

ZZ/ZW Sex Chromosomes in the Endemic Puerto Rican Leaf-Toed Gecko (*Phyllodactylus wirshingi*)

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Abstract

Investigating the evolutionary processes influencing the origin, evolution, and turnover of vertebrate sex chromosomes requires the classification of sex chromosome systems in a great diversity of species. Among amniotes, squamates (lizards and snakes) – and gecko lizards in particular – are worthy of additional study. Geckos possess all major vertebrate sex-determining systems, as well as multiple transitions among them, yet we still lack data on the sex-determining systems for the vast majority of species. We here utilize restriction-site associated DNA sequencing (RADseq) to identify the sex chromosome system of the Puerto Rican endemic leaf-toed gecko (Phyllodactylidae: *Phyllodactylus wirshingi*), in order to confirm a ZZ/ZW sex chromosome system within the genus, as well as to better categorize the diversity within this poorly characterized family. RADseq has proven an effective alternative to cytogenetic methods for determining whether a species has an XX/XY or ZZ/ZW sex chromosome system – particularly in taxa with non-differentiated

sex chromosomes – but can also be used to identify which chromosomes in the genome are the sex chromosomes. We here identify a ZZ/ZW sex chromosome system in *P. wirshingi*. Furthermore, we show that 4 of the female-specific markers contain fragments of genes found on the avian Z and discuss homology with *P. wirshingi* sex chromosomes.

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Investigating the number and directionality of transitions among sex-determining systems is a vital prerequisite for studying sex chromosome evolution. This involves not only determining whether a species has a heterogametic male (XX/XY) or female (ZZ/ZW) sex chromosome system, but also identifying which chromosomes in the genome are the sex chromosomes. However, we still lack basic knowledge of sex-determining mechanisms for many species [Bachtrog et al., 2014], let alone the genomic homology of said sex chromosomes. Cytogenetic methods, like karyotyping, have long been the principal means of identifying an organism's sex chromosome system, yet most vertebrate species possess morphologically indistinguishable sex chromosomes [Devlin and Nagahama, 2002; Matsubara et al., 2006; Stöck et al.,

2011; Gamble and Zarkower, 2014; Otto, 2014]. Furthermore, such data do not address which chromosomes in the genome are the sex chromosomes in a comparative framework. However, recent advances in both cytogenetics and DNA sequencing techniques have facilitated the identification of sex chromosomes in many additional species, spawning a renewed interest in ascertaining and classifying the sex chromosome systems of previously intractable taxa [Pokorná et al., 2011; Deakin et al., 2016; Gamble et al., 2017, 2018; Nielsen et al., 2018].

Even within a group as varied as squamates, the geckos (Squamata: Gekkota) are a stand-out clade worthy of more detailed study. Geckos are a species-rich (>1,700 species [Uetz et al., 2017]), near globally distributed clade of lizards, who possess all major vertebrate sex-determining systems, as well as multiple transitions among them [Moritz, 1990; Ezaz et al., 2009; Gamble, 2010; Gamble et al., 2015a]. The high diversity of geckos makes them an ideal vertebrate model to study the origins and evolution of sex chromosomes. However, huge swaths of the gecko phylogeny lack any information about sex-determining systems, and fewer than 3% of gecko species have a sex-determining system known with high confidence [Gamble et al., 2015a]. Despite this paucity of data, roughly one-half to two-thirds of all observed transitions among squamate sex-determining systems are in geckos [Gamble et al., 2015a]. Fostering an enhanced knowledge concerning gekkotan, and thus squamate, sex-determining systems will increase their utility as a model clade to study the origins and evolution of sex-determining systems [Janzen and Krenz, 2004]. Therefore, a concentrated effort to identify sex chromosome systems in additional clades will serve to fill crucial gaps in our knowledge, and permit more comprehensive hypothesis testing.

