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Abstract

Current understanding of sex chromosome evolution is largely dependent on species with highly degenerated, heteromorphic sex chromosomes, but by studying species with recently evolved or morphologically indistinct sex chromosomes we can greatly increase our understanding of sex chromosome origins, degeneration and turnover. Here, we examine sex chromosome evolution and stability in the gecko genus *Aristelliger*. We used

RADseq to identify sex-specific markers and show that four *Aristelliger* species, spanning the phylogenetic breadth of the genus, share a conserved ZZ/ZW system syntenic with avian chromosome 2. These conserved sex chromosomes contrast with many other gecko sex chromosome systems by showing a degree of stability among a group known for its dynamic sex-determining mechanisms. Cytogenetic data from *A. expectatus* revealed homomorphic sex chromosomes with an accumulation of repetitive elements on the W chromosome. Taken together, the large number of female-specific *A. praesignis* RAD markers and the accumulation of repetitive DNA on the *A. expectatus* W karyotype suggest that the Z and W chromosomes are highly differentiated despite their overall morphological similarity. We discuss this paradoxical situation and suggest that it may, in fact, be common in many animal species.

1 INTRODUCTION

Sex chromosomes evolve from an autosomal chromosome pair that acquires a sex-determining locus. The canonical model of sex chromosome evolution suggests linkage between the sex-determining locus and some neighbouring sexually antagonistic allele occurs via recombination suppression between the X and Y (or Z and W) chromosomes (Charlesworth, **1991**; Muller, **1914**; Ohno, **1967**). However, recombination suppression between the proto-sex chromosomes (the X/Y and Z/W) prevents DNA repair and can lead to an accumulation of deleterious mutations and repetitive DNAs on the sex-specific chromosome (the Y or W; Charlesworth, **1991**; Charlesworth & Charlesworth, **2000**). Given sufficient time, this degeneration can result in a pair of morphologically distinct, or heteromorphic, sex chromosomes. Historically, cytogenetic methods were used to identify a species' sex chromosome system based on the presence of these heteromorphic sex chromosomes in one sex and not the other. However, when taxa exhibit morphologically similar (homomorphic) sex chromosomes, classic cytogenetic methods fall short. This has led to large knowledge gaps concerning the taxonomic distribution of sex chromosomes (Bachtrog et al., **2014**; Devlin & Nagahama, **2002**; Ezaz, Sarre, O'Meally, Graves, & Georges, **2009**; Gamble & Zarkower, **2014**; Matsubara et al., **2006**; Schmid & Steinlein, **2001**; Stöck et al., **2011**).

The advent of high-throughput DNA sequencing has led to new methods for identifying homomorphic sex chromosomes, resulting in a rapid expansion of our knowledge of sex chromosome systems across the tree of life (Gamble, **2016**). One such method uses genetic markers generated via restriction site-associated DNA sequencing (RADseq; Baird et al., **2008**), which utilizes the naturally occurring restriction enzyme cut sites distributed throughout the genome to generate tens of thousands of markers (RADtags). Comparing RADtags from multiple males and females of a species can identify a small fraction of markers that corresponds to the sex-specific sex chromosomes (i.e. the Y or the W). Species with an abundance of male-specific RAD markers have an XX/XY sex chromosome system, and species with an abundance of female-specific RAD markers have a ZZ/ZW system (Gamble et al., **2015**; Gamble & Zarkower, **2014**; Pan et al., **2016**). This method has been used to identify homomorphic sex chromosomes across a wide range of taxa (Fowler & Buonaccorsi, **2016**; Gamble et al., **2015**; Jeffries et al., **2018**; Nielsen, Daza, Pinto, & Gamble, **2019**; Pan et al., **2016**) and detect important and unexpected transitions among sex chromosomes (Gamble et al., **2017**; Nielsen, Banks, Diaz, Trainor, & Gamble, **2018**). Such methods, particularly when combined with modern cytogenetics (Deakin et al., **2019**), are building a greater foundation on which to study the evolutionary processes governing sex chromosome origins, degeneration and stability.

Gecko lizards exhibit both male and female heterogamety as well as temperature-dependent sex determination (TSD), wherein egg incubation temperature determines sex. Notably, geckos exhibit the highest number of identified sex-determining system transitions of any amniote group with between 17 and 25 transitions identified thus far (Gamble, **2010**; Gamble et al., **2015**). The gecko family Sphaerodactylidae consists of over 200 species in 12 genera broadly distributed in South America, the Caribbean, North Africa, the Middle East and

