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XX/XY Sex Chromosomes in the South American Dwarf Gecko (*Gonatodes humeralis*)

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Abstract

Sex-specific genetic markers identified using restriction site-associated DNA sequencing, or RADseq, permits the recognition of a species' sex chromosome system in cases where standard cytogenetic methods fail. Thus, species with male-specific RAD markers have an XX/XY sex chromosome system (male heterogamety) while species with female-specific RAD markers have a ZZ/ZW sex chromosome (female heterogamety). Here, we use RADseq data from 5 male and 5 female South American dwarf geckos (*Gonatodes humeralis*) to identify an XX/XY sex chromosome system. This is the first confidently known sex chromosome system in a *Gonatodes* species. We used a low-coverage de novo *G. humeralis* genome assembly to design PCR primers to validate the male-specificity of a subset of the sex-specific RADseq markers and describe how even modest genome assemblies can facilitate the design of sex-specific PCR primers in species with diverse sex chromosome systems.

Keywords

genome, lizard, Neotropics, PCR-RFLP, RADseq, Squamata

There are an incredible variety of sex-determining mechanisms among multicellular organisms (Bachtrog et al. 2014). However, to understand the evolutionary mechanisms that have produced this diversity, we must first identify and catalog the sex-determining systems of major plant and animal lineages. Historically this work involved cytogenetics to karyotype males and females and identify morphologically distinct or heteromorphic sex chromosomes (Valenzuela et al. 2003). Species where males are the heterogametic sex have an XX/XY sex chromosome system, while species where females are the heterogametic sex have a ZZ/ZW system (Bull 1983; Graves 2008). Unfortunately, many species lack heteromorphic sex chromosomes and instead have morphologically identical or homomorphic sex chromosomes (Ezaz et al. 2009; Bachtrog et al. 2014; Gamble and Zarkower 2014). Sex chromosomes in these species cannot be identified using traditional cytogenetic methods but can only be recognized via breeding experiments involving sex-reversed animals (Wallace et al. 1999), advanced cytogenetic techniques such as comparative genomic hybridization or florescent in situ hybridization (FISH) of repetitive elements (Ezaz et al. 2005; Pokorná et al. 2011; Gamble et al. 2014), copy number variation of sex-linked markers (Gamble et al. 2014; Rovatsos et al. 2014), or the identification of sex-specific genetic markers (Devlin et al. 2001; Berset-Brandli et al. 2006; Gamble and Zarkower 2014). Recently, restriction site-associated DNA sequencing, or RADseq, has been used to generate tens of thousands of molecular genetic markers from multiple confidently sexed males and females to identify genetic markers found in one sex but not the other (Baxter et al. 2011; Palaiokostas et al. 2013; Gamble and Zarkower 2014; Gamble et al. 2015; Gamble 2016; Pan et al. 2016). A species' sex chromosome system, either XX/XY or ZZ/ZW, can be inferred via the identification of these sex-specific genetic markers. Species with an excess of male-specific RAD markers have an XX/XY system, with markers occurring on the Y chromosome, and conversely an excess of female-specific RAD markers indicates a ZZ/ZW system, those markers occurring on the W chromosome (Gamble and Zarkower 2014; Gamble 2016).

Once identified, sex-specific RAD markers can be further validated via PCR (Gamble and Zarkower 2014; Gamble et al. 2015). Restricted recombination around the sex-determining locus and linked sexually antagonistic alleles results in increasing sequence divergence between the X and Y (or Z and W) chromosomes over time (Bull 1983; Charlesworth and Charlesworth 2000; Graves 2008). Thus, PCR primers designed for most sex-specific RAD markers should amplify only in the heterogametic sex, males in XX/XY systems and females in ZZ/ZW systems. However, some Y (or W) linked RAD sequences, particularly in young or newly evolved sex chromosomes, can have high sequence similarity to homologous regions on the X (or Z) and PCR primers that are intended to be

sex-specific may instead amplify in both males and females. Thus, PCR validation will be an overly conservative test of sex-specificity in these species (Gamble 2016). There are, however, 2 ways to overcome this problem.

