

It's a trap?! Escape from an ancient, ancestral sex chromosome system and implication of *Foxl2* as the putative primary sex-determining gene in a lizard (Anguimorpha; Shinisauridae)

Brendan J. Pinto^{1,2,3,*}, Stuart V. Nielsen^{4,5}, Kathryn A. Sullivan^{3,6}, Ashmika Behere⁶, Shannon E. Keating⁶, Mona van Schingen-Khan⁷, Truong Q. Nguyen^{8,9}, Thomas Ziegler^{10,11}, Jennifer Pramuk¹², Melissa A. Wilson^{1,2,13}, Tony Gamble^{3,6,14,*}

¹School of Life Sciences, Arizona State University, Tempe, AZ, United States

²Center for Evolution and Medicine, Arizona State University, Tempe, AZ, United States

³Department of Zoology, Milwaukee Public Museum, Milwaukee, WI, United States

⁴Department of Biological Sciences, Museum of Life Sciences, Louisiana State University-Shreveport, Shreveport, LA, United States

⁵Florida Museum of Natural History, University of Florida, Gainesville, FL, United States

⁶Department of Biological Sciences, Marquette University, Milwaukee, WI, United States

⁷Federal Agency for Nature Conservation, CITES Scientific Authority, Bonn, Germany

⁸Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology, Hanoi, Vietnam

⁹Graduate University of Science and Technology, Vietnam Academy of Science and Technology, Hanoi, Vietnam

¹⁰Cologne Zoo, Cologne, Germany

¹¹Department of Biology, Institute of Zoology, University of Cologne, Cologne, Germany

¹²Former affiliation: Woodland Park Zoo, Seattle, WA, United States

¹³Center for Mechanisms of Evolution, Biodesign Institute, Tempe, AZ, United States

¹⁴Bell Museum of Natural History, University of Minnesota, St Paul, MN, United States

*Corresponding author: Department of Zoology, Milwaukee Public Museum, Milwaukee, WI, United States. Email: brendanjohnpinto@gmail.com; tgamble@geckoevolution.org

Abstract

Although sex determination is ubiquitous in vertebrates, mechanisms of sex determination vary from environmentally to genetically influenced. In vertebrates, genetic sex determination is typically accomplished with sex chromosomes. Groups like mammals maintain conserved sex chromosome systems, while sex chromosomes in most vertebrate clades are not conserved across similar evolutionary timescales. One group inferred to have an evolutionarily stable mode of sex determination is Anguimorpha, a clade of charismatic taxa including monitor lizards, Gila monsters, and crocodile lizards. The common ancestor of extant anguimorphs possessed a ZW system that has been retained across the clade. However, the sex chromosome system in the endangered, monotypic family of crocodile lizards (Shinisauridae) has remained elusive. Here, we analyze genomic data to demonstrate that *Shinisaurus* has replaced the ancestral anguimorph ZW system with a novel ZW system on LG3. The linkage group, LG3, corresponds to chromosome 9 in chicken, and this is the first documented use of this syntenic block as a sex chromosome in amniotes. Additionally, this ~1 Mb region harbors approximately 10 genes, including a duplication of the sex-determining transcription factor, *Foxl2*, critical for the determination and maintenance of sexual differentiation in vertebrates, and thus a putative primary sex-determining gene for *Shinisaurus*.

Keywords: sex chromosomes, candidate gene, primary sex determiner, sex determination, squamates, Shinisauridae

Introduction

The evolution of sex determination in vertebrates is impressive in its ability to combine a highly conserved developmental network that can be initiated by quite distinct molecular mechanisms in different species (Bachtrog et al., 2014; Graves, 2008). In vertebrates, sex is commonly determined via either environmental and/or genetic cues at critical points in development. In vertebrate groups that use genetic mechanisms, the most common mechanism is sex chromosomes;

either a male or female heterogametic system where the male or female inherits the sex-limited (Y or W) chromosome, respectively (Bachtrog et al., 2014; Gamble et al., 2015). Sex chromosomes have been traditionally identified by comparing male and female karyotypes under the light microscope. The presence of morphological differences between the X and Y (or Z and W) chromosomes (i.e., heteromorphic sex chromosomes) identifies a species' sex chromosome system (Bull, 1983; Stevens, 1905). However, many species possess sex

Received July 7, 2023; revisions received October 25, 2023; accepted November 8, 2023

Associate editor: Beatriz Vicoso; Handling editor: Jason Wolf

© The Author(s) 2023. Published by Oxford University Press on behalf of The Society for the Study of Evolution (SSE). All rights reserved. For permissions, please e-mail: journals.permissions@oup.com

chromosomes that cannot be identified via light microscopy because the X and Y (or Z and W) chromosomes are not morphologically distinguishable from each other (i.e., homomorphic sex chromosomes). Other methods must be employed, such as advanced cytogenetic techniques or high-throughput DNA sequencing technologies, to identify sex chromosome systems in these taxa (Gamble & Zarkower, 2014; Gamble et al., 2017; Pinto et al., 2022).

Squamate reptiles (lizards and snakes) demonstrate high variability in modes of sex determination: where some clades have conserved, often heteromorphic, sex chromosomes, while others display extraordinary lability in their modes of sex determination and a high incidence of homomorphic sex chromosomes (Augstenová et al., 2021a; Gamble et al., 2015; Kratochvíl et al., 2021). One hypothesis of sex chromosome evolution is that ancient, degenerated sex chromosome systems may act as an “evolutionary trap,” where the existence of highly differentiated (i.e., heteromorphic) sex chromosomes preclude transitions to other sex-determining systems (Bull, 1983; Bull & Charnov, 1985; Pokorná & Kratochvíl, 2009). The stability of old sex chromosome systems in mammals, birds, caenophidian snakes, and others provides anecdotal support for this hypothesis (Bull & Charnov, 1985; Gamble et al., 2015; Pokorná & Kratochvíl, 2009). As more and more sex chromosome transitions are identified, it remains unclear whether all ancient sex chromosome systems are destined to become traps, but examples of taxa transitioning away from ancient, degenerated sex chromosome systems are rare in amniotes (Acosta et al., 2019; Nielsen et al., 2019; Rovatsos et al., 2019a). Previous phylogenetic studies have supported the trap hypothesis in squamates (Gamble et al., 2015; Pokorná & Kratochvíl, 2009), but also suffered from incomplete taxonomic sampling, which might have biased the conclusions. In other words, testing this hypothesis is contingent upon having sufficient data necessary to identify transitions away from an ancient sex chromosome system, which typically requires (1) a reference genome to coordinate linkage groups (which are rare in squamates; Pinto et al., 2023), (2) genome-scale data from both sexes (e.g., Gamble et al., 2015; Pinto et al., 2022; Vicoso et al., 2013), and (3) a robust phylogenetic hypothesis to establish relationships within the focal taxa (Nielsen et al., 2019). Thus, the burden of proof is higher for identifying escapees from these ancient sex chromosome systems, which may be responsible for the dearth of examples and the previous lack of conclusive examinations of the evolutionary trap hypothesis. Future identification of additional escapees will permit more conclusive analyses of whether or not ancient sex chromosome systems truly act as evolutionary traps across a broader phylogenetic scale.

The sex chromosomes of the infraorder Anguimorpha (lizards including monitor lizards, Gila monsters, alligator lizards, and their allies) have long been a topic of interest, likely resulting from the paucity of genetic and cytogenetic data for this group. In recent years, advanced cytogenetic techniques (FISH) have facilitated karyotypic analysis and identification of ZW sex chromosomes in the Gila monster (*Heloderma suspectum*; Pokorná et al., 2014) and Komodo dragon (*Varanus komodoensis*; Pokorná et al., 2016) leading to expanded interest in studying chromosome evolution in this enigmatic group. More recently, RNAseq and qPCR analysis, in conjunction with draft genomes of these same two anguimorph species [Gila monster Webster et al. (2023) and Komodo dragon Lind et al., (2019)], has provided some additional insights into this

system (Rovatsos et al., 2019b), namely, identifying homology of the heteromorphic ZW systems in the anguimorph genera *Abronia*, *Heloderma*, and *Varanus* (Rovatsos et al., 2019b; Webster et al., 2023). The presence of a ZW sex chromosome in the same linkage group—syntenic with chromosome 28 in the chicken genome—in these three genera, spanning the phylogenetic breadth of extant Anguimorpha, is strong evidence that this is the ancestral sex chromosome system in the clade. Ancient sex chromosome systems, like those ancestral to anguimorphs (115–180 million years old), fit the criteria that should render them as an evolutionary trap (Pokorná & Kratochvíl, 2009; Rovatsos et al., 2019b). However, the sex chromosomes of many anguimorph taxa remain unknown, including the monotypic family Shinisauridae, which is nested within the anguimorph phylogeny (Figure 1).

