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ORIGINAL ARTICLE

Mammalian X homolog acts as sex chromosome in lacertid lizards

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Among amniotes, squamate reptiles are especially variable in their mechanisms of sex determination; however, based largely on cytogenetic data, some lineages possess highly evolutionary stable sex chromosomes. The still very limited knowledge of the genetic content of squamate sex chromosomes precludes a reliable reconstruction of the evolutionary history of sex determination in this group and consequently in all amniotes. Female heterogamety with a degenerated W chromosome typifies the lizards of the family Lacertidae, the widely distributed Old World clade including several hundreds of species. From the liver transcriptome of the lacertid *Takydromus sexlineatus* female, we selected candidates for Z-specific genes as the loci lacking single-nucleotide polymorphisms. We validated the candidate genes through the comparison of the copy numbers in the female and male genomes of *T. sexlineatus* and another lacertid species, *Lacerta agilis*, by quantitative PCR that also proved to be a reliable technique for the molecular sexing of the studied species. We suggest that this novel approach is effective for the detection of Z-specific and X-specific genes in lineages with degenerated W, respectively Y chromosomes. The analyzed gene content of the Z chromosome revealed that lacertid sex chromosomes are not homologous with those of other reptiles including birds, but instead the genes have orthologs in the X-conserved region shared by viviparous mammals. It is possible that this part of the vertebrate genome was independently co-opted for the function of sex chromosomes in viviparous mammals and lacertids because of its content of genes involved in gonad differentiation.
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INTRODUCTION

Amniote vertebrates as a whole possess variability in sex-determining systems ranging from environmental sex determination (ESD), where sexes do not differ in genotype, to genotypic sex determination (GSD) with highly differentiated sex chromosomes. The ancestral sex-determining system for this important animal clade has not yet been satisfactorily reconstructed. Some authors recently argued that it was likely to be ESD (Pokorná and Kratochvíl, 2009; Johnson Pokorná and Kratochvíl, 2016), and that GSD, and hence sex chromosomes, evolved multiple times within this clade and remained notably stable after their emergence (Pokorná and Kratochvíl, 2009; Johnson Pokorná and Kratochvíl, 2016; Gamble *et al.*, 2015). However, evolutionary transitions from GSD to ESD are theoretically possible and have been experimentally induced in a reptile in the laboratory (Quinn *et al.*, 2007; Holleley *et al.*, 2015). In any case, more robust testing of the hypotheses of the ancestral sex determination and on the evolution of sex-determining mechanisms in amniotes is largely precluded by our very limited knowledge about the homology of sex chromosomes and sex-determining genes among particular groups. The homology of sex chromosomes can be inferred from the knowledge of the gene content of sex chromosomes; however, it has been identified in only a few amniote lineages such as viviparous mammals (see, for example, Kohn *et al.*, 2004) and monotremes (see, for example, Rens *et al.*, 2007), birds (see, for example, Zhou *et al.*, 2014), several turtle species (see, for example, Kawagoshi *et al.*, 2012, 2014), advanced snakes (Matsubara *et al.*, 2006; Vicoso *et al.*, 2013; Rovatsos *et al.*, 2015), iguanas (see, for example, Alföldi *et al.*, 2011; Rovatsos *et al.*, 2014a–c), a gecko (Kawai *et al.*, 2009) and recently also a lacertid lizard (Srikulnath *et al.*, 2014).

The family Lacertidae represents a very important section of diurnally active reptiles in Europe, Asia, Africa and adjacent islands. Lacertids occupy an extensive range of environments, from rain forests through to grasslands and deserts, and a single species even exists north of the Polar Circle. Species of the family are mostly terrestrial, but several lineages are saxicolous or even arboreal. Currently, 321 species categorized into 42 genera have been recognized (Uetz and Hošek, 2014). Studies using conventional cytogenetic techniques revealed that most lacertids have an all-acrocentric karyotype with 2n = 38 chromosomes that is rather unusual among reptiles (reviewed by Olmo and Signorino, 2005) and that wherever known, they possess ZZ/ZW sex chromosomes (Pokorná and Kratochvíl, 2009).