The first involves situations where the restriction site itself is sex specific, that is, the restriction site occurs on the Y (or W) allele but is lacking on the X (or Z) allele, and the regions flanking the restriction site are identical or nearly so. In these instances, the bioinformatic analyses will identify these RAD markers as sex-specific but subsequent PCR validation would fail because primers would produce amplicons in both males and females due to the similarity between the X and Y (or Z and W) regions flanking the restriction site to which the primers bind (Fowler and Buonaccorsi 2016; Gamble 2016). One solution is to align sex-specific reads to the species genome (or to the genome of a closely related species), pairing adjacent RAD markers and then design PCR primers that flank either side of the restriction site. PCR amplicons can then be restriction digested, a technique known as PCR-RFLP. The X (or Z) amplicon will remain unchanged while the Y (or W) amplicon will be cut, resulting in different banding patterns on a gel between males and females (Figure 1). This strategy has been effective in validating sex-specific RAD markers in both fish and snakes (Fowler and Buonaccorsi 2016; Gamble et al. 2017).

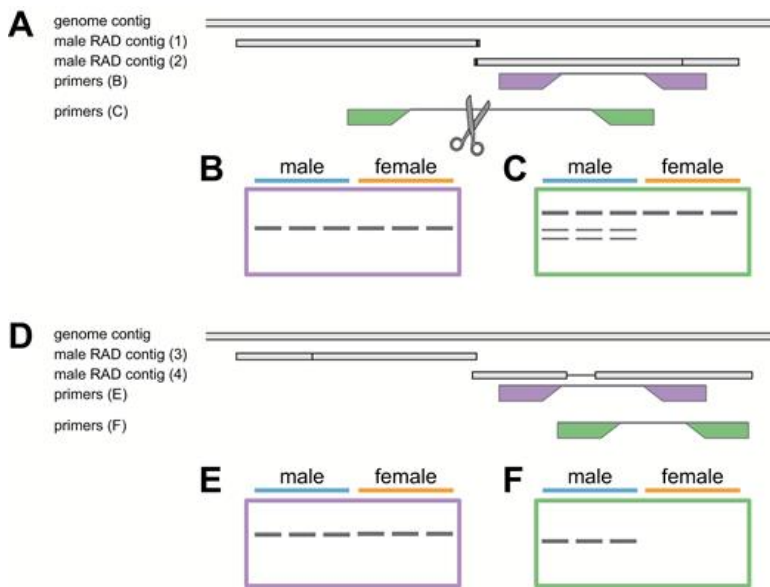


Figure 1. Conceptual examples using genomic resources to validate sex-specific RAD markers via PCR and PCR-RFLP. Examples illustrate an XX/XY sex chromosome system but results are similar in species with a ZZ/ZW system. Male RAD contigs are presumed to be Y alleles while the genomic contig is presumed to be the X allele. Black vertical segments indicate Y-specific SNPs that do not occur on the X. Thin horizontal segments in the contigs represent insertions or deletions (indels) that differ between X and Y alleles. **(A)** Alignment of 2 adjacent, male-specific RAD contigs to the homologous region of the female genome. The restriction site is male-specific (as indicated by SNPs in the restriction site on both male RAD contigs). With no prior genomic resources, primer pair B would be designed. However, the sequence similarity between the X and Y alleles would result in PCR amplification in both sexes. In light of the genomic alignment, primer pair C can be developed and used for PCR-RFLP with cartoon scissors indicating where the Y allele would be cut via restriction digest. This is the approach developed by Fowler and Buonaccorsi (2016). **(B)** Gel electrophoresis of primer pair B showing PCR amplification in both sexes. **(C)** Gel electrophoresis of PCR amplicons using primer pair C after restriction digest (PCR-RFLP) showing different banding patterns between male and female samples. **(D)** Alignment of 2 adjacent, male-specific RAD contigs to the homologous region of the female genome. The restriction site is not male-specific but instead found in both sexes. With no further genomic resources, primer pair E would be designed. However, the sequence similarity between the X and Y alleles would lead to PCR amplification in both sexes. In light of the genomic alignment, primer pair F can be designed for the Y allele, which places one of the primers on top of a male-specific indel. This primer placement should restrict PCR amplification to just the Y allele. This is the approach taken in this study. **(E)** Gel electrophoresis of primer pair E showing PCR amplification in both sexes. **(F)** Gel electrophoresis of primer pair F showing male-specific PCR amplification. See online version for full colors.