The crocodile lizard (*Shinisaurus crocodilurus*) is the sole living member of the family Shinisauridae and native to small disjunct regions of southeastern China and northern Vietnam (Le & Ziegler, 2003; Huang et al., 2008; Nguyen et al., 2015). It is one of the rarest lizard species in the world and is listed as Endangered in the IUCN Red List (Nguyen et al., 2014). Due to anthropogenic pressures, populations have experienced severe declines in recent years with less than 1,000 individuals in the wild in China and less than 100 adults in Vietnam (Huang et al., 2008; van Schingen et al., 2014a). They are semi-aquatic habitat specialists and depend upon clean streams in broad-leaf evergreen forest (Ning et al., 2006; van Schingen et al., 2016a), and their restricted ecological niche is predicted to all but disappear due to climate change by the end of this century (Li et al., 2013; van Schingen et al., 2014a; see also van Schingen-Khan et al., 2022). Habitat destruction threatens remaining populations, as well as overcollection for food and the international pet trade (Huang et al., 2008; van Schingen et al., 2014b, 2016a). Although still recognized as a single species, there exist multiple conservation units, with *S. crocodilurus vietnamensis* from Vietnam and the nominal subspecies from China consisting of several distinct lineages (Ngo et al., 2020; Nguyen et al., 2022; van Schingen et al., 2016b). Crocodile lizards do not have a clear sexual dimorphism. While morphological traits, such as coloration or body morphometry, may provide some indication of the sex, it remains difficult for most people to identify the sex of individuals (van Schingen et al., 2016b). Relevant to the present study, examination of male and female *S. crocodilurus* karyotypes have revealed no heteromorphic sex chromosomes (Augstenová et al., 2021b; Zhang et al., 1996). To identify sex chromosomes in this species, we analyzed whole-genome re-sequencing data for approximately 50 sexed, individual crocodile lizards (Xie et al., 2022) using whole-genome re-sequencing to show that the sex-determining system in *S. crocodilurus* is a novel ZW system that has eluded previous analyses, at least in part, due to the small size (<1 Mb) of its sex-determining region (SDR).

Methods

Whole-genome Illumina re-sequencing analysis

We downloaded low-coverage whole-genome Illumina re-sequencing (WGS) reads from NCBI SRA for multiple male and female individuals (see *Data availability* for accessions). We constructed a Snakemake [v6.10.0] (Mölder et al., 2021) workflow in an isolated conda environment [v4.11.0] (<https://docs.anaconda.com/>) containing relevant packages: BBmap [v38.93] (Bushnell, 2014), FastQC [v0.11.9] (Andrews, 2010),

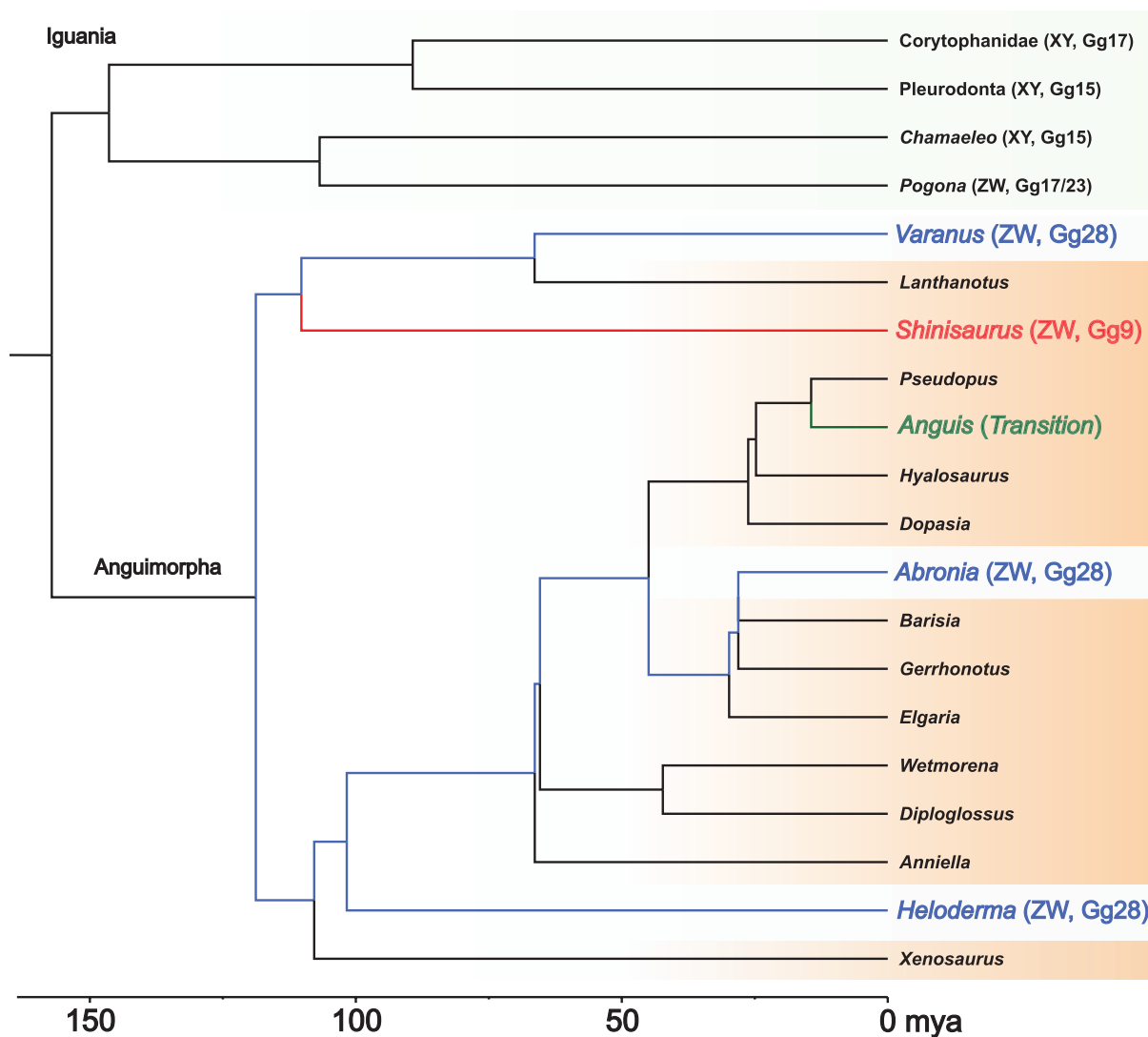


Figure 1. Summary of current anguimorph sex chromosome knowledge summarized from Rovatsos et al. (2019b) indicated by blue and green tips/branches; information identified in this study indicated by red tips/branches and what remains unknown across the phylogeny indicated by black tips/branches. Phylogeny from TimeTree using a representative species from each clade (Kumar et al., 2017) and visualized using Figtree [v1.4.4] (https://github.com/rambaut/figtree). Of note, “pleurodonta” represents “non-corytophanid pleurodonta” and “Gg” stands for chicken (*Gallus gallus*) linkage group.

