Current Biology

Report

The Discovery of XY Sex Chromosomes in a *Boa* and *Python*

Highlights

- All snakes were thought to possess the same ZW sex chromosome system
- There is no evidence that boas and pythons have ZW sex chromosomes
- Male-specific genetic markers in boa and python indicate XY sex chromosomes
- Comparative genomics reveals boa and python independently evolved XY systems

Authors

Tony Gamble, Todd A. Castoe, Stuart V. Nielsen, ..., Drew R. Schield, Gordon W. Schuett, Warren Booth

Correspondence

tgamble@geckoevolution.org

In Brief

Gamble et al. identify XY sex chromosomes in a boa and a python, overturning the long-held assumption that all snake species possess ZW sex chromosomes.

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The Discovery of XY Sex Chromosomes in a *Boa* and *Python*

Tony Gamble,[1,5,6,*] Todd A. Castoe,[2] Stuart V. Nielsen,[1] Jaison L. Banks,[1] Daren C. Card,[2] Drew R. Schield,[2] Gordon W. Schuett,[3] and Warren Booth[4]
[1]Department of Biological Sciences, Marquette University, Milwaukee, WI 53201, USA
[2]Department of Biology, University of Texas at Arlington, Arlington, TX 76019, USA
[3]Department of Biology and Neuroscience Institute, Georgia State University, Atlanta, GA 30303, USA
[4]Department of Biological Science, The University of Tulsa, Tulsa, OK 74104, USA
[5]Bell Museum of Natural History, University of Minnesota, St. Paul, MN 55108, USA
[6]Lead Contact
*Correspondence: tgamble@geckoevolution.org
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SUMMARY

For over 50 years, biologists have accepted that all extant snakes share the same ZW sex chromosomes derived from a common ancestor [1–3], with different species exhibiting sex chromosomes at varying stages of differentiation. Accordingly, snakes have been a well-studied model for sex chromosome evolution in animals [1, 4]. A review of the literature, however, reveals no compelling support that boas and pythons possess ZW sex chromosomes [2, 5]. Furthermore, phylogenetic patterns of facultative parthenogenesis in snakes and a sex-linked color mutation in the ball python (*Python regius*) are best explained by boas and pythons possessing an XY sex chromosome system [6, 7]. Here we demonstrate that a boa (*Boa imperator*) and python (*Python bivittatus*) indeed possess XY sex chromosomes, based on the discovery of male-specific genetic markers in both species. We use these markers, along with transcriptomic and genomic data, to identify distinct sex chromosomes in boas and pythons, demonstrating that XY systems evolved independently in each lineage. This discovery highlights the dynamic evolution of vertebrate sex chromosomes and further enhances the value of snakes as a model for studying sex chromosome evolution.

RESULTS AND DISCUSSION

Reevaluation of Sex Chromosome Evolution in Snakes

Sex chromosomes have evolved repeatedly and independently in various plant and animal lineages [8, 9]. Sex chromosomes evolve from autosomes, and the first step in this transition is the evolution of a sex-determining gene followed by restricted recombination around the sex-determining locus and linked sexually antagonistic alleles [8, 10]. Under this model, newly evolved sex chromosomes are cytogenetically similar to each other or homomorphic. Inversions and other rearrangements, and the loss or gain of genetic material related to restricted recombination, can, over time, result in morphological differences between the X and Y (or Z and W), leading to karyotypically distinct heteromorphic sex chromosomes [10–13]. This model of sex chromosome evolution represents the current dominant paradigm and explains differences in gene content between the X and Y (or Z and W), the presence of gene dosage differences involving hemizygous alleles on the heteromorphic sex chromosomes and the subsequent evolution of dosage compensation to correct for those differences, and the evolutionary stability of sex chromosomes in certain lineages [8, 13–15]. This hypothesis was originally derived from studying *Drosophila* [16], although it was the discovery of sex chromosomes at all stages of differentiation in both snakes and birds that suggested that this process may occur universally across taxa with genetic sex-determining systems [1, 4].

