.; Blom, M.P.K.; Christidis, L.; Prost, S.; et al. Dynamic evolutionary history and gene content of sex chromosomes across diverse songbirds. *Nat. Ecol. Evol.* **2019**, *3*, 834–844. [CrossRef] [PubMed]
19. Acosta, M.J.; Marchal, J.A.; Fernández-Espartero, C.; Romero-Fernández, I.; Rovatsos, M.T.; Giagia-Athanasopoulou, E.B.; Gornung, E.; Castiglia, R.; Sánchez, A. Characterization of the satellite DNA Msat-160 from species of *Terricola* (*Microtus*) and *Arvicola* (Rodentia, Arvicolinae). *Genetica* **2010**, *138*, 1085–1098. [CrossRef]
20. Hughes, J.F.; Skaletsky, H.; Pyntikova, T.; Graves, T.A.; van Daalen, S.K.M.; Minx, P.J.; Fulton, R.S.; McGrath, S.D.; Locke, D.P.; Friedman, C.; et al. Chimpanzee and human Y chromosomes are remarkably divergent in structure and gene content. *Nature* **2010**, *463*, 536–539. [CrossRef] [PubMed]
21. Cortez, D.; Marin, R.; Toledo-Flores, D.; Froidevaux, L.; Liechti, A.; Waters, P.D.; Grützner, F.; Kaessmann, H. Origins and functional evolution of Y chromosomes across mammals. *Nature* **2014**, *508*, 488–493. [CrossRef]
22. Gamble, T.; Geneva, A.J.; Glor, R.E.; Zarkower, D. *Anolis* sex chromosomes are derived from a single ancestral pair. *Evolution* **2014**, *68*, 1027–1041. [CrossRef]
23. Rovatsos, M.T.; Marchal, J.A.; Romero-Fernández, I.; Cano-Linares, M.; Fernández, F.J.; Giagia-Athanasopoulou, E.B.; Sánchez, A. Molecular and physical characterization of the complex pericentromeric heterochromatin of the vole species *Microtus thomasi*. *Cytogenet. Genome Res.* **2014**, *144*, 131–141. [CrossRef] [PubMed]
24. Soh, Y.Q.S.; Alföldi, J.; Pyntikova, T.; Brown, L.G.; Graves, T.; Minx, P.J.; Fulton, R.S.; Kremitzki, C.; Koutseva, N.; Mueller, J.L.; et al. Sequencing the mouse Y chromosome reveals convergent gene acquisition and amplification on both sex chromosomes. *Cell* **2014**, *159*, 800–813. [CrossRef] [PubMed]
25. Zhou, Q.; Zhang, J.; Bachtrog, D.; An, N.; Huang, Q.; Jarvis, E.D.; Gilbert, M.T.P.; Zhang, G. Complex evolutionary trajectories of sex chromosomes across bird taxa. *Science* **2014**, *346*, 1246338. [CrossRef]
26. Altmanová, M.; Rovatsos, M.; Kratochvíl, L.; Johnson Pokorná, M. Minute Y chromosomes and karyotype evolution in Madagascan iguanas (Squamata: Iguania: Opluridae). *Biol. J. Linn. Soc.* **2016**, *118*, 618–633. [CrossRef]
27. Morgan, A.P.; Pardo-Manuel de Villena, F. Sequence and structural diversity of mouse Y chromosomes. *Mol. Biol. Evol.* **2017**, *34*, 3186–3204. [CrossRef]
28. Augstenová, B.; Mazzoleni, S.; Kratochvíl, L.; Rovatsos, M. Evolutionary dynamics of the W chromosome in caenophidian snakes. *Genes* **2018**, *9*, 5. [CrossRef] [PubMed]
29. Iannucci, A.; Altmanová, M.; Ciofi, C.; Ferguson-Smith, M.; Milan, M.; Pereira, J.C.; Pether, J.; Rehák, I.; Rovatsos, M.; Stanyon, R.; et al. Conserved sex chromosomes and karyotype evolution in monitor lizards (Varanidae). *Heredity* **2019**, *123*, 215–227. [CrossRef] [PubMed]
30. Rovatsos, M.; Altmanová, M.; Augstenová, B.; Mazzoleni, S.; Velenský, P.; Kratochvíl, L. ZZ/ZW sex determination with multiple neo-sex chromosomes is common in Madagascan chameleons of the genus *Furcifer* (Reptilia: Chamaeleonidae). *Genes* **2019**, *10*, 1020. [CrossRef] [PubMed]
31. Deakin, J.E.; Potter, S.; O'Neill, R.; Ruiz-Herrera, A.; Cioffi, M.B.; Eldridge, M.D.B.; Fukui, K.; Marshall Graves, J.A.; Griffin, D.; Grutzner, F.; et al. Chromosomics: Bridging the gap between genomes and chromosomes. *Genes* **2019**, *10*, 627. [CrossRef]
32. Ezaz, T.; Deakin, J.E. Repetitive sequence and sex chromosome evolution in vertebrates. *Adv. Evol. Biol.* **2014**, *2014*, 104683. [CrossRef]
33. Hobza, R.; Kubat, Z.; Cegan, R.; Jesionek, W.; Vyskot, B.; Kejnovsky, E. Impact of repetitive DNA on sex chromosome evolution in plants. *Chromosome Res.* **2015**, *23*, 561–570. [CrossRef] [PubMed]