The Phyllodactylidae are distributed throughout the New World, North Africa, Europe, and the Middle East. Of the more than 135 species, only 14 have published karyotypes and just 2 species, *Phyllodactylus lanei* and *Thecadactylus rapicauda*, exhibit evidence of heteromorphic sex chromosomes [King, 1981; Pellegrino et al., 2004, 2005, 2010; Olmo and Signorino, 2005; Murphy et al., 2009; Schmid et al., 2014]. However, in both species, the story is complex. For example, in *P. lanei*, different karyotypic formulas between sexes were indicative of a ZZ/ZW sex chromosome system, yet more recent work could not replicate these findings [Castiglia et al., 2009]. Similarly in *T. rapicauda*, the authors conclude that sex chromosomes are in a “nascent state of differentiation” as not all sampled populations were heteromorphic. Using a recently developed restriction-site associated DNA sequencing (RAD-

Table 1. *Phyllodactylus wirshingi* samples used in this study

ID	Sex	Locality
TG2007	Male	Isla de Caja de Muertos, Puerto Rico
TG3210	Male	Bosque Estatal de Guánica, Puerto Rico
TG3219	Male	Bosque Estatal de Guánica, Puerto Rico
TG3220	Male	Bosque Estatal de Guánica, Puerto Rico
TG3221	Male	Bosque Estatal de Guánica, Puerto Rico
TG3223	Male	Bosque Estatal de Guánica, Puerto Rico
TG3225	Male	Bosque Estatal de Guánica, Puerto Rico
TG3228	Male	Bosque Estatal de Guánica, Puerto Rico
TG2016 ^a	Male	Bosque Estatal de Guánica, Puerto Rico
TG3208 ^a	Male	Bosque Estatal de Guánica, Puerto Rico
TG2004	Female	Isla de Caja de Muertos, Puerto Rico
TG2008	Female	Isla de Caja de Muertos, Puerto Rico
TG2009	Female	Isla de Caja de Muertos, Puerto Rico
TG2385	Female	Bosque Estatal de Guánica, Puerto Rico
TG3209	Female	Bosque Estatal de Guánica, Puerto Rico
TG3222	Female	Bosque Estatal de Guánica, Puerto Rico
TG3224	Female	Bosque Estatal de Guánica, Puerto Rico
TG3226	Female	Bosque Estatal de Guánica, Puerto Rico
TG3227	Female	Bosque Estatal de Guánica, Puerto Rico
TG2017 ^a	Female	Bosque Estatal de Guánica, Puerto Rico

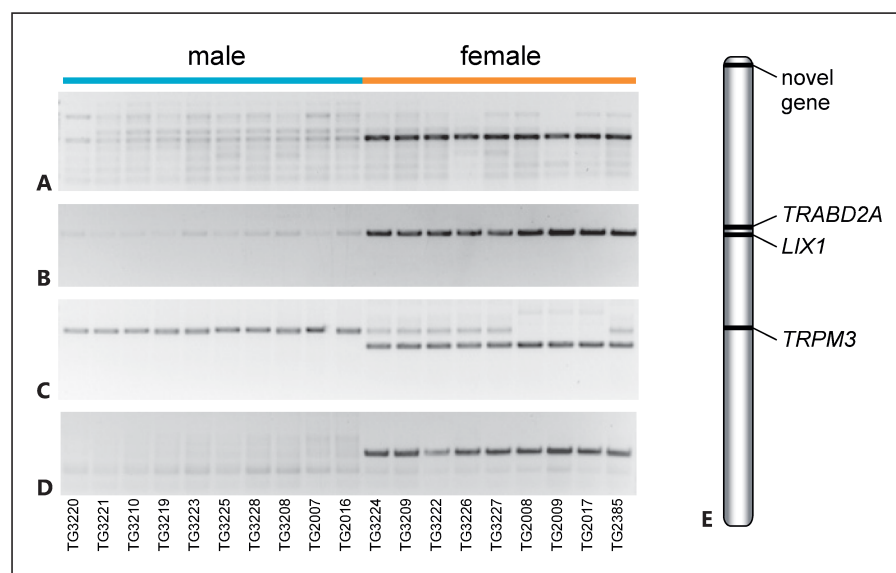
^a These samples were used for PCR validation only.