Cytogenetic data from early work on snakes were crucial in formulating the prevailing theory of sex chromosome evolution [1]. The "advanced" snakes (caenophidians) exhibited a heteromorphic ZW sex chromosome system at various stages of differentiation, whereas boas and pythons (henophidians) were purported to have a homomorphic ZW sex chromosome system [1, 4]. Claims that boas and pythons have a ZW sex chromosome system were not, however, supported by empirical data, and despite decades of study and numerous published karyotypes, there is no direct evidence that boas and pythons have a ZW sex chromosome system (Table S1). Lack of robust evidence is not surprising, however, because detecting male or female heterogamety in species with homomorphic sex chromosomes is not possible with standard cytogenetic methods; instead, it requires other kinds of experimental evidence, such as breeding experiments involving sex-reversed individuals or the development of sex-specific genetic markers [17]. A review of the cytogenetic literature (Table S1) confirms that, with one exception [18], there are no published reports of cytogenetically identifiable sex chromosomes in a boa or python species. That exception involved finding a heteromorphic pair of chromosomes in a single Dumeril's boa (*Acrantophis dumerili*) from Madagascar. Most of the snakes in that study, however, were sampled nonlethally and their sexes were not recorded, rendering the results ambiguous [18]. Consequently, the heteromorphic chromosomes in *A. dumerili* could represent either a ZW or XY system.

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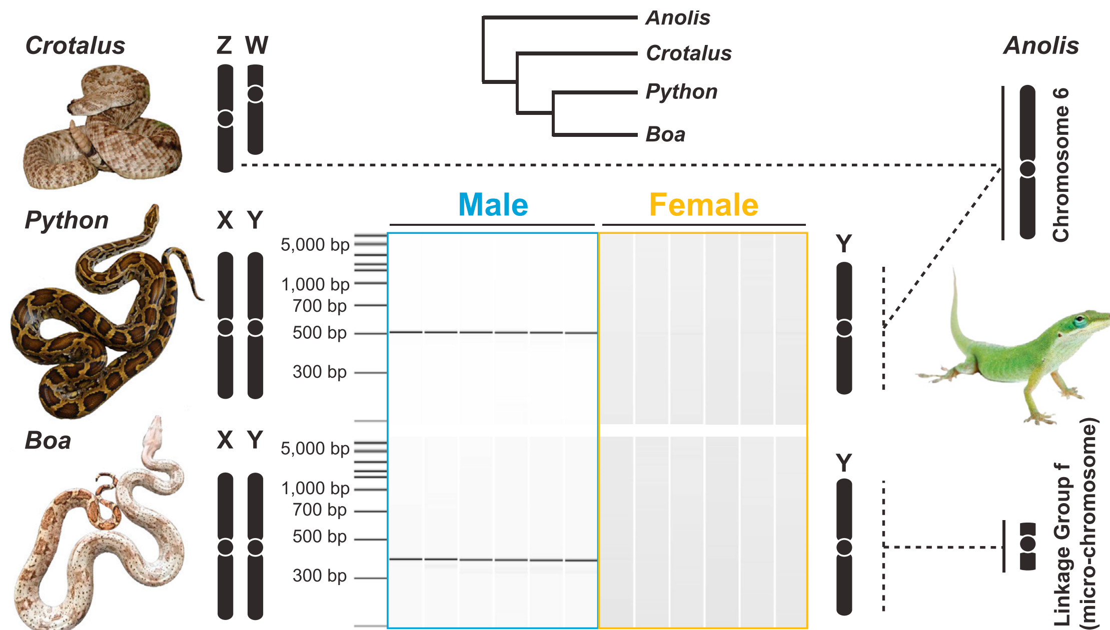

Figure 1. Male-Specific RAD Markers in *Boa* and *Python*

Bioanalyzer results show male-biased PCR amplification of locus TCBoa_2918 in six male and six female *B. imperator* and locus M3 in six male and six female *P. bivittatus* (individuals sampled here are a subset of individuals used in Figure S1). Primers for all loci are listed in STAR Methods. Inferred synteny with *Anolis* and rattlesnake (*Crotalus*) chromosomes is also shown. Phylogenetic relationships are based on Zheng and Wiens [24]. See also Figure S1 and Tables S2 and S3.