Central Asia (Gamble, Bauer, et al., **2011**; Gamble, Bauer, Greenbaum, & Jackman, **2008**; Gamble, Daza, Colli, Vitt, & Bauer, **2011**). Despite this rich species diversity, sex-determining systems are known in only a handful of species. Of the 12 sphaerodactylid species that have been karyotyped (Table **S1**), only one, the XX/XY species *Eupletes europaea*, can confidently be said to possess heteromorphic sex chromosomes (Gamble, **2010**; Gornung, Mosconi, Annesi, & Castiglia, **2013**). *Gonatodes ceciliae* has heteromorphic chromosomes, which have been interpreted as XX/XY sex chromosomes (McBee, Bickham, & Dixon., **1987**). However, the diverse karyotypes among sampled individuals, lack of published female karyotypes and failure of the heteromorphic chromosomes to form sex bivalents raise doubts that these are sex chromosomes (Schmid et al., **2014**). The remaining karyotyped species have homomorphic sex chromosomes (Schmid et al., **2014**). More recently, RADseq methods have been used to successfully identify additional XX/XY species (*Sphaerodactylus nicholsi*, *S. inigoi* and *Gonatodes ferrugineus)* as well as the first (and currently only) sphaerodactylid species with a ZZ/ZW sex chromosome system, *Aristelliger expectatus* (Gamble et al., **2015**, **2018**). This suggests a minimum of one sex chromosome transition in the Sphaerodactylidae. Based on the high level of sex chromosome turnover observed in other gecko clades, additional transitions are likely to be uncovered as more data are generated.

The sphaerodactylid genus *Aristelliger*, commonly known as croaking geckos, is comprised of nine species distributed in the Caribbean and Central America (Bauer & Russell, **1993**; Diaz & Hedges, **2009**; Schwartz & Henderson, **1991**). This charismatic group diverged from its sister genus *Quedenfeldtia* approximately 70 million years ago (Gamble, Bauer, et al., **2011**) and differs from other New World sphaerodactylids in being nocturnal, arboreal, possessing large basal adhesive toepads, and exhibiting the largest range of body sizes of all sphaerodactylids (Griffing, Daza, DeBoer, & Bauer, **2018**; Henderson & Powell, **2009**; Schwartz & Henderson, **1991**). As previously mentioned, *Aristelliger expectatus* represents the only sphaerodactylid species with a confirmed ZZ/ZW sex chromosome system (Gamble et al., **2015**). Here, we combine newly generated RADseq data with traditional cytogenetics to identify and characterize sex chromosomes in three additional *Aristelliger* species, *A. praesignis*, *A. lar* and *A. barbouri*. We focus on two main questions: (1) Do other species of *Aristelliger* possess a ZZ/ZW sex chromosome system? And, if so, (2) are the sex chromosomes homologous across the genus? In contrast with what has been observed in other geckos, a group noted for dynamic sex chromosome turnover, we here recover a conserved sex chromosome system among all sampled *Aristelliger* species.

2 MATERIALS AND METHODS

2.1 RADseq

We extracted DNA from eleven males and ten females of *A. praesignis* using the QIAGEN DNeasy Blood and Tissue Kit (Table **S2**). We generated single-digest RADseq libraries using a modified protocol from Etter, Bassham, Hohenlohe, Johnson, and Cresko (**2012**) as described in Gamble et al. (**2015**). Briefly, we digested genomic DNA using a high-fidelity *Sbf1* restriction enzyme (New England Biolabs) and ligated individually barcoded P1 adapters to each sample. We pooled samples into multiple libraries, sonicated and size-selected for 200- to 500-bp fragments using magnetic beads in a PEG/NaCl buffer (Rohland & Reich, **2012**). We then blunt-end-repaired, dA-tailed and ligated pooled libraries with a P2 adapter containing unique Illumina barcodes. Pooled libraries were amplified using NEBNext Ultra II Q5 polymerase (New England Biolabs) for 16 cycles and size-selected a second time for 250- to 650-bp fragments that now contained Illumina adapters and unique barcodes. Libraries were sequenced using paired-end 125 bp reads on an Illumina HiSeq 2500 at the Medical College of Wisconsin.

2.2 Bioinformatic analysis

We analysed the RADseq data using a previously described bioinformatics pipeline (Gamble et al., **2015**). Raw Illumina reads were demultiplexed, trimmed and filtered using the process_radtags function in STACKS (1.41,

Catchen, Amores, Hohenlohe, Cresko, & Postlethwait, **2011**). We used RADtools (1.2.4, Baxter et al., **2011**) to generate RADtags for each individual and identified candidate loci and alleles from the forward reads. We then used a custom Python script (Gamble et al., **2015**) to identify putative sex-specific markers from the RADtools output, that is markers found in one sex but not the other. The script also generated a list of 'confirmed' sex-specific RAD markers that excluded any sex-specific markers found in the original read files of the opposite sex. Finally, we used Geneious (R11, Kearse et al., **2012**) to assemble the forward and reverse reads of 'confirmed' sex-specific RAD markers. These loci should correspond to genomic regions unique to a single sex, the Y or W chromosome, such that female-specific markers denote a ZZ/ZW system, whereas male-specific markers suggest a XX/XY system.