34. De Smet, W.H. Description of the orcein stained karyotypes of 36 lizard species (*Lacertilia, Reptilia*) belonging to the families Teiidae, Scincidae, Lacertidae, Cordylidae and Varanidae (Autarchoglossa). *Acta Zool. Pathol. Antverp.* **1981**, *76*, 73–118.
35. Olmo, E.; Odierna, G.; Capriglione, T. The karyology of Mediterranean lacertid lizards. In *Lacertids of the Mediterranean Region: A Biological Approach*; Valakos, E.D., Böhme, W., Perez Mellado, V., Maragou, P., Eds.; Hellenic Zoological Society: Athens, Greece, 1993; pp. 61–84.
36. Odierna, G.; Caprigilone, T.; Kupriyanova, L.A.; Olmo, E. Further data on sex chromosomes of Lacertidae and a hypothesis on their evolutionary trend. *Amphib.-Reptil.* **1993**, *14*, 1–11. [CrossRef]
37. Olmo, E.; Cobror, O.; Morescalchi, A.; Odierna, G. Homomorphic sex chromosomes in the lacertid lizard *Takydromus sexlineatus*. *Heredity* **1984**, *53*, 457–459. [CrossRef]
38. Pokorná, M.; Kratochvíl, L.; Kejnovský, E. Microsatellite distribution on sex chromosomes at different stages of heteromorphism and heterochromatinization in two lizard species (Squamata: Eublepharidae: *Coleonyx elegans* and Lacertidae: *Eremias velox*). *BMC Genet.* **2011**, *12*, 90. [CrossRef]
39. Rojo Oróns, V. Cytogenetic and Molecular Characterization of Lacertid Lizard Species from the Iberian Peninsula. Ph.D. Thesis, Universidade da Coruña, Coruña, Spain, 2015.
40. Matsubara, K.; Uno, Y.; Srikulnath, K.; Matsuda, Y.; Miller, E.; Olsson, M. No interstitial telomeres on autosomes but remarkable amplification of telomeric repeats on the W sex chromosome in the sand lizard (*Lacerta agilis*). *J. Hered.* **2015**, *106*, 753–757. [CrossRef]
41. Lisachov, A.P.; Borodin, P.M. Microchromosome polymorphism in the sand lizard, *Lacerta agilis* Linnaeus, 1758 (Reptilia, Squamata). *Comp. Cytogenet.* **2016**, *10*, 387–399. [CrossRef]
42. Giovannotti, M.; Nisi Cerioni, P.; Slimani, T.; Splendiani, A.; Paoletti, A.; Fawzi, A.; Olmo, E.; Caputo Barucchi, V. Cytogenetic characterization of a population of *Acanthodactylus lineomaculatus* Duméril and Bibron, 1839 (Reptilia, Lacertidae), from Southwestern Morocco and insights into sex chromosome evolution. *Cytogenet. Genome Res.* **2017**, *153*, 86–95. [CrossRef]
43. Giovannotti, M.; Nisi Cerioni, P.; Rojo, V.; Olmo, E.; Slimani, T.; Splendiani, A.; Caputo Barucchi, V. Characterization of a satellite DNA in the genera *Lacerta* and *Timon* (Reptilia, Lacertidae) and its role in the differentiation of the W chromosome. *J. Exp. Zool.* **2018**, *330*, 83–95. [CrossRef]
44. Lisachov, A.P.; Giovannotti, M.; Pereira, J.C.; Andreyushkova, D.A.; Romanenko, S.A.; Ferguson-Smith, M.A.; Borodin, P.M.; Trifonov, V.A. Chromosome painting does not support a sex chromosome turnover in *Lacerta agilis* Linnaeus, 1758. *Cytogenet. Genome Res.* **2020**, *160*, 134–140. [CrossRef] [PubMed]
45. Olmo, E.; Signorino, G. Chromorep: A Reptile Chromosomes Database. 2005. Available online: http://chromorep.univpm.it/ (accessed on 25 March 2020).
46. Lisachov, A.P.; Galkina, S.A.; Saifitdinova, A.F.; Romanenko, S.A.; Andreyushkova, D.A.; Trifonov, V.A.; Borodin, P.M. Identification of sex chromosomes in *Eremias velox* (Lacertidae, Reptilia) using lampbrush chromosome analysis. *Comp. Cytogenet.* **2019**, *13*, 121–132. [CrossRef] [PubMed]
47. Andrade, P.; Pinho, C.; Pérez i de Lanuza, G.; Afonso, S.; Brejcha, J.; Rubin, C.-J.; Wallerman, O.; Pereira, P.; Sabatino, S.J.; Bellati, A.; et al. Regulatory changes in pterin and carotenoid genes underlie balanced color polymorphisms in the wall lizard. *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 5633–5642. [CrossRef] [PubMed]
48. Vertebrate Genome Project. Genome of *Lacerta agilis*. 2019. Available online: https://www.ncbi.nlm.nih.gov/genome/?term=txid80427 (accessed on 25 March 2020).
49. Rovatsos, M.; Vukić, J.; Altmanová, M.; Johnson Pokorná, M.; Moravec, J.; Kratochvíl, L. Conservation of sex chromosomes in lacertid lizards. *Mol. Ecol.* **2016**, *25*, 3120–3126. [CrossRef] [PubMed]
50. Pokorná, M.; Rens, W.; Rovatsos, M.; Kratochvíl, L. A ZZ/ZW sex chromosome system in the thick-tailed gecko (*Underwoodisaurus milii*; Squamata: Gekkota: Carphodactylidae), a member of the ancient gecko lineage. *Cytogenet. Genome Res.* **2014**, *142*, 190–196. [CrossRef] [PubMed]
51. Sumner, A.T. A simple technique for demonstrating centromeric heterochromatin. *Exp. Cell Res.* **1972**, *75*, 304–306. [CrossRef]
52. Rovatsos, M.; Johnson Pokorná, M.; Kratochvíl, L. Differentiation of sex chromosomes and karyotype characterisation in the dragonsnake *Xenodermus javanicus* (Squamata: Xenodermatidae). *Cytogenet. Genome Res.* **2015**, *147*, 48–54. [CrossRef]
53. Oguma, K. Studies on the sauropsid chromosomes. II. The cytological evidence proving female heterogamety in the lizard (*Lacerta vivipara*). *Arch. Biol.* **1934**, *45*, 27–46.
54. Gorman, G.C. New chromosome data for 12 species of lacertid lizards. *J. Herpet.* **1969**, *3*, 49–54. [CrossRef]

55. Ivanov, V.G.; Fedorova, T.A. Sex heteromorphism of chromosomes in *Lacerta strigata*. *Tsitologiia* **1970**, *12*, 1582–1585.
56. Ivanov, V.; Bogdanov, O.; Anisimov, E.Y.; Fedorova, T. Studies into karyotypes of 3 lizard species (Sauria, Scincidae, Lacertidae). *Tsitologiia* **1973**, *15*, 1291–1296.
57. Cano, J.; Baez, M.; Lopez-Jurado, L.F.; Ortega, G. Karyotype and chromosome structure in the lizard, *Gallotia galloti* in the Canary Islands. *J. Herpetol.* **1984**, *18*, 344–346. [CrossRef]
58. Olmo, E.; Odierna, G.; Capriglione, T. Evolution of sex-chromosomes in lacertid lizards. *Chromosoma* **1987**, *96*, 33–38. [CrossRef]
59. Pyron, R.; Burbrink, F.T.; Wiens, J.J. A phylogeny and revised classification of Squamata, including 4161 species of lizards and snakes. *BMC Evol. Biol.* **2013**, *13*, 93. [CrossRef]
60. Garcia-Porta, J.; Irisarri, I.; Kirchner, M.; Rodríguez, A.; Kirchhof, S.; Brown, J.L.; MacLeod, A.; Turner, A.P.; Ahmadzadeh, F.; Albaladejo, G.; et al. Environmental temperatures shape thermal physiology as well as diversification and genome-wide substitution rates in lizards. *Nat. Commun.* **2019**, *10*, 4077. [CrossRef] [PubMed]
61. Rutkowska, J.; Lagisz, M.; Nakagawa, S. The long and the short of avian W chromosomes: No evidence for gradual W shortening. *Biol. Lett.* **2012**, *8*, 636–638. [CrossRef] [PubMed]
62. Matsubara, K.; Sarre, S.D.; Georges, A.; Matsuda, Y.; Marshall Graves, J.A.; Ezaz, T. Highly differentiated ZW sex microchromosomes in the Australian *Varanus* species evolved through rapid amplification of repetitive sequences. *PLoS ONE* **2014**, *9*, e95226. [CrossRef]
63. Oguiura, N.; Ferrarezzi, H.; Batistic, R.F. Cytogenetics and molecular data in snakes: A phylogenetic approach. *Cytogenet. Genome Res.* **2009**, *127*, 128–142. [CrossRef]
64. Rovatsos, M.; Vukić, J.; Kratochvíl, L. Mammalian X homolog acts as sex chromosome in lacertid lizards. *Heredity* **2016**, *117*, 8–13. [CrossRef]
65. Kejnovsky, E.; Kubat, Z.; Hobza, R.; Lengerova, M.; Sato, S.; Tabata, S.; Fukui, K.; Matsunaga, S.; Vyskot, B. Accumulation of chloroplast DNA sequences on the Y chromosome of *Silene latifolia*. *Genetica* **2006**, *128*, 167–175. [CrossRef]
66. Schartl, M.; Schmid, M.; Nanda, I. Dynamics of vertebrate sex chromosome evolution: From equal size to giants and dwarfs. *Chromosoma* **2016**, *125*, 553–571. [CrossRef] [PubMed]
67. Matsubara, K.; O'Meally, D.; Azad, B.; Georges, A.; Sarre, S.D.; Graves, J.A.M.; Matsuda, Y.; Ezaz, T. Amplification of microsatellite repeat motifs is associated with the evolutionary differentiation and heterochromatinization of sex chromosomes in Sauropsida. *Chromosoma* **2016**, *125*, 111–123. [CrossRef] [PubMed]
68. Singh, L.; Purdom, I.F.; Jones, K.W. Satellite DNA and evolution of sex chromosomes. *Chromosoma* **1976**, *59*, 43–62. [CrossRef] [PubMed]
69. Singh, L.; Purdom, I.F.; Jones, K.W. Sex chromosome associated satellite DNA: Evolution and conservation. *Chromosoma* **1980**, *79*, 137–157. [CrossRef] [PubMed]
70. Rovatsos, M.; Altmanová, M.; Johnson Pokorná, M.; Augstenová, B.; Kratochvíl, L. Cytogenetics of the Javan file snake (*Acrochordus javanicus*) and the evolution of snake sex chromosomes. *J. Zool. Syst. Evol. Res.* **2018**, *56*, 117–125. [CrossRef]
71. Johnson Pokorná, M.; Altmanová, M.; Rovatsos, M.; Velenský, P.; Vodička, R.; Rehák, I.; Kratochvíl, L. First description of the karyotype and sex chromosomes in the Komodo dragon (*Varanus komodoensis*). *Cytogenet. Genome Res.* **2016**, *148*, 284–291. [CrossRef] [PubMed]
72. Matsubara, K.; Tarui, H.; Toriba, M.; Yamada, K.; Nishida-Umehara, C.; Agata, K.; Matsuda, Y. Evidence for different origin of sex chromosomes in snakes, birds, and mammals and step-wise differentiation of snake sex chromosomes. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 18190–18195. [CrossRef]
73. Rovatsos, M.; Kratochvíl, L.; Altmanová, M.; Johnson Pokorná, M. Interstitial telomeric motifs in squamate reptiles: When the exceptions outnumber the rule. *PLoS ONE* **2015**, *10*, e0134985. [CrossRef]
74. Rykena, S.; Nettmann, H.K. The karyotype of Lacerta princeps kurdistanica and its meaning in phylogeny. In *Studies in Herpetology*; Roček, Z., Ed.; Prague, Czech Republic, 1986; pp. 193–196. Available online: https://www.lacerta.de/AF/Bibliografie/BIB_4026.pdf (accessed on 8 May 2020).
75. Odierna, G.; Olmo, E.; Capriglione, T.; Caputo, V. Karyological differences between *Lacerta lepida* and *Lacerta pater*. *J. Herpet.* **1990**, *24*, 97–99. [CrossRef]