Table 2. PCR primers used to validate female-specific RADseq markers in *Phyllodactylus wirshingi*

Primer ID	Sequence (5'→3')	Annealing temperature
Pw84-F	CAGAAGGCATGAGACTGGAGAG	57°C
Pw84-R	CAAATCTCCAGGAGCAGAGTGG	
Pw116-F	CGATTCCCTTGCCTTAATCGGT	56°C
Pw116-R	AGATTCTGACCCAGGAAGAGGA	
Pw186-F	ACTTTCCACTAAGGTGATCCCC	56°C
Pw186-R	GGGCCAAGGACTATGACTTGAA	
Pw187-F	GACTGAGGAGGGTCTGCTCT	56°C
Pw187-R	GTCTTCTGGGCTCTGACTGG	

seq) methodology [Gamble and Zarkower, 2014], another population of *T. rapicauda* from Trinidad and Tobago was found to also have ZZ/ZW sex chromosomes [Gamble et al., 2015a]. In addition to ZZ/ZW sex chromosomes, a number of species in the genus *Tarentola* show strong evidence of environmentally determined sex (i.e., temperature-dependent sex determination or TSD) [Nettmann and Rykena, 1985; Hielen, 1992]. Thus, there are some interesting inferences one can draw within this family. First, we know very little about the diversity of sex chro-

Fig. 1. A–D PCR validation of 4 female-specific RADseq markers in *Phyllodactylus wirshingi*. **A** Pw84. **B** Pw116. **C** Pw186. **D** Px187. Primers amplified in a female-specific manner in all examined male and female samples (see Table 1), generating a single (presumably W-specific) band in all but one locus, Pw186. The latter produced both Z- and W-specific bands. Specimen ID numbers are listed below each lane. **E** Cytogenetic map of the *Gallus gallus* Z chromosome depicting the location of 4 genes identified by BLAST of *P. wirshingi* female-specific RAD contigs (Table 3).



mosome systems within Phyllodactylidae, and given the diversity observed in other gekkotan groups, data from additional species are essential before more definitive conclusions can be drawn. Second, based on our limited evidence, there is at least 1 transition among sex-determining systems within Phyllodactylidae – between TSD and female heterogamety. Lastly, both *T. rapicauda* and *P. lanei* possess ZZ/ZW sex chromosome systems, but are their sex chromosomes homologous?

Here, we used RADseq to discover the sex chromosome system of the Puerto Rican endemic leaf-toed gecko (*Phyllodactylus wirshingi*), to confirm the ZZ/ZW sex chromosome system within the genus, as well as to better categorize sex chromosome diversity within the Phyllodactylidae. By utilizing paired-end Illumina sequencing, we generated large RADseq contigs and successfully identified 4 genes putatively linked to the sex chromosomes. We discuss sex chromosome homology within Gekkota and among amniotes, with particular attention paid to synteny between the newly discovered *P. wirshingi* ZZ/ZW chromosomes and the avian Z.

Materials and Methods

Using the Qiagen® DNeasy Blood and Tissue Kit, we extracted genomic DNA from tail clips, or liver, from 8 adult male and 9 adult female *P. wirshingi* collected near Guánica in Puerto Rico and the island of Caja de Muertos (Table 1). RADseq libraries were constructed following a modified protocol from Etter et al. [2011] as described in Gamble et al. [2015a]. Genomic DNA was digested

with 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 200–500-bp fragments using magnetic beads in a PEG/NaCl buffer [Rohland and Reich, 2012]. Libraries were blunt-end repaired and dA-tailed before ligating a P2 adapter containing unique Illumina barcodes to each pooled library. We amplified libraries via PCR (16 cycles) with Q5 high-fidelity DNA polymerase (New England Biolabs) and cleaned/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 Medical College of Wisconsin.

We demultiplexed, trimmed, and filtered raw Illumina reads using the `process_radtags` function in STACKS (v1.4.8) [Catchen et al., 2011]. We applied 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 employing previously described parameters [Gamble et al., 2015a, 2017]. We identified putative sex-specific markers from the RADtools output using a custom python script [Gamble et al., 2015a]. 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 read files from the opposite sex from further consideration [Gamble and Zarkower, 2014; Gamble et al., 2015a]. 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 the confirmed female-specific markers by PCR (Table 2).