We used BLAST (Altschul, Gish, Miller, Myers, & Lipman, **1990**) to query the sex-specific *A. expectatus* RAD markers from Gamble et al. (**2015**) to the assembled *A. praesignis* RAD markers. We then aligned sex-specific markers found in both species to assess homology and design PCR primers.

2.3 Validating sex-specific markers

We PCR-validated a subset of sex-specific markers for four *Aristelliger* species and visualized the results with gel electrophoresis. PCR primers (Table **S3**) were designed in Geneious (R11, Kearse et al., **2012**). We used ten males and ten females of *A. praesignis*, six males and three females of *A. lar*, eight males and seven females of *A. expectatus*, and one male and two females of *A. barbouri*.

One putative W-linked markers appeared to retain high-sequence similarity to homologous regions on the Z chromosome, a pattern detectable by amplification in both males and females (Fowler & Buonaccorsi, **2016**; Gamble et al., **2018**). To overcome this, we used a PCR-RFLP assay where primers were designed to span a diagnostic female-specific restriction site. The marker is PCR-amplified in both males and females, but only the female-specific W allele contains the restriction site. Thus, amplified female PCR amplicons will be cut by the restriction enzyme and display multiple bands, whereas male PCR amplicons will display a single band. Following amplification, we cleaned the PCR solution with serapure beads, washed it thoroughly with freshly prepared 85% EtOH and resuspended the amplicon in buffer. We added 1µl of high-fidelity *Sbf1* restriction enzyme (New England Biolabs) and 5µl of CutSmart Buffer (New England Biolabs), and added nuclease-free water to increase the solution volume to 50µl. We incubated the solution at 37ºC for 30 min and 80ºC for 20 min. Following restriction digest, we visualized the results on a 1% agarose gel.

We determined synteny of the *Aristelliger* sex chromosomes by comparison with the chicken (*Gallus gallus*) genome using BLAST to query female-specific *A. expectatus* and *A. praesignis* RAD markers to chicken protein-coding genes (International Chicken Genome Sequencing Consortium, **2004**; Warren et al., **2017**). In some cases, we used a modest draft assembly of the *Gonatodes ferrugineus* genome (Gamble et al., **2018**) as an intermediary BLAST step to identify larger scaffolds before aligning to the chicken genome. *Gonatodes ferrugineus* is the only sphaerodactylid gecko with a genome-scale resource currently available. By identifying syntenic regions, we can assess the homology of sex-linked loci among the *Aristelliger* species; if the markers are found on different chicken chromosomes, then a *cis*-turnover occurred such that a ZZ/ZW (or XX/XY) sex chromosome system in one species transitioned to a different ZZ/ZW (or XX/XY) sex chromosome system in another species. If the markers map to the same chicken chromosome, this suggests a shared homologous sex chromosome system.

2.4 *Aristelliger* phylogeny

Interpreting our results in an evolutionary framework required a phylogenetic hypothesis for sampled *Aristelliger* species. We PCR-amplified and Sanger-sequenced the mitochondrial gene NADH dehydrogenase subunit 2 (ND2) and adjacent tRNAs with the primers L4437b (Macey, Larson, Ananjeva, & Papenfuss, **1997**), L5005 (Jennings, Pianka, & Donnellan, **2003**) and H5934a (Arevalo, Davis, & Sites, **1994**). We

aligned these new sequences with previously published sequences (Gamble, Greenbaum, Jackman, Russell, & Bauer, **2012**) in Geneious (R9.1.6, Kearse et al., **2012**) using MUSCLE (Edgar, **2004**) and inspected the resulting alignment by eye for errors. We constructed a maximum likelihood tree using RAxML (V8.2, Stamatakis, **2014**) with a GTR + GAMMA model. Branch support was generated using 1,000 bootstrap replicates. We included *Quedenfeldtia trachyblepharus* and *Quedenfeldtia moerens* as outgroups. Finally, we calculated between-group genetic p-distance for putative species using MEGA X (V10.1, Kumar, Stecher, Li, Knyaz, & Tamura, **2018**).

2.5 Cytogenetics

Chromosome spreads from two males and two females of *A. expectatus* were prepared from fibroblasts established from tail clips following published lizard cell culture protocols (Ezaz et al., **2008**; Gamble, Geneva, Glor, & Zarkower, **2014**; Main, Scantlebury, Zarkower, & Gamble, **2012**). Fibroblasts were grown at 28–31°C in media containing DMEM 1X (Invitrogen) with 4.5 g/L glucose and L-glutamine without sodium pyruvate, 20% foetal bovine serum and anti–anti (Invitrogen), which contains penicillin, streptomycin and amphotericin. Cells were arrested in metaphase using vinblastine sulphate (1 mg/ml), collected after trypsin digestion and incubated in a 0.07 M KCl hypotonic solution for 20 min in a 37°C water bath. Cells were fixed and washed in methanol:acetic acid (3:1). Cell suspensions were then dropped onto clean glass slides, allowed to air-dry and dehydrated in an ethanol series (70%, 95%, 100%). Slides were stained with DAPI (4,6-diamidino-2-phenylindole) and mounted with PermaFluor (Lab Vision) and a cover slip. All slides were photographed on a Zeiss Imager Z1 microscope using a Zeiss MRm camera. Images were captured using Zeiss AxioVision software.

