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Brief Communication

First Report of Sex Chromosomes in Night Lizards (Scincoidea: Xantusiidae)

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Abstract

Squamate reptiles (lizards, snakes, and amphibians) are an outstanding group for studying sex chromosome evolution—they are old, speciose, geographically widespread, and exhibit myriad sex-determining modes. Yet, the vast majority of squamate species lack heteromorphic sex chromosomes. Cataloging the sex chromosome systems of species lacking easily identifiable, heteromorphic sex chromosomes, therefore, is essential before we are to fully understand the evolution of vertebrate sex chromosomes. Here, we use restriction site-associated DNA sequencing (RADseq) to classify the sex chromosome system of the granite night lizard, *Xantusia henshawi*. RADseq is an effective alternative to traditional cytogenetic methods for determining a species' sex chromosome system (i.e., XX/XY or ZZ/ZW), particularly in taxa with non-differentiated sex chromosomes. Although many xantusiid lineages have been karyotyped, none possess heteromorphic sex chromosomes. We identified a ZZ/ZW sex chromosome system in *X. henshawi*—the first such data for this family. Furthermore, we report that the *X. henshawi* sex chromosome contains fragments of genes found on *Gallus gallus* chromosomes 7, 12, and 18 (which are homologous to *Anolis carolinensis* chromosome 2), the first vertebrate sex chromosomes to utilize this linkage group.

Subject areas: Genomics and gene mapping, Reproductive strategies and kinship analysis
Keywords: karyotype evolution, RADseq, reptile, sex chromosome evolution, Squamata

Sex chromosomes have evolved repeatedly and independently in various animal lineages. Species, where males are the heterogametic sex, are said to have an XX/XY sex chromosome system, and the inverse, female heterogamety, is called ZZ/ZW (Bull 1983; Graves 2008). The majority of what we know about sex chromosomes is chiefly based on a few extraordinary taxa that exhibit heteromorphic sex chromosomes (such as mammals and *Drosophila* [XX/XY] or birds and lepidopterans [ZZ/ZW]). Yet most animal species possess morphologically similar, or homomorphic, sex chromosomes, or lack sex chromosomes altogether (Devlin and Nagahama 2002; Matsubara et al. 2006; Stöck et al. 2011; Gamble and Zarkower 2014; Otto 2014). Because traditional cytogenetic techniques fail to identify instances of homomorphic sex chromosomes, sex chromosome systems across much of the tree of life remain largely unknown (Charlesworth and Mank 2010). Recently, improved cytogenetic and sequencing technologies have permitted the identification of sex

chromosome systems in taxa with homomorphic sex chromosomes, generating a renewed interest in the discovery and classification of sex chromosome systems across previously intractable vertebrate taxa.

Squamates (>10 000 species of lizards, snakes, and amphisbaenians; Uetz et al. 2017) are an exceptional clade for studying sex chromosome evolution. They exhibit myriad sex-determining modes, including temperature-dependent (TSD) and genetic (GSD) sex determination, with both male and female heterogamety, and many independent transitions among them (Bull 1980; Wapstra et al. 2007; Ezaz et al. 2009; Pokorná and Kratochvíl 2009; Gamble 2010; Gamble et al. 2015). Unfortunately, even at the family level, we still lack this basic information for the vast majority of squamate lineages (Pokorná and Kratochvíl 2009; Gamble et al. 2015). For example, within the Scincomorpha, a clade comprised of skinks, cordylids, plated lizards, and night lizards, we only know the sex chromosome systems in a handful of species, all within a single family, Scincidae (skinks; ~1660 sp.). Yet within this clade, both male and female heterogamety occur (albeit only one instance of the latter; see Patawang et al. 2018), as well as a few accounts of TSD in at least 2 families (see references in Pokorná and Kratochvíl 2009; Gamble et al. 2015), although it is possible that these findings may need reevaluation given that extreme temperatures can override an underlying genetic sex-determining mechanism in some squamate species (Sarre et al. 2004; Radder et al. 2008; Holleley et al. 2015). We still lack any data for the Cordylidae (girdled lizards; 68 sp.), Gerrhosauridae (plated lizards; 37 sp.) or–the focus of the present study–Xantusiidae (night lizards; 35 sp.), and this paucity of data limits our ability to study macro-evolutionary patterns of sex chromosome evolution both across this clade, and in squamates as a whole. Consequently, a concerted effort to categorize sex chromosome systems in these and other data deficient clades is essential.