The recent study by Srikulnath *et al.* (2014) used physical gene mapping in a single lacertid species, the sand lizard (*Lacerta agilis* (LAG)), in order to reconstruct the homology of individual lacertid chromosomes with chromosomes of other sauropsids. Based on the cytogenetic mapping of nine tested genes, the authors concluded that the Z chromosome of LAG is the fifth pair of the complement, and its gene content is partially homologous to the chicken (*Gallus gallus* (GGA)) chromosomes 6 (GGA6) and 9 (GGA9) and to the green anole (*Anolis carolinensis* (ACA)) chromosome 3 (ACA3).

In order to examine the gene content of the lacertid Z chromosome in more detail, we applied a new methodology to another lacertid

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species, the six-striped long-tailed lizard (*Takydromus sexlineatus* (TSE)). We determined candidates for Z-specific genes based on the analysis of partial liver transcriptome in one TSE female and validated them via quantitative real-time PCR (qPCR). We discovered that the Z chromosome in TSE was homologous with GGA chromosomes other than those previously reported in LAG and subsequently tested the homology of sex chromosomes between these two species.

MATERIALS AND METHODS

Studied species, material origin and ethics statement

The six-striped long-tailed lizard (TSE) is a medium-sized lacertid. It is widely distributed throughout southeast Asia inhabiting mainly high grasslands and is registered as 'Least Concern' in the IUCN (International Union for Conservation of Nature and Natural Resources) list (http://www.iucnredlist.org). Previous cytogenetic studies have reported individuals of this species with 2n = 38, 40 and 42 acrocentric chromosomes (Olmo *et al*., 1986). In contrast to Z, the W chromosome is highly heterochromatic, as revealed from C-banding staining (Olmo *et al*., 1984, 1986; Odierna *et al*., 1985).

Three male and three female wild-caught individuals of TSE were obtained directly from legal import from Indonesia. The sex of the individuals was determined by their external morphology, that is, the coloration and thickness of the base of the tail. DNA was sourced from blood taken nondestructively from the tail vein using a sterile heparinized syringe. One female specimen was killed to provide RNA samples. In addition, muscle tissue samples from three male and three female wild-caught sand lizards (LAG) were obtained from the National Museum of Prague (specimen numbers NMP6V 70883, NMP6V 74105-107). 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 Committee for Animal Welfare of the Ministry of Agriculture of the Czech Republic (permission no. 29555/2006-30).

RNA isolation and Illumina sequencing

Total RNA was extracted from the liver tissue of one female of TSE using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and according to the manufacturer's protocol. We selected liver tissue for RNA sequencing as liver of adult animals has a high transcriptional activity and it usually expresses a large number of genes (see, for example, Eckalbar *et al*., 2013 in anoles). In addition, liver largely consists of a single type of cells, the hepatocytes, in contrast to other tissues, such as blood, muscle or skin. Such relative homogeneity has the advantage that it could be expected that less transcript variants would be present per locus, making the assembly/mapping procedure easier. However, other tissues with a wide scope of transcripts could be used for our procedure as well. The quantity and the purity of the extracted RNA were estimated using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific Inc, Waltham, MA, USA). The quality of the RNA was examined in 1% agaroze gel. An RNA sample with an absorbance ratio at 260 and 280 nm of ~2.0 and a concentration of 800 ng μl^{-1} was sent to GeneCore (EMBL, Heidelberg, Germany). A single lane of Illumina (San Diego, CA, USA) HiSeq 2000 was used to sequence 2 × 50 bp pair-end fragments of complementary DNA libraries. After sequencing, the raw Illumina reads were processed using Geneious v. R7.1 software (http://www.geneious.com, Kearse *et al*., 2012). The data were checked for quality and filtered and subsequently mapped to the reference transcriptome of the green anole (http://www.ensembl.org/index.html), the closest related lizard with a well-annotated genome. The parameters for the 'map to reference' option in Geneious are presented in the Supplementary File S1. The consensus sequences of all transcripts were exported from Geneious and were further analyzed for the presence/absence of single-nucleotide polymorphisms (SNPs) for identification of candidate Z-specific genes (Figure 1).