More recently, a series of increasingly sophisticated methods have been used to study caenophidian sex chromosomes, which found differences in the genetic content of the Z and W. These include cytogenetic mapping of cDNAs and repetitive sequences using fluorescence in situ hybridization (FISH) [3, 19], qPCR of sex chromosome genes [5], and even whole-genome sequencing [2]. Nonetheless, no differences were identified between the putative Z and W in the boas and pythons sampled for these studies. The failure to find differences in the boa and python ZW has thus far been attributed to the homomorphic nature of their sex chromosomes and presumed sequence similarity, consistent with Ohno's original claims [1]. An alternative explanation is that boas and pythons do not share the same sex chromosome system as members of Caenophidia. So, although it is true that nearly all species of boas and pythons studied thus far lack heteromorphic sex chromosomes, there is no evidence that they have the same ZW sex chromosomes as caenophidian snakes, or that they even possess ZW sex chromosomes.

Beyond a lack of evidence for female heterogamety in boas and pythons, there is compelling indirect evidence that they may, in fact, have an XY sex chromosome system. Examining facultative parthenogenesis across vertebrates, a pattern has emerged in which ZW species produce only ZZ male offspring and XY species produce only XX female offspring [6]. Consistent with this pattern, all confirmed parthenogens from caenophidian snakes reported to date have been male [6]. However, neonates of boas and pythons resulting from facultative parthenogenesis have been female [6]. These parthenogens were initially suspected of being WW, to conform with the dogma that all snakes possess a ZW sex chromosome system [20]. Nonetheless, an XY sex chromosome system in boas and pythons provides a far more parsimonious explanation of these results. Furthermore, breeding a female parthenogenetic *Boa imperator* [20] with a sexually produced wild-type male yielded both male and female offspring (W.B. and G.W.S., unpublished data). The expected outcome under a ZW system and sexual reproduction would be all females (i.e., ZZ male paired with a WW female, resulting in all ZW female offspring), whereas secondary automictic parthenogenesis would produce only WW female offspring. Hence, under both of these reproductive mechanisms, ZZ males would not be possible [6]. Additional evidence for XY sex chromosomes in boas and pythons is based on the observation of an incomplete-dominant color mutation in the ball python (*Python regius*), whose inheritance suggests sex linkage and an XY (and not a ZW) sex-determining system [7].

An XY Sex Chromosome System in a Boa and Python

Several recent studies have used reduced-representation sampling of genomes (e.g., restriction-site-associated DNA sequencing, RAD-seq) to identify sex-specific markers in species lacking heteromorphic sex chromosomes and to differentiate between male (XY) and female (ZW) heterogamety [17, 21]. The approach involves sequencing thousands of RAD markers from multiple confidently sexed males and females to identify sex-specific markers, that is, RAD markers found in one sex, but not the other [17]. These sex-specific RAD markers are presumed to be in sex-linked regions, i.e., the Y or W. Thus, species with an excess of male-specific markers have an XY system, whereas species with an excess of female-specific markers have a ZW system [17, 21, 22]. Here we used RAD-seq data to identify sex-specific genetic markers in boa constrictor (*B. imperator*) and Burmese python (*Python bivittatus*). As a