The second strategy is to design PCR primers in particular regions of the Y (or W) allele that are distinct from the X (or Z) allele. However, as with the PCR-RFLP example, identifying these regions is only possible if additional genomic resources are available. Here, we utilized an approach that used paired-end Illumina sequence reads from an individual of the homogametic sex (XX or ZZ individual) to produce a de novo genome assembly. We subsequently aligned sex-specific RAD contigs from the heterogametic sex (XX/XY or ZZ/ZW individuals) to these genomic contigs and designed PCR primers in regions that should amplify in a sex-specific manner, such as over insertions or deletions (indels) (Figure 1). We implemented this approach using the South American dwarf gecko (*Gonatodes humeralis*), a species we identified as having an XX/XY sex chromosome system but were unable to PCR validate using standard methods.

Dwarf geckos of the genus *Gonatodes* (Gekkota: Sphaerodactylidae) consist of 31 described species distributed across Central and South America and some Caribbean islands (Gamble et al. 2008; Schargel et al. 2017). Nine species in the genus, including our focal species *G. humeralis*, have published karyotypes but, with one exception, no heteromorphic sex chromosomes have been observed (Schmid et al. 2014). That exception is *Gonatodes ceciliae* from Trinidad. McBee et al. (1987) karyotyped 2 males, which both had a pair of heteromorphic chromosomes that were interpreted as sex chromosomes. However, both individuals had different numbers of chromosomes, $2N = 26$ and $2N = 22$. The subsequent karyotyping of an additional male *G. ceciliae* exhibited a third, unique karyotype, $2N = 24$ (Schmid et al. 2014). This individual also had a pair of heteromorphic chromosomes although they did not form a sex bivalent in meiosis (Schmid et al. 2014). Multiple, unique male karyotypes and lack of published female karyotypes suggests more evidence is needed before the *G. ceciliae* sex chromosome system can be confirmed. Thus, there are no *Gonatodes* species with a confidently known sex-determining system. Furthermore, the Sphaerodactylidae overall are poorly known with regards to sex determining systems although at least one transition between XX/XY and ZZ/ZW systems is presumed to have occurred; 2 *Sphaerodactylus* species and *Euleptes europeae* have XX/XY sex chromosomes, while *Aristelliger expectatus* has a ZZ/ZW system (Gamble 2010; Gornung et al. 2013; Schmid et al. 2014; Gamble et al. 2015). Thus, sex chromosome data from additional sphaerodactylid species are needed to better understand sex chromosome evolution in this group. This need for sex chromosome data from additional species that motivated the current study.

Methods

We extracted genomic DNA using the Qiagen® DNeasy Blood and Tissue extraction kit from tail clips of 5 adult male and 5 adult female *G. humeralis* collected on Trinidad ([Supplementary Table 1](#)). RADseq libraries were constructed following a modified protocol from Etter et al. (2011) as described in Gamble et al. (2015). Briefly, we digested genomic DNA using high-fidelity *SbfI* restriction enzyme (New England Biolabs). We ligated individually barcoded P1 adapters onto the *SbfI* cut site for each sample and pooled samples into multiple libraries, sonicated, and size selected into 200–500 bp fragments using magnetic beads in a PEG/NaCl buffer (Rohland and Reich 2012). We blunt-end repaired libraries and added a dA tail before ligating a P2 adapter containing unique Illumina barcodes to each of these pooled libraries. We used 16 PCR cycles with Phusion high-fidelity DNA polymerase (New England Biolabs) and size-selected a second time into 250–600 bp library fragments using magnetic beads in PEG/NaCl buffer. Libraries were pooled and sequenced using paired-end 125 bp reads on an Illumina HiSeq2500 at the Institute for Integrative Genome Biology, University of California—Riverside ([Supplementary Table 1](#)).