Identification of candidate Z-specific genes from transcriptome

The W chromosome in lacertid lizards is highly degenerated and heterochromatic and contains accumulations of repetitive motives and hence is largely different sequentially from the Z chromosome (Pokorná *et al*., 2011b). Degenerated W or Y chromosomes lack in the non-recombining region that most of the genes present on their Z or X counterparts, respectively (see, for example. Rovatsos *et al*., 2014c, 2015; Zhou *et al*., 2014). Therefore, we can assume that the female gene pool in TSE should contain just a single allele for all single-copy Z-specific loci because they are hemizygous in this sex. The transcripts from hemizygous loci should not have any SNPs. On the contrary, the autosomal and pseudoautosomal loci should be either heterozygous (presence of SNPs) or homozygous (absence of SNPs). Taking into account that TSE has a lot of chromosomes in its karyotype (2n = 42) and only a single of them is the Z chromosome, the vast majority of TSE transcripts should be autosomal. Our TSE female was wild-caught, and hence we did not expect any effect of inbreeding that could be present more likely in captive populations and we assumed that a significant portion of its transcripts should exhibit SNPs. In our procedure, we filtered out all TSE transcripts with SNPs, as they are most likely autosomal or pseudoautosomal. After this filtering we got the smaller data set of TSE transcripts without SNPs that included not only Z-specific genes but also homozygous autosomal or pseudoautosomal transcripts. To further select the most likely candidates for the Z-specific genes, we grouped the transcripts without SNPs according to chromosomal position of their orthologous genes in GGA genome. We would have preferred to use the ACA instead of the GGA genome in this step, but physical mapping in ACA is still not sufficient. However, using GGA genome is substantiated by the high level of conservation in gene synteny between GGA and squamates (see, for example, Alföldi *et al*., 2011; Pokorná *et al*., 2011a, 2012; Srikulnath *et al*., 2014). In the next step, we identified the unusually numerous groups of the TSE transcripts without SNPs grouped according to the linkage of their orthologs to particular GGA chromosomes. The selected genes from these groups were further tested by qPCR for differences in gene dose between sexes in order to accurately identify the Z-specific loci.

Identification of Z-specific genes by qPCR

In organisms with degenerated W chromosomes, males (ZZ) have twice as many copies of most genes linked to the Z-specific part of sex chromosomes

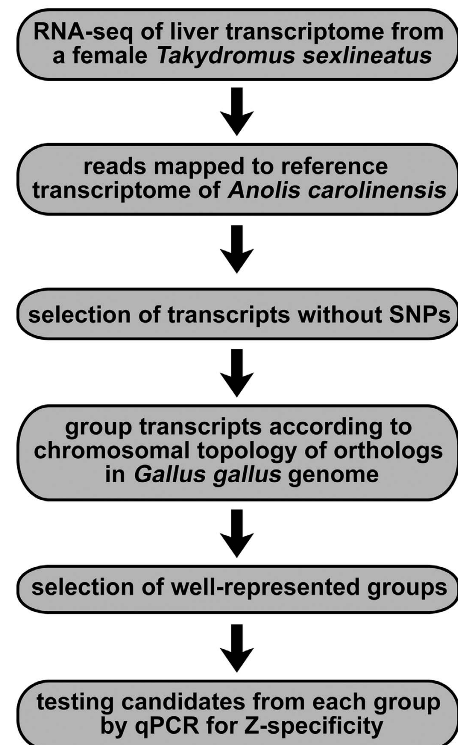

Figure 1 The schematic depiction of the pipeline used to uncover Z-specific genes with the combination of RNA sequencing (RNA-seq) and qPCR approaches.

than females (ZW), whereas 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 sex-specific genes (Rovatsos *et al*., 2014a–c, 2015; for similar application of qPCR see also Nguyen *et al*., 2013; Gamble *et al*., 2014; Literman *et al*., 2014).