Xantusiidae is composed of 3 genera endemic to the New World: the monotypic and biogeographically enigmatic *Cricosaura*, restricted to southwestern Cuba; *Lepidophyma* (20 spp.) broadly distributed throughout Middle America; and *Xantusia* (14 spp.) equally broadly distributed (but entirely nonoverlapping with *Lepidophyma*) in the southwestern United States and northwestern Mexico (Noonan et al. 2013). Published karyotype data across Xantusiidae has identified that diploid chromosome complements vary from 2n = 24 to 2n = 40, although within *Xantusia* the karyotypic formula is highly conserved with all assessed species displaying the latter (as a side note, there was a single report of 2n = 42 in *X. henshawi* [Matthey 1931], yet Bezy [1972] suggests this was very likely an error and all subsequent work, albeit limited, has only recovered 2n = 40 karyotypic formulas in the genus [Bezy and Villela 1999]). Within *Lepidophyma* there are also 2, independently derived, all-female—and presumably parthenogenetic—lineages, *L. reticulatum*, and *L. flavimaculatum*. Unlike other parthenogenetic, "asexual" lizards, these lineages do not appear to be of hybrid origin (Bezy and Sites Jr. 1987; Sinclair et al. 2010; Noonan et al. 2013) and Bezy (1972) reported a karyotype of 3n = 57 in one all-female population of *L. flavimaculatum*. There is no evidence of heteromorphic sex chromosomes within the family (Bezy 1972; Bull 1980; Janzen and Paukstis 1991).

Here, we employ restriction site-associated DNA sequencing (RADseq) to identify the sex chromosome system in *Xantusia henshawi*. RADseq is useful to identify sex chromosome systems in a variety of taxa, particularly for species that lack cytogenetically distinct sex chromosomes—for example, most squamate lizards (Ezaz et al. 2009; Baxter et al. 2011; Gamble et al. 2015, 2017; Gamble 2016). This methodology involves generating RADseq data from multiple males and females, then isolating sex-specific RAD markers found in only one of the 2 sexes (Willing et al. 2011; Gamble and Zarkower 2014). Logically, such sex-specific RAD markers must be on sex-specific regions of the genome (i.e., the Y or W chromosomes) and taxa exhibiting a disproportionate number of male-specific markers are presumed to have an XX/XY system, and vice versa for species with a ZZ/ZW system (Gamble et al. 2015, 2017; Nielsen et al. 2018). We here identify a ZZ/ZW sex chromosome system in *X. henshawi*—the first such data for any xantusiid lizard—which reveals a previously unknown transition in sex chromosome systems within Scincomorpha. We also discuss homology with other vertebrate sex chromosomes.

Materials and Methods

We extracted genomic DNA using the Qiagen® DNeasy Blood and Tissue extraction kit from tail clips, or liver, from 10 adult male and 9 adult female *X. henshawi* collected from Imperial and San Diego counties in southern California (Figure 1, Supplementary Table S1). RADseq libraries were constructed following a modified protocol from Etter et al. (2011), as described in Gamble et al. (2015). Genomic DNA was digested using high-fidelity *SbfI* restriction enzyme (New England Biolabs). Individually barcoded P1 adapters were ligated to the *SbfI* cut site for each sample. We pooled samples into multiple libraries, sonicated, and size selected into 200- to 500-bp fragments using magnetic beads in a PEG/NaCl buffer (Rohland and Reich 2012). Libraries were blunt-end repaired and dA-tailed before ligating P2 adapters containing unique Illumina® barcodes to each pooled library. We amplified libraries via polymerase chain reaction (PCR) (16 cycles) with Q5 high-fidelity DNA polymerase (New England Biolabs®) and cleaned/size selected a second time using the Qiagen® GeneRead Size Selection Kit. Libraries were