We assessed the accumulation of repetitive DNA sequences on the putative sex chromosome by hybridizing a fluorescently labelled GATA satellite repeat onto metaphase spreads. The GATA satellite repeat, also called the Bkm satellite repeat, has been shown to accumulate onto the sex chromosomes of multiple animal species and is a good candidate marker for identifying sex chromosomes (Jones & Singh, **1981**; Nanda et al., **1990**; O'Meally et al., **2010**; Perry et al., **2018**; Singh, Purdom, & Jones, **1980**). $(GATA)_n$ probes were generated by PCR in the absence of template DNA (Ijdo, Wells, Baldini, & Reeders, **1991**) using $(GATA)_7$ and $(TATC)_7$ primers. Probes were labelled via nick translation with ChromaTide/Alexa Fluor fluorescently labelled dUTP 488-5 (Life Technologies). We confirmed the sizes of the nick translated fragments by electrophoresis on a 1% TBE gel. Labelled DNA was ethanol precipitated and resuspended in 100 µl hybridization buffer (Ezaz et al., **2005**), denatured at 72°C for 10 min and snap-cooled on ice for five minutes. We added 20 µl of probe to each slide, affixed a cover slip using rubber cement, heated slides again to 72°C for 5 min and incubated overnight at 37°C. Slides were washed once at 60°C in 0.4% SSC and 0.3% Igepal CA-630 (Sigma-Aldrich) for two minutes, followed by a second two-minute wash in 2% SSC and 0.1% Igepal CA-630 at room temperature. Slides were dehydrated in an ethanol series (70%, 95%, 100%) and air-dried. Slides were stained with 4,6-diamidino-2-phenylindole (DAPI) and mounted with a cover slip using PermaFluor (Lab Vision). We performed GATA fluorescent in situ hybridization (FISH) experiments on male and female slides in parallel and in four replicates to control for batch-related variation in hybridization conditions.

3 RESULTS

3.1 Identifying ZW sex chromosomes

We identified 127,937 RAD loci with two or fewer alleles in *A. praesignis*. Of these, we found 878 female-specific markers and no male-specific markers. After checking the female markers against the original male reads, we retained 743 'confirmed' female-specific markers. We designed PCR primers for eleven of them, and seven amplified in a female-specific pattern **(**Figure **1**), whereas the remaining four failed to amplify in a sex-specific manner or did not amplify at all. We found three of these markers also amplified in a sex-specific manner in *A. lar* (Figure **1**), which shows both *A. praesignis* and *A. lar* share the same ZZ/ZW sex chromosome system.

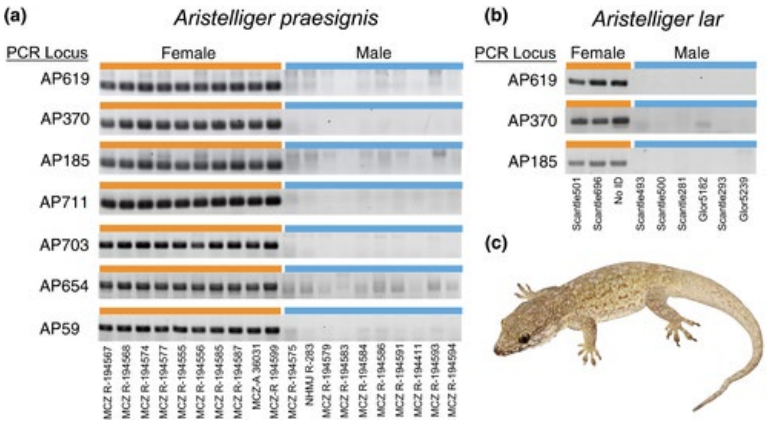

FIGURE 1 PCR validation of female-specific RADseq markers in (a) *Aristelliger praesignis* and (b) *Aristelliger lar*. Specimen identification numbers are listed below. (c) Photograph of *A. praesignis*