Blood samples were collected from six individuals of TSE and genomic DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA). Primer pairs were designed for the amplification of the 120–200 bp exon fragment of autosomal 'control' genes, candidate Z-specific genes from the transcriptome of TSE and of genes identified as Z-linked in LAG in the previous study by Srikulnath *et al.* (2014) using the Primer-BLAST software (Ye *et al*., 2012). As this was not technically possible in several cases, we designed primers from genes with the closest topology in the chromosomes/scaffolds of the green anole. In addition, primers were designed for *elongation factor 1a* (*EF1a*) that was used for normalization of values in the qPCR experiments (Table 1). The primers used in this study are listed in Supplementary Table S2. The qPCR with DNA template was carried out in a LightCycler II 480 (Roche Diagnostics, Basel, Switzerland) 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 papers (Rovatsos *et al*., 2014a, b, 2015). A relative female-to-male gene dosage ratio (r) of 0.5 is expected for Z-specific genes, that is, the genes lacking in the degenerated part of the W chromosome, and 1.0 for (pseudo)autosomal genes. For adequate biological replicates, the Z-specificity of the tested genes was determined in three pairs of TSE, and also in three pairs of LAG for comparison with the previous study (Srikulnath *et al*., 2014).

Table 1 Chromosomal position of the genes tested for differences in gene dose between sexes in the six-striped long-tailed lizard (*Takydromus sexlineatus* (TSE)), the green anole (*Anolis carolinensis* (ACA)), the chicken (*Gallus gallus* (GGA)) and human (*Homo sapiens* (HSA))

Gene symbol	*Position in*			
	TSE	*ACA*	*GGA*	*HSA*
adarb2	(Pseudo)autosomal	6	2	10
fbxw7	(Pseudo)autosomal	5	4	4
mecom	(Pseudo)autosomal	3	9	3
mgea5	(Pseudo)autosomal	NW_003338786.1	6	10
mynn	(Pseudo)autosomal	3	9	3
sh3pxd2a	(Pseudo)autosomal	3	6	10
slc7a14	(Pseudo)autosomal	3	9	3
anapc7	(Pseudo)autosomal	X	15	12
atp2a2	(Pseudo)autosomal	X	15	12
bag5	(Pseudo)autosomal	1	5	14
dnajb14	(Pseudo)autosomal	NW_003338870.1	4	4
eif4e	(Pseudo)autosomal	NW_003338854.1	4	4
metap1	(Pseudo)autosomal	NW_003338854.1	4	4
ppp3ca	(Pseudo)autosomal	NW_003338870.1	4	4
btk	Z	NW_003338998.1	4	X
dock11	Z	NW_003338950.1	4	X
enox2	Z	NW_003338998.1	4	X
f8	Z	NW_003339075.1	4	X
faah2	Z	NW_003339075.1	4	X
klhl13	Z	NW_003338950.1	4	X
LOC100559614	Z	NW_003338998.1	4	—
lpar4	Z	NW_003339075.1	4	X
mars2	Z	NW_003338998.1	4	2
ocrl	Z	NW_003338998.1	4	X
zdhhc9	Z	NW_003338998.1	4	X
angptl2	Z	NW_003339049.1	17	9
cdc26	Z	NW_003339637.1	17	9
slc31a1	Z	NW_003339637.1	17	9

In ACA, most genes can be localized only to scaffolds (scaffold numbers are thus presented in the table).

RESULTS

Transcriptome sequencing and assembling

The transcriptome sequencing of a female of TSE in a single lane of Illumina HiSeq 2000 produced 185.3 million pair-end reads (50 bp each direction). The raw reads have been made available in the NCBI (National Center for Biotechnology Information) Short Read Archive (SRA) database under the accession number SRP069196. Consensus sequences from the assembly were exported, treating polymorphic sites (for example, SNPs) in all sequences as ambiguous bases. Sequences with a coverage below 10× and those containing ambiguous bases were filtered out, giving a data set with 296 transcripts of >150 bp size. We then selected transcripts of a relatively large size (>500 bp) because, in general, it is easier to uncover polymorphism in a longer sequence and our aim was to keep only the transcripts that had a greater chance of being truly hemizygous (Z-specific) or homozygous (autosomal or pseudoautosomal). This final data set contained 88 sequences of >500 bp (Supplementary Table S1).