Freebayes [v1.3.5] (Garrison & Marth, 2012), GFF utilities [v0.10.1] (Pertea & Pertea, 2020), IQ-TREE [v2.0.6] (Nguyen et al., 2015), Minimap2 [v2.22] (Li, 2018), Mosdepth [v0.3.2] (Pedersen & Quinlan, 2018), MultiQC [v1.11] (Ewels et al., 2016), Parallel [v20211022] (Tange, 2018), pixy [v1.2.5.beta1] (Korunes & Samuk, 2021), RTGTools [v3.12.1] (Cleary et al., 2015), Sambamba [v0.8.1] (Tarasov et al., 2015), Samtools [v1.12] (Li et al., 2009), seqkit [v0.11.0] (Shen et al., 2016), and Trim Galore! [v0.6.7] (https://doi.org/10.5281/zenodo.5127899). To process the raw sequencing data, we trimmed adapters and low-quality regions using Trim Galore!, and then removed PCR duplicates using BBmap. Quality assessment using FastQC and MultiQC was conducted at each step, and we subsequently removed samples with fewer than 5 million paired-end reads after filtering PCR duplicates. The final WGS dataset possessed 50 sexed samples (27 male and 23 female individuals) sourced from China and Vietnam. We proceeded to map reads for each individual to the female

reference genome (Xie et al., 2022) with minimap2 and calculated read depth and read-mapping statistics using mosdepth and samtools, respectively. Then, we generated an all-sites VCF file with freebayes parallel and calculated F_{ST} between males and females and nucleotide diversity statistics using pixy at 500 kb resolution and, for LG3 only, also at 100 kb resolution (Weir & Cockerham, 1984).

Validation of the putative ZW system in *S. crocodilurus*

Male vs. female F_{ST} values are agnostic to which sex is heterogametic (i.e., XY vs. ZW). Therefore, we generated a dataset of “in silico PoolSeq” reads by subsampling each WGS sample to 10 million paired reads (20 million total reads per sample) using seqkit and combined into male and female pools. We analyzed the pools using Pooled Sequencing Analyses for Sex Signal [PSASS; v3.1.0] (https://doi.org/10.5281/zenodo.3702337). We then generated PCR primers targeting the

annotated version of *Foxl2*'s second exon [FOXL2-ex2-F2 5'-CAGAGCTCGTCCCATTCACCTT-3' and FOXL2-ex2-R2 5'-GAGAGATGTACCACCGGGAG-3'] and sequenced the resultant amplicon using Sanger sequencing (Psomagen). Individuals used in Sanger sequencing are detailed in [Supplementary Table 1](#).

Genome annotation

We used previously lifted over annotations ([Pinto et al., 2023](#); <https://doi.org/10.6084/m9.figshare.20201099.v1>) via Liftoff [v1.6.3] ([Shumate & Salzberg, 2021](#)) from the draft genome of a male *S. crocodilurus* (Gao et al., 2017) to the new, unannotated female reference genome ([Xie et al., 2022](#); GCA_021292165.1). We pulled coding transcripts from the genome using GFF utilities. We used the 10 genes within the putative ~1 Mb SDR on LG3 to perform a high-stringency tBLASTx query ([Altschul et al., 1990](#)) to the chicken genome on Ensembl ([Howe et al., 2020](#)) with a word size of three, maximum of 10 hits, e-value cutoff of 1e⁻⁵⁰, using the BLOSUM62 scoring matrix. These queries received hits on seven of the 10 total genes ([Table 1](#)).

Results

Across WGS experiments, read-mapping efficiency ranged from 80.60% (for SRR5019740) to 99.40% (for SRR14583318). After variant calling, the WGS dataset contained 6,202,005 biallelic variants (see *Data availability* section for additional VCF statistics). We identified a region of high *F*_{ST} between males and females on linkage group 3 (LG3; [Figure 2](#)); however, comparing male/female (M/F) *F*_{ST} values does not necessarily diagnose which sex is heterogametic (i.e., XY vs. ZW). Therefore, we composed a dataset of “in silico PoolSeq” reads to identify an excess of female-associated single-nucleotide polymorphisms (SNPs) aligning to the previously identified region of high M/F *F*_{ST} ([Supplementary Figure 1](#)). Taken together, these data suggest that *S. crocodilurus* possesses a female heterogametic system (ZW) with an SDR located in a ~900 kb region on LG3.

Upon further investigation of the SDR, we identified a total of 10 genes annotated within this region of high *F*_{ST} and an excess of female-specific SNPs. To better characterize these genes, we BLAST-ed each to the chicken genome. We

recovered high-quality BLAST hits for seven of the 10 annotated *S. crocodilurus* SDR genes in chicken ([Table 1](#)). Six out of the seven queries hit genes located on chicken chromosome 9, while the other landed on a chicken chromosome 30 ([Table 1](#)). In our PoolSeq analysis, one of these genes possessed half the read depth in females relative to males ([Supplementary Table 2](#)) and, upon closer inspection, we identified a duplicated, unannotated copy of that gene Forkhead Box L2 (*Foxl2*), located approximately 70 kb upstream—with 99% sequence identity, also located within the putative SDR. We included this *Foxl2* copy in a BLAST search against chicken, where it was again identified as a *Foxl2* homolog ([Table 1](#)). We also BLAST-ed *Foxl2* to the earlier male *S. crocodilurus* draft genome ([Gao et al., 2017](#)) and found only a single copy of *Foxl2* in this genome matching one copy in the updated reference genome with 100% sequence identity, consistent with both (1) the duplicated version being W-specific and (2) the female reference being chimeric for Z and W alleles ([Xie et al., 2022](#)). Lastly, we generated a gene tree using both copies of *Foxl2* from across reptiles to confirm its duplicated origination was within the shinisaurid lineage ([Supplementary Figure 2](#)). Thus, in the chimeric female reference genome, this putative W-linked *Foxl2* copy was located approximately 70 kb upstream of the annotated Z-linked copy of *Foxl2* on the other side of an assembly gap.

To confirm our WGS findings in a different set of samples, we generated PCR primers for a segment of *Foxl2*'s second exon and Sanger sequenced multiple females (Vietnam) and males (China and Vietnam) ([Supplementary Figure 3](#)). We identified one SNP only present in the female Vietnamese samples in this region and tested its association with sex using Fisher's exact test (*p* = .0003***). Thus, the ZW SDR containing *Foxl2* appears to be conserved between populations of *S. crocodilurus* from both China and Vietnam present in both Sanger and WGS datasets.

Discussion

Escaping the “evolutionary trap”

An open question within sex chromosome evolution is whether ancient, degenerated sex chromosomes act as evolutionary traps ([Nielsen et al., 2019](#); [Pinto et al., 2023](#); [Pokorná & Kratochvíl, 2009](#)). The most recent common ancestor of

Table 1. Top tBLASTx hits in chicken for the CDS of each gene present in the *Shinisaurus* 900 kb SDR. Results for the two copies of the *Foxl2* gene are bolded.

<i>Shinisaurus</i> gene ID	Chicken gene Id	Location (chicken)	E-value
ENSACAP00000003394-D1	ENSGALG000000026187	9:6115461–6115832	1.42E-95
ENSGALP000000008531-D1	<i>RBP1</i>	9:6110534–6110713	7.54E-54
ENSACAP000000003392-D1	No hits		
ENSACAP000000003371-D1	ENSGALG000000034575	30:1402166–1402369	2.51E-78
ENSACAP000000003355-D1	ENSGALG000000005357	9:6041607–6041822	0
ENSACAP000000003221-D1	ENSGALG000000005367	9:6037018–6037164	1.03E-80
ENSGALP0000000033127-W	<i>FOXL2W</i>	9:5875297–5875587	2.44E-76
ENSGALP0000000033127-D1	<i>FOXL2</i>	9:5875297–5875587	6.78E-77
ENSACAP000000003172-D1	<i>PIK3CB</i>	9:5800560–5800790	0
ENSGALP0000000040175-D1	No hits		
ENSACAP000000002765-D1	No hits		

Note. The duplicated *Foxl2* copy is dubbed ENSGALP0000000033127-W.