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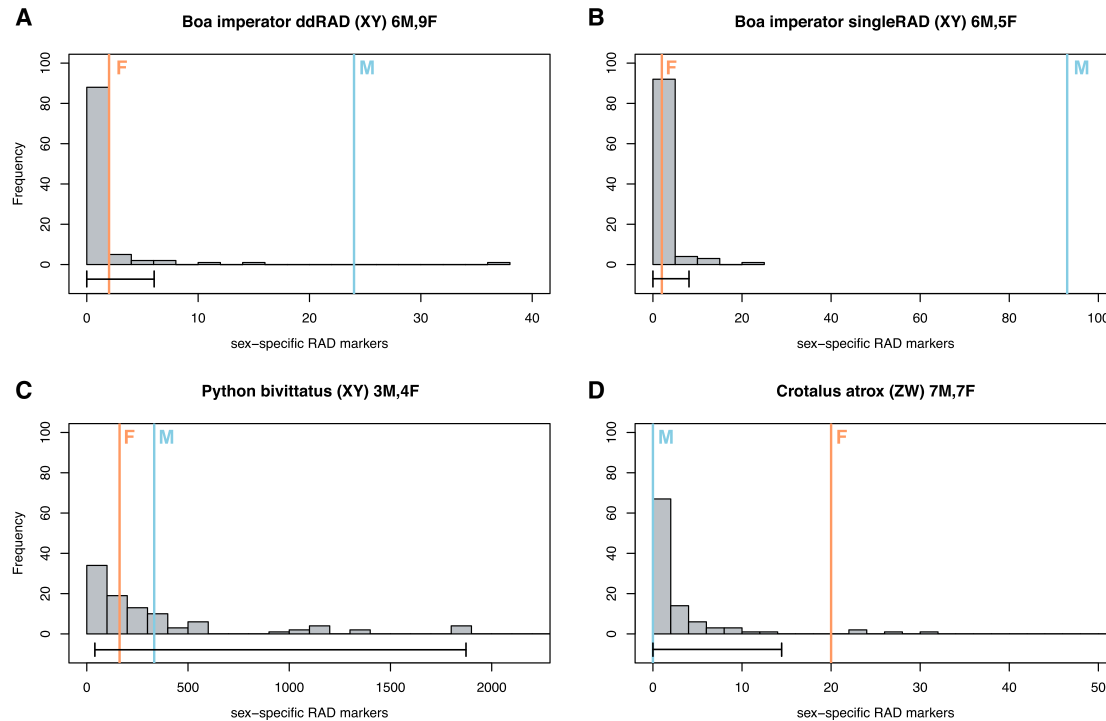

Figure 2. Permutations of the Number of Sex-Specific Markers Expected Solely by Chance for Each of the Four RAD-Seq Datasets

Blue and orange vertical lines denote the observed number of male- and female-specific RAD markers in each dataset, respectively. These values are also listed in Table 1. Observed numbers of sex-specific markers outside the 95% confidence interval of the null distribution (the horizontal line below each histogram) are considered significantly different from that expected by chance alone.
(A) The boa ddRAD dataset with six males and nine females.
(B) The boa single-digest RAD dataset with six males and five females.
(C) The python dataset with three males and four females.
(D) The rattlesnake dataset with seven males and seven females.

positive control for the method, we also analyzed RAD-seq data from the western diamondback rattlesnake (*Crotalus atrox*), a caenophidian with heteromorphic ZW sex chromosomes [23] (Figures 1, 2, and S1; Table 1). RAD-seq data from multiple male and female samples (Table S4) were compared using a previously published bioinformatic pipeline for identifying sex-specific RAD markers [17, 21]. These analyses identified an excess of male-specific RAD markers in the python and boa (Table 1), indicating an XY sex chromosome system in these species. We recovered the expected inverse result from the rattlesnake—an excess of female-specific RAD markers—confirming a ZW sex chromosome system (Figure 2; Table 1). Permutation tests demonstrated that the observed number of sex-specific RAD markers identified in boa and rattlesnake was larger than expected by chance alone, falling outside of the 95% confidence interval of the null distribution (Figure 2). We confirmed the sex specificity of one of these boa RAD markers using PCR (Figures 1 and S1). PCR validation included additional individuals that were not used to generate the original RAD-seq data, thereby constituting an independent validation of the bioinformatic results (Figures 1 and S1; Table S4). Furthermore, primers designed from male-specific RAD markers for *B. imperator* also amplified in males of *Boa constrictor*, demonstrating a conserved sex chromosome system between the two species (Figure S1). We identified an excess of male-specific markers in the python RAD-seq data (Table 1). However, only a small number of individuals were used to generate the python dataset (Table 1), which most likely explains why the observed number of sex-specific RAD markers did not fall outside the null distribution in the permutation test (Figure 2). Although initial python results may seem ambiguous, previous work has shown that sex-specific markers can still be identified when sample sizes are small [17]. However, as the sample size decreases, the true sex-specific markers will be contained within an increasingly larger number of false positives and require subsequent PCR validation. Therefore, we again confirmed the sex specificity of two python RAD markers using PCR and PCR-RFLP (restriction fragment-length polymorphism) (Figures 1 and S1).