Identification of putative Z-specific genes

The transcriptome of the green anole was used for mapping our reads as this species is the phylogenetically closest relative of TSE with a well-annotated genome. Unfortunately, only about half of the anole genes have been mapped to chromosomes, whereas the rest are distributed in scaffolds. Therefore, we examined the chromosome linkage of the genes from our final data set with GGA, whose gene content is more comprehensibly mapped to chromosomes (see Supplementary Table S1). The 88 filtered transcripts of TSE corresponded to 85 distinct genes, with 23 of them having orthologs with the fourth chicken chromosome (GGA4) and 11 genes with the 17th chicken chromosome (GGA17), and the rest having orthologs scattered to 14 other chromosomes (Supplementary Figure S1). Therefore, we assume that the orthologs of genes localized in GGA4 and GGA17 are good candidates for the Z-specific genes in lacertids.

qPCR validation of putative Z-specific genes

With qPCR, we tested 16 genes with orthologs linked to GGA4 and 3 genes with orthologs linked to GGA17 (Table 1, Figure 2 and Supplementary Tables S2 and S3). Among them, 5 genes linked to the part of GGA4 homologous to the fourth chromosome of *Homo sapiens* (HSA) had equal gene dose values between sexes of lacertids, whereas all 10 genes linked to the part of GGA4 homologous to the X chromosome of HSA were Z-specific in both tested species of lacertids. In addition, the gene *mars2* linked to GGA4 and HSA2 appeared to be Z-specific in lacertid lizards. All three genes with orthologs linked to GGA17 were also Z-specific. Thus, we conclude that the Z-specific region in both lacertid species is homologous to the part of GGA17 and the part of GGA4 homologous with HSAX (Figure 3).

In addition, we tested five genes reported to be Z-linked in LAG (Srikulnath *et al*., 2014) or which are localized in the proximity of the previously reported Z-linked genes: *sh3pxd2a*, *mgea5* (GGA6), *mecom*, *mynn* and *slc7a14* (GGA9). Surprisingly, all five genes proved to be autosomal or pseudoautosomal in TSE, and also in LAG (Figure 2).

The genes *adarb2* (GGA2), *bag5* (GGA5), *anapc7* and *atp2a2* (GGA15) selected from the genome parts outside candidate Z-specific regions served as autosomal controls. All these genes gave equal gene dose values in both sexes and are therefore indeed autosomal or pseudoautosomal.