76. Ruiz-Herrera, A.; Nergadze, S.G.; Santagostino, M.; Giulotto, E. Telomeric repeats far from the ends: Mechanisms of origin and role in evolution. *Cytogenet. Genome Res.* **2008**, *122*, 219–228. [CrossRef]
77. Schmid, M.; Steinlein, C.; Reiter, A.M.; Rovatsos, M.; Altmanová, M.; Mazzoleni, S.; Johnson Pokorná, M.; Kratochvíl, L. 5-Methylcytosine-rich heterochromatin in reptiles. *Cytogenet. Genome Res.* **2019**, *157*, 53–64. [CrossRef] [PubMed]

Chapter 3

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Little evidence for switches to environmental sex determination and turnover of sex chromosomes in lacertid lizards

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Amniotes possess variability in sex determination, from environmental sex determination (ESD), where no sex chromosomes are present, to genotypic sex determination (GSD) with highly differentiated sex chromosomes. Some evolutionary scenarios postulate high stability of differentiated sex chromosomes and rare transitions from GSD to ESD. However, sex chromosome turnovers and two independent transitions from highly differentiated ZZ/ZW sex chromosomes to ESD were previously reported in the lacertid lizards. Here, we examined the homology of sex chromosomes in the wide phylogenetic spectrum of lacertids and their outgroups by comparing gene copy numbers between sexes in genes previously found to be Z-specific in some lacertids. Our current sampling covers 45 species from 26 genera including lineages supposed to possess a derived sex determining systems. We found that all tested lacertids share homologous differentiated ZZ/ZW sex chromosomes, which were present already in their common ancestor living around 85 million years ago. These differentiated sex chromosomes are not present in amphisbaenians and teiid lizards, the close relatives of lacertids. Our study demonstrates how inaccuracies in data can influence the outcome of phylogenetic reconstructions of evolution of sex determination, in this case they overestimated the number of shifts from GSD to ESD and the rate in turnovers of sex chromosomes.

Sex determination, the process that decides the sex of an individual, is variable among lineages of amniotes[1–3]. Despite the great effort and recent advances, the reconstruction of the ancestral state and transitions between particular sex determination modes in amniotes is still equivocal. Some authors argue that the ancestral state was environmental sex determination (ESD), where sexes do not differ in genotype consistently. According to this scenario, the ancestral ESD is still present in recent crocodiles, the majority of turtles and a few squamate lineages[4]. Furthermore, the transitions from ESD to genotypic sex determination (GSD), where sexes differ in genotypes, should be frequent, but transitions in the opposite direction should be rare. This view might be supported by the shared parts of the molecular machinery of sex determination across several ESD lineages[5], which can, however, also reflect independent co-option of the same epigenetic, thermally-sensitive process. Notably, other authors suggested that GSD, and not ESD, was the ancestral state in amniotes[6]. This alternative was supported by the finding that the same syntenic blocks play the role of sex chromosomes in several lineages, which was interpreted as evidence for a homology of these sex-determining systems. But again, homoplasy, in this case independent co-options of the same part of genome as sex chromosomes, cannot be excluded. Sex chromosomes likely evolved independently many times in amniotes and the repeated independent co-option of the same blocks might be a result of a multiple random selection from a limited number of syntenic blocks, or a higher tendency of a syntenic block to be co-opted due to its gene content, particularly due to enrichment of genes involved in gonad differentiation[7]. The ancestral GSD hypothesis suggests repeated transitions from GSD to ESD.

The two scenarios differ in the predictions on the stability of GSD with respect to ESD and hence the frequency of GSD to ESD transitions. Several transitions from GSD to ESD expected under the ancestral GSD hypothesis

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were suggested in some phylogenetic reconstructions of the evolution of sex determination systems[8–12], but many of them were put into doubt by some authors[1,3,13]. Two such putative transitions were reported in the lacertid lizards based on published data in *Podarcis pityusensis*[14] and *Eremias multiocellata*[15–17]. In lacertids, differentiated ZZ/ZW sex chromosomes containing genes with orthologs linked to the shorter arm of chicken (*Gallus gallus*; GGA) chromosome 4 (GGA4p), homologous to the ancestral X chromosome of viviparous mammals, and GGA17 were documented in 18 species. However, differences in morphology of sex chromosomes among lacertids led to the hypothesis that the differentiation of their sex chromosomes occurred repeatedly and independently in different taxa within the family[18]. Furthermore, recent cytogenetic evidence from comparative chromosome painting points to the non-homology of sex chromosomes between members of the genera *Iberolacerta* and *Timon* versus *Lacerta schreiberi*, suggesting that there has been a turnover of sex chromosomes within lacertids[19].

In the current study, we performed a molecular test of homology of sex chromosomes using up to now the densest sampling of lacertids. We aimed to clarify the stability and the age of differentiated sex chromosomes in lacertids and to explore the putative exceptions to the general ZZ/ZW pattern. We included the lineages where derived sex determining system was previously reported, which in the case of the genera *Eremias* and *Podarcis* led to the reconstruction of the transitions from the ancestral GSD to ESD within lacertids[8–10,12], undermining the ancestral ESD hypothesis for amniotes.