We attempted to assess synteny between the newly identified sex-specific RAD markers in *P. wirshingi* with chicken chromosomes. The chicken genome is well annotated and widely used as a reference for comparative genomics in nonavian reptiles [Inter-

Fig. 2. A time-calibrated genus-level phylogeny of the Phyllodactylidae, modified from Gamble et al. [2015a]. Sex chromosome systems, if known, are indicated by colored circles to the left of taxon names. Series of numbers under taxon names indicate diploid (2n) chromosomal complement (when known), the number of described species within the genus, the subset that have been karyotyped, and the number that exhibit heteromorphic sex chromosomes. Karyotype data from Olmo and Signorino [2005], Pellegrino et al. [2010], and Gamble et al. [2015a]. * The 2n number for *Homonota* is currently unpublished. Photographs of *Asaccus* and *Thecadactylus* by T. Pierson and A. Snyder, respectively.

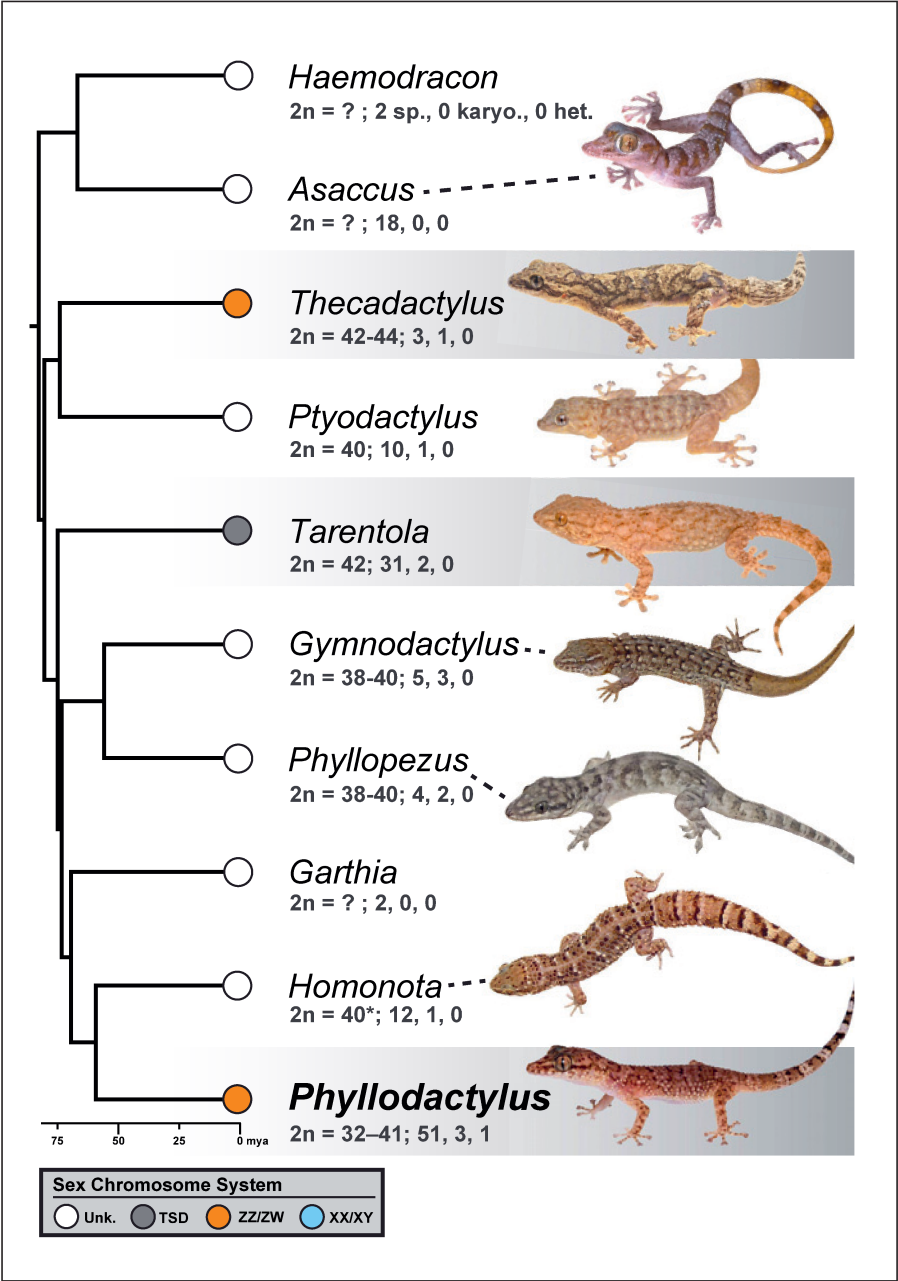


Table 3. The 4 hits from the BLAST of the 539 female-specific *Phyllodactylus wirshingi* RAD contigs against chicken genes demonstrating synteny with avian sex chromosomes