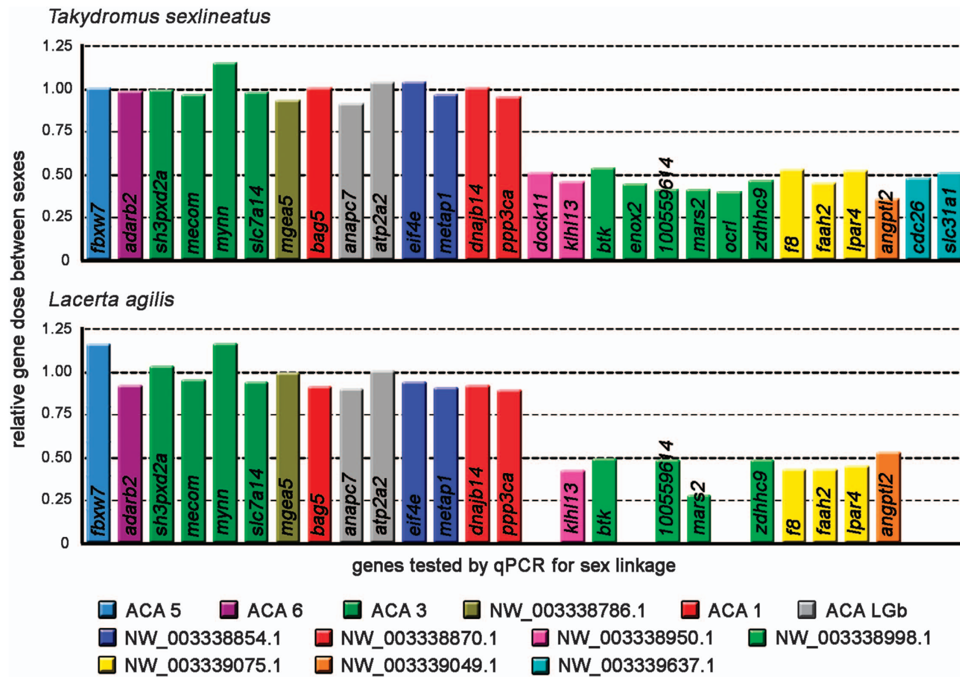

Figure 2 Relative gene dose ratios between females and males for 28 genes in two species of lacertid lizards. Value 1.0 is expected for autosomal or pseudoautosomal genes, whereas the value 0.5 is consistent with Z-specificity. Linkage to genome parts in *Anolis carolinensis* (ACA) is given.

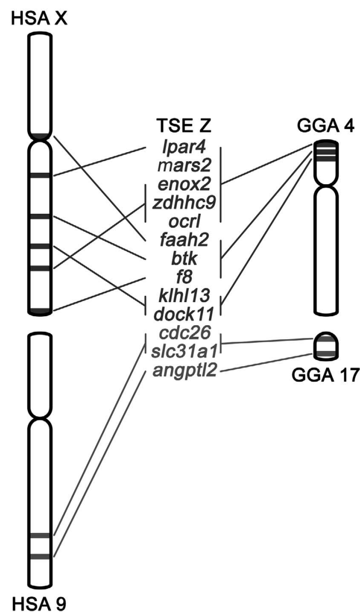

Figure 3 Position of orthologs of genes specific for the Z chromosome in lacertid lizards in human (HSA) and chicken (GGA).

DISCUSSION

Our technique (Figure 1) of the identification of Z- or X-specific genes in organisms with degenerated unpaired sex chromosomes based on the combination of transcriptome analysis in a heterogametic individual and subsequent validation of candidates by qPCR technique proved to be reliable, relatively cheap and effective. However, we should keep in mind that it is not able to distinguish autosomal and pseudoautosomal regions (see, for example, Smeds *et al.*, 2014; Zhang *et al.*, 2015 for molecular approaches devoted to the analysis of gene content of pseudoautosomal regions). In addition, not all Z- or X-specific genes are lost from the degenerated W- and Y-specific region, respectively, and nonfunctional remnants of the ancestral genes (pseudogenes) may still persist in the degenerated part of the W or Y chromosomes (see Smeds *et al.*, 2014). Such genes are likely to be heterozygous in transcriptome and thus filtered out in our analysis. Of course, using our strategy, we are able to find just the candidate genes that are expressed in the tissue used for transcriptome sequencing, which is only a subset of coding genes in the genome, and the success of the technique depends on the heterozygosity of the individuals used in transcriptome sequencing. Nevertheless, although we are likely to find only a fraction of the Z-specific genes in our model lizard species, those loci are informative for comparative studies, testing sex chromosome homology across reptilian lineages. The number of revealed Z-linked genes can be easily increased in follow-up studies. As chromosomes in sauropsids possess a relatively high level of conserved synteny (see, for example, Pokorná *et al.*, 2011a, 2012; Johnson Pokorná *et al.*, 2016), it is very likely that knowledge of several validated Z- or X-specific genes would lead to the discovery of many other such genes in loci linked in annotated genomes of model sauropsids (see Rovatsos *et al.*, 2014c for such follow-up approach in ACA).