We demultiplexed, trimmed, and filtered raw Illumina reads using the `process_radtags` function in STACKS [v1.23] (Catchen et al. 2011). We used RADtools 1.2.4 (Baxter et al. 2011) to generate candidate alleles for each individual and candidate loci across all individuals from the forward reads with parameters as previously described (Gamble et al. 2015, 2017). We used a python script (Gamble et al. 2015) to identify putative sex-

specific markers from the RADtools output. This script also produces a second list of “confirmed” sex-specific RAD markers, which are a subset of the initial list of sex-specific RAD markers, but excludes from further consideration any sex-specific markers that also appear in the original reads files from the opposite sex. We assembled forward and reverse reads from the confirmed sex-specific RAD markers into sex-specific RAD contigs using Geneious R9 (Kearse et al. 2012).

Given some large number of confidently sexed male and female samples, the preceding methods will correctly identify sex-specific RAD markers. However, with small sample sizes, as we have here, we cannot rule out that some number of sex-specific RAD markers may be identified by chance. To address this, we permuted the sex among sampled individuals to create an expected null distribution of the number of sex-specific RAD markers when none are actually present (Gamble et al. 2017). We then compared the observed number of sex-specific markers to this null distribution to test whether it is larger than expected by chance or contained within the lower 95% confidence interval of the null distribution. We performed 100 permutations using parameters from our original dataset including the total number of RAD markers and the same number of males and females. Note that permutations were evaluated using the total number of sex-specific markers, not the number of confirmed sex-specific markers.

We attempted to use PCR to validate the sex specificity of a subset of confirmed male-specific RAD markers, as done in previous studies (Gamble and Zarkower 2014; Gamble et al. 2015, 2017; Fowler and Buonaccorsi 2016; Hayashi et al. 2017), but initial attempts were unsuccessful and every PCR resulted in amplification in both male and female samples (data not shown). This can result when PCR primers are designed on parts of the RAD marker that exhibit high sequence similarity between the X and Y (or Z and W) (Gamble et al. 2015; Fowler and Buonaccorsi 2016; Gamble 2016). One solution to this problem is to align sex-specific RAD markers to the species’ genome, if available, and then: 1) determine whether the restriction site is sex-specific and design primers that flank the restriction site for subsequent PCR RFLP (Fowler and Buonaccorsi 2016); or 2) design primers on regions of the sex-specific RAD marker that exhibit considerable divergence from the reference genome, such as indels, that could amplify in a sex-specific fashion. While there are 2 published gecko genomes (Liu et al. 2015; Xiong et al. 2016), they are too phylogenetically distant from *G. humeralis* to be useful for our purposes. Therefore, we sequenced and assembled the genome of a female *G. humeralis* which we aligned male-specific RAD markers, and used these additional data to design new sex-specific PCR primers. We made an Illumina library from sheared DNA of a female *G. humeralis* (TG2241) from Trinidad using the NEBNext Ultra DNA Library Prep Kit with a 350 bp insert size. We sequenced 634 million paired-end 150 bp reads on an Illumina NextSeq at the Institute for Integrative Genome Biology, University of California—Riverside. Raw Illumina reads were trimmed and filtered using the `process_shortreads` function in STACKS [v1.23] (Catchen et al. 2011). We de novo assembled reads into contigs using CLC Genomics Workbench [v10.1.1]. We estimated genome size and sequencing coverage from k-mer frequencies (Li et al. 2010). Thirty-one basepair k-mers were counted from cleaned, paired-end Illumina reads with Jellyfish [v2.2.6] (Marçais and Kingsford 2011). We used perl scripts (https://github.com/josephryan/estimate_genome_size.pl) to generate a histogram of k-mer frequencies from the Jellyfish output, identify the peak k-mer frequency, and subsequently estimate genome size and sequence coverage.