Query	Gene name	Transcript ID	Chicken ^a	E value	Hit start	Hit end
PwF_429	<i>TRPM3</i>	ENSGALT00000024411	Z	1.21e-56	3,246	3,446
PwF_381	<i>LIX1</i>	ENSGALT00000024678	Z	3.87e-44	241	388
PwF_203	Novel gene	ENSGALT00000028259	Z	1.25e-44	442	683
PwF_37	<i>TRABD2A</i>	ENSGALT00000043645	Z	2.51e-47	809	656

^a The location of these genes on the chicken Z chromosome is shown in Figure 1.

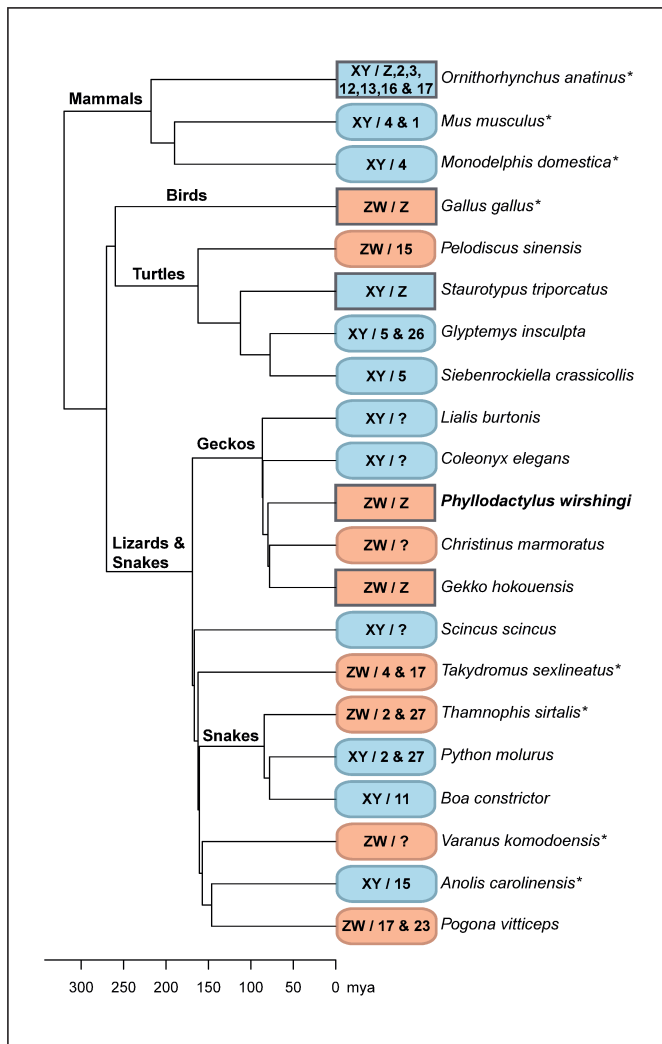


Fig. 3. Phylogenetic distribution and chromosomal synteny of the avian Z with the sex chromosomes of a selection of taxa spanning the amniote tree of life. Boxes at the tips of the phylogeny indicate the sex chromosome system, followed by sex chromosome synteny, if known, with birds. For example, the ZZ/ZW sex chromosomes of the turtle, *Pelodiscus sinensis*, are homologous with chicken (*Gallus gallus*) chromosome 15. Rectangular boxes share sex chromosome synteny with birds while curved boxes do not. XY and ZW indicate male and female heterogamety, respectively. Several taxa have unknown sex chromosome synteny (indicated by “?”), but comparative FISH experiments have shown that their sex chromosomes do not share homology with the avian Z [Pokorná et al., 2011; Matsubara et al., 2014]. When indicated by an asterisk, the pattern is reflective of the broader clade (e.g., *Takydromus sexlineatus* and all other lacertid lizards examined to date share a homologous ZW sex chromosome system). For more details on sex chromosome synteny data, we refer the reader to the original data sources [Matsubara et al., 2006; Graves, 2008; Veyrunes et al., 2008; Kawagoshi et al., 2009, 2012, 2014; Kawai et al., 2009; Alföldi et al., 2011; Deakin et al., 2016; Rovatsos et al., 2016a, b; Montiel et al., 2016; Gamble et al., 2017]. Phylogeny modified from Anderson and Wiens [2017].