Surprisingly, we found completely different gene content in the Z chromosome in TSE than was previously reported for LAG based on physical gene mapping (Srikulnath *et al.*, 2014). We excluded the possibility that the sex chromosomes of these two species are not homologous by testing the Z-linkage of the Z-specific genes detected by us in TSE in LAG. In qPCR, both species exhibited the same pattern, proving Z-linkage of the same genes (Figure 2 and Supplementary Table S3). Moreover, the genes from the Z-linked region suggested by Srikulnath *et al.* (2014) appeared to be (pseudo)autosomal in both species, with many of them being heterozygous in liver transcriptome of the TSE female, supporting their location outside the Z-specific region. One possible explanation of the discrepancies between the studies is that Srikulnath *et al.* (2014) used sand lizards from Sweden, whereas we

tested samples from the Czech populations. Thus, we could assume that an interspecific polymorphism might exist in sex chromosomes of sand lizards. However, sand lizards colonized southern Sweden recently, after the last glacial events (Gullberg *et al.*, 1998), and the populations from central Europe and southern Scandinavia are phylogenetically very closely related (Andres *et al.*, 2014). Most lacertids, including LAG, share morphologically similar karyotypes, with only acrocentric chromosomes, gradually decreasing in size. Thus, it is difficult to apply comparative gene mapping in such karyotypes, as individual chromosome pairs might not be accurately identified in chromosomal spreads, even after banding staining such as Hoechst used by Srikulnath *et al.* (2014). The genes we identified as Z-specific based on our approach seem to be linked to microchromosomes (chromosomes 11–18) based on the results by Srikulnath *et al.* (2014). Therefore, we tentatively suggest that the Z chromosome in LAG is a microchromosome. Nevertheless, this situation must be clarified in subsequent studies of the sex chromosomes of Swedish sand lizards. Taking into account the recent phylogeny of the European sand lizards, the large evolutionary stability of female heterogamety in lacertids (Pokorná and Kratochvíl, 2009) and the shared gene content of the Z chromosomes in TSE and the Czech populations of LAG demonstrated in this study, we find it probable that sex chromosomes could be shared at least in the lacertid clade derived from the last common ancestor of these two species, that is, the clade including the genera *Takydromus*, *Zootoca*, *Timon*, *Lacerta*, *Teira*, *Podarcis*, *Scelarcis*, *Iberolacerta*, *Apathya*, *Archaeolacerta*, *Hellenolacerta*, *Dalmatolacerta*, *Iranolacerta*, *Algyroides*, *Dinarolacerta*, *Anatololacerta*, *Parvilacerta* and *Darevskia* (see phylogeny by Pyron *et al.*, 2013).

The genes linked to the Z chromosome of lacertids have orthologs on the p-arm of GGA4 (GGA4p) and on GGA17 (Table 1 and Figure 3). The part of vertebrate genomes orthologous to GGA4p has a long and interesting history. A chromosome homologous to GGA4p was very likely already present in the common ancestor of tetrapods (see, for example, Mácha *et al.*, 2012). In the common ancestor of viviparous mammals (Theria), with the emergence of the sex-determining gene *Sry* that is homologous to the *Sox3* gene linked to GGA4p, this chromosomal pair started to function as sex chromosomes. In the common ancestor of placentals, the ancestral therian X chromosome (the X-conserved region of placentals, including humans) fused another chromosome homologous to part of GGA1 (the X-added region) forming the eutherian neo-sex X chromosome (Delgado *et al.*, 2009).

As far as we know, sex chromosomes of lacertids are not homologous with those of other sauropsid lineages with known gene content, such as advanced snakes (Matsubara *et al.*, 2006; Vicoso *et al.*, 2013), iguanas (see, for example, Alföldi *et al.*, 2011; Rovatsos *et al.*, 2014a-c), geckos (Kawai *et al.*, 2009), turtles (see, for example, Kawagoshi *et al.*, 2012, 2014) and birds (see, for example, Zhou *et al.*, 2014). The lacertid Z chromosome partially shares gene content with the ancestral therian X, and thus we assume that these sex chromosome systems probably evolved from the same ancestral chromosome (syntenic block). However, it is likely that the mammalian and lacertid sex chromosomes are at the same time homoplasic in the sense that the same ancestral autosome became independently co-opted for the function of sex chromosomes in the ancestor of therian mammals and in lacertids. These two lineages are phylogenetically distant and are separated by nested lineages with different sex-determining mechanisms such as geckos, tuataras, archosaurs and turtles, with a possibility for amniotes of ancestral ESD, or monotremes with non-homologous sex chromosomes (reviewed in Johnson Pokorná and Kratochvíl, 2016).