Material and Methods

Material collection and DNA isolation. Blood or tissue material were collected from both sexes from 27 species of lacertids and their outgroups, i.e., two species of the legless amphisbaenians of the family Blanidae and three species of the family Teiidae (Table S1). When needed, specimens were temporarily maintained in the Animal Facilities of Faculty of Science, Charles University (Accreditation No. 13060/2014-MZE-17214). All experimental procedures were carried out under the supervision and with the approval of the Ethics Committee of the Faculty of Science, Charles University, followed by the Committee for Animal Welfare of the Ministry of Agriculture of the Czech Republic (Accreditation No. 24773/2008-10001). Genomic DNA was extracted from samples using a DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol. DNA concentration and quality were measured by Nanodrop 2000 Spectrophotometer (Thermo Scientific).

Test of homology based on quantitative real-time PCR (qPCR). In ZZ/ZW sex chromosome systems genes linked to the Z chromosome and missing on its degenerated W counterpart differ in gene copy numbers between sexes. In such genes, males (ZZ) have twice as many copies than females (ZW), whereas genes in autosomal or pseudoautosomal regions have equal copy numbers in both sexes. Quantitative Real-Time PCR (qPCR) is a useful tool to estimate a difference in copy number between a male and a female individual of the same species. A relative female-to-male gene dose ratio (r) of 0.5 is expected for the Z-specific genes and 1.0 for the (pseudo)autosomal genes. Thus qPCR analysis can be performed in species across a lineage to test whether the same genes are Z-specific in them as a test of homology and degree of differentiation of sex chromosomes. In previous studies, it was shown that 18 species of lacertids have homologous sex chromosomes and their gene content is homologous to a part of GGA4p and GGA17[20,21]. Here we expand these studies by inclusion of other 27 lacertid and five outgroup species to reliably date the origin of the lacertid sex chromosomes. For qPCR measurement, we used previously designed primers targeting two autosomal genes (*adarb2*, *mecom*) and four putative Z-specific genes in lacertids (*mars2*, *lpar4*, *klhl13*, *angptl2*)[21]. In addition, we designed new primers for one autosomal gene with an ortholog in GGAZ (*smad7*), and four candidate Z-linked genes with orthologs linked to GGA4p (*gab3*, *mbnl3*) and GGA17 (*hspa5*, *lrrc8a*). The gene *mecom* was used as a reference gene for the normalization of the qPCR values. The primer sequences are given in Table S2. For detailed methodology on primer design and qPCR calculations see[22]. The qPCR was performed using a LightCycler II 480 (Roche Diagnostics, Basel, Switzerland) and all samples were run in triplicates. The loci with female-to-male gene dose ratio values in the range 0.25–0.75 were considered Z-specific, and in the range 0.75–1.25 autosomal or pseudoautosomal. We tested significance of deviations of the gene dose ratios in lacertids and outgroups in each gene from the values 0.5 expected for Z-specific genes and 1.0 expected for (pseudo-)autosomal genes by t-test.

Results

A relative gene dose was tested by qPCR in four genes previously showed to be Z-linked in lacertids, three of which are orthologous to genes in GGA4p and one to GGA17[20,21], in 27 species of lacertids and five species of their closest outgroups (two species of blanids and three species of teiids). Two additional genes with orthologs linked to GGA4p and two genes with orthologs linked to GGA17 were also tested in all species using the primers newly designed for this study. Although not all loci amplified successfully in all the tested species, at least two putative Z-specific genes were tested in all species. Overall, all putative Z-specific genes show the expected female-to-male gene dose ratio value of approximately 0.5 across lacertids, although individual genes depart from this pattern in some species (Tables S3 and S4). Therefore, it seems that all tested lacertid species share at least partially the same Z chromosome, with gene content homologous mainly to two major syntenic chromosome regions in chicken (GGA4p, GGA17) and human (HSA9, HSAX) (Fig. 1). The exception is the gene *mars2*, which has orthologs linked to GGA4p and to HSA2[20]. For the comparison of the partial gene content of the Z-specific part of sex chromosomes across major lineages of lacertid lizards and the estimation of the age of sex chromosomes in lacertids, we included the results of the previous study for 18 lacertid species[21], and thus obtained the densest dataset so far, including altogether 45 lacertid species and five close outgroup species from the families Blanidae and Teiidae. We found that differentiated sex chromosomes are shared across all 45 species of lacertids, including both lacertid subfamilies (Gallotinae and Lacertinae), and broadly covering the phylogenetic diversity of the group (see Fig. 2). We showed that *Podarcis pityusensis*, previously reported to possess ESD[14] and thus supposed to lack sex chromosomes, shares the same ZZ/ZW sex chromosomes with other lacertids. Also *Lacerta schreiberi*,

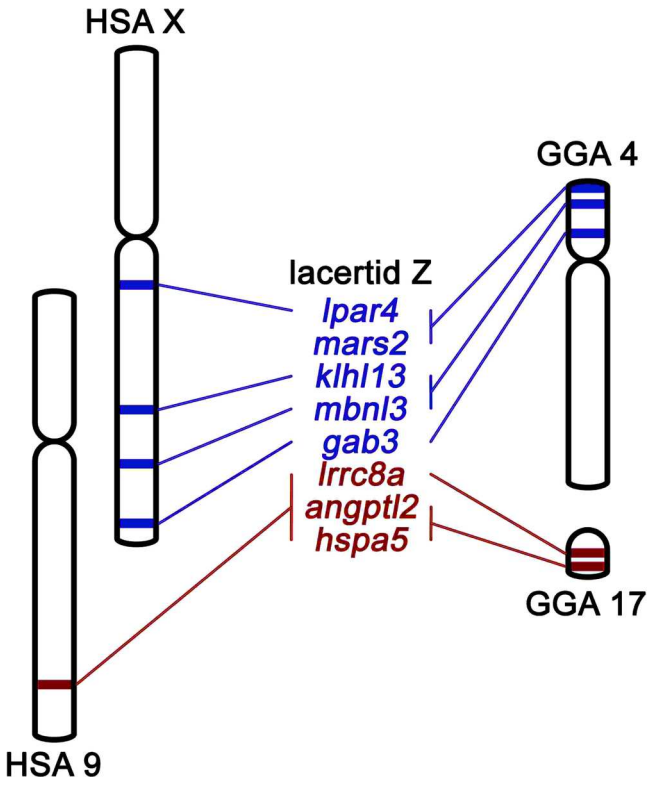

Figure 1. Position of lacertid Z-linked genes in human (HSA) and chicken (GGA) chromosomes.

previously thought to possess different sex chromosomes[19], has the same partial gene content of the Z-specific part of sex chromosomes, and thus possess the same sex chromosomes as other lacertids. Although we strongly supported the stability of the differentiated sex chromosomes across the lacertids, some Z-linked genes, both those with orthologs linked to GGA4p and those to GGA17, return a (pseudo)autosomal pattern in a few cases in different species (Table S3). Our results showed that the Z-specific genes of lacertids are (pseudo)autosomal in their closest outgroups, blanids and teiids (Tables S3 and S4). This allows the estimation of the age of lacertid differentiated sex chromosomes, placing their origin between the split between lacertids and amphisbaenians approximately 150 MYA and the basal split of lacertids, i.e., the split between Gallotinae and Lacertinae, approximately 85 MYA (the dating of these events follows ref.[23]).