national Chicken Genome Sequencing Consortium, 2004; Alföldi et al., 2011; Pokorná et al., 2011; O’Meally et al., 2012]. We performed BLAST of the assembled female-specific RAD contigs to the chicken transcriptome (using Ensembl [Zerbino et al., 2018]), 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 RADtool analysis recovered 143,649 RAD markers with 2 or fewer alleles including 1 male-specific RAD marker and 574 female-specific RAD markers. Of these, we identified zero confirmed male-specific RAD markers and 539 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 which occurred in the raw read files of the opposite sex and likely are false positives. From this pool of confirmed female-specific RAD contigs, we designed 11 primer pairs, 4 of which amplified in a sex-specific manner (Fig. 1). These loci produced either a single band in each of the female samples with no amplification in male samples (Pw84, Pw116, and Pw187; i.e., a RAD marker presumably on the W chromosome) or 2 bands in females and a single band in males (Pw186; i.e., different-sized Z and W alleles). The combined results – an excess of female-specific RAD markers and PCR amplification of a subset of these markers only in females – is indicative of a ZZ/ZW sex chromosome system within *Phyllodactylus* (Fig. 2).

BLAST queries of the 539 female-specific RAD contigs against chicken genes resulted in 4 hits (Table 3). All 4 matching genes are on the chicken Z chromosome, revealing homology between the avian and the *P. wirshingi* ZZ/ZW sex chromosomes (Fig. 3).

Discussion

The discovery of ZZ/ZW sex chromosomes in *P. wirshingi* makes it the second member of the genus with female heterogamety, the other being *P. lanei* [King, 1981]. The exact identity of the species King [1981] examined, however, is up for debate since subsequent karyotypes of *P. lanei* revealed different chromosomal arrangements and no heteromorphic sex chromosomes [Castiglia et al., 2009]. Because King [1981] apparently did not keep vouchered specimens from his study, the exact identity of the species examined remains unknown. Castiglia et al. [2009] obtained their samples from Jalisco, Mexico, while

King's [1981] samples were from Guerrero, Mexico. Preliminary genetic data suggest that *P. lanei* is likely a species complex composed of multiple, undescribed species [Blair et al., 2015]. Thus, differences between studies may reflect karyotypic variation among cryptic taxa. Further complicating matters, 5 *Phyllodactylus* species (*P. delcampoi*, *P. bordai*, *P. tuberculosus*, *P. papenfussi*, and *P. lanei*) all occur in Guerrero where King's samples originated [Dixon and Kluge, 1964; Murphy et al., 2009; Gamble, 2010], and it is possible that King's samples were misidentified.

The large number of female-specific RAD markers identified in *P. wirshingi* suggests significant differentiation between the Z and W chromosomes. Indeed, compared to other squamate species, using similarly prepared *SbfI* RADseq libraries, the 539 sex-specific *P. wirshingi* RAD markers are second only to the 855 female-specific RAD markers identified in the ZZ/ZW gecko *Christinus marmoratus* [Gamble et al., 2015a]. Because *C. marmoratus* has heteromorphic sex chromosomes [King and Rofe, 1976], it is probable that *P. wirshingi* also has cytogenetically differentiated Z and W chromosomes. However, since it is not clear whether there is a direct relationship between the sequence similarity of sex chromosomes and the degree of chromosomal heteromorphism [Vicoso et al., 2013; Gamble et al., 2014], this would need to be verified cytogenetically.