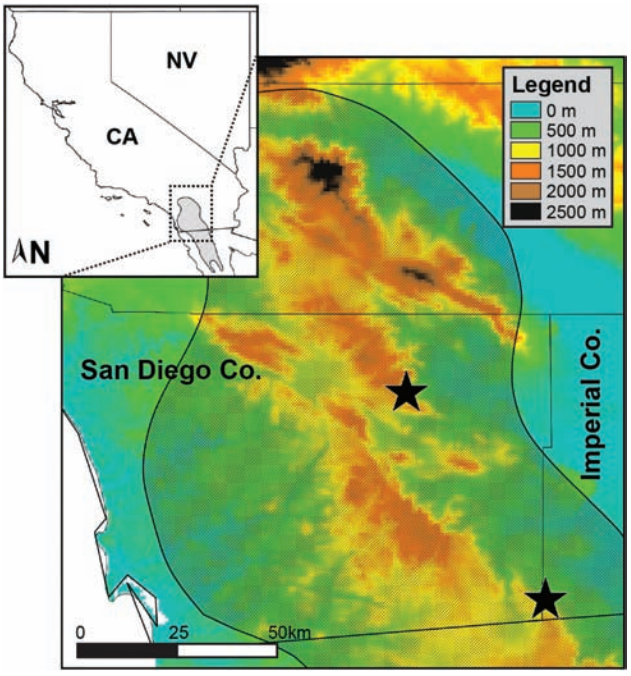

Figure 1. A digital elevation map showing the distribution of *Xantusia henshawi* in southern California (shaded portion in inset) and the 2 sampling localities mentioned in the text (black stars). Please see the online version for full colors.

pooled and sequenced using paired-end 150 bp reads on an Illumina HiSeqX at the Novogene Corporation (Davis, CA).

We demultiplexed, trimmed, and filtered raw Illumina reads using the process_radtags function in STACKS [v2.2] (Catchen et al. 2011). We used RADtools [v1.2.4] (Baxter et al. 2011) to generate candidate alleles for each individual and candidate loci across all individuals from the forward reads using previously described parameters (Gamble et al. 2015, 2017). From these reads, we identified putative sex-specific markers from the RADtools output using a custom python script (Gamble et al. 2015; Nielsen et al. 2019b). This script also produced a second list of "confirmed" sex-specific RAD markers, which are a subset of the initial list of sex-specific RAD markers that excludes any sex-specific marker that also appears in the original raw reads files from the opposite sex from further consideration (Gamble and Zarkower 2014; Gamble et al. 2015). We assembled forward and reverse reads from the confirmed sex-specific RAD markers into sex-specific RAD contigs using Geneious® v10 (Kearse et al. 2012). We then used these confirmed RAD contigs to design sex-specific PCR primers, also in Geneious® v10, and validated the sex specificity of a subset of confirmed female-specific markers using PCR (Supplementary Table S2). We performed a touchdown (TCHDN) PCR where the initial annealing temperature was 67 °C, then decreased by 0.4 °C per cycle for 15 cycles, followed by 20 additional cycles at 61 °C. All other PCR conditions followed the standard GoTaq® Green master mix protocol (Promega® Corporation).

Due to differences in sex-specific PCR amplification between localities, we performed additional population demographic analyses using STACKS. We split the individuals into 2 "populations" representing the 2 collection localities in Imperial and San Diego counties (a straight-line distance of approx. 70 km; see Figure 1, Supplementary Table S1) and estimated F_{ST}, Φ_{ST}, and F_{IS} for each population in order to approximate the divergence and allelic diversity within and between populations. To confirm that these populations were genetically distinct sub-populations, and not artifacts of binning-by-locality, we de novo assembled RAD markers under a stringent set of assembly criteria using ipyrad [v0.7.29] (Eaton and Overcast 2016; https://github.com/dereneaton/ipyrad) and conducted an unbiased population genetic assessment using STRUCTURE [v2.3.4] (Falush et al. 2007) to confirm whether allelic populations were strictly subdivided by locality. We tested values of K (from 1 to 4) repeating 3 independent MCMC chains of 150 000 replicates, each with a 10% burn-in.

We attempted to assess synteny between the newly identified sex-specific RAD markers in *X. henshawi* with chicken (*Gallus gallus*) and anole (*Anolis carolinensis*) chromosomes. These genomes are well-annotated and widely used as references for comparative genomics among amniotes (Hillier et al. 2004; Alföldi et al. 2011; Pokorná et al. 2011; O'Meally et al. 2012). We performed BLAST (Altschul et al. 1990) of the assembled female-specific RAD contigs to chicken CDSs (using Ensembl; Zerbino et al. 2017), implemented in Geneious® [v10] (Kearse et al. 2012) with a maximum E-value cutoff of $1e^{-50}$ and word size of 15 bp.