We used BLASTn to align male-specific RAD contigs to the female genome assembly and designed primers using Geneious R9 (Kearse et al. 2012). Primers were designed to amplify in males and not females by placing one primer onto an aligned region with a sex-specific indel (Figure 1). We used PCR of 6 males and 5 females ([Supplementary Table 1](#)) to validate sex-specificity and visualized the PCR amplicons using gel electrophoresis.

Results

We recovered 151073 RAD markers, which included 156 male-specific and 2 female-specific markers. A subset of these RAD markers was excluded from further consideration because they occurred in the original reads files of the opposite sex. The RAD markers that remained are called “confirmed” sex-specific markers. We identified 25 confirmed male-specific RAD markers and 2 confirmed female-specific RAD markers. This excess of male-specific RAD markers indicates an XX/XY sex chromosome system. Permutations showed the observed number of male-specific markers (156) was greater than the lower 95% confidence interval of the null distribution (Figure 2), whereas the observation of female-specific markers was contained within the lower 95% null distribution and considered an artifact of our small sample size.

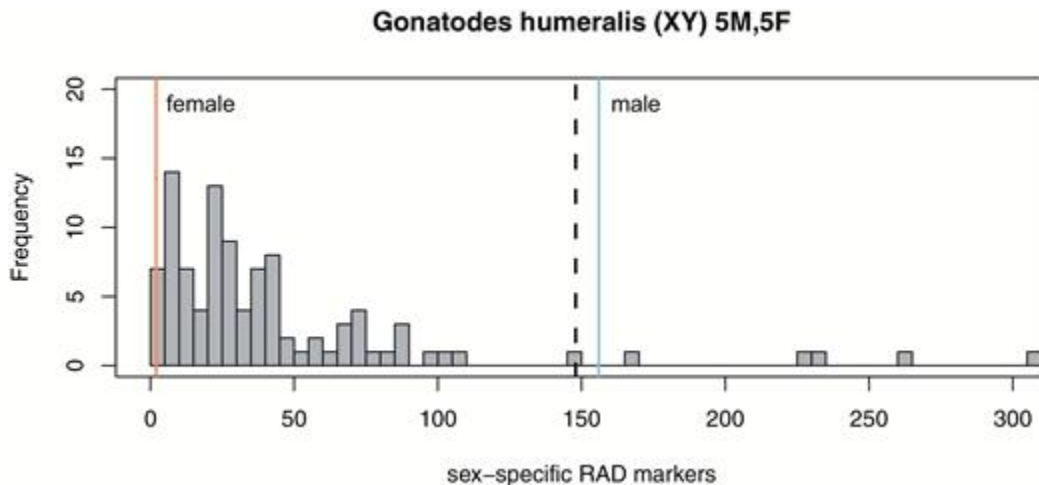


Figure 2. Permutations of the number of sex-specific markers expected by chance for the gecko *Gonatodes humeralis*. Blue and orange vertical lines denote the observed number of male- and female-specific RAD markers, respectively. The observed number of male-specific markers, 156, is greater than the upper 95% confidence interval of the null distribution (dashed vertical line) and is considered significantly different than expected by chance. See online version for full colors.

The female genome was sequenced to about 36× from 629 million trimmed and cleaned Illumina reads. The de novo genome assembly resulted in 1.42 million contigs with an N50 of 2879 bp, 43.7% GC content, and estimated genome size of 1.3 Gb.