Discussion

As far as we know, differentiated sex chromosomes (ZZ/ZW or derived multiple neo-sex chromosomes) were uncovered by previous cytogenetic work in 21 species present in our sample, the qPCR results are in agreement with these cytogenetic observations and moreover suggest that these cytogenetically detectable sex chromosomes are homologous. The qPCR results presented here and in our previous studies[20,21] represent the first evidence for female heterogamety in further 24 species of lacertids. Furthermore, female heterogamety was uncovered up to now solely by cytogenetics in 27 other lacertid species (reviewed in Table 1). Therefore, evidence for female heterogamety exists in *c*. 20% of the recently recognized species of lacertids. The current analysis supports the long-term stability of differentiated ZZ/ZW sex chromosomes across the whole family Lacertidae (Fig. 2). Lacertid sex chromosomes can be dated back to the Mesozoic epoch and are of comparable age to avian sex chromosomes[24]. The former studies documenting the variability in sex chromosomes in lacertids[18,25] were based on cytogenetic descriptions without any molecular or cytogenetic marker for testing of homology of sex chromosomes. Differentiated W chromosomes are highly variable in sequence content and heterochromatin distribution[26–28], which can explain the differences in morphology of lacertid sex chromosomes. Interestingly, we found that the Z chromosome of *Lacerta schreiberi* is homologous to Z of other lacertids, although it was previously reported that the flow-sorted probe containing the Z of *Iberolacerta monticola* hybridized to even number of chromosomes in metaphases from both sexes of *L. schreiberi*, which indicated that the ancestor of this species had a turnover of sex chromosomes[19].

The loci originally revealed to be Z-specific in *Takydromus sexlineatus*, the first lacertid with known partial gene content of sex chromosomes[20], are Z-specific in other lacertids as well, but several exceptions exist. In some species, putative Z-specific genes gave (pseudo)autosomal pattern in qPCR (Table S3). The distribution of these values with (pseudo)autosomal pattern does not seem to have any clear phylogenetic pattern. According to phylogenetic position of their bearers, these genes seem to be ancestrally Z-specific in lacertids. Analogous situation was found in the genomic analysis of the differentiation of Z and W chromosomes across birds, demonstrating that the sex chromosome evolution might be unexpectedly complex[24]. There are several, up to now purely

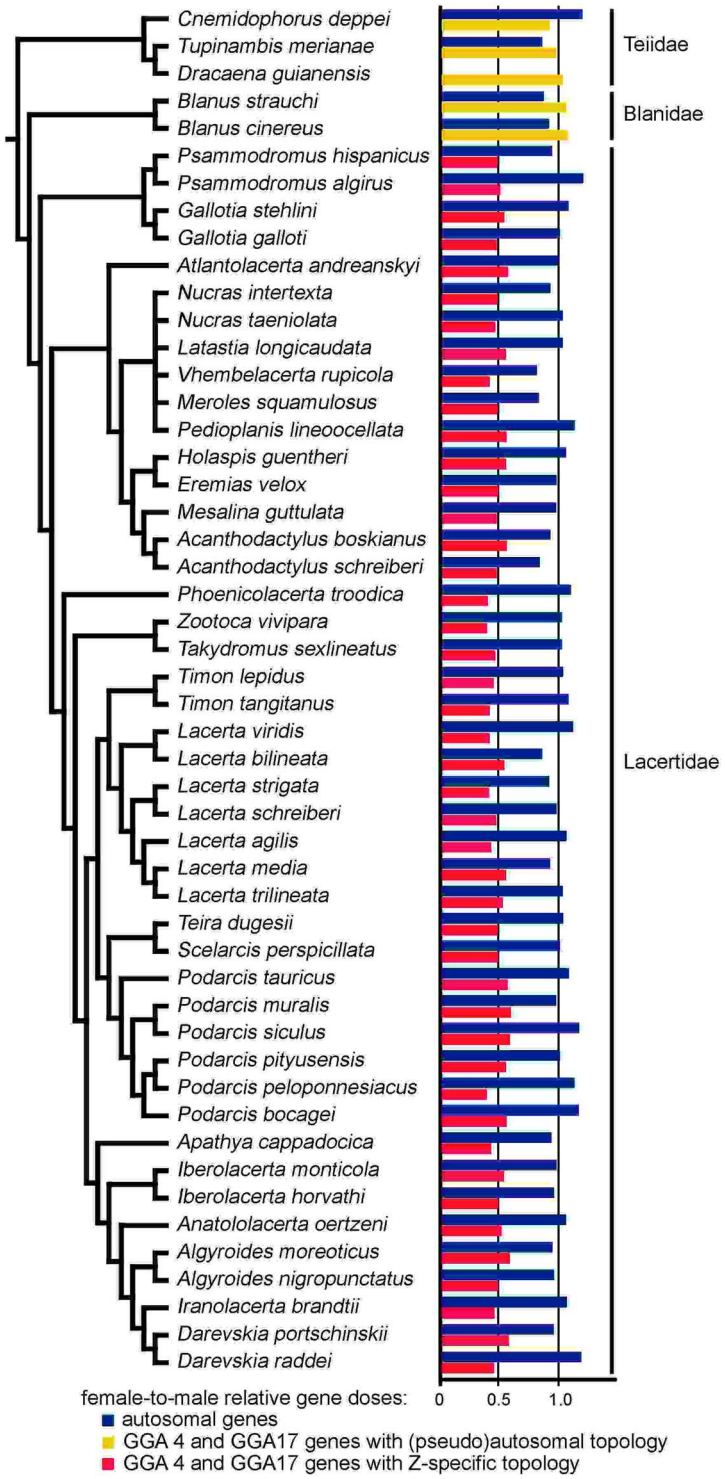

Figure 2. Relative gene dose ratios between sexes in 45 species of lacertids and five species representing outgroups (teiids and amphisbaenians from the family Blanidae). Red and yellow bars correspond to average Z-specific and (pseudo)autosomal values, respectively. Blue bars correspond to the average values for (pseudo) autosomal control loci. Value 1.0 is expected for (pseudo)autosomal genes, while value 0.5 is consistent with Z-specificity. Our results suggest that sex chromosomes are highly conserved and homologous across lacertids, although in some species several genes, which are Z-linked in the majority of lacertids, have a (pseudo) autosomal pattern. These genes were not included in the figure, but were assigned in Table S3. Data from[20,21] were included. Phylogeny follows[68]. Not all sub-Saharan species studied here were included in this phylogenetic hypothesis, which led to the soft polytomy in this clade.

Species	Cytogenetic evidence	qPCR evidence
Algyroides moreoticus	yes[18]	yes
Algyroides nigropunctatus	yes[18]	yes
Atlantolacerta andreanskyi	yes[46]	yes
Darevskia portschinskii	yes[47]	yes
Darevskia raddei	yes[47]	yes
Eremias velox	yes[48]	yes
Gallotia galloti	yes[49]	yes
Iberolacerta horvathi	yes[50]	yes
lberolacerta monticola	yes[51]	yes
Lacerta agilis	yes[52]	yes
Lacerta bilineata	yes[49]	yes
Lacerta schreiberi	yes[19]	yes
Lacerta strigata	yes[48]	yes
Lacerta trilineata	yes[53]	yes
Lacerta viridis	yes[51]	yes
Podarcis siculus	yes[51]	yes
Podarcis tauricus	yes[54]	yes
Psammodromus algirus	yes[51]	yes
Takydromus sexlineatus	yes[55]	yes
Teira dugesii	yes[25]	yes
Timon lepidus	yes[25]	yes
Zootoca vivipara	yes[56]	yes
Acanthodactylus boskianus	no	yes
Acanthodactylus schreiberi	no	yes
Anatololacerta oertzeni	no	yes
Apathya cappadocica	no	yes
Gallotia stehlini	no	yes
Holaspis guentheri	no	yes
Iranolacerta brandtii	no	yes
Lacerta media	no	yes
Latastia longicaudata	no	yes
Meroles squamulosus	no	yes
Mesalina guttulata	no	yes
Nucras intertexta	no	yes
Nucras taeniolata	no	yes
Pedioplanis lineoocellata	no	yes
Phoenicolacerta troodica	no	yes
Podarcis bocagei	no	yes
Podarcis muralis	no	yes
Podarcis peloponnesiaca	no	yes
Podarcis pityusensis	no	yes
Psammodromus hispanicus	no	yes
Scelarcis perspicillata	no	yes
Timon tangitanus	no	yes
Teira dugesii	no	yes
Vhembelacerta rupicola	no	yes
Acanthodactylus erythrurus	yes[25]	no
Acanthodactylus lineomaculatus	yes[39]	no
Darevskia armeniaca	yes[57]	no
Darevskia dahli	yes[58]	no
Darevskia mixta	yes[59]	no
Darevskia rostombekovi	yes[47]	no
Darevskia unisexualis	yes[47]	no
Darevskia valentini	yes[59]	no
Dinarolacerta mosorensis	yes[60]	no