Identification of the Boa and Python Sex Chromosomes

RAD-seq data and subsequent PCR validation confirmed XY systems in the boa and python species examined here. Our next objective was to use additional analyses of recently published boa and python genomes [25, 26] to identify which chromosomes are the sex chromosomes and evaluate chromosomal synteny with other vertebrate species. Overall, the data indicate that boas and pythons have evolved XY sex chromosomes independently on different linkage groups. The caenophidian sex chromosome is the fourth largest chromosome pair, which is orthologous to chromosome 6 in the green anole (*Anolis*

Table 1. Summary of the Analyses of Three RAD-Seq Libraries Identifying Male-Specific Markers in Boa and Python and One RAD-Seq Library Identifying Female-Specific Markers in a Rattlesnake

Species	Library Preparation	Samples	Total Number of RAD Markers	Male-Specific RAD Markers	Female-Specific RAD Markers	Confirmed Male-Specific RAD Markers	Confirmed Female-Specific RAD Markers	Sex Chromosome System
B. imperator	single-digest RAD	6 males, 5 females	66,866	93	2	16	0	XY
B. imperator	ddRAD	6 males, 9 females	349,348	24	2	17	0	XY
P. bivittatus	ddRAD	3 males, 4 females	240,316	333	162	58	16	XY
C. atrox	ddRAD	7 males, 7 females	22,096	0	20	0	3	ZW

Confirmed sex-specific markers are a subset of the sex-specific RAD markers, but they exclude from consideration any RAD markers that also appear in the original read files from the opposite sex. Female-specific loci in the python data are most likely false positives, due to the small number of individuals sampled. ddRAD, double-digest RAD libraries. See also Tables S1 and S4.

carolinensis) and chromosomes 2 and 27 in birds [3, 5, 27]. Putative sex-linked genome scaffolds were identified in the boa by mapping male and female RAD-seq reads to the boa genome, calling SNPs, and using Fisher's exact test to identify the sex-specific SNPs. We identified 46 putative sex-linked scaffolds in the boa, and the majority of scaffolds with identifiable genes correspond to human chromosome 19(q) and *Anolis* linkage group f (LGf), as well as several unmapped *Anolis* scaffolds (Table S3). The small number of individuals used to generate the python RAD-seq data precluded using RAD-seq SNPs to identify python sex-linked scaffolds. Therefore, we used two other approaches. First, using BLAST of the published python genome, we found a match to python RAD marker M10 (one of the two male-specific RAD markers validated using PCR; see Figure S1; we found no matches to the other PCR-validated python RAD marker, M3) in scaffold KE954149, which corresponds to *Anolis* chromosome 6. Second, we mapped intestinal RNA-seq reads from six male and two female pythons to the python transcriptome and identified five python transcripts with sex-specific SNPs, three of which also map to *Anolis* chromosome 6 (Table S4). Taken together, these data suggest that the python sex chromosome is also homologous to *Anolis* chromosome 6, which raises the possibility that within snakes, pythons and caenophidians independently recruited the same chromosomes into both an XY and ZW system, respectively.