Results

Output from the RADtools analysis recovered 133 388 RAD markers with 2 or fewer alleles, including 0 male-specific and 296 female-specific RAD markers. Of these, we identified 0 confirmed male-specific RAD markers and 267 confirmed female-specific RAD markers. "Confirmed" sex-specific markers, as described above, are a subset of the total number of sex-specific RAD markers that excludes RAD markers that occurred in the raw reads files of the opposite sex. From this pool of confirmed, female-specific RAD contigs, we designed 15 primer pairs, only one of which amplified in a sex-specific manner across all samples (Figure 2). One additional primer pair (Xan96) amplified in a sex-specific manner, but only in individuals from the San Diego Co. population.

Differences in PCR amplification between populations led us to postulate about the degree of divergence between "San Diego" and "Imperial" populations (Supplementary Table S1). First, we analyzed these 2 populations as distinct entities. Using STACKS software, we

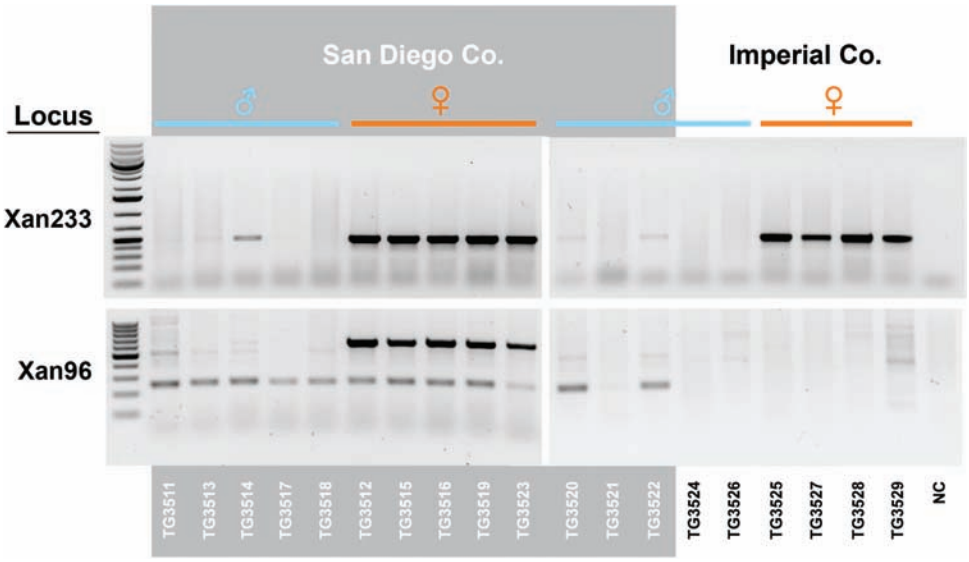

Figure 2. PCR validation of 2 female-specific RAD markers in *Xantusia henshawi*. Marker Xan233 amplified in a female-specific manner in both populations, generating a strong, single (W-specific) band in females. Marker Xan96 in San Diego individuals had a weak, presumably Z-specific, band in most individuals and a strong, W-specific band in females, but failed to amplify in females from Imperial Co (Table 1). Specimen ID numbers are listed below each lane. NC = negative control. Please see the online version for full colors.

estimated the mean F_{ST} and Φ_{ST} between populations as 0.38 and 0.57, respectively, indicating that these populations are highly divergent. Additionally, the inbreeding coefficient (F_{IS}) approached 0 for each population (at 0.01 and −0.05, respectively), indicating that each is close to Hardy-Weinberg equilibrium. To confirm that our sampling was concordant with these population demographic inferences, we analyzed the population genetic sub-structure under 3 alternative hypotheses using STRUCTURE. Indeed, the most-likely value was $K = 2$ and enforcing higher values of K (3 and 4) yielded no additional allelic populations (Supplementary Figure S1). These metrics of population structure provide support that these sampled *Xantusia* populations are divergent, and under Hardy-Weinberg equilibrium, confirming this divergence as a plausible explanation for the population-biased, sex specificity in PCR amplification.

