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Conservation of sex chromosomes in lacertid lizards

MICHAIL ROVATSOS,* JASNA VUKIĆ,* MARIE ALTMANOVÁ,* MARTINA JOHNSON POKORNÁ,*† JIŘÍ MORAVEC‡ and LUKÁŠ KRATOCHVÍL*

*Department of Ecology, Faculty of Science, Charles University in Prague, Viničná 7, 128 44 Prague, Czech Republic, †Institute of Animal Physiology and Genetics, The Czech Academy of Sciences, Liběchov, Czech Republic, ‡Department of Zoology, National Museum, Václavské nám. 68, 115 79 Prague, Czech Republic

Abstract

Sex chromosomes are believed to be stable in endotherms, but young and evolutionary unstable in most ectothermic vertebrates. Within lacertids, the widely radiated lizard group, sex chromosomes have been reported to vary in morphology and heterochromatinization, which may suggest turnovers during the evolution of the group. We compared the partial gene content of the Z-specific part of sex chromosomes across major lineages of lacertids and discovered a strong evolutionary stability of sex chromosomes. We can conclude that the common ancestor of lacertids, living around 70 million years ago (Mya), already had the same highly differentiated sex chromosomes. Molecular data demonstrating an evolutionary conservation of sex chromosomes have also been documented for iguanas and caenophidian snakes. It seems that differences in the evolutionary conservation of sex chromosomes in vertebrates do not reflect the distinction between endotherms and ectotherms, but rather between amniotes and anamniotes, or generally, the differences in the life history of particular lineages.

Keywords: lizards, molecular sexing, reptiles, sex chromosomes

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Introduction

In vertebrates, the gonad is not differentiated early in ontogeny and only later develops into testicular or ovarian structures. Although the genetic framework for the differentiation of testes or ovaries is highly conserved, the process of sex determination which decides whether the undifferentiated gonad will turn into a testis or an ovary, is surprisingly variable. Also, the rate of turnovers of sex-determining mechanisms is notably different among particular vertebrate lineages. Some ectothermic lineages, such as several well-studied groups of fish or frogs, possess a rapid turnover of sex chromosomes (Miura 2007; Kikuchi & Hamaguchi 2013; Dufresnes *et al.* 2015), while endotherms, that is mammals and birds, have highly conserved sex chromosomes (e.g. Shetty *et al.* 1999; Graves 2006). As ectotherms, reptiles are usually considered as a group with a rapid turnover of sex-determining mechanisms (Sarre *et al.* 2004; Organ & Janes 2008; Grossen *et al.* 2011), and as a whole, they indeed exhibit a large variability in sex-determining systems. However, this variability seems to be distributed unequally among particular reptile lineages. As far as is known, all crocodiles share environmental sex determination (ESD; Valenzuela & Lance 2004), where the sex of an individual is decided by environmental conditions during the sensitive period of embryonic development. In comparison, turtles and lepidosaurs (tuataras and squamates) possess variability in sex-determining systems, and both ESD and genotypic sex determination (GSD, where the sex of an individual is set by its sex-specific genotype) can be found in different species of these lineages (Janzen & Phillips 2006; Pokorná & Kratochvíl 2009; Valenzuela & Adams 2011; Gamble *et al.* 2015; Johnson Pokorná & Kratochvíl 2016). Nevertheless, based on the phylogenetic distribution of the types of sex chromosomes given by classical cytogenetic data (Pokorná & Kratochvíl 2009; Gamble *et al.* 2015), some lineages of squamates might possess evolutionary highly conserved sex chromosomes, although the cytogenetic data might not always be reliable in