Continued

Species	Cytogenetic evidence	qPCR evidence
Eremias arguta	yes[61]	no
Eremias grammica	yes[62]	no
Heliobolus lugubris	yes[63]	no
Hellenolacerta graeca	yes[18]	no
Iberolacerta aranica	yes[64]	no
Iberolacerta aurelioi	yes[64]	no
Iberolacerta bonnali	yes[64]	no
Iberolacerta cyreni	yes[64]	no
Iberolacerta galani	yes[65]	no
Meroles cuneirostris	yes[25]	no
Mesalina olivieri	yes[52]	no
Omanosaura jayakari	yes[66]	no
Ophisops elegans	yes[67]	no
Phoenicolacerta kulzeri	yes[69]	no
Phoenicolacerta laevis	yes[69]	no
Podarcis melisellensis	yes[52]	no
Podarcis hispanica	yes[18]	no
Podarcis wagleriana	yes[25]	no

Table 1. Overview of lacertid lizards with cytogenetic and qPCR[20,21,this study] evidence for female heterogamety.

speculative explanations for this variability. The observed (pseudo)autosomal pattern in otherwise Z-specific genes in lacertids might reflect a different rate of independent differentiation of the W-specific regions from the ancestral pseudoautosomal state, as was suggested for birds[24]. However, we cannot exclude independent secondary re-emergence of recombination between particular Z and W regions recreating locally the pseudoautosomal state or independent translocations of the genes to pseudoautosomal region or autosomes. Alternatively, the scattered pseudoautosomal pattern in certain genes can reflect convergence, for instance by gene conversion, of gametolog sequences leading to binding of qPCR primers otherwise specific to Z gametologs to both Z- and W-linked gametologs. These possibilities should be evaluated in future when more data on genomics of sex chromosomes in lacertids are available. As criticized already by Harlow[13], the evidence that *Podarcis pityusensis* possesses ESD is extremely poor. It is based on a single description of the production of one male to '10–15 females' at a single temperature without validation of sexing of juveniles[14,29]. However, this species is still included as having ESD in the majority of phylogenetic analyses of sex determination[8–10,12] but see[1,3]. The results of our analysis strongly suggest that this species has the same sex-linked region as all other tested lacertids. The shared sex-linkage demonstrates that *Podarcis pityusensis* does not have any derived sex determination system, but instead relies on the ancestral ZZ/ZW sex chromosomes of the lacertids.

More recently, ESD was reported in another lacertid, the viviparous species *Eremias multiocellata*[15–17,30]. This information was included in the subsequent comparative phylogenetic analysis, which led to a reconstruction of the second transition from GSD to ESD in the family Lacertidae[12]. Highly biased sex ratio related to constant temperatures during gestation was reported in the first experimental study in *E. multiocellata*[15]. In the follow-up study, the differences in sex ratios among temperatures were much less pronounced in the same species and equal sex ratios were reported from the females that went through gestation in the field and from moderate gestation temperatures[16]. The norm of reaction with equal sex ratios in non-extreme temperatures itself questions the presence of ESD[31]. Neither of these two studies were able to exclude differential mortality of sexes at certain temperatures (known for example in snakes)[32] or temperature-induced sex reversals (reported in the skink *Bassiana duperreyi* or dragon lizard *Pogona vitticeps*)[33–35]. Moreover, juveniles were sexed by examination of hemipene size (in[15] also by histology, but methodological details and data from histological sections were not presented), which was not validated, e.g., it was not tested whether hemipene size is not phenotypically plastic in relation to temperature. But the most important argument against ESD in *E. multiocellata* is the finding of highly differentiated ZZ/ZW sex chromosomes in this species by molecular cytogenetics. The highly differentiated W chromosome was found in all females, but not in any male examined[17] and the sample size was adequate to document clear, statistically significant sex-linkage of the genotype to sex. Due to unavailability of genetic samples, we were not able to include *E. multiocellata* in the recent study, but other congeneric species possess the typical lacertid well-differentiated sex chromosomes[36] and homologous sex chromosomes between *E. velox* and other lacertids was demonstrated[21] based on our Z-specific molecular markers.

The diversity in sex determination is unequally distributed among amniotes. Traditionally, it was assumed that unlike birds and mammals, reptiles, i.e., the paraphyletic group of non-avian sauropsids, exhibit rapid and frequent transitions in sex-determining systems[37]. Here, we document the gross stability in homology of sex chromosomes in lacertids since the Mesozoic era. Their subsequent evolutionary change can be documented by three reconstructed origins of multiple sex chromosomes in this lineage[38], here shown variation in the (pseudo) autosomal versus Z-specific pattern revealed for some genes, and highly dynamic nature of repetitive elements on lacertid W chromosome[19,36,39]. Nevertheless, the previously suggested large variability in sex determination and

sex chromosomes in lacertids seems to be inaccurate. Furthermore, it is important to keep in mind that earlier data for some reptile lineages might be questionable. In some cases, sex chromosomes were misidentified and confused with autosomes[40,41] and in other species the earlier reports on the presence of ESD later appeared to be unreliable. Recently, cytogenetic or molecular evidence for sex chromosomes and (hence GSD) was found for previously assumed "ESD" chameleons[42,43], varanids[44], skinks[45] and lacertids[16], this study. The inclusion of such erroneous "ESD" species in previous phylogenetic comparative studies[8–10,12], caused an overestimation of the number of GSD to ESD transitions among amniotes, and undermined the long-term stability of GSD systems. We stress that phylogenetic comparative analyses are sensitive to errors in character states and to make them robust, we have to not only fill in the gaps in species with no data, but also to check and critically evaluate the original data.

Data Availability

All data are available in the Supplementary Material.