As mentioned previously, PCR amplicons from primers designed from male-specific RAD markers amplified in both sexes (not shown). Because PCR is likely to be an overly conservative means of validating sex-specificity (Gamble 2016) we designed new PCR primers that could accurately validate male-specificity after aligning male-specific RAD contigs to the de novo female genome assembly using BLASTn. All 25 of the confirmed male-specific contigs had BLAST hits to the female genome with 8 of the markers mapping in matched pairs to 4 separate genome contigs. The 4 female contigs that corresponded to these 8 RAD markers all had an *Sbf*I restriction site and we subsequently found the corresponding RAD markers, which are presumably X-linked, in our RAD dataset. Thus, PCR-RFLP was not possible for these 8 RAD markers. However, 2 of these alignments had indel differences between putative X and Y alleles that allowed us to design primers that produced male-specific PCR amplicons in the same individuals for both primer sets (Figure 3), confirming an XX/XY sex chromosome system. The following primer pairs amplified in a male-specific manner: Ghum_1and9-F 5'-GAGCAGATGATTGGGGCTGAT-3' and Ghum_1and9-R 5'-ATACCTGGGTGAGACCAGGA-3'; Ghum_23and28-F1 5'-CGCAGCAAGGTTCTTGTACA-3' and Ghum_23and28-R1 5'-ACCGATAGTGTAACCTTGCTTTG-3'.

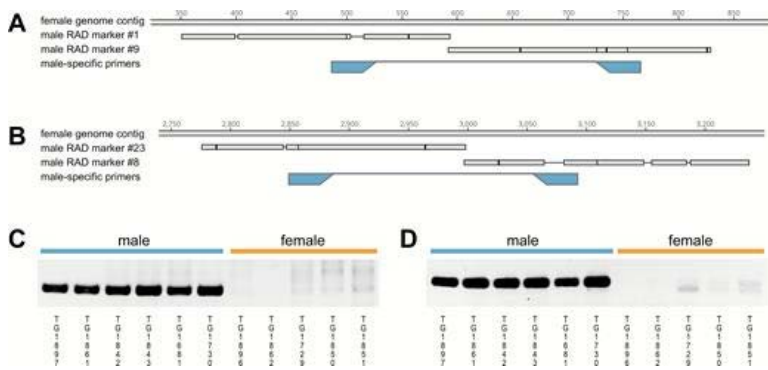


Figure 3. Primer design and PCR validation of male-specific RAD markers in the gecko *Gonatodes humeralis*. Both (A) and (B) show the alignment of 2 adjacent, male-specific RAD contigs to the homologous region of the female genome on the X chromosome. In light of this genomic information, one primer at each locus was designed on top of a male-specific indel. This primer placement should restrict PCR amplification to just the male-specific Y allele. Sequence alignments and PCR primers were implemented in Geneious R9 (Kearse et al. 2012). (A) Alignment of male-specific RAD markers 1 and 9, to design primers Ghum_1and9-F/Ghum_1and9-R. (B) Alignment of male-specific RAD markers 23 and 8, to design primers Ghum_23and8-F/Ghum_23and8-R. (C) Male-specific PCR amplification of primers Ghum_1and9-F/Ghum_1and9-R in 6 male and 5 female *G. humeralis*. (D) Male-specific PCR amplification of primers Ghum_23and8-F/Ghum_23and8-R in 6 male and 5 female *G. humeralis*. Specimen ID numbers are listed below every well for both gel images. See online version for full colors.