BLAST queries of the 267 female-specific RAD contigs against chicken genes resulted in 4 hits, matching genes on chicken chromosomes 7, 12, and 18 (Table 1). BLAST queries against anole genes resulted in 16 hits, with half matching genes on chromosome 2 (homologous to chicken 12 and 18), and additional singletons matching genes on chromosomes 1, 3, and unmapped scaffolds (Table 1). The 4 hits in chicken were a subset of the anole matches and matched a chicken homolog in anole, that is, anole chromosome 2.

Discussion

The combined results–an excess of female-specific RAD markers and PCR amplification only in females—are indicative of a ZZ/ZW sex chromosome system in *X. henshawi* (Figure 2). This is the first evidence of sex chromosomes in the genus *Xantusia*, and family Xantusiidae, increasing our scant knowledge concerning the phylogenetic distribution of sex chromosomes within Scincomorpha (Figure 3). Though recent work has noted that a few, particularly speciose squamate lineages possess highly conserved sex chromosomes (Vicoso et al. 2013; Gamble et al. 2014; Rovatsos et al. 2014, 2015, 2016, 2019), many other squamate clades show a high incidence of turnover among sex-determining mechanisms (Sarre et al. 2004; Ezaz et al. 2009; Gamble et al. 2015; Rovatsos et al. 2016; Gamble et al. 2017; Nielsen et al. 2018, 2019b), particularly when compared to mammals and birds—clades that possess highly conserved, heteromorphic sex chromosome systems (e.g., Shetty et al. 1999; Graves 2006; Ellegren 2010). The discovery of ZZ/ZW sex chromosomes in Xantusiidae implies that at least 2 transitions between XX/XY and ZZ/ZW systems have occurred within the Scincomorpha, implicating this group as another that may possess high incidence of sex chromosome turnover.

It is worth noting that several male samples exhibit secondary or "ghost" bands on the gel that are the same size as female-specific RAD markers (Figure 2; e.g., RAD marker Xan233; males TG3514, TG3520, and TG3522). These weakly amplified, secondary products have been observed in the PCR of sex-specific RAD markers in other species (Gamble et al. 2015; Nielsen et al. 2019a) and are not altogether unexpected. While the PCR primers were designed using W-specific sequences, these secondary bands likely result from sequence similarities in the primer binding sites on the Z and W chromosomes (Fowler and Buonaccorsi 2016; Gamble 2016; Gamble et al. 2018). Sequence similarities are expected given that the Z and W evolved from a single autosomal pair and may share considerable sequence similarity, particularly in young, newly evolved sex chromosomes. Thus, PCR primers designed to amplify W-specific regions share sequence homology with Z-linked regions and, in the absence of their preferential binding sites on the W chromosome, may bind degenerately to these Z regions and produce low-quality amplicons. Nevertheless, clear differences in band intensity on the gel make it easy to distinguish male and female samples.

The RADseq methodology used herein has been pivotal in discovering previously unknown sex chromosome systems across vertebrates (Gamble and Zarkower 2014; Gamble et al. 2015; Fowler and Buonaccorsi 2016; Gamble et al. 2017, 2018; Nielsen et al. 2018,

Table 1. Results from BLAST of the female-specific *Xantusia henshawi* RAD contigs against chicken (*Gallus gallus*) and anole (*Anolis carolinensis*) genes demonstrating synteny with anole chromosome 2 and avian chromosomes 7, 12, and 18

Xantusia RAD marker	*Gallus* transcript	*Gallus* gene	*Gallus* chromosome	*E*-value
195	ENSGALT00000020272	LRP1B	7	2.67E-22
237	ENSGALT00000036256	FLNB	12	8.52E-29
217	ENSGALT00000013031	CASKIN2	18	3.89E-89
235	ENSGALT00000007292	NPLOC4	18	4.67E-51
Xantusia RAD marker	*Anolis* transcript	*Anolis* gene	*Anolis* chromosome	*E*-value
137	ENSACAT00000004974	KDM5C	1	3.24E-41
32	ENSACAT00000021082	AVPR2	2	1.44E-44
132	ENSACAT00000013455	AGAP2	2	4.13E-50
208	ENSACAT00000001506	ANKRD40	2	1.09E-59
217	ENSACAT00000006694	CASKIN2	2	5.28E-107
235	ENSACAT00000015187	NPLOC4	2	5.94E-63
237	ENSACAT00000010200	FLNA	2	0
253	ENSACAT00000009195	CCDC130	2	3.27E-28
260	ENSACAT00000009600	IQSEC2	2	2.51E-143
244	ENSACAT00000030745	novel gene	3	4.12E-27
19	ENSACAT00000030588	novel gene	AAWZ02039583	3.10E-41
195	ENSACAT00000006242	LRP1	GL343212	1.75E-69
261	ENSACAT00000028183	MBD6	GL343212	1.04E-78
13	ENSACAT00000029180	novel gene	GL343255	1.54E-88
88	ENSACAT00000029773	novel gene	GL343263	1.03E-27
169	ENSACAT00000029755	novel gene	GL343303	2.07E-30