Correspondence: Lukáš Kratochvíl, Fax: +420 221951673;
E-mail: lukas.kratochvil@natur.cuni.cz

demonstrating the homology of sex chromosomes. Cytogenetically similar sex chromosomes might appear to be nonhomologous in related lineages (Vicoso & Bachtrog 2015), and on the other hand, sex chromosomes can be homologous in spite of observed variability in morphology and heterochromatinization (Rovatsos *et al*. 2014a,b; Altmanová *et al*. 2016). Despite significant progress in recent years, molecular data on the evolutionary stability of sex chromosomes among squamates exist only for iguanas (Pleurodonta; Rovatsos *et al*. 2014a,b) and caenophidian snakes (e.g. Matsubara *et al*. 2006; Vicoso *et al.* 2013; Rovatsos *et al*. 2015). These studies show that at least in some cases, conservation of sex chromosomes in ectothermic vertebrates can be comparable to endotherms, but little is known whether snakes and iguanas are rules or exceptions to the more general pattern.

The lizards of the family Lacertidae represent a very important part of diurnally active reptiles in Europe, Asia, Africa and adjacent islands (for instance, they play a very important ecological role in many Mediterranean islands and in the Canary Islands). They occupy an extensive range of environments, from rain forests through to deserts, with the notable case of the lizard *Zootoca vivipara*, having a wide distribution across the Palearctic region, from Europe to Japan and even north of the Polar Circle. Lacertids are mostly terrestrial, but several species are saxicolous or even arboreal and partly fossorial (Pough *et al*. 2003). Currently, 322 species categorized into 42 genera have been recognized (Uetz & Hošek 2015). The phylogenetic relationships among lacertids are not fully resolved, and conflicting topologies can be found among recent phylogenetic studies based on molecular data (Arnold *et al.* 2007; Mayer & Pavlicev 2007; Pyron *et al.* 2013). However, the splitting of the family into two subfamilies (Gallotiinae and Lacertinae with two tribes: Eremiadini and Lacertini) has been well supported and now accepted. The precise age of the group is not known, but the split between lacertids and their likely sister group, limbless fossorial amphisbaenians, has been estimated at approximately 110–130 Mya (Hedges *et al.* 2006), while the basal divergence just within the tribe Lacertini based on molecular clocks was estimated to be the surprisingly young age of 12–16 Mya (Arnold *et al.* 2007).

Studies have revealed that lacertids possess GSD (reviewed, e.g. in Pokorná & Kratochvíl 2009, one older report of ESD in a single species, *Podarcis pityusensis*, is dubious; see, e.g. critical review in Pokorná & Kratochvíl 2009). Obligatory unisexuality exists within the genus *Darevskia* (e.g. Kupriyanova 2010). Wherever known, sex chromosomes of lacertids point to a female heterogamety (reviewed in Olmo & Signorino 2015). With the exception of several lineages with multiple neo-sex chromosomes (Rojo *et al*. 2014; reviewed in Pokorná *et al.* 2014a), the Z and W sex chromosomes are believed to be rather homomorphic and are only cytogenetically distinguishable by C-banding detection of the notable heterochromatin accumulation on the W chromosomes (Olmo *et al.* 1987; Pokorná *et al.* 2011a). The heterochromatization of the W chromosome is likely a result of its considerable degeneration. In some species, the W chromosome contains an enormous accumulation of repetitive elements (Pokorná *et al.* 2011a in *Eremias velox*), while sex chromosome size differs across lacertid species and W chromosomes are euchromatic in certain lacertid lineages (Olmo *et al.* 1987). The latter fact led to the hypothesis that differentiation of sex chromosomes took place repeatedly and independently in different taxa within the family (Odierna *et al.* 1993). Alternatively, nonhomologous sex chromosomes may be present in different lineages in lacertids. The gene content of the Z chromosome was reported in the Swedish population of *Lacerta agilis* (Srikulnath *et al.* 2014), where the lacertid Z chromosome was suggested to be homologous to a part of the third largest chromosome pair of *Anolis carolinensis*, the model species for reptile genomics. However, the analysis of transcriptome in *Takydromus sexlinaeatus* and the test of Z-specificity based on qPCR in this species and in the Czech population of *Lacerta agilis* revealed that the genes from this region are in fact not Z-specific (Rovatsos *et al.* 2016). This finding suggests either a turnover of sex chromosomes within *Lacerta agilis*, several turnovers of sex chromosomes in lacertids as a whole or the misidentification of the Z-specific region in the previous study (Srikulnath *et al.* 2014). Only, further comparative study within lacertids can resolve this issue and uncover the degree of conservation of sex chromosomes in this clade. In this study, we tested the competing hypotheses on homology and differentiation of sex chromosomes across lacertids.