Discussion

These results provide the first robust evidence of sex chromosomes in the genus *Gonatodes* and increase our limited knowledge concerning the phylogenetic distribution of sex chromosomes in the gecko family Sphaerodactylidae (Figure 4). While it is clear that at least one transition between XX/XY and ZZ/ZW systems has occurred in Sphaerodactylidae, additional work is needed to assess whether the XX/XY systems in *Sphaerodactylus*, *Gonatodes*, and *Euleptes* are derived from the same ancestral XY system and thus homologous with each other. A shared XX/XY system among these genera would be quite ancient, as their most recent common ancestor is at least 95 mya (Gamble et al. 2015). Independently derived XX/XY systems would further increase the number of transitions in this clade making them an ideal model to study sex chromosome transitions and evolution. The strong sequence similarity between X and Y alleles coupled with the relatively few confirmed male-specific markers implies limited differentiation between the *G. humeralis* X and Y and suggests that these sex chromosomes are relatively young (Rice 1996; Charlesworth and Charlesworth 2000). This scenario would support the independent origin of XX/XY sex chromosomes among the Sphaerodactylidae. However, poorly differentiated sex chromosomes are not necessarily newly evolved and a variety of processes may prevent Y degeneration in older sex chromosomes, for example, occasional recombination between the X and Y; weak natural selection to restrict recombination between the X and Y; or the inability to evolve dosage compensating mechanisms (Rice 1987; Perrin 2009; Stöck et al. 2011; Adolfsson and Ellegren 2013; Bachtrog et al. 2014). Determining whether XX/XY Sphaerodactylidae share a common sex chromosome system would resolve the age of sex chromosomes in the family, as could the identification of sex chromosomes in additional species and genera. Methods such as RADseq can be used to increase the number of sphaerodactylid species with known sex chromosome systems and targeting additional sphaerodactylid genera should be a high priority.

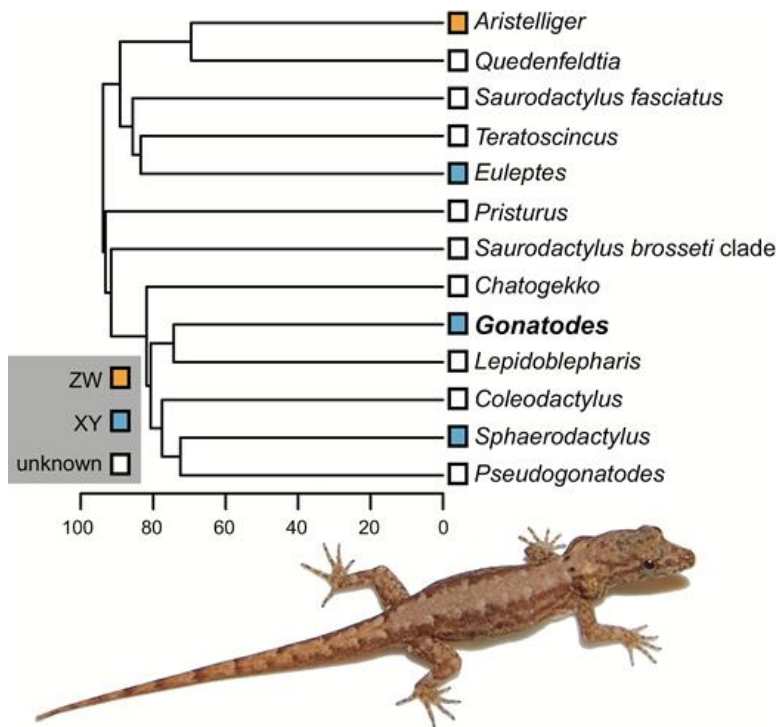


Figure 4. Sex chromosome evolution in the gecko family Sphaerodactylidae. Time-calibrated phylogeny (Gamble et al. 2015) shows relationships among sphaerodactylid genera and sex chromosome systems, if known, are indicated by colored boxes to the left of generic names. An XX/XY sex chromosome system in the genus *Gonatodes* (in bold) is confirmed here for the first time. Image: Female *G. humeralis* from Trinidad (T. Gamble). Phylogenetic time, along the x-axis, is measured in millions of years. See online version for full colors.