Snakes represent important models to advance our understanding of sex chromosome evolution. Indeed, reptiles more generally have been of particular interest because they exhibit repeated independent origins of diverse sex-determining systems [9, 21, 28]. Despite this diversity, emerging evidence suggests that most transitions in reptile sex-determining systems are among—as opposed to within—major clades [21, 29]. It is, therefore, surprising to identify an XY system in boas and pythons, given that snakes are well studied and long assumed to have a stable sex chromosome system [2, 3, 19, 21]. The historical failure to correctly identify the boa and python sex chromosomes was most likely due to uncritical evaluation of the earliest claims of ZW homomorphy [1, 4]. Our findings require a reexamination of decades of comparative sex chromosome research in snakes, and the existence of multiple XY/ZW transitions within snakes makes them even more valuable than previously thought for studying sex chromosome evolution. These include the processes that govern the origin and evolution of XY and ZW systems [30–32] and differences between male and female heterogamety, including possible differences in dosage compensation between XY and ZW taxa [33]. Furthermore, XY sex chromosomes may not be a shared trait among all boas and pythons, and our results are currently restricted to *B. imperator*, *B. constrictor*, and *P. bivittatus*. Thus, searching for sex chromosomes in additional "primitive" snake species—including the blind snakes (Scolecophidia)—is sorely needed, as there is most likely much more to discover about snake sex chromosomes. Finally, our results highlight ongoing efforts to both document and catalog the astonishing diversity of sex-determining systems across the tree of life [9] and the importance of newly developed sequence-based methods to identify the sex chromosome systems in species lacking heteromorphic sex chromosomes [17, 21].

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHOD DETAILS
  - An XY sex chromosome system and a boa and python
  - Identification of the boa and python sex chromosomes
  - A comment on the number of sex-specific markers
- QUANTIFICATION AND STATISTICAL ANALYSIS
- DATA AND SOFTWARE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2017.06.010.

AUTHOR CONTRIBUTIONS

T.G., G.W.S., and W.B. formulated the theory and predictions; T.G., T.A.C., and W.B. contributed to the experimental conception and design; T.G., S.V.N., J.L.B., T.A.C., D.C.C., D.R.S., and W.B. were involved in sample acquisition, analysis, and interpretation of data; and T.G., T.A.C., G.W.S., and W.B. wrote the manuscript. All authors read and approved the final manuscript.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological Samples		
Snake tissue/skin samples used for DNA isolation	This paper, [34]	Table S4
Chemicals, Peptides, and Recombinant Proteins		
restriction enzyme - *SpeI*	New England BioLabs	R3133S
restriction enzyme - *Sau3AI*	New England BioLabs	R0169S
restriction enzyme - *PstI*	New England BioLabs	R3140T
restriction enzyme - *SbfI*	New England BioLabs	R3642S
T4 DNA Ligase	New England BioLabs	M0202T
50 bp DNA ladder	New England BioLabs	N0556S
GoTaq Green Master Mix	Promega	M8291
Q5 High-Fidelity 2X Master Mix	New England BioLabs	M0492S
Sera-mag Beads	Fisher	09-981-123
Polyethylene glycol	Fisher	BP233-1
Tween	Fisher	BP337-100
Sodium Chloride	VWR	0241-500G
Tri-reagent	Molecular Research Center	TB 126
BCP – Phase Separation Reagent	Molecular Research Center	BP 151
DEPC H20	Fisher	5532-18-5
Isopropanol	Fisher	67-63-0
100% Ethanol	Fisher	64-17-5
Agarose I	VWR	0710-500G
Tris base	Acros Organics	14050-0010
Boric acid	Amresco	M139-1kg
EDTA	VWR	0105-500G
Ethidium bromide	Amresco	X328-10ML
Critical Commercial Assays		
Dneasy Blood and Tissue Kit	QIAGEN	69504
MinElute Reaction Cleanup Kit	QIAGEN	28204
NEBNext Ultra II End Repair/dA-Tailing Module	New England BioLabs	E7546S
TruSeq Stranded mRNA kits	Illumina	RS-122-2103
Qubit RNA BR Assay	ThermoFisher	Q10211
Qubit dsDNA BR Assay	ThermoFisher	Q32850
Bioanalyzer chip: RNA 6000 Nano	Agilent	5067-1511
Bioanalyzer chip: DNA 7500	Agilent	5067-1506
Deposited Data		
Boa imperator (ddRAD)	[34]	NCBI SRA: PRJNA382366
Boa imperator (single-digest RAD)	This paper	NCBI SRA: PRJNA387612
Python bivittatus (ddRAD)	This paper	NCBI SRA: PRJNA382347
Python bivittatus (RNA-seq)	This paper	NCBI SRA: PRJNA382362
Crotalus atrox (ddRAD)	This paper	NCBI SRA: PRJNA269607
Oligonucleotides		
Boa imperator:TCBoa2918-F:TGCAGAGCAAGACCTACCCTA	This paper	N/A
Boa imperator:TCBoa2918-R:TTCCACCTGGAAGAACAACC	This paper	N/A
Python bivittatus:Python_M10rflp-F:TGCATACATCTACACA ACCCCT	This paper	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Python bivittatus:Python_M10rflp-R:TACCACTGAGAACTG CTGCA	This paper	N/A
Python bivittatus:Python_M3-F:GCTGATTATTCCAGCGGCAT	This paper	N/A
Python bivittatus:Python_M3-R:GGATTCCAAGTCCACAACGG	This paper	N/A
Software and Algorithms		
Stacks-1.41	[35]	http://catchenlab.life.illinois.edu/stacks/
RADtools 1.2.4	[36]	https://github.com/johnomics/RADtools
sex-specific markers python script	[21]	http://datadryad.org/bitstream/handle/10255/dryad.80848/rsw.py
Geneious R9	[37]	https://www.geneious.com
BLAST	[38]	https://blast.ncbi.nlm.nih.gov/Blast.cgi
CLC Genomics workbench v.9.0	QIAGEN	https://www.qiagenbioinformatics.com/products/clc-genomics-workbench/
R 3.3.2	The R Foundation	https://www.r-project.org
Primer 3	[39, 40]	http://primer3.ut.ee