Materials and methods

Material and ethics statement

Tissue or blood samples in ethanol were acquired from 32 individuals from 16 species of lacertids (one male and one female per species), covering all major lacertid clades (Table S1, Supporting information). Particular attention was taken to include as many species from the genus *Lacerta* and its close relatives (genera *Timon* and *Podarcis*) as possible, as previous studies have suggested a variation in sex chromosomes within this genus (Srikulnath *et al.* 2014; *cf.* to Rovatsos *et al.* 2016).

All experimental procedures were 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 (permission No. 35484/2015-14). Permissions were granted for collecting lacertid species in Greece in the jurisdiction area of the Management Body of Mt Parnonas and Moustos Wetlands (Protocol No. 474, 29/5/2013) and Management Body of Chelmos-Vouraikos (Protocol No. 746, 11/8/2014), in Yemen (permission No. 10/2007 issued by the Environment Protection Agency, Sana'a, Republic of Yemen) and in France (permissions Nos. 29/2012 and 11/DDTM/657-SERN-NB issued by Direction Régionale de l'Environnement, de l'Aménagement et du Logement).

Test of homology of sex chromosomes by qPCR

In organisms with degenerated W chromosomes, the males (ZZ) have twice as many copies of most genes linked to the Z-specific part of sex chromosomes than the females (ZW), while genes in autosomal or pseudoautosomal regions have equal copy numbers in both sexes. This difference in copy number between sexes can be determined by qPCR, allowing the reliable identification of Z- (or X-)specific genes (Rovatsos *et al.* 2014a,b,c, 2015, 2016; for similar application of qPCR, see also Nguyen *et al.* 2013; Gamble *et al.* 2014; Literman *et al.* 2014).

Genomic DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen). Primer pairs (see Table S2, Supporting information for list) were designed for the amplification of the 120–200 bp exon fragment of the single-copy gene elongation factor 1a (*eef1a1*), two autosomal 'control' genes (*fbxw7*, *adarb2*), five Z-specific genes (*mars2*, *lpar4*, *klhl13*, *angptl2*, *slc31a1*) previously identified in *Takydromus sexlineatus* and in the Czech population of *Lacerta agilis* (Rovatsos *et al.* 2016) and three genes (*mecom*, *mynn*, *sh3pxd2a*) recently identified as Z-linked in *Lacerta agilis* in the study by Srikulnath *et al.* (2014), using Primer-BLAST software (Ye *et al.* 2012). The control genes have orthologs linked to chromosomes 2 and 4 in the zebrafinch (*Taeniopygia guttata*, TGU), the five Z-specific genes found in the two lacertids have orthologs linked to TGU 4A and TGU 17, and the candidate genes from the study by Srikulnath *et al.* (2014) are linked to TGU 6 and 9. Instead of the green anole or the chicken genomes, the topology of the genome of the zebrafinch is used for this study. This is because many of the genes in the green anole are still only on scaffolds not linked to particular chromosomes (Alföldi *et al.* 2011; www.ensembl.org) and chromosomes 4 and 4A of the zebrafinch are fused in the chicken genome leading to a lower resolution of the physical localization in chicken in comparison with TGU. The qPCR with DNA template was carried out in a LightCycler II 480 (Roche Diagnostics) with all samples run in triplicate. The detailed qPCR protocol and the formula for the calculation of the relative gene dose between sexes have been presented in our previous articles (Rovatsos *et al.* 2014a,b). A relative female-to-male gene dosage ratio (*r*) of 0.5 is expected for Z-specific genes and 1.0 for pseudoautosomal or autosomal genes.