Using sex-specific RAD markers to identify a species' sex chromosomes typically requires additional genomic resources because determining chromosomal synteny involves comparing gene identity and order among species. Although most RAD markers map to noncoding fragments, and thus provide little information as to their genomic location absent in a sequenced genome, a small number of RAD markers – usually less than 15% of RAD contigs – may overlap with a gene or other coding region [Amores et al., 2011; Baxter et al., 2011; Chutimanitsakun et al., 2011; Bruneaux et al., 2013]. Sequencing paired-end reads, as we have done here, will generate larger RAD contigs, which in turn increases the probability of identifying genes [Amores et al., 2011; Baxter et al., 2011; Gamble and Zarkower, 2014]. Sex-specific RAD markers with gene fragments can be used to query genomic assemblies of related species to identify the sex chromosomes [Bruneaux et al., 2013; Gamble and Zarkower, 2014; Fowler and Buonaccorsi, 2016; Qiu et al., 2016]. This is the approach we successfully applied here to identify synteny between the avian Z and the *P. wirshingi* ZZ/ZW.

Among amniotes, the ancestral autosome that became the avian Z has been recruited into a sex-determining role

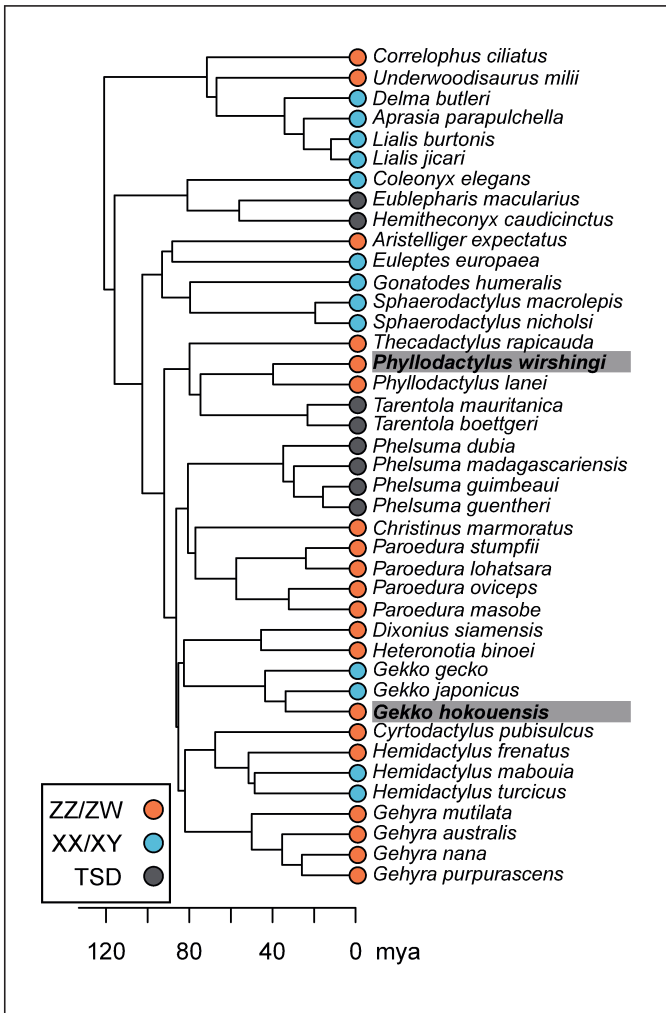


Fig. 4. Evolution of sex-determining mechanisms in geckos. Colored circles at the tips of the phylogenetic tree indicate sex-determining systems of selected species. Sex chromosomes in *Phyllodactylus wirshingi* and *Gekko hokouensis* (highlighted in gray) both share homology with the avian Z chromosome. Given their phylogenetic placement, it is likely that these have evolved independently. Sex chromosome data were taken from multiple sources [Gamble, 2010, and citations therein; Gornung et al., 2013; Gamble et al., 2015a, 2018, and citations therein; Rovatsos et al., 2016a]. Phylogeny modified from Gamble et al. [2015b].