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Tony Gamble (tgamble@geckoevolution.org).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Boa constrictor, Boa imperator, Crotalus atrox, Python bivittatus (see Table S4 for details).

METHOD DETAILS

An XY sex chromosome system and a boa and python

The identification of sex-specific genetic markers can be used to infer a species' sex chromosome system [17, 41, 42]. Here we identified sex-specific markers from RAD-seq data. RAD-seq uses Illumina sequencing to produce paired-end reads from libraries made from restriction digested DNA [43]. The process involves sequencing thousands of RAD markers from multiple confidently sexed males and females to identify the sex-specific markers, that is, RAD markers found in one sex but not the other [17]. These sex-specific RAD markers are presumed to be on the Y or W. Thus, species with an excess of male-specific markers have an XY system while species with an excess of female-specific markers have a ZW system [17, 21, 22].

We produced four groups of multiplexed RAD-seq libraries that each included multiple male and females samples. These were (1) double digest or ddRAD libraries for Western Diamondback Rattlesnake (*Crotalus atrox*); (2) ddRAD libraries for Burmese python (*Python bivittatus*); (3) ddRAD libraries for the Central American Boa constrictor (*Boa imperator*); and (4) single digest RAD libraries for the Central American Boa constrictor (*Boa imperator*) (Tables 1 and S4). Double-digest RAD-seq (ddRAD) libraries for boas (six males and nine females) and pythons (three males and four females) were constructed following the protocol of Peterson et al. [44] with minor modifications following Card et al. [34]. We used enzymes *PstI* and *Sau3AI* and a size selection of 570 to 690 bp (including adapters) for boa ddRAD libraries. For pythons, we used enzymes *SpeI* and *Sau3AI* and size selected 300 to 625 bp. Libraries were sequenced on an Illumina HiSeq2500 using 100 bp paired-end reads. Rattlesnake ddRAD libraries (seven males and seven females) also followed Peterson et al. [44] and used enzymes *SbfI* and *Sau3AI* and a size selection of 590 to 640 bp [45]. Libraries were sequenced on an Illumina HiSeq2500. We also made a single digest RAD library for additional boa samples (six males and five females), all siblings from a single litter, using the *SbfI* enzyme and size selection of 300 to 550 bp [21, 46] and sequenced these on an Illumina HiSeq2500 using 125 bp paired-end reads. Sequencing reads are available at the NCBI Short Read Archive (*Boa*, NCBI SRA: PRJNA382366, PRJNA387612; *Python*, NCBI SRA: PRJNA382347; *Crotalus*, NCBI SRA: PRJNA269607).

Sex-specific markers were identified using a previously published bioinformatic pipeline [17, 21]. We used the "process radtags" script from Stacks