We used BLAST to identify four female-specific *A. praesignis* RAD markers that contained fragments of chicken protein-coding genes (Table **1**). Three of these genes are on chicken chromosome 2 and one on chicken chromosome 33. Using the *G. ferrugineus* genome as an intermediate step, we discovered one additional *A. praesignis* female-specific RAD marker near the *ZNRF2* gene, also on chicken chromosome 2. PCR primers amplified in a sex-specific manner for two of these genes: *MSANTD3* and *ZNRF2*. A third gene, *EPC1*, amplified in both females and males but contained a diagnostic female-specific restriction site, and we used PCR-RFLP to visual differences between males and females. *EPC1* and *ZNRF2* are found on the p arms of chicken chromosome 2, and *MSANTD3* is found on the q arms of chicken chromosome 2 (Figure **2**). All three genes, *EPC1*, *ZNRF2* and *MSANTD3*, are located on chromosome 6 in *Anolis carolinensis*. Two of the three loci, *MSANTD3* and *EPC1*, were sex-linked in all four *Aristelliger* species, although with slightly different banding patterns; *MSANTD3* produced a single band only in females (Figure **2c**), whereas *EPC1*, following a post-PCR restriction digest with enzyme *Sbf1*, produced two bands in females (the smaller of which varied in intensity) and a single band in males (Figure **2d**). *ZNRF2* amplified in a female-specific manner for *A. lar* and *A. praesignis* but failed to amplify in *A. expectatus* or *A. barbouri* (Figure **2b**).

TABLE 1. Results from BLAST of the female-specific *Aristelliger praesignis* RAD contigs queried against chicken (*Gallus gallus*) genes demonstrating synteny with avian chromosome 2

Ensembl ID	Gene	Gallus chromosome	E value	A. praesignis query
ENSGALG00000013403	MSANTD3	2	4.29E−88	Ap_727
ENSGALG00000048612	RPS26	33	6.07E−87	Ap_289
ENSGALG00000007435	RAB18	2	4.15E−76	Ap_450
ENSGALG00000007169		2	3.80E−70	Ap_138

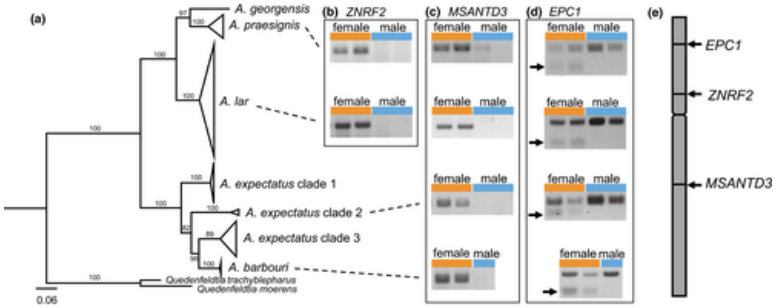

FIGURE 2 (a) Maximum likelihood phylogeny of the mitochondrial ND2 gene showing phylogenetic relationships among sampled *Aristelliger* taxa. Bootstrap values are shown on branches. The phylogeny was rooted

with *Quedenfeldtia*. PCR validation for sex-specific markers *ZNRF2* (b), *MSANTD3* (c) and *EPC1* (d) in *A. praesignis*, *A. lar*, *A. expectatus* and *A. barbouri*. Sex-specific amplification is denoted by the presence of a band in females and lack of a band in males (b and c), or by the presence of a second band in females following a *Sbf1* restriction digest (d, shown by black arrow). *MSANTD3* and *EPC1* were sex-specific in all four species, and *ZNRF2* was sex-specific in *A. praesignis* and *A. lar*. For ease of viewing, two males and two females were used for validation in all species except for *A. barbouri*, for which only a single male tissue was available. (e) Approximate location of *EPC1*, *ZNRF2* and *MSANTD3* on chicken chromosome 2

3.2 Phylogenetic results

The mitochondrial maximum likelihood phylogeny inferred two major *Aristelliger* clades (Figure **2a**). One corresponds to the subgenus *Aristelligella* (Noble & Klingel, **1932**), containing *A. expectatus* and *A. barbouri*. The second clade, subgenus *Aristelliger*, contains *A. praesignis*, *A. lar* and *A. georgeensis* (due to a lack of available samples, the latter was not included in the sex chromosome system part of this study). We recovered three clades corresponding to *A. expectatus* that are rendered paraphyletic by *A. barbouri*. The *A. expectatus* clade 3 is most closely related to *A. barbouri*, and these two sister clades had the lowest between-group p-distance (0.159, Table **2**). All branches were highly supported (BS > 90), except for the divergence of *A. barbouri* and *A. expectatus* clade 3 from *A. expectatus* clade 2 (Figure **2**).