at least 5 times (Fig. 3): in birds; in the ZZ/ZW gecko, *Gekko hokouensis*; in an XX/XY clade of turtles, *Staurotypus salvinii* and *S. triporcatus*; as part of the multiple sex chromosomes in monotremes; and finally, the ZZ/ZW sex chromosomes of *P. wirshingi* [Veyrunes et al., 2008; Kawai et al., 2009; Kawagoshi et al., 2014]. Comparative analyses, including cytogenetics and genome-scale datasets, have identified the sex chromosomes in at least 8

other amniote lineages, which were recruited from ancestral autosomes not homologous with the avian Z [Matsubara et al., 2006; Veyrunes et al., 2008; Graves, 2008; Kawagoshi et al., 2009, 2012, 2014; Kawai et al., 2009; Alföldi et al., 2011; Deakin et al., 2016; Montiel et al., 2016; Rovatsos et al., 2016b; Gamble et al., 2017]. Furthermore, comparative FISH analyses using fluorescently labeled chromosome paints have shown that the sex chromosomes of additional geckos, *Lialis burtonis* (XXXX/XXY), *Coleonyx elegans* (XXXX/XXY), and *C. marmoratus* (ZZ/ZW), are not homologous to the avian ZZ/ZW [Pokorná et al., 2011; Matsubara et al., 2014]. Because *P. wirshingi* and *G. hokouensis* possess ZZ/ZW sex chromosomes that are homologous with the avian Z, it is worth asking whether their ZZ/ZW sex chromosomes are derived from a common ancestor with “avian ZW” sex chromosomes [Ezaz et al., 2017]. While not impossible, it appears unlikely in this case. The phylogenetic distribution of these taxa, coupled with the numerous transitions among sex chromosome systems across geckos more generally, strongly suggests that the *P. wirshingi* and *G. hokouensis* ZZ/ZW systems derived independently (Fig. 4).

The repeated recruitment of the avian Z as a sex chromosome in amniotes can inform the search for factors that determine which ancestral chromosome will become a sex chromosome. There are several competing hypotheses to address this. For example, it has been hypothesized that one or more of these extant sex chromosome systems are ancestral and thus exist in multiple lineages due to inheritance from a common ancestor [Graves and Peichel, 2010; O’Meally et al., 2012; Ezaz et al., 2017]. As mentioned above, this seems unlikely, at least in geckos. However, there remain 2 other hypotheses that we can consider here. First, some autosomes, because of their gene content, may be “better” at being sex chromosomes than others. These chromosomes may host genes playing a role in the sex-determining pathway that can be co-opted into controlling sex determination [Graves and Peichel, 2010; O’Meally et al., 2012]. The avian Z, for example, contains *DMRT1*, a member of a gene family involved in sex determination and sexual differentiation in all animals, and the likely sex-determining gene in birds and several other vertebrates [Raymond et al., 1998; Matsuda et al., 2002; Nanda et al., 2002; Yoshimoto et al., 2008; Smith et al., 2009; Matson and Zarkower, 2012; Chen et al., 2014; Hirst et al., 2017]. Under this scenario, those chromosomes that are “better at being sex chromosomes” should be preferentially recruited into a sex-determining role in different lineages. Second, laboratory experiments with the roundworm *Caenorhabditis elegans* suggest that al-

most any kind of gene can become a sex-determining gene and every chromosome a sex chromosome [Hodgkin, 2002]. Thus, sex chromosome recruitment should be random, and there should be no biases when sex chromosome synteny is examined in a phylogenetic context. Differentiating between these 2 alternatives is not straightforward because we only know the sex chromosomes of a small number of taxa. Indeed, the sex chromosome identity of nearly two-thirds of amniote sex chromosome systems remains unknown (Fig. 3). To resolve this requires an approach that integrates phylogenetic, cytogenetic, and genomic analyses, and exploits species-rich vertebrate model clades in which many transitions among sex chromosome systems have occurred, e.g., amniotes and/or squamates. Only after identifying the sex chromosomes in most or all of the relevant lineages, we can say with any certainty whether there have been biases in which chromosomes get recruited into a sex-determining role. The current study makes an incremental step in achieving this objective.

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Statement of Ethics

All experiments were carried out in accordance with animal use protocols at University of Minnesota (0810A50001 and 1108A03545) and Marquette University (AR279 and AR288). Samples were collected under Puerto Rico permits DRNA: 2013-IC-006, 2014-IC-042, and 2016-IC-091.

Disclosure Statement

The authors declare no conflicts of interest.

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