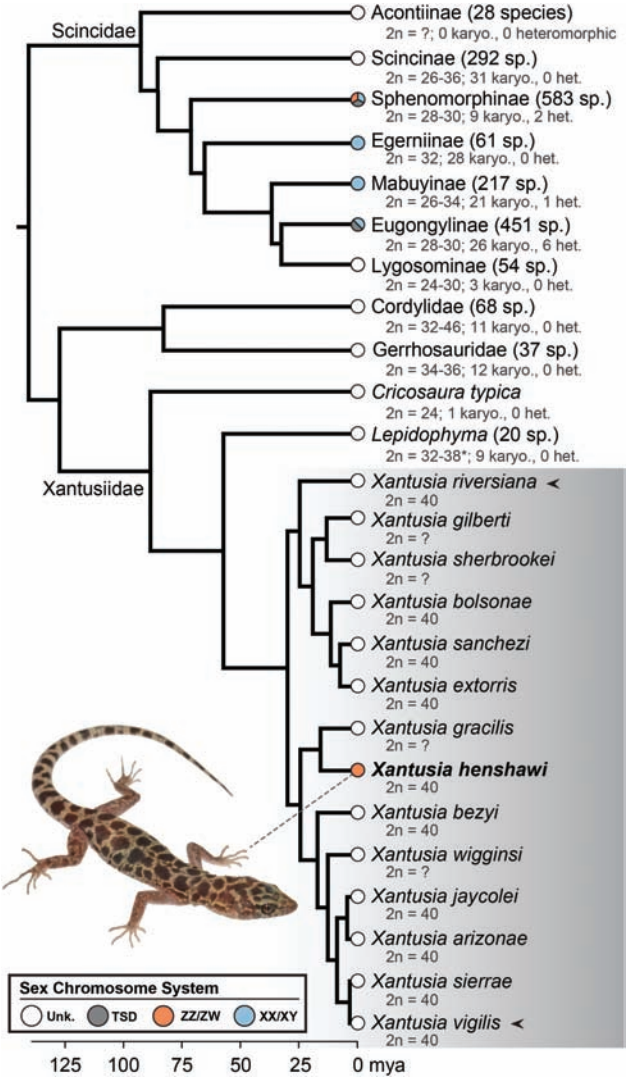

Figure 3. A time-calibrated phylogeny of the Scincomorpha (skink lizards and their allies), modified from Tonini et al. (2016). Sex chromosome systems, if known, are indicated by colored circles to the left of taxon names (pie segments indicate presence not frequency). Series of numbers under taxon names indicate diploid (2n) chromosomal complement (when known), the subset that have been karyotyped, and the number that exhibit heteromorphic sex chromosomes (*indicates the peculiar formula, 3n = 57, observed in a parthenogenetic lineage of *Lepidophyma*). Data from: Bezy 1972; Bezy and Villela 1999; Olmo and Signorino 2005; Hass and Hedges 2006; Bezy et al. 2008; Pokorná and Kratochvíl 2009; Gamble et al. 2015; Patawang et al. 2018. Note that the TSD reported in some skink species may be coincident with sex chromosomes and, upon closer examination, may represent temperature-influenced sex reversal (Valenzuela et al. 2003; Pokorná and Kratochvíl 2009; Gamble et al. 2015). Arrows indicate additional taxa that were evaluated using the sex-specific loci we developed in this study (see text). Please see the online version for full colors.