TABLE 2. Between-group genetic p-distances for a fragment of the mitochondrial ND2 gene. *Aristelliger expectatus* was split into three clades corresponding to the mtDNA phylogeny (Figure 2)

	Q. moerens	Q. trachyblepharus	A. georgensis	A. praesignis	A. lar	A. expectatus clade 1	A. expectatus clade 2	A. expectatus clade 3	A. barbouri
Q. Moerens									
Q. trachyblepharus	0.140								
A. georgensis	0.310	0.310							
A. praesignis	0.291	0.284	0.130						
A. lar	0.300	0.292	0.155	0.139					
A. expectatus clade 1	0.305	0.303	0.218	0.215	0.233				
A. expectatus clade 2	0.318	0.297	0.239	0.228	0.229	0.166			
A. expectatus clade 3	0.331	0.312	0.235	0.226	0.224	0.163	0.165		
A. barbouri	0.412	0.393	0.259	0.292	0.292	0.196	0.188	0.159	

3.3 Cytogenetics

Examination of mitotic cells from four *A. expectatus* individuals revealed a diploid number of 30, with three pairs of large metacentric chromosomes, one large acrocentric pair and eleven pairs of acrocentric/biarmed chromosomes gradually decreasing in size (Figure **3**). Roughly half of the examined cells had a complete chromosomal complement: females—TG1447 (13 of 24) and TG1449 (1 of 2); males—TG1448 (12 of 24) and TG1451 (4 of 6).

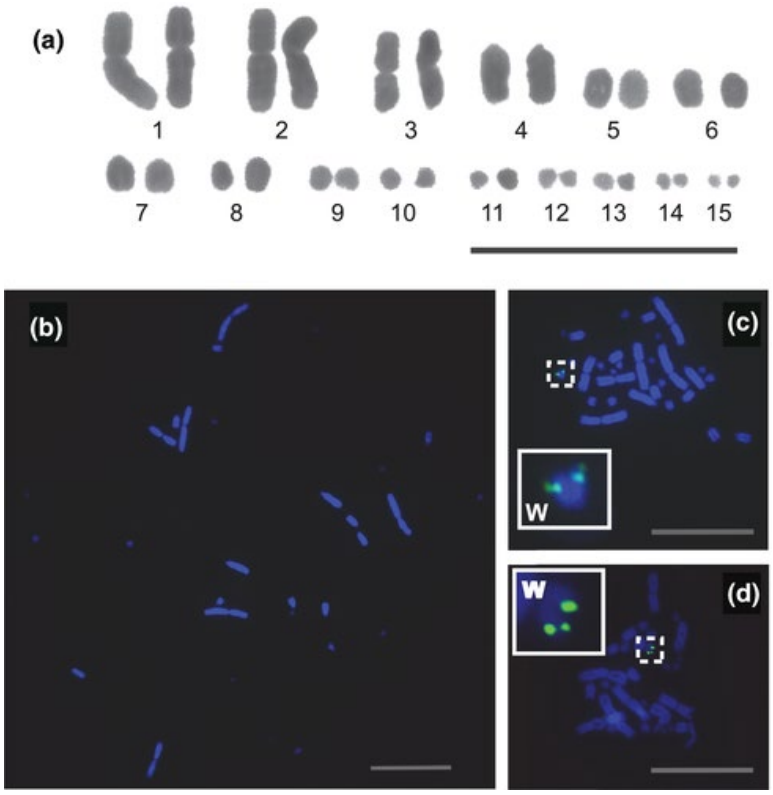

FIGURE 3 (a) Karyotype (2*n* = 30) of a male *Aristelliger expectatus* (TG1448). Fluorescent in situ hybridization (FISH) of the GATA minisatellite to chromosomes of a male (b; TG1451) and two female *Aristelliger expectatus* (c; TG1447; d; TG1449). GATA hybridization (green) is sex-specific, occurring only on the distal arms of the W chromosome. Solid lines indicate magnified views of areas in dashed lines. Scale bars = 20 μm

The GATA satellite repeat hybridized to the distal arms of a single small, biarmed chromosome in female cells with no GATA hybridization in male cells (Figure **3**). This pattern held for incomplete cells as well, and we did not observe any GATA hybridization in any male cells: TG1448 (8 cells) and TG1451 (6 cells). Similarly, we did not observe more than a single chromosome with GATA hybridization in nonoverlapping female cells: TG1447 (16 cells) and TG1449 (2 cells). The observed pattern of a single chromosome possessing GATA signal in females with no concomitant signal in males is consistent with a ZZ/ZW sex chromosome system with the W being the GATA hybridized chromosome. The size and shape of the ZW pair are most consistent with pair eight but given that there are no other apparent differences among the Z and W chromosomes exact identification is not possible at this time.