Results

We tested the relative gene dose (*r*) between sexes for 10 loci in 16 species of lacertid lizards with qPCR (see Fig. 1; Table S3, Supporting information). Although not all of the loci were successfully amplified, a minimum of 5 loci (median 9 loci) were tested in each species. The two autosomal 'control' genes (*fbxw7*, *adarb2*) and the three genes (*mecom*, *mynn*, *sh3pxd2a*) identified as Z-linked in *Lacerta agilis* in the previous study (Srikulnath *et al.* 2014) provided equal gene doses between sexes in all 16 of the tested species here (see Fig. 1; Table S3, Supporting information) and in two species tested previously (Rovatsos *et al.* 2016), indicating that these loci have autosomal or pseudoautosomal topology in the lacertid genomes.

In contrast, the five genes (*mars2*, *lpar4*, *klhl13*, *angptl2*, *slc31a1*) identified as Z-linked in *Takydromus sexlineatus* and in the Czech population of *Lacerta agilis* (Rovatsos *et al.* 2016; three pairs were tested in each of these two species) show relative gene dose ratios of approximately 0.5, indicating their Z-specific topology (see Fig. 1; Table S3, Supporting information). Exceptions to this pattern were found for the gene *mars2* with (pseudo)autosomal topology in *Gallotia galloti* and *Podarcis tauricus*, and the gene *angptl2* with (pseudo)autosomal topology in *Podarcis muralis* (tested also with the same result in the second pair of *Podarcis muralis*).

Discussion

Our sampling included all major lineages of lacertids covering both subfamilies (Gallotiinae and Lacertinae) and both tribes (Eremiadini and Lacertini) of the subfamily Lacertinae. Based on the Z-specificity of the tested genes, all included species demonstrated homologous sex chromosomes. As our sampling included both lineages arisen from the basal splitting of the recent lacertids (Gallotiinae and Lacertinae), we can conclude that the common ancestor of lacertids living *c.* 70 Mya (Hedges *et al.* 2006) possessed the same, already highly differentiated ZZ/ZW sex chromosomes currently found in the recent species. Female heterogamety has been documented in a single species of amphisbaenas (Cole & Gans 1987), the first outgroup to lacertids (e.g. Pyron *et al.* 2013), while the second outgroup (families Gymnophthalmidae and Teiidae) possesses male