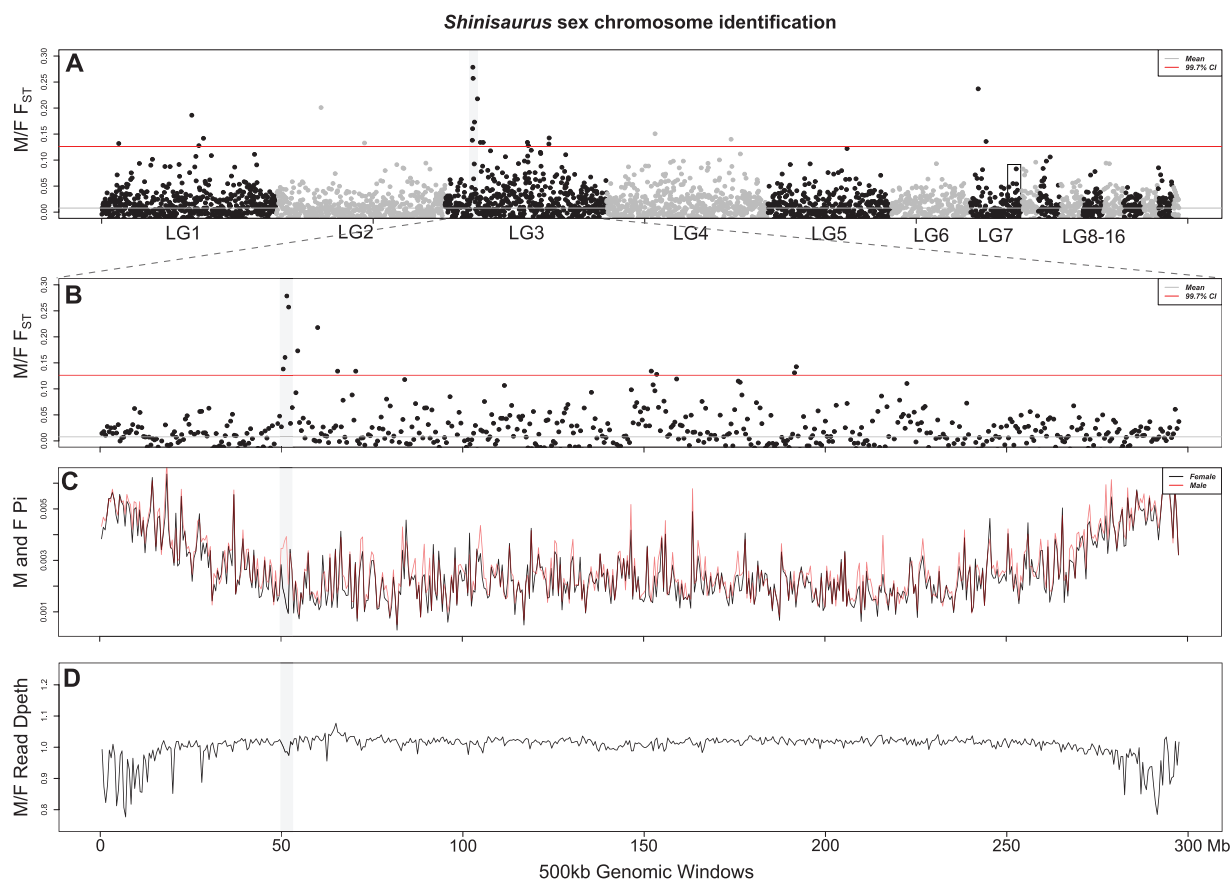


Figure 2. Identification of the ZW sex chromosome system in *Shinisaurus crocodilurus*. (A) Whole-genome F_{ST} scan with a clear peak in a ~1 Mb region on LG3. The square block on LG7 is syntenic with the SDR in *Varanus* and *Heloderma* (Webster et al., 2023). (B) Isolation and magnification of LG3 F_{ST} peak. (C) Modest increase in male, relative to female, nucleotide diversity and (D) decrease in male/female read depth in the region corresponding to the F_{ST} peak on LG3.

extant anguimorphs is thought to have possessed a ZW system on the linkage group syntenic with chicken chromosome 28, which is located on the distal region of LG7 in the *S. crocodilurus* reference genome (Rovatsos et al., 2019b; Webster et al., 2023). The SDR in *S. crocodilurus* is located on LG3, a region syntenic with chicken chromosome 9. Of note, however, it is difficult at present to assess the precise genomic coordinates and gene content of the SDR due to the chimeric nature of the reference genome assembly. To the best of our knowledge, this is the first demonstration in a tetrapod of the syntenic region of chicken chromosome 9 being recruited in a sex-determining role (Kratochvíl et al., 2021), lending further support to the idea that all chromosomes will likely be recruited into a sex-determining role given thorough enough phylogenetic sampling (Graves & Peichel, 2010; Hodgkin, 2002; O'Meally et al., 2012; Pinto et al., 2022).

It is clear from these genomic data that *S. crocodilurus* possesses a distinct sex chromosome system from all other known anguimorphs. Unlike the case of Corytophanidae and other pleurodonts, where phylogenetic relationships among taxa were inconclusive (Nielsen et al., 2019), the relationship of *S. crocodilurus* to all other anguimorphs is far less divisive. Indeed, *S. crocodilurus* is well supported as nested within Anguimorpha—either sister to Varanidae as a member of the “Paleoanguimorpha” (Burbrink et al., 2020) or as sister to a clade containing Varanidae and Lanthanotidae (Singhal et al., 2021), depending on taxonomic sampling. Thus, assuming

the hypothesis that an ancient origin of the ZW sex chromosome system possessed by extant *Varanus*, *Heloderma*, and *Abronia* is correct, then *S. crocodilurus* has successfully escaped the evolutionary trap of their ancestral, degenerated sex chromosome system—a system nearly as ancient as those systems found in both mammals and birds (Rovatsos et al., 2019b; Webster et al., 2023). It is worth noting that there remains another putative escape from the ancestral anguimorph sex chromosome system in *Anguis* that has yet to be explored further (Rovatsos et al., 2019b) and more recent phylogenetic work has implicated that Corytophanidae is likely nested somewhere within other pleurodonts, rather than being sister to all other species (Burbrink et al., 2020; Singhal et al., 2021). This suggests that there may be a minimum of two evolutionary escapes within Toxicofera (snakes, iguanians, and anguimorphs)—and perhaps two or more within the infraorder Anguimorpha alone.

Primary sex determination in Shinisauridae

In many vertebrate groups where the primary sex determiner (PSD) is known, a relatively short list of commonly recruited PSDs have been identified (i.e., the “usual suspects”; Adolphi et al., 2021; Dor et al., 2019; Herpin & Schartl, 2015). Indeed, the same genes, or their paralogs, have been independently co-opted to function as the PSD in many taxa, examples including *Sox3* in placental mammals and some medaka (members of the *Oryzias celebensis* and *O. javanicus* groups);

Amb in tilapia, northern pike, and potentially other anguimorphs (Li et al., 2015; Myosho et al., 2015; Pan et al., 2019; Rovatsos et al., 2019b; Webster et al., 2023; and see Pan et al., 2021 for recent review); and *Dmrt1* in birds, a frog (*Xenopus laevis*), tongue sole, and other medaka fish (members of the *Oryzias latipes* group) (Chen et al., 2014; Ioannidis et al., 2021; Matsuda et al., 2002; Nanda et al., 2002; Smith et al., 2009). This is the first time Forkhead Box L2 (*Foxl2*) has been implicated as a PSD in a vertebrate, although it has been predicted to be one (e.g., Ma et al., 2022).

The transcription factor, *Foxl2*, is a direct transcriptional activator of aromatase, involved in development of the ovaries and its loss in mice during embryogenesis leads to abnormal ovarian development and infertility (Fleming et al., 2010; Pannetier et al., 2006; Schmidt et al., 2004; Uda et al., 2004). After primary sex determination and sexual development have concluded, *Dmrt1* and *Foxl2* antagonize each other transcriptionally in gonadal tissue, where sustained *Dmrt1* and *Foxl2* expression is required for adult maintenance of testis and ovary tissue, respectively (Garcia-Ortiz et al., 2009; Matson et al., 2011; Uhlenhaut et al., 2009). Indeed, *Foxl2* also behaves in a dose-dependent manner in some turtle species where its overexpression at the embryonic stage can induce male-to-female sex reversal in ZZ soft-shelled turtles (*Pelodiscus sinensis*) and female differentiation in male-temperature-incubated red-eared sliders (*Trachemys scripta*) (Jin et al., 2022; Ma et al., 2022). Importantly, *Dmrt1* has been recruited to act as a primary sex-determining gene in multiple taxa (Matson & Zarkower, 2012), while *Foxl2* has remained mysteriously absent from this list—with the singular putative exception being recently described in some species of bivalve mollusks (Han et al., 2022). Thus, the identification of both *Foxl2* and a duplicated *Foxl2* copy in the W-limited region of the *Shinisaurus* genome supports the expanded list of the “usual suspects” that might act as the PSD in vertebrates.