4 DISCUSSION

Our results demonstrate that all *Aristelliger* species studied to date possess a ZZ/ZW sex chromosome system. Furthermore, because the species studied here encompass the phylogenetic breadth of the genus (Hecht, **1952**), we can conservatively hypothesize that all *Aristelliger* species share an ancestral ZZ/ZW sex chromosome system

homologous to chicken chromosome 2 and *Anolis* chromosome 6. The large number of female-specific markers in *A. praesignis* (743 'confirmed' female-specific RAD markers) suggests that the Z and W chromosomes are highly differentiated from one another in this species. Additionally, the accumulation of GATA repeats on the W chromosome of *A. expectatus* is indicative of nascent deterioration, and similar patterns have been observed on the sex chromosomes of many plant and animals species (Gamble et al., **2014**; Jones & Singh, **1981**; Marais et al., **2008**; Nanda et al., **1990**; O'Meally et al., **2010**; Parasnis, Ramakrishna, Chowdari, Gupta, & Ranjekar, **1999**; Schäfer, Böltz, Becker, Bartels, & Epplen, **1986**; Singh et al., **1980**). This suggests that although the *Aristelliger* W chromosome has begun to accumulate repetitive sequences and sex-specific mutations, it has yet to purge mutation-rich regions to produce a morphologically distinct, or heteromorphic, W chromosome as seen in some other groups such as birds or Lepidoptera (Charlesworth & Charlesworth, **2000**).

Homomorphic sex chromosomes are not necessarily young or lacking degeneration but are simply cytogenetically indistinguishable. For example, flightless ratite birds, such as ostriches and emus, have maintained homomorphic sex chromosomes for approximately 100 million years (Ogawa, Murata, & Mizuno, **1998**; Zhou et al., **2014**), whereas the homologous sex chromosomes of other bird taxa are heteromorphic (Zhou et al., **2014**). It is thus possible for a sex chromosome pair to have distinct gene content and significant allelic differences even though they appear to be morphologically similar (Conte, Gammerdinger, Bartie, Penman, & Kocher, **2017**; Fontaine et al., **2017**; Gamble et al., **2014**; Perry et al., **2018**; Toups, Rodrigues, Perrin, & Kirkpatrick, **2019**; Zhou et al., **2014**), including the *Aristelliger* sex chromosomes presented here. Indeed, there are examples in the literature of sex chromosomes exhibiting various combinations of genetic differentiation and chromosomal morphology (Darolti et al., **2019**; Gamble et al., **2014**; Kamiya et al., **2012**; Kottler et al., **2020**; Lahn & Page, **1999**; Zhou et al., **2014**). Thus, the overly simplistic distinction between homomorphic and heteromorphic sex chromosomes that emerged when cytogenetics was the prevailing technology may fail to describe the continuum of differences between gametologs that can be distinguished using DNA sequence data (Furman et al., **2020**). As the sex chromosomes of more and more species are identified using DNA sequences, it may be more useful and accurate to simply describe the degree of genetic differentiation between gametologs. The terms homomorphic and heteromorphic may then resume their historical cytogenetic definition.

Previous work reported that *A. expectatus* possessed a ZZ/ZW sex chromosome system based on 10 'confirmed' female-specific RAD markers (Gamble et al., **2015**). Comparatively, we here report 743 'confirmed' female-specific markers for *A. praesignis*. The substantially lower number of female-specific markers within *A. expectatus* might be explained by cryptic diversity within the former as samples from Gamble et al. (**2015**) can be allocated to all three *A. expectatus* clades. Although Gamble et al. (**2015**) analysed all samples as a single taxon, our phylogenetic results suggest that *A. expectatus sensu lato* is composed of at least three distinct (species-level) lineages (Figure **2**—see below). Although the RADseq methodology used herein has a fairly high success rate, the bioinformatic pipeline may be sensitive to overly divergent samples. The sex-specific chromosomes (the Y and W) have a higher evolutionary rate than autosomes due to a lack of recombination and smaller effective population sizes that allow deleterious or nonsynonymous mutations to become fixed (Berlin & Ellegren, **2006**; Ellegren, **2011**). Thus, the *Aristelliger* W chromosome may accumulate species-specific mutations faster than the autosomes. The bioinformatic pipeline identifies W alleles shared among all sampled females and will not identify sex-specific alleles unique to any one of the three putative species when analysed together. We do not have sufficient sampling to analyse the *A. expectatus* clades individually but we suspect that there are many more lineage-specific sex-specific RAD markers that remain unidentified. However, we here show that this methodology can prove successful even with highly divergent samples (see also Hundt, Liddle, Nielsen, Pinto, & Gamble, **2019**; Nielsen, Pinto, Guzmán-Méndez, & Gamble, **2020**). Although we would not expect to find shared sex-specific RAD markers in all cases, these results should be encouraging in cases where samples are of uncertain taxonomic placement, not geographically proximate, or might have elevated genetic diversity.

However, in species with highly polymorphic Y or W chromosomes, these methods may result in a lower number of sex-specific RAD markers or, worst-case scenario, no markers at all. Further investigation is needed to examine the power of this approach among divergent populations and/or species.