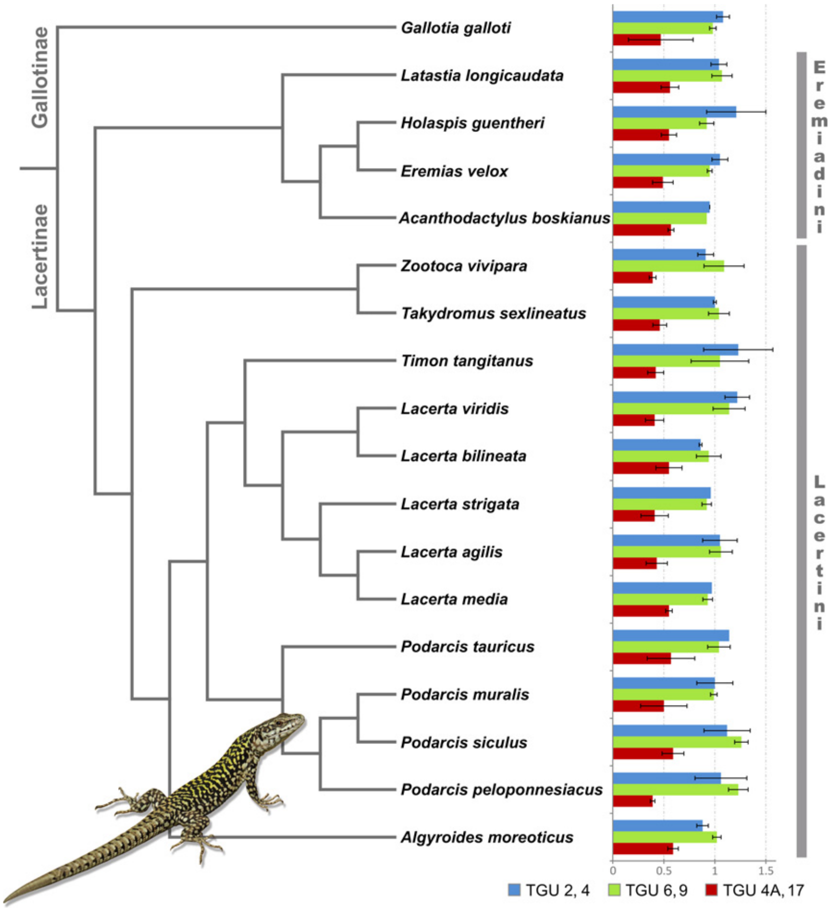

Fig. 1 Relative gene dose ratios between female and male genomes in 18 species of lacertid lizards. Means + SD are depicted. Value 1.0 is expected for autosomal or pseudoautosomal genes, while the value 0.5 is consistent with Z-specificity. The exceptional values consistent with (pseudo)autosomal position in the gene *mars2* in *Gallotia galloti* and *Podarcis tauricus*, and in the gene *angptl2* in *Podarcis muralis* were excluded for simplicity (see text for details). Phylogenetic relationships follow Pyron *et al.* (2013). The legend shows the linkage of genes to zebrafinch (TGU) chromosomes. These data suggest that the differentiated ZZ/ZW sex chromosomes were already present in the common ancestor of extant lacertids and that they have been conserved across the evolution of the group.

heterogamety (reviewed in Pokorná & Kratochvíl 2009). The homology of sex chromosomes between lacertids and their outgroups has yet to be studied and would be necessary to determine the age of lacertid sex chromosomes more precisely. It is possible that the lacertid sex chromosomes are older than the basal splitting of the subfamilies Gallotiinae and Lacertinae. Nevertheless, even this age and the phylogenetic coverage of the present study demonstrate a strong conservation of sex chromosomes in lacertids, the highly radiated and morphologically and ecologically diversified lizard clade.

Sex chromosomes in lacertids are homologous and highly differentiated in all of the tested species regardless of the reported differences in the heterochromatization of the W chromosome and in size of the Z and W chromosome (e.g. Odierna *et al.* 1993). Lacertids have largely conserved karyotypes with mostly acrocentric chromosomes varying only in size. Such chromosomes are difficult to distinguish morphologically even after differential staining (see, e.g. Pokorná *et al.* 2014b). Highly degenerated W or Y chromosomes contain dynamic repetitive sequences (Pokorná *et al.* 2011a; Matsubara *et al.* 2016) and are rather variable in size (Rutkowska *et al.* 2012). Assuming that sex chromosomes are usually homomorphic in reptiles, which used to be a common belief, one can easily assemble the chromosomes into pairs where the W chromosome is paired with an autosome of a similar size, which is mistakenly assigned as the Z chromosome. Our present study demonstrates that Z chromosomes, or at least the parts of them containing the tested genes in lacertids, are highly conserved, and we therefore predict that lacertid Z chromosomes might in fact also be similar in morphology. Improved cytogenetic characterization of the Z chromosomes across lacertids would be beneficial in further studies. The similarity of chromosomes in lacertid karyotypes might also be responsible for a possible error in the determination of the genetic content of the Z chromosome in the Swedish population of *Lacerta agilis* in the cytogenetic study by Srikulnath *et al.* (2014). It seems unlikely that the sex chromosomes widely conserved across lacertids as shown in the present study would differ only between our tested Czech samples and the previously studied Swedish populations of a single species. Nevertheless, the pattern consistent with the exceptional pseudoautosomal or autosomal position from qPCR in the gene *angptl2* in *Podarcis muralis* and in the gene *mars2* in *Gallotia galloti* and *Podarcis tauricus* (Table S3, Supporting information) suggests that although generally highly conserved, sex chromosomes in lacertids might have been subjected to certain rearrangements such as independent translocations of these loci from the ancestral Z chromosome to autosomes. The further study of the situation in *Lacerta agilis* is therefore warranted.