Pragmatically, the identification of a novel ZW system in *S. crocodilurus* may present an important juncture in the conservation efforts of this endangered lizard species, which are urgently needed (Nguyen et al., 2015). Body morphometrics in mature specimens may provide an indication of the sex, i.e., males tend to have a relatively larger head, relative to abdomen length than females (van Schingen et al., 2016b). However, definitive sexually dimorphic characters are lacking in the species, especially in hatchlings, juveniles, and subadults. Therefore, a molecular genetic sex test could assist in well-managed captive breeding efforts in this species (Ziegler et al., 2019). This is vital as it is estimated that only ~1,000 individuals remain in the wild populations in China and Vietnam during the last census (Huang et al., 2008; van Schingen et al., 2016a), while loss of remaining habitats and poaching are considered ongoing. This information may play a vital role in conservation efforts of this species and should be incorporated into ongoing captive breeding work (Ziegler et al., 2019).

In conclusion, using a combination of sequencing and validation techniques, we identified the elusive ZW system in the endangered crocodile lizard, *S. crocodilurus*. This ZW system is located on LG3 and, although interpretation inherits strong reference bias (a chimeric ZW reference genome assembly), the SDR appears to be <1 Mb in size and contains approximately 10 genes. One of these genes, *Foxl2*, possesses a duplicated copy and is important in ovarian development and fertility in vertebrates. Because of its sequence conservation (either strictly age-related or via ongoing Z-W gene

conversion) and possibly its putative proximity to the original Z copy of *Foxl2*, we hypothesize that if *Foxl2* is the PSD in this system, it may be a gene dosage-dependent mechanism—where ZW females possess three copies of *Foxl2* instead of the two copies of ZZ males. This specific hypothesis assumes that there remains the Z copy of *Foxl2* in the pseudoautosomal region of the W chromosome (the annotated copy 70 kb downstream of the unannotated W-linked copy of *Foxl2* on the other side of the assembly gap). However, currently available data are unable to disentangle this from an alternative of only two gametologous (ZW) copies in females and two orthologous (ZZ) copies in males, and phased Z and W sequences are needed to provide additional support to this hypothesis. The hypothetical mechanism suggested here would then essentially be the inverse of the dose-dependent *Dmrt1* sex determination in birds, where a lack of *Dmrt1* on the W decreases *DMRT1* expression in females, allowing for *Foxl2* to proceed with ovarian development (Ioannidis et al., 2021; Smith et al., 2009). Here, the extra gene copy of *Foxl2* would increase *FOXl2* expression to downregulate *DMRT1* expression and initiate ovarian development in the developing gonad. Thus, we provide a putative sex-determining gene for the crocodile lizard (*S. crocodilurus*) and speculate as to its potential mechanism of action in this system.

Supplementary material

Supplementary material is available online at *Evolution*.

Data availability

The WGS data used in this study are available on NCBI, SRA accessions for WGS data are: *SRR14583317*, *SRR14583321*, *SRR14583324-26*, *SRR14583330*, *SRR14583333*, *SRR14583340-49*, *SRR14583351*, *SRR14583353-54*, *SRR14583356*, *SRR14583360-66*, *SRR5019733-45*, *SRR14583318-20*, *SRR14583322-23*, *SRR14583331*, *SRR14583334-39*, *SRR14583346*, *SRR14583350*, *SRR14583352*, *SRR14583355*, *SRR14583357-59*. Sequence data generated in this study are available on SRA under BioProject *PRJNA975696*, detailed in [Supplementary Table 1](#), and code, including and VCF statistics and gene alignments, are available on GitHub: https://github.com/DrPintoThe2nd/Shinisaurus_ZW.

Author contributions

B.J.P., S.V.N., K.A.S., A.B., and S.E.K. conducted wet lab work; M.V., T.Q.N., T.Z., J.P. provided samples and organismal expertise; B.J.P. and T.G. conducted analyses; B.J.P. wrote the manuscript; T.G. and M.A.W. acquired funding in support of this project. All authors edited, read, and approved the submission of the manuscript.

Funding

This work was funded by the Morris Animal Foundation (study grant D19ZO-021) for their generous funding of this project (T.G.) and also supported by the National Institute of General Medical Sciences (NIGMS) of the National Institutes of Health (grant no. R35GM124827 to M.A.W.).

Conflict of interest: The authors declare no conflict of interest.

Acknowledgments

The authors would like to acknowledge Research Computing at Arizona State University for providing high-performance computing and storage resources that have contributed to the research results reported within this paper (<https://cores.research.asu.edu/research-computing>). We thank Anna Rauhaus (Cologne Zoo) for her help with the application and preparation of tissue sending and the Woodland Park Zoo for their respective assistance. Many thanks CITES Management Authority of Vietnam for issuing permits (CITES permits No. 13VN1246N/CT-KL and 16VN0920N/CT-KL).