Aristelliger expectatus is likely comprised of cryptic diversity. Genetic distances among the sampled *A. expectatus* mitochondrial clades were comparable to genetic distances between recognized gecko sister species, which typically range from 4.1% to 35.5% using the same fragment of the mitochondrial ND2 gene (Botov et al., **2015**; Grismer et al., **2014**; Oliver, Hutchinson, & Cooper, **2007**; Pepper, Doughty, & Keogh, **2006**; Portik, Travers, Bauer, & Branch, **2013**). This discovery is not unusual as molecular phylogenies have regularly uncovered undescribed species in other Neotropical gecko taxa (Daza et al., **2019**; Hedges & Conn, **2012**; Pinto et al., **2019**). Integrative taxonomy combining multi-locus phylogenetics and a re-examination of morphology is needed to formally describe these taxa.

The 2*n* = 30 karyotype of *A. expectatus* is unique among sphaerodactylid geckos (Table **S3**). It can be derived from a 2*n* = 36 karyotype of all acrocentric chromosomes, like that of the sphaerodactylid genera *Teratoscincus* (Manilo, **1993**; Zeng et al., **1998**) or *Chatogekko* (dos Santos, Bertolotto, Pellegrino, Rodrigues, & Yonenaga-Yassuda, **2003***)*, by a series of three centric fusions. This would result in three pairs of large metacentric chromosomes and a reduction of chromosome number from 2*n* = 36 to 2*n* = 30. King (**1987**) indicated the lack of cytogenetic data for sphaerodactylid genera made it premature to evaluate cytogenetic evolution and ancestral chromosome number in the clade and the same is still true today. Collecting cytogenetic data for additional sphaerodactylid genera should be a high priority to better understand the chromosomal evolution in the group.

Chicken chromosome 2/*Anolis* 6 is homologous to the sex-linked chromosome in *Python bivittatus* (Gamble et al., **2017**) and caenophidian snakes (Matsubara et al., **2006**; Vicoso, Emerson, Zektser, Mahajan, & Bachtrog, **2013**). Having become sex-linked in three independent squamate lineages, this linkage group appears to be a frequent sex chromosome candidate. It is possible the ancestral autosome contained some genomic content that makes it more likely to serve a sex determination function in multiple unrelated taxa (Graves & Peichel, **2010**; O'Meally, Ezaz, Georges, Sarre, & Graves, **2012**). This linkage group may harbour a gene (or multiple genes) involved in the sex differentiation pathway that has been co-opted to act as the master sex determination gene controlling the entire cascade of regulatory networks (Herpin & Schartl, **2015**). Among other reptiles, this potential sex-determining gene (or genes) might merely work within the confines of the sex differentiation pathway, but within *Aristelliger*, pythons and caenophidian snakes it has moved to the top of the determination pathway. Chicken chromosome 2 does not have any of the 'usual suspects' for sex determination, such as *DMRT1*, *FOXL2* or *SOX3*, but it does contain *CTNNB1*, a gene required for ovarian development (Liu, Bingham, Parker, & Yao, **2008**), and *SRD5A1*, required for spermatogenesis and sexual differentiation (O'Donnell, Stanton, Wreford, Robertson, & McLachlan, **1996**). However, for the moment, the sex-determining genes for all three groups remain unknown.

We here confirm a common origin of sex chromosomes in the most recent common ancestor of extant *Aristelliger* species, although the exact timing and circumstances of their origin remain unknown. Given that *Aristelliger* boast a ZZ/ZW sex chromosome system, yet other members of the Sphaerodactylidae exhibit the XX/XY condition, at least one turnover has occurred within the family, and possibly more if the XX/XY sex chromosome systems of *Sphaerodactylus*, *Gonatodes* and *Euleptes* are independently derived. In order to infer the age of the *Aristelliger* ZZ/ZW sex chromosomes and the directionality of sphaerodactylid sex chromosome turnovers, future work should aim to increase the sampling of sex chromosome systems among sphaerodactylid geckos, particularly for *Quedenfeldtia*, the closest relative of *Aristelliger* (Gamble et al., **2008**). Such an investigation will allow us to better understand the behaviour of gecko sex chromosomes and the evolution of sex chromosomes as a whole.

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CONFLICT OF INTEREST

We declare no conflicts of interest in regard to this manuscript.

AUTHOR CONTRIBUTIONS

T.G., D.P.S. and S.V.N. designed the study. A.H.G. and D.P.S. conducted fieldwork. S.E.K. and S.V.N. conducted the laboratory research. S.E.K., S.V.N. and T.G. analysed the data. S.E.K., A.H.G., S.V.N. and T.G. wrote the manuscript. All authors have read and approved the manuscript.

DATA AVAILABILITY STATEMENT

DNA sequences are deposited at GenBank accession numbers MN694928-MN694967 and NCBI Short Read Archive Bioproject PRJNA639828. The ND2 sequence alignment, ND2 maximum likelihood phylogeny and fasta file of 743 female-specific *A. praesignis* RAD markers are available on Dryad **https://doi.org/10.5061/dryad.573n5tb51**.

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