Squamate genomes range in size from 1.05 to 3.93 pg (1 pg of DNA = 978 Mb; (DeSmet 1981; Doležal et al. 2003; Gregory 2017). Thus, the estimated genome size of *G. humeralis* (1.3 Gb) is among the smallest of all squamate genomes and smaller than recent genome size measurements for other *Gonatodes* species (1.6 pg to an astonishing 7.8 pg; Schmid et al. 2014). Previously published *Gonatodes* genome size estimates should be interpreted with caution, however, as they were generated with flow cytometry using DAPI (4',6-diamidino-2-phenylindole) staining instead of the more commonly used propidium iodide (PI) or ethidium bromide (EB) staining (Schmid et al. 2014). DAPI preferentially binds AT-rich sites and can result in significantly different genome size estimates than estimates made from PI or EB, which lack such biases (Doležal et al. 1992; Kapuscinski 1995; Johnston et al. 1999). K-mer-based genome size estimates, as presented here, should also be interpreted cautiously as they can be biased by a variety of issues including sequencing errors, high heterozygosity, uneven sequence coverage, the overrepresentation of organelle sequences, for example, mitochondrial sequence and repetitive elements (Williams et al. 2013; Simpson 2014; Sun et al. forthcoming; Vurture et al. 2017). Improving the *G. humeralis* genome assembly through additional sequencing and scaffolding will facilitate more accurate genome size estimates.

The draft *G. humeralis* genome assembly, while modest, proved indispensable for designing PCR primers to validate the male-specificity of the RAD markers identified here. RADseq has been promoted as an efficient, cost-effective way to identify sex-specific genetic markers in species lacking genomic resources (Gamble and Zarkower 2014; Gamble et al. 2015; Gamble 2016). In a subset of cases though, PCR primers designed from the assembled RAD contigs will fail to amplify in a sex-specific manner (Gamble et al. 2015; Fowler and Buonaccorsi 2016). Inclusion of additional genomic resources can resolve these problems if initial attempts to perform PCR

validation are unsuccessful. Similar efforts to design and validate sex-specific RAD markers in other taxa could see benefits from sequencing low coverage genomes to facilitate primer development. While additional uses for modest genome assemblies like this are limited, we are currently generating more data that will improve this initial assembly and elucidate which chromosomes are the *G. humeralis* sex chromosomes.

Sex-specific genetic markers have proven useful in identifying the sex chromosome systems in many plant and animal species (Charlesworth and Mank 2010; Gamble and Zarkower 2014; Gamble 2016). However, these markers have many other uses, including identifying an individual's sex in species lacking recognizable secondary sexual traits (Griffiths et al. 1998; Rovatsos et al. 2015), sexing embryonic material in developmental studies (Hacker et al. 1995; Smith et al. 1999), identifying sex-reversed individuals (Quinn et al. 2007; Hayes et al. 2010; Holleley et al. 2015), and the breeding and management of endangered species (Griffiths and Tiwari 1995; Ortega et al. 2004; Robertson et al. 2006; Literman et al. 2017). Therefore, the identification of sex-specific markers and subsequent development of accurate PCR-based sex assays has important practical uses on top of their importance in studying sex chromosome evolution. The utilization of low-coverage whole genome sequencing to facilitate sex-specific PCR primer design should be considered in cases where the development and validation of PCR-based sex assays from RADseq data is problematic. Not only do the additional genomic resources enable the development of PCR primers in challenging cases, like *G. humeralis*, but the alignment of sex-specific RAD markers to a genome could enable primer design at many more independent loci than would otherwise be discovered. Having multiple PCR-based assays for a single species is useful as it can reduce sexing inaccuracies due to technical errors and other mistakes (Robertson and Gemmell 2006). The combination of RADseq with whole genome sequencing appears to offer the greatest flexibility in identifying sex-specific markers and sex chromosomes for a wide range of uses.

Supplementary Material

Supplementary data are available at *Journal of Heredity* online.

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Data Availability

Demultiplexed *G. humeralis* RADseq reads were deposited to NCBI SRA: SAMN06827899–06827908; cleaned paired-end Illumina reads used in whole genome sequencing were submitted to NCBI SRA: SAMN07634392 ([Supplementary Table 1](#)).

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