References

- Adolfi, M. C., Herpin, A., & Scharl, M. (2021). The replaceable master of sex determination: Bottom-up hypothesis revisited. *Philosophical Transactions of the Royal Society of London, Series B: Biological Sciences*, 376(1832), 20200090. <https://doi.org/10.1098/rstb.2020.0090>
- Acosta, A., Suárez-Varón, G., Rodríguez-Miranda, L. A., Lira-Noriega, A., Aguilar-Gómez, D., Gutiérrez-Mariscal, M., Hernández-Gallegos, O., Méndez-de-la-Cruz, F., & Cortez, D. (2019). Corytophanids replaced the pleurodont XY system with a new pair of XY chromosomes. *Genome Biology and Evolution*, 11(9), 2666–2677. <https://doi.org/10.1093/gbe/evz196>
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, 215(3), 403–410. [https://doi.org/10.1016/S0022-2836\(05\)80360-2](https://doi.org/10.1016/S0022-2836(05)80360-2)
- Andrews, S. (2010). *FastQC: A quality control tool for high throughput sequence data*. <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>
- Augstenová, B., Pensabene, E., Kratochvíl, L., & Rovatsos, M. (2021b). Cytogenetic evidence for sex chromosomes and karyotype evolution in anguimorph lizards. *Cells*, 10(7), 1612. <https://doi.org/10.3390/cells10071612>
- Augstenová, B., Pensabene, E., Veselý, M., Kratochvíl, L., & Rovatsos, M. (2021a). Are geckos special in sex determination? Independently evolved differentiated ZZ/ZW sex chromosomes in carphodactylid geckos. *Genome Biology and Evolution*, 13(7), evab119. <https://doi.org/10.1093/gbe/evab119>
- Bachtrog, D., Mank, J. E., Peichel, C. L., Kirkpatrick, M., Otto, S. P., Ashman, T. L., Hahn, M. W., Kitano, J., Mayrose, I., Ming, R., Perin, N., Ross, L., Valenzuela, N., & Vamori, J. C.; Tree of Sex Consortium (2014). Sex determination: Why so many ways of doing it? *PLoS Biology*, 12(7), e1001899. <https://doi.org/10.1371/journal.pbio.1001899>
- Bull, J., & Charnov, E. (1985). On irreversible evolution. *Evolution*, 39, 1149–1155.
- Bull, J. J. (1983). *Evolution of sex determining mechanisms*. Benjamin Cummings.
- Burbrink, F. T., Grazziotin, F. G., Pyron, R. A., Cundall, D., Donnellan, S., Irish, F., Keogh, J. S., Kraus, F., Murphy, R. W., Noonan, B., Raxworthy, C. J., Ruane, S., Lemmon, A. R., Lemmon, E. M., & Zaher, H. (2020). Interrogating genomic-scale data for Squamata (Lizards, Snakes, and Amphisbaenians) shows no support for key traditional morphological relationships. *Systematic Biology*, 69(3), 502–520. <https://doi.org/10.1093/sysbio/syzy062>
- Bushnell, B. (2014). *BBMap: A fast, accurate, splice-aware aligner* (No. LBNL-7065E). Lawrence Berkeley National Lab (LBNL).
- Chen, S., Zhang, G., Shao, C., Huang, Q., Liu, G., Zhang, P., Song, W., An, N., Chalopin, D., Volff, J. -N., Hong, Y., Li, Q., Sha, Z., Zhou, H., Xie, M., Yu, Q., Liu, Y., Xiang, H., Wang, N., ... Wang, J. (2014). Whole-genome sequence of a flatfish provides insights into ZW sex chromosome evolution and adaptation to a benthic lifestyle. *Nature Genetics*, 46(3), 253–260. <https://doi.org/10.1038/ng.2890>
- Cleary, J. G., Braithwaite, R., Gaastra, K., Hilbush, B. S., Inglis, S., Irvine, S. A., & Francisco, M. (2015). Comparing variant call files for performance benchmarking of next-generation sequencing variant calling pipelines. *BioRxiv*. 023754; <https://doi.org/10.1101/023754>, 03 August 2015, preprint: not peer reviewed.
- Dor, L., Shirak, A., Kohn, Y. Y., Gur, T., Weller, J. I., Zilberg, D., Serousi, E., & Ron, M. (2019). Mapping of the sex determining region on linkage group 12 of guppy (*Poecilia reticulata*). *G3 (Bethesda, Md.)*, 9(11), 3867–3875. <https://doi.org/10.1534/g3.119.400656>
- Ewels, P., Magnusson, M., Lundin, S., & Käller, M. (2016). MultiQC: Summarize analysis results for multiple tools and samples in a single report. *Bioinformatics*, 32(19), 3047–3048. <https://doi.org/10.1093/bioinformatics/btw354>
- Fleming, N. I., Knowler, K. C., Lazarus, K. A., Fuller, P. J., Simpson, E. R., & Clyne, C. D. (2010). Aromatase is a direct target of FOXL2: C134W in granulosa cell tumors via a single highly conserved binding site in the ovarian specific promoter. *PLoS One*, 5(12), e14389. <https://doi.org/10.1371/journal.pone.0014389>
- Gamble, T., Castoe, T. A., Nielsen, S. V., Banks, J. L., Card, D. C., Schield, D. R., Schuett, G. W., & Booth, W. (2017). The discovery of XY sex chromosomes in a *Boa* and *Python*. *Current Biology*, 27(14), 2148–2153.e4. <https://doi.org/10.1016/j.cub.2017.06.010>
- Gamble, T., Coryell, J., Ezaz, T., Lynch, J., Scantlebury, D., & Zarkower, D. (2015). Restriction site-associated DNA sequencing (RAD-seq) reveals an extraordinary number of transitions among gecko sex-determining systems. *Molecular Biology and Evolution*, 32, 1296–1309.
- Gamble, T., & Zarkower, D. (2014). Identification of sex-specific molecular markers using restriction site-associated DNA sequencing. *Molecular Ecology Resources*, 14(5), 902–913. <https://doi.org/10.1111/1755-0998.12237>
- Gao, J., Li, Q., Wang, Z., Zhou, Y., Martelli, P., Li, F., & Zhang, G. (2017). Sequencing, de novo assembling, and annotating the genome of the endangered Chinese crocodile lizard *Shinisaurus crocodilurus*. *GigaScience*, 6, gix041.
- García-Ortiz, J. E., Pelosi, E., Omari, S., Nedorezov, T., Piao, Y., Karmazin, J., & Ottolenghi, C. (2009). *Foxl2* functions in sex determination and histogenesis throughout mouse ovary development. *BMC Developmental Biology*, 9, 36.
- Garrison, E., & Marth, G. (2012). Haplotype-based variant detection from short-read sequencing. *arXiv*. <https://arxiv.org/abs/1207.3907>, 20 Jul 2012, preprint: not peer reviewed.
- Graves, J. (2008). Weird animal genomes and the evolution of vertebrate sex and sex chromosomes. *Ann Rev Genet*, 42, 565–586.
- Graves, J., & Peichel, C. (2010). Are homologies in vertebrate sex determination due to shared ancestry or to limited options? *Genome Biology*, 11, 205. <https://doi.org/10.1186/gb-2010-11-4-205>
- Han, W., Liu, L., Wang, J., Wei, H., Li, Y., Zhang, L., & Wang, S. (2022). Ancient homomorphy of molluscan sex chromosomes sustained by reversible sex-biased genes and sex determiner translocation. *Nature Ecology & Evolution*, 6(12), 1891–1906.
- Herpin, A., & Scharl, M. (2015). Plasticity of gene-regulatory networks controlling sex determination: of masters, slaves, usual suspects, newcomers, and usurpators. *EMBO Reports*, 16(10), 1260–1274. <https://doi.org/10.15252/embr.201540667>
- Hodgkin, J. (2002). Exploring the envelope: systematic alteration in the sex-determination system of the nematode *Caenorhabditis elegans*. *Genetics*, 162(2), 767–780. <https://doi.org/10.1093/genetics/162.2.767>
- Howe, K. L., Achuthan, P., Allen, J., Allen, J., Alvarez-Jarreta, J., Amodé, M. R., Armean, I. M., Azov, A. G., Bennett, R., Bhai, J., Billis, K., Boddu, S., Charkhchi, M., Cummins, C., Da Rin Fioretto, L., Davidson, C., Dodiya, K., El Houdaigui, B., Fatima, R., & Flicek, P. (2020). Ensembl 2021. *Nucleic Acids Research*, 49, 884–891. <https://doi.org/10.1093/nar/gkaa942>
- Huang, C. M., Yu, H., Wu, Z. J., Li, Y. B., Wei, F. W., & Gong, M. H. (2008). Population and conservation strategies for the Chinese crocodile lizard (*S. crocodilurus*) in China. *Animal Biodiversity and Conservation*, 31(2), 63–70.
- Ioannidis, J., Taylor, G., Zhao, D., Liu, L., Idoko-Akoh, A., Gong, D., Lovell-Badge, R., Guoli, S., McGrew, M. J., & Clinton, M. (2021). Primary sex determination in birds depends on DMRT1 dosage,