References

1. Pokorná, M. & Kratochvíl, L. Phylogeny of sex-determining mechanisms in squamate reptiles: are sex chromosomes an evolutionary trap? *Zool. J. Linn. Soc.* **156**, 168–183 (2009).
2. Ashman, T. L. *et al*. Tree of sex: a database of sexual systems. *Sci. Data* **1**, 140015 (2014).
3. Gamble, T. *et al*. Restriction site-associated DNA Sequencing (RAD-seq) reveals an extraordinary number of transitions among gecko sex-determining systems. *Mol. Biol. Evol.* **32**, 1296–1309 (2015).
4. Johnson Pokorná, M. & Kratochvíl, L. What was the ancestral sex-determining mechanism in amniote vertebrates? *Biol. Rev.* **91**, 1–12 (2016).
5. Deveson, I. W. *et al*. Differential intron retention in Jumonji chromatin modifier genes is implicated in reptile temperature-dependent sex determination. *Sci. Adv.* **3**, e1700731 (2017).
6. Ezaz, T., Srikulnath, K. & Marshall-Graves, J. A. Origin of amniote sex chromosomes: an ancestral super-sex chromosome, or common requirements? *J. Hered.* **108**, 94–105 (2017).
7. Marshall Graves, J. A. & Peichel, C. L. Are homologies in vertebrate sex determination due to shared ancestry or to limited options? *Genome Biol.* **11**, 205 (2010).
8. Janzen, F. J. & Phillips, P. C. Exploring the evolution of environmental sex determination, especially in reptiles. *J. Evol. Biol.* **19**, 1775–1784 (2006).
9. Organ, C. L. & Janes, D. Evolution of sex chromosomes in Sauropsida. *Integr. Comp. Biol.* **48**, 512–519 (2008).
10. Janes, D. E., Organ, C. L. & Edwards, S. V. Variability in sex-determining mechanisms influences genome complexity in reptiles. *Cytogenet. Genome Res.* **127**, 242–248 (2010).
11. Valenzuela, N. & Adams, D. C. Chromosome number and sex determination coevolve in turtles. *Evolution* **65**, 1808–1813 (2011).
12. Pennell, M. W., Mank, J. E. & Peichel, C. L. Transitions in sex determination and sex chromosomes across vertebrate species. *Mol. Ecol.* **27**, 3950–3963 (2018).
13. Harlow, P. S. Temperature-dependent sex determination in lizards. In: *Temperature-Dependent Sex Determination in Vertebrates*, pp. 42–52, Smithsonian Books, Washington, DC (2004).
14. Eichenberger, P. Smaragdhagedissen kweken op bestelling. *Lacerta* **39**, 72–76 (1981).
15. Zhang, D. J. *et al*. Effect of gestation temperature on sexual and morphological phenotypes of offspring in a viviparous lizard, *Eremias multiocellata*. *J. Therm. Biol.* **35**, 129–133 (2010).
16. Tang, X. L. *et al*. Effects of gestation temperature on offspring sex and maternal reproduction in a viviparous lizard (*Eremias multiocellata*) living at high altitude. *J. Therm. Biol.* **37**, 438–444 (2012).
17. Wang, C. *et al*. Identification of sex chromosomes by means of comparative genomic hybridization in a lizard, *Eremias multiocellata*. *Zool. Sci.* **32**, 151–156 (2015).
18. Odierna, G., Kupriyanova, L. A., Capriglione, T. & Olmo, E. Further data on sex chromosomes of Lacertidae and a hypothesis on their evolutionary trend. *Amph.-Rept.* **14**, 1–11 (1993).
19. Rojo Oróns, V. Cytogenetic and molecular characterization of lacertid lizard species from the Iberian Peninsula. Doctoral Thesis, Universidade da Coruña (2015).
20. Rovatsos, M., Vukić, J. & Kratochvíl, L. Mammalian X homolog acts as sex chromosome in lacertid lizards. *Heredity* **117**, 8–13 (2016).
21. Rovatsos, M. *et al*. Conservation of sex chromosomes in lacertid lizards. *Mol. Ecol.* **25**, 3120–3126 (2016).
22. Rovatsos, M., Altmanová, M., Johnson Pokorná, M. & Kratochvíl, L. Novel X-linked genes revealed by quantitative polymerase chain reaction in the green anole, *Anolis carolinensis*. *G3* **34**, 2107–2113 (2014).
23. Zheng, Y. & Wiens, J. J. Combining phylogenomic and supermatrix approaches, and a time-calibrated phylogeny for squamate reptiles (lizards and snakes) based on 52 genes and 4162 species. *Mol. Phyl. Evol.* **94**, 537–547 (2016).
24. Zhou, Q. *et al*. Complex evolutionary trajectories of sex chromosomes across bird taxa. *Science* **346**, 1246338 (2014).
25. Olmo, E., Odierna, G. & Capriglione, T. Evolution of sex chromosomes in lacertid lizards. *Chromosoma* **96**, 33–38 (1987).
26. Altmanová, M., Rovatsos, M. T., Kratochvíl, L. & Johnson Pokorná, M. Minute Y chromosomes and karyotype evolution in Madagascan iguanas (Squamata: Iguania: Opluridae). *Biol. J. Linn. Soc.* **118**, 618–633 (2016).
27. Matsubara, K. *et al*. Amplification of microsatellite repeat motifs is associated with the evolutionary differentiation and heterochromatinization of sex chromosomes in Sauropsida. *Chromosoma* **125**, 111–123 (2016).
28. Augstenová, B., Mazzoleni, S., Kratochvíl, L. & Rovatsos, M. Evolutionary dynamics of the W chromosome in caenophidian snakes. *Genes* **9**, 5 (2017).
29. Viets, B. E., Ewert, M. A., Talent, L. G. & Nelson, C. E. Sex-determining mechanisms in squamate reptiles. *J. Exp. Zool.* **270**, 45–56 (1994).
30. Holleley, C. E., Sarre, S. D., O'Meally, D. & Georges, A. Sex reversal in reptiles: reproductive oddity or powerful driver of evolutionary change? *Sex Dev.* **10**, 279–287 (2016).
31. Ospina-Alvarez, N. & Piferrer, F. Temperature-dependent sex determination in fish revisited: prevalence, a single sex ratio response pattern, and possible effects of climate change. *PLoS One* **3**, e2837 (2008).
32. Burger, J. & Zappalorti, R. T. Effects of incubation temperature on sex ratios in pine snakes: differential vulnerability of males and females. *Am. Nat.* **132**, 492–505 (1988).
33. Quinn, A. E. *et al*. Temperature sex reversal implies sex gene dosage in a reptile. *Science* **316**, 411 (2007).
34. Radder, R. S., Quinn, A. E., Georges, A., Sarre, S. D. & Shine, R. Genetic evidence for co-occurrence of chromosomal and thermal sex-determining systems in a lizard. *Biol. Lett.* **4**, 176–178 (2008).
35. Ehl, J., Vukić, J. & Kratochvíl, L. Hormonal and thermal induction of sex reversal in the bearded dragon (*Pogona vitticeps*, Agamidae). *J. Comp. Zool.* **271**, 1–5 (2017).
36. Pokorná, M., Altmanová, M. & Kratochvíl, L. Multiple sex chromosomes in the light of female meiotic drive in amniote vertebrates. *Chromosome Res.* **22**, 35–44 (2014).