2019a). One of the desired byproducts of the RADseq methodology is a species- and sex-specific PCR assay (Gamble 2016). Yet, in this study, the population genetic structure of *X. henshawi* reduced the efficiency of developing a PCR-based molecular marker that works across sampled populations (Figure 2). Population-specific changes can accumulate quickly on the non-recombining region of the Y or W chromosome due to their smaller effective population size (N_e) and lack of gene flow, increasing the strength and effects of drift (Bachtrog et al. 2011; Gamble et al. 2015; Wilson 2018). Sampling across populations has the potential to produce molecular markers that target more conserved regions of the Y or W chromosome, and these conserved regions are more likely to amplify in a sex-specific pattern across closely related species. Thus, by sampling across populations, at the cost of the total number of RAD loci, one can potentially develop PCR primers that have a higher probability of amplifying sex-specifically in more divergent populations, and even other species (Gamble and Zarkower 2014; Fowler and Buonaccorsi 2016; Hundt et al. 2019). However, as we show here, success is difficult to predict.

The lack of karyotypic diversity within *Xantusia* (as opposed to across all xantusiids) is remarkable, as all species with known karyotypes possess 2n = 40 (Figure 3). Based on similar patterns in other vertebrate clades (e.g., birds; Ellegren 2010), this may be indicative of a conserved sex chromosome system. However, when we attempted to use the sex-specific loci we developed in this study on a limited number of samples of known sex for 2 additional *Xantusia* species (*X. vigilis* and *X. riversiana*; taxa with arrows in Figure 3) that span the diversity of the genus, results were inconclusive; the markers either failed to amplify, or there was no difference in amplification between sexes (Xan96 and Xan233, respectively; results not shown). Although this outcome unfortunately highlights one of the shortcomings of this and other molecular marker generating methodologies—that is, occasionally, sex-specific loci only amplify in the species for which they were developed—it suggests that the rapid evolution of Y (and W) chromosomes (Wilson 2018) and perhaps the presence of multiple substitutions/indels in primer binding sites (Gamble et al. 2018), cumulatively lower the success of interspecific PCR. However, such results are perhaps anticipated given the overly conservative nature of the PCR validation step (Gamble et al. 2015; Gamble 2016). With the addition of a modest genome assembly, we suspect these markers could be refined to work across multiple related taxa (Gamble et al. 2018). Such data would substantially improve our ability to test whether karyotype stability is disassociated with rates and patterns of speciation in this clade.

The *X. henshawi* Z chromosome is composed of genes syntenic to chicken chromosomes 7, 12, and 18 (which in turn are syntenic with anole chromosome 2; Deakin et al. 2016). To our knowledge, this is the first time this combination of chicken chromosomes have been reported to have a role in sex determination (Nielsen et al. 2019a). Although some research suggests that certain linkage groups might be more likely to be recruited into a sex-determining role (supported by the homology of gene content and arrangement across divergent lineages; Graves and Peichel 2010; O'Meally et al. 2012), limited empirical work suggests that any linkage group can be recruited into a sex-determining role, and thus any chromosome could become a sex chromosome (Hodgkin 2002). Distinguishing between these hypotheses is, at present, difficult given our scant knowledge of sex chromosome identity across amniotes (Graves 2008; Deakin and Ezaz 2019). Although there is no known master sex-determining genes in squamate reptiles, *Sox9*, a gene crucial for testis differentiation (Da Silva et al. 1996), is located on chicken chromosome 18 and most likely occurs on anole chromosome 2 (Srikulnath et al. 2015; Deakin et al. 2016; Zerbino et al. 2017). Future work ascertaining the possible role of *Sox9* in *Xantusia* sex determination could be illuminating. Although we are just scratching the surface as to whether some chromosomes may be "better" at being sex chromosomes than others,

the current results continue to build the groundwork to ask further questions about the nature of sex chromosome evolution.

Supplementary Material

Supplementary material is available at *Journal of Heredity* online.
Table S1. Samples used in this study.
Table S2. PCR primers used to validate female-specific RAD markers in *Xantusia henshawi*. PCR followed a touchdown (TCHDN) protocol where initial annealing temp was set at 67°C but then decreased 0.2°C per cycle for 35 cycles (see Methods for more details). (*successfully amplified only in females from San Diego Co.)
Fig. S1. Distruct plots from K=1–4 showing the clear population subdivision between *Xantusia* populations from San Diego and Imperial counties in California.

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Conflict of Interest

The authors declare no conflict of interests.

Data Availability

Sequencing reads are deposited with NCBI's SRA under the BioProject PRJNA527120.

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