Such situation was previously reported among the sex chromosomes of *Staurotypus* turtles (XY; Kawagoshi *et al.*, 2014), gecko *Gekko hokouensis* (ZW; Kawai *et al.*, 2009), birds (ZW; Zhou *et al.*, 2014) and partially monotremes (multiple sex chromosomes, male heterogamety; Rens *et al.*, 2007). It was suggested that thanks to their gene content, particularly due to the inclusion of genes related to gonad differentiation, some chromosomes are more likely than others to start functioning as sex chromosomes (O'Meally *et al.*, 2012). The case of the therian X and lacertid Z supports this hypothesis. However, it was estimated that sex chromosomes evolved around 35 times just in squamate reptiles (Gamble *et al.*, 2015) and thus probably more than 40 times in amniotes (reviewed in Johnson Pokorná and Kratochvíl, 2016) and we still do not have enough information on the homology of sex chromosomes to enable us to test whether some chromosomes are indeed more likely to evolve into sex chromosomes than others. Taking into account the relatively small number of chromosomes in the ancestral amniote karyotype and the large number of reconstructed independent emergences of sex chromosomes, it cannot yet be rejected whether some chromosomes, such as those homologous to avian Z or mammalian X, evolved repeatedly into sex chromosomes more often than just by chance.

Little is known about the molecular pathway of sex determination in lacertids. The male phenotype of tetraploid rock lizards from the genus *Darevskia* with ZZZW sex chromosomes and the observation of male, female or intersexual phenotypes in triploid ZZW individuals (Danielyan *et al.*, 2008) suggest that sex determination can be based on the gene dose of Z-specific loci rather than on the dominant W-specific gene. No sex-determining gene has been identified in sauropsids yet, with the exception of *dmrt1* that is considered a strong candidate of this function in birds (Smith *et al.*, 2009; Lambeth *et al.*, 2014). Known sex-determining genes in vertebrates are usually homologs of genes involved in gonad differentiation (see, for example, Kikuchi and Hamaguchi, 2013; Bachtrog *et al.*, 2014), and thus a better knowledge of the function of many genes in the ancestral mammalian X could allow the future identification of the sex-determining gene in lacertids. Promising candidates might be genes homologous to *Sox3* as in therian mammals and the fish *Oryzias dancena* (Takehana *et al.*, 2014), or an androgen receptor, the alleles of which play a role in the sex determination of the Japanese frog *Rana rugosa* (Fujii *et al.*, 2014). Candidates for the sex-determining gene in lacertids might also be found in the part of the lacertid Z chromosome homologous to GGA17. This chromosome for example contain the gene *NR5A1* (also known as *steroidogenic factor 1* or *Sf1*), the mutation of which is known to alter sexual phenotype in humans (Shabsovich and Tirado, 2014).

In conclusion, our study demonstrates the useful application of transcriptome analysis for the derivation of candidate Z- and X-specific genes in lineages with degenerated sex chromosomes. These candidates can be validated by the comparison of gene copy numbers between sexes using qPCR. We applied this technique to sex chromosomes in lacertid lizards and found that they share genes with GGA17 and GGA4p (Table 1 and Figure 2). The latter genome part is syntenic with ancestral therian X chromosomes. In further studies, this would permit the tracking of evolutionary changes of genes in a single homologous block of the amniote genome that is autosomal in ESD lineages such as crocodiles or many turtles, as well as in some GSD lineages such as birds or iguanas, and became the X chromosome in therian mammals and, correspondingly, part of the Z chromosome in lacertids. Apart from these findings important for the further development of our understanding of sex chromosome evolution, our study also presents a technique for molecular sexing in lacertids based on qPCR with identified Z-specific genes that will no doubt prove to be useful for subsequent molecular ecological studies of this group, as well as for conservation biology.

DATA ARCHIVING

The raw reads from RNA sequencing of *Takydromus sexlineatus* are available in the NCBI SRA database under the accession number

SRP069196. The qPCR results are available in the Supplementary Information online.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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