37. Ezaz, T., Sarre, S. D., O'Meally, D., Graves, J. A. & Georges, A. Sex chromosome evolution in lizards: independent origins and rapid transitions. *Cytogenet. Genome Res.* **127**, 249–260 (2009).
38. Pokorná, M., Kratochvíl, L. & Kejnovský, E. Microsatellite distribution on sex chromosomes at different stages of heteromorphism and heterochromatinization in two lizard species (Squamata: Eublepharidae: *Coleonyx elegans* and Lacertidae: *Eremias velox*). *BMC Genet.* **12**, 90 (2011).
39. Giovannotti *et al*. Cytogenetic characterization of a population of *Acanthodactylus lineomaculatus* Duméril and Bibron, 1839 (Reptilia, Lacertidae), from southwestern Morocco and insights into sex chromosome evolution. *Cytogenet. Genome Res.* **153**, 86–95 (2017).
40. Rovatsos, M., Johnson Pokorná, M., Altmanová, M. & Kratochvíl, L. Mixed-up sex chromosomes: identification of sex chromosomes in the $X_1X_1X_2X_2/X_1X_2Y$ system of the legless lizards of the genus *Lialis* (Squamata: Gekkota: Pygopodidae). *Cytogenet. Genome Res.* **149**, 282–289 (2016).
41. Mazzoleni, S. *et al*. Turtles of the genera *Geoemyda* and *Pangshura* (Testudines: Geoemydidae) lack differentiated sex chromosomes: the end of a 40-year error cascade for *Pangshura*. *Peer J* **7**, e6241 (2019).
42. Rovatsos, M., Johnson Pokorná, M., Altmanová, M. & Kratochvíl, L. Female heterogamety in Madagascar chameleons (Squamata: Chamaeleonidae: *Furcifer*): differentiation of sex and neo-sex chromosomes. *Sci. Rep.* **5**, 13196 (2015).
43. Nielsen, S. V., Banks, J. L., Diaz, R. E. Jr., Trainor, P. A. & Gamble, T. Dynamic sex chromosomes in Old World chameleons (Squamata: Chamaeleonidae). *J. Evol. Biol.* **31**, 484–490 (2018).
44. Iannucci, A. *et al*. Conserved sex chromosomes and karyotype evolution in monitor lizards (Varanidae). *Heredity* In press, https://doi.org/10.1038/s41437-018-0179-6 (2019).
45. Hill, P. L., Burridge, C. P., Ezaz, T. & Wapstra, E. Conservation of sex-linked markers among conspecific populations of a viviparous skink, *Niveoscincus ocellatus*, exhibiting genetic and temperature-dependent sex determination. *Genome Biol. Evol.* **10**, 1079–1087 (2018).
46. Volobouev, V., Pasteur, G., Bons, J., Guillaume, C. P. & Dutrillaux, B. Sex chromosome evolution in reptiles: divergence between two lizards long regarded as sister species, *Lacerta vivipara* and *Lacerta andreanskyi*. *Genetica* **83**, 85–91 (1990).
47. Darevsky, I. S., Kupriyanova, L. A. & Danielyan. F. D. New evidence or hybrid males of parthenogenetic lizards. In *Studies in Herpetology: Proceedings of the European Herpetological Meeting* (3rd Ordinary General Meeting of the Societas Europaea Herpetologica) (ed. Roček, Z.) 207–211 (S.E.H., 1986).
48. Ivanov, V. G. & Fedorova, T. A. Sex heteromorphism of chromosomes in *Lacerta strigata*. *Tsitologiia* **12**, 1582–1585 (1970).
49. Olmo, E., Cobror, O., Morescalchi, A. & Odierna, G. Homomorphic sex chromosomes in the lacertid lizard *Takydromus sexlineatus*. *Heredity* **53**, 457–459 (1984).
50. Capula, M., Lapini, L. & Capanna, E. The karyotype of *Lacerta horvathi* (Reptilia, Sauria, Lacertidae). *Genetica* **79**, 11–16 (1989).
51. Rojo, V. *et al*. Karyological characterization of the endemic Iberian rock lizard, *Iberolacerta monticola* (Squamata, Lacertidae): insights into sex chromosome evolution. *Cytogenet. Genome Res.* **142**, 28–39 (2014).
52. De Smet, W. H. O. Description of the orcein stained karyotypes of 36 lizard species (Lacertilia, Reptilia) belonging to the family Teiidae, Scincidae, Lacertidae, Cordylidae and Varanidae (Autarchoglossa). *Acta Zool. et Pathol. Antverp.* **76**, 73–118 (1981).
53. Gorman, G. C. New chromosome data for 12 species of lacertid lizards. *J. Herpetol.* **3**, 49–54 (1969).
54. Orlova, V. F. & Orlov, N. F. Chromosome complements and some questions of systematics of lizards of the genus *Lacerta*. *Russ. Zool. Zhurn.* **48**, 1056–1060 (1969).
55. Oguma, K. Studies on sauropsid chromosomes. II. Cytogenetical evidence proving female heterogamety in the lizard (*Lacerta vivipara* J.). *Arch. Biol.* **45**, 27–46 (1934).
56. Odierna, G. *et al*. The karyology of the Iberian rock lizards. *Herpetologica* **52**, 542–550 (1996).
57. Darevsky, I. S., Kupriyanova, L. A. & Bakradze, M. A. Occasional males and intersexes in parthenogenetic species of Caucasian rock lizards (genus *Lacerta*). *Copeia* **1978**, 201–207 (1978).
58. Kupriyanova, L. A. Occasional males and intersexes in parthenogenetic species of Caucasian rock lizards (genus *Lacerta*). *Evolution and Ecology of Unisexual Vertebrates* **466**, 236–240 (1989).
59. Ivanov, V. G. & Fedorova, T. A. Heterochromosomes in the karyotype of *Eremias arguta* Pall. *Tsitologiia* **15**, 762–765 (1973).
60. Capula, M., Lapini, L. & Capanna, E. The karyotype of *Lacerta mosorensis* (Reptilia: Lacertidae): evidence for a new case of female heterogamety in a lacertid lizard. *Rend. Fis. Acc. Lincei* **9**, 51–57 (1991).
61. Ivanov, V. G., Bogdanov, O. P., Anislmova, E. Y. & Fedorova, T. A. Studies of the karyotypes of three lizard species (Sauria, Scincidae, Lacertidae). *Tsitologiia* **15**, 1291–1296 (1973).
62. Kupriyanova, L. A. Diversity in parthenogenetic lacertid lizards: cytogenetic studies. In *Proceedings of the 6th Ordinary General Meeting of the Societas Europaea Herpetologica* (eds Korsós, Z. & Kiss, Z.) 273–279 (S.E.H., 1992).
63. Kupriyanova, L. A. Structure, localization and stability of chromosomes in karyotype evolution in lizards of the lacertidae family. *Russ. J. Herpetol.* **1**, 161–168 (1994).
64. Odierna, G. *et al*. The karyology of some South African lacertids belonging to the genera *Heliobolus*, *Merolles* and *Pedioplanis*. *J. Afr. Zool.* **104**, 541–547 (1990).
65. Arribas, O., Carranza, S. & Odierna, G. Description of a new endemic species of mountain lizard from northwestern Spain: *Iberolacerta galani* sp. nov. (Squamata: Lacertidae). *Zootaxa* **1240**, 1–55 (2006).
66. Fritz, B., Bischoff, W. & Fritz, J. P. Karyologische Untersuchungen an der Omaneidechse *Lacerta jayakari* Boulenger, 1887. *Bonn. Zool. Beiträge* **42**, 67–73 (1991).
67. Bhatnagar, A. N. & Yoniss, Y. T. H. A proof of female heterogamety in a lacertid lizard *Ophisops elegans*. *Cytologia* **41**, 507–511 (1976).
68. Pyron, R. A., Burbrink, F. T. & Wiens, J. J. A phylogeny and revised classification of Squamata, including 4161 species of lizards and snakes. *BMC Evol. Biol.* **13**, 93 (2013).
69. Bosch, I. N. *et al*. Karyological and genetic variation in middle eastern lacertid lizards, *Lacerta laevis* and the *Lacerta kulzeri* complex: a case of chromosomal allopatric speciation. *Chromosome Res.* **11**, 165–178 (2003).

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Author Contributions

M.R. and L.K. designed the project, M.R., J.V., A.M., G.S. performed the experiments, P.L. provided a large part of the studied material and sexed most of the specimens, L.K., J.V., M.R. wrote the first draft of the manuscript, all authors read and approved the manuscript.

Additional Information

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