- but gonadal sex does not determine adult secondary sex characteristics. *Proceedings of the National Academy of Sciences of the United States of America*, 118(10), e2020909118. <https://doi.org/10.1073/pnas.2020909118>
- Jin, L., Sun, W., Bao, H., Liang, X., Li, P., Shi, S., Wang, Z., Qian, G., & Ge, C. (2022). The forkhead factor *Foxl2* participates in the ovarian differentiation of Chinese soft-shelled turtle *Pelodiscus sinensis*. *Developmental Biology*, 492, 101–110. <https://doi.org/10.1016/j.ydbio.2022.10.001>
- Korunes, K. L., & Samuk, K. (2021). pixy: Unbiased estimation of nucleotide diversity and divergence in the presence of missing data. *Molecular Ecology Resources*, 21(4), 1359–1368. <https://doi.org/10.1111/1755-0998.13326>
- Kratochvíl, L., Gamble, T., & Rovatsos, M. (2021). Sex chromosome evolution among amniotes: Is the origin of sex chromosomes non-random? *Philosophical Transactions of the Royal Society B: Biological Sciences*, 376(1833), 20200108. <https://doi.org/10.1098/rstb.2020.0108>
- Kumar, S., Stecher, G., Suleski, M., & Hedges, S. B. (2017). TimeTree: A resource for timelines, timetrees, and divergence times. *Molecular Biology and Evolution*, 34(7), 1812–1819. <https://doi.org/10.1093/molbev/msx116>
- Le, K. Q., & Ziegler, T. (2003). First record of the Chinese crocodile lizard from outside of China: report on a population of *S. crocodilurus* Ahl, 1930 from North-Eastern Vietnam. *Hamadryad*, 27(2), 193–199.
- Li, H. (2018). Minimap2: Pairwise alignment for nucleotide sequences. *Bioinformatics*, 34(18), 3094–3100. <https://doi.org/10.1093/bioinformatics/bty191>
- Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., & Durbin, R. (2009). The sequence alignment/map format and SAM-tools. *Bioinformatics*, 25, 2078–2079.
- Li, M., Sun, Y., Zhao, J., Shi, H., Zeng, S., Ye, K., Jiang, D., Zhou, L., Sun, L., Tao, W., Nagahama, Y., Kocher, T. D., & Wang, D. (2015). A tandem duplicate of anti-Müllerian hormone with a missense SNP on the Y chromosome is essential for male sex determination in Nile tilapia, *Oreochromis niloticus*. *PLoS Genetics*, 11(11), e1005678. <https://doi.org/10.1371/journal.pgen.1005678>
- Li, X., Tian, H., Wang, Y., Li, R., Song, Z., Zhang, F., Xu, M., & Li, D. (2013). Vulnerability of 208 endemic or endangered species in China to the effects of climate change. *Regional Environmental Change*, 13, 843–852.
- Lind, A. L., Lai, Y. Y. Y., Mostovoy, Y., Holloway, A. K., Iannucci, A., Mak, A. C. Y., Fondi, M., Orlandini, V., Eckalbar, W. L., Milan, M., Rovatsos, M., Kichigin, I. G., Makunin, A. I., Johnson Pokorná, M., Altmanová, M., Trifonov, V. A., Schijlen, E., Kratochvíl, L., Fani, R., ... Bruneau, B. G. (2019). Genome of the Komodo dragon reveals adaptations in the cardiovascular and chemosensory systems of monitor lizards. *Nature Ecology & Evolution*, 3(8), 1241–1252. <https://doi.org/10.1038/s41559-019-0945-8>
- Ma, X., Liu, F., Chen, Q., Sun, W., Shen, J., Wu, K., Zheng, Z., Huang, J., Chen, J., Qian, G., & Ge, C. (2022). *Foxl2* is required for the initiation of the female pathway in a temperature-dependent sex determination system in *Trachemys scripta*. *Development*, 149(13), dev200863.
- Matson, C. K., Murphy, M. W., Sarver, A. L., Griswold, M. D., Bardwell, V. J., & Zarkower, D. (2011). *DMRT1* prevents female programming in the postnatal mammalian testis. *Nature*, 476(7358), 101–104. <https://doi.org/10.1038/nature10239>
- Matson, C. K., & Zarkower, D. (2012). Sex and the singular DM domain: Insights into sexual regulation, evolution and plasticity. *Nature Reviews Genetics*, 13(3), 163–174. <https://doi.org/10.1038/nrg3161>
- Matsuda, M., Nagahama, Y., Shinomiya, A., Sato, T., Matsuda, C., Kobayashi, T., Morrey, C. E., Shibata, N., Asakawa, S., Shimizu, N., Hori, H., Hamaguchi, S., & Sakaizumi, M. (2002). DMY is a Y-specific DM-domain gene required for male development in the medaka fish. *Nature*, 417(6888), 559–563. <https://doi.org/10.1038/nature751>
- Mölder, F., Jablonski, K. P., Letcher, B., Hall, M. B., Tomkins-Tinch, C. H., Sochat, V., Forster, J., Lee, S., Twardziok, S. O., Kanitz, A., Wilm, A., Holtgrewe, M., Rahmann, S., Nahnsen, S., & Köster, J. (2021). Sustainable data analysis with Snakemake. *F1000Res*, 10, 33. <https://doi.org/10.12688/f1000research.29032.2>
- Myosho, T., Takehana, Y., Hamaguchi, S., & Sakaizumi, M. (2015). Turnover of sex chromosomes in celebensis group medaka fishes. *G3: Genes|Genomes|Genetics*, 5(12), 2685–2691. <https://doi.org/10.1534/g3.115.021543>
- Nanda, I., Kondo, M., Hornung, U., Asakawa, S., Winkler, C., Shimizu, A., Shan, Z., Haaf, T., Shimizu, N., Shima, A., Schmid, M., & Scharl, M. (2002). A duplicated copy of *DMRT1* in the sex-determining region of the Y chromosome of the medaka, *Oryzias latipes*. *Proceedings of the National Academy of Sciences of the United States of America*, 99(18), 11778–11783. <https://doi.org/10.1073/pnas.182314699>
- Ngo, H. T., Nguyen, T. T., Le, M. D., van Schingen-Khan, M., Nguyen, T. Q., Rauhaus, R., Vences, M., & Ziegler, T. (2020). Genetic screening of captive crocodile lizards (*Shinisaurus crocodilurus*) in Europe. *Der Zoologische Garten*, 88, 17–30.
- Nguyen, L. -T., Schmidt, H. A., von Haeseler, A., & Minh, B. Q. (2015). IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Molecular Biology and Evolution*, 32(1), 268–274. <https://doi.org/10.1093/molbev/msu300>
- Nguyen, T. Q., Hamilton, P., & Ziegler, T. (2014). *Shinisaurus crocodilurus*. The IUCN red list of threatened species. <https://doi.org/10.2305/IUCN.UK.2014-1.RLTS.T57287221A57287235.en>. accessed March 4, 2022.
- Nguyen, T. T., Ngo, H. T., Ha, Q. Q., Nguyen, T. Q., Le, T. Q., Nguyen, S. H., Pham, C. T., Ziegler, T., van Schingen-Khan, M., & Le, M. D. (2022). Molecular phylogenetic analyses and ecological niche modeling provide new insights into threats to the endangered Crocodile Lizard (*Shinisaurus crocodilurus*). *Frontiers of Biogeography*, 14(1), e54779.
- Nielsen, S. V., Guzmán-Mendez, I. A., Gamble, T., Blumer, M., Pinto, B. J., Kratochvíl, L., & Rovatsos, M. (2019). Escaping the evolutionary trap? Sex chromosome turnover in basilisks and related lizards (Corytophanidae: Squamata). *Biology Letters*, 15, 20190498.
- Ning, J., Huang, C., Yu, H., Dai, D., Wu, Z., & Zhong, Y. (2006). Summer habitat characteristics of the Chinese crocodile lizard (*Shinisaurus crocodilurus*) in the Loukeng Nature Reserve, Guangdong. *Zoological Research*, 27, 419–426.
- O'Meally, D., Ezaz, T., Georges, A., Sarre, S. D., & Graves, J. A. M. (2012). Are some chromosomes particularly good at sex? Insights from amniotes. *Chromosome Research*, 20(1), 7–19. <https://doi.org/10.1007/s10577-011-9266-8>
- Pan, Q., Feron, R., Yano, A., Guyomard, R., Jouanno, E., Vigouroux, E., Wen, M., Busnel, J. M., Bobe, J., Concordet, J. P., Guiguen, Y., Journot, L., Klopp, C., Lluch, J., Roques, C., Postlethwait, J., Scharl, M., Herpin, A., & Guiguen, Y. (2019). Identification of the master sex determining gene in Northern pike (*Esox lucius*) reveals restricted sex chromosome differentiation. *PLoS Genetics*, 15(8), e1008013. <https://doi.org/10.1371/journal.pgen.1008013>
- Pan, Q., Kay, T., Depincé, A., Adolphi, M., Scharl, M., Guiguen, Y., & Herpin, A. (2021). Evolution of master sex determiners: TGF- β signalling pathways at regulatory crossroads. *Philosophical Transactions of the Royal Society of London, Series B: Biological Sciences*, 376(1832), 20200091. <https://doi.org/10.1098/rstb.2020.0091>
- Pannetier, M., Fabre, S., Batista, F., Kocer, A., Renault, L., Jolivet, G., Mandon-Pépin, B., Cotinot, C., Veitia, R., & Pailhoux, E. (2006). FOXL2 activates P450 aromatase gene transcription: Towards a better characterization of the early steps of mammalian ovarian development. *Journal of Molecular Endocrinology*, 36(3), 399–413. <https://doi.org/10.1677/jme.1.01947>
- Pedersen, B. S., & Quinlan, A. R. (2018). Mosdepth: Quick coverage calculation for genomes and exomes. *Bioinformatics*, 34(5), 867–868. <https://doi.org/10.1093/bioinformatics/btx699>
- Pertea, G., & Pertea, M. (2020). GFF utilities: GffRead and GffCompare. *F1000Research*, 9, ISCB Comm J–ISCB Com304. <https://doi.org/10.12688/f1000research.23297.2>