Although reptiles as a whole are often viewed as a group with a frequent turnover of sex-determining systems, the emerging evidence (Pokorná & Kratochvíl 2009; Gamble *et al.* 2015; Johnson Pokorná & Kratochvíl 2016) suggests that in actual fact the variability can only be found in three lineages: in turtles (Valenzuela & Adams 2011), geckos (Pokorná & Kratochvíl 2009; Gamble 2010; Pokorná *et al.* 2010, 2011b, 2014b; Koubová *et al.* 2014; Gamble *et al.* 2015) and in dragon lizards (Ezaz *et al.* 2009). In these three ancient lineages, this variability might be explained by the presence of ancestral ESD and repeated independent emergences of sex chromosomes (Gamble *et al.* 2015; Johnson Pokorná & Kratochvíl 2016). Sex-determining systems, particularly GSD systems and hence sex chromosomes, might be stable in many other reptile lineages (Pokorná & Kratochvíl 2009; Gamble *et al.* 2015), but currently there is a lack of molecular evidence to determine this. So far among amniotes a high evolutionary stability of sex chromosomes has been confirmed by molecular evidence in birds (ZW; Shetty *et al.* 1999), viviparous mammals (XY; Graves 2006), iguanas (XY; Rovatsos *et al.* 2014b), caenophidian snakes (ZW; Matsubara *et al.* 2006; Vicoso *et al.* 2013; Rovatsos *et al.* 2015) and lacertids (ZW, this study). It is evident that evolutionary conservation of sex chromosomes in amniotes is not connected with heterogamety as lineages with both male and female heterogamety show comparable conservation of sex chromosomes, although XY and ZW sex chromosomes generally differ in many important aspects such as in the tendency to evolve global dosage compensation (e.g. Vicoso & Bachtrog 2009; Mank 2009, 2013) or to form multiple neo-sex chromosomes (Pokorná *et al.* 2014a; Pennell *et al.* 2015). It can be also concluded that the stability of sex chromosomes has nothing to do with endothermy versus ectothermy as was previously suggested (e.g. Grossen *et al.* 2011). In contrast to several lineages of anamniotes (*cf.* the situation in sticklebacks: Ross *et al.* 2009; medaka fish: Kikuchi & Hamaguchi 2013; the frog genera *Hyla*: Dufresnes *et al.* 2015 and *Rana*: Miura 2007), up to now no case of frequent and rapid turnovers of sex chromosomes has ever been reported among amniotes. Therefore, we suggest that the difference in the stability of sex chromosomes does not follow the distinction between endotherms and ectotherms, but more likely between amniotes versus anamniotes. Surprising recent evidence has shown that effective population size, intensity of sexual selection and possibly also the rate of molecular evolution reflected by intraspecific genetic polymorphism might differ between lineages with different mortality of juvenile stages versus adults, respectively, different parental investment to individual offspring reflected by propagule size (Romiguier *et al.* 2014; Pischedda *et al.* 2015). The putative link between the life history and the evolutionary stability of sex chromosomes deserves further theoretical and empirical studies.

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Data accessibility

All data are presented in the Table S3 (Supporting information).

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Lacertid specimens used in the study and their origin.

Table S2 Primers used for the measurement of relative gene dosage by qPCR.

Table S3 Relative gene dose ratios (*r*) between female and male genomes in lacertids.