- 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(5), 445–458. <https://doi.org/10.1093/jhered/esad023>
- Pinto, B. J., Keating, S. E., Nielsen, S. V., Scantlebury, D. P., Daza, J. D., & Gamble, T. (2022). Chromosome-level genome assembly reveals dynamic sex chromosomes in Neotropical leaf-litter geckos (Sphaerodactylidae: *Sphaerodactylus*). *The Journal of Heredity*, 113(3), 272–287. <https://doi.org/10.1093/jhered/esac016>
- Pokorná, M., & Kratochvíl, L. (2009). Phylogeny of sex-determining mechanisms in squamate reptiles: Are sex chromosomes an evolutionary trap? *Zoological Journal of the Linnean Society*, 156(1), 168–183. <https://doi.org/10.1111/j.1096-3642.2008.00481.x>
- Rovatsos, M., Farkačová, K., Altmanová, M., Johnson Pokorná, M., & Kratochvíl, L. (2019a). The rise and fall of differentiated sex chromosomes in geckos. *Molecular Ecology*, 28(12), 3042–3052. <https://doi.org/10.1111/mec.15126>
- Rovatsos, M., Rehák, I., Velenský, P., & Kratochvíl, L. (2019b). Shared ancient sex chromosomes in varanids, bearded lizards, and alligator lizards. *Molecular Biology and Evolution*, 36(6), 1113–1120. <https://doi.org/10.1093/molbev/msz024>
- Schmidt, D., Ovitt, C. E., Anlag, K., Fehsenfeld, S., Gredsted, L., Treier, A. -C., & Treier, M. (2004). The murine winged-helix transcription factor *Foxl2* is required for granulosa cell differentiation and ovary maintenance. *Development*, 131(4), 933–942. <https://doi.org/10.1242/dev.00969>
- Shen, W., Le, S., Li, Y., & Hu, F. (2016). SeqKit: A cross-platform and ultrafast toolkit for fasta/q file manipulation. *PLoS One*, 11(10), e0163962. <https://doi.org/10.1371/journal.pone.0163962>
- Shumate, A., & Salzberg, S. L. (2021). Liftoff: Accurate mapping of gene annotations. *Bioinformatics*, 37(12), 1639–1643. <https://doi.org/10.1093/bioinformatics/btaa1016>
- Singhal, S., Colston, T. J., Grundler, M. R., Smith, S. A., Costa, G. C., Colli, G. R., & Rabosky, D. L. (2021). Congruence and conflict in the higher-level phylogenetics of squamate reptiles: An expanded phylogenomic perspective. *Systematic Biology*, 70(3), 542–557.
- Smith, C. A., Roeszler, K. N., Ohnesorg, T., Cummins, D. M., Farlie, P. G., Doran, T. J., & Sinclair, A. H. (2009). The avian Z-linked gene DMRT1 is required for male sex determination in the chicken. *Nature*, 461(7261), 267–271. <https://doi.org/10.1038/nature08298>
- Stevens, N. M. (1905). A study of the germ cells of *Aphis rosae* and *Aphis oenotherae*. *Journal of Experimental Zoology*, 2(3), 313–333. <https://doi.org/10.1002/jez.1400020302>
- Tange, O. (2018). Gnu parallel 2018. *Zenodo*, pp. 112. <https://doi.org/10.5281/zenodo.1146014>
- Tarasov, A., Vilella, A. J., Cuppen, E., Nijman, I. J., & Prins, P. (2015). Sambamba: Fast processing of NGS alignment formats. *Bioinformatics*, 31(12), 2032–2034. <https://doi.org/10.1093/bioinformatics/btv098>
- Uda, M., Ottolenghi, C., Crisponi, L., Garcia, J. E., Deiana, M., Kimber, W., Forabosco, A., Cao, A., Schlessinger, D., & Pilia, G. (2004). *Foxl2* disruption causes mouse ovarian failure by pervasive blockage of follicle development. *Human Molecular Genetics*, 13(11), 1171–1181. <https://doi.org/10.1093/hmg/ddh124>
- Uhlenhaut, N. H., Jakob, S., Anlag, K., Eisenberger, T., Sekido, R., Kress, J., Treier, A. C., Klugmann, C., Klasen, C., Holter, N. I., Riethmacher, D., Schütz, G., Cooney, A. J., Lovell-Badge, R., & Treier, M. (2009). Somatic sex reprogramming of adult ovaries to testes by *FOXL2* ablation. *Cell*, 139(6), 1130–1142. <https://doi.org/10.1016/j.cell.2009.11.021>
- van Schingen, M., Duc Le, M., Thi Ngo, H., The Pham, C., Quy Ha, Q., Quang Nguyen, T., & Ziegler, T. (2016b). Is there more than one crocodile lizard? An integrative taxonomic approach reveals Vietnamese and Chinese *Shinisaurus crocodilurus* represent separate conservation and taxonomic units. *Der Zoologische Garten*, 85(5), 240–260. <https://doi.org/10.1016/j.zoolgart.2016.06.001>
- van Schingen, M., Ha, Q. Q., Pham, C. T., Le, T. Q., Nguyen, T. Q., Bonkowski, M., & Ziegler, T. (2016a). Discovery of a new crocodile lizard population in Vietnam: Population trends, future prognoses and identification of key habitats for conservation. *Revue Suisse de Zoologie*, 123(2), 241–251.
- van Schingen, M., Ihlow, F., Nguyen, T. Q., Ziegler, T., Bonkowski, M., Wu, Z., & Rödder, D. (2014a). Potential distribution and effectiveness of the protected area network for the crocodile lizard, *Shinisaurus crocodilurus* (Reptilia: Squamata: Sauria). *Salamandra*, 50(2), 71–76.
- van Schingen, M., Pham, C. T., Thi, H. A., Bernardes, M., Hecht, V., Nguyen, T. Q., Bonkowski, M., & Ziegler, T. (2014b). Current status of the crocodile lizard *Shinisaurus crocodilurus* Ahl, 1930 in Vietnam with implications for conservation measures. *Revue Suisse de Zoologie*, 121(3), 1–15.
- van Schingen-Khan, M., Barthel, L. M. F., Pham, D. T. K., Pham, C. T., Nguyen, T. Q., Ziegler, T., & Bonkowski, M. (2022). Will climatic changes affect the Vietnamese crocodile lizard? Seasonal variation in microclimate and activity pattern of *Shinisaurus crocodilurus vietnamensis*. *Amphibia-Reptilia*, 43(2), 155–167. <https://doi.org/10.1163/15685381-bja10089>
- Vicoso, B., Emerson, J. J., Zektser, Y., Mahajan, S., & Bachtrog, D. (2013). Comparative sex chromosome genomics in snakes: Differentiation, evolutionary strata, and lack of global dosage compensation. *PLoS Biology*, 11(8), e1001643. <https://doi.org/10.1371/journal.pbio.1001643>
- Webster, T. H., Vannan, A., Pinto, B. J., Denbrock, G., Morales, M., Dolby, G. A., Fiddes, I. T., DeNardo, D. F., & Wilson, M. A. (2023). Incomplete dosage balance and dosage compensation in the ZZ/ZW Gila monster (*Heloderma suspectum*) revealed by de novo genome assembly. *BioRxiv*. <https://doi.org/10.1101/2023.04.26.538436> April 28, 2023.
- Weir, B. S., & Cockerham, C. C. (1984). Estimating F-statistics for the analysis of population structure. *Evolution*, 38(6), 1358–1370. <https://doi.org/10.1111/j.1558-5646.1984.tb05657.x>
- Xie, H. X., Liang, X. X., Chen, Z. Q., Li, W. M., Mi, C. R., Li, M., Wu, Z. -J., Zhou, X. -M., Du, W. -G., & Du, W. G. (2022). Ancient demographics determine the effectiveness of genetic purging in endangered lizards. *Molecular Biology and Evolution*, 39(1), msab359. <https://doi.org/10.1093/molbev/msab359>
- Zhang, Y., Ban, R., Wu, C., & Zhijian, C. (1996). *Studies on ultrastructure and karyotypes of crocodilian lizard*. China Guanxi Teachers University Press, pp.77.
- Ziegler, T., Van Schingen, M., Rauhaus, A., Dang, P. H., Pham, D. T. K., Pham, C. T., & Nguyen, T. Q. (2019). New insights into the habitat use and husbandry of crocodile lizards (Reptilia: Shinisauridae) including the conception of new facilities for Vietnamese crocodile lizards *Shinisaurus crocodilurus vietnamensis* in Vietnam and Germany. *International Zoo Yearbook*, 53(1), 250–269. <https://doi.org/10.1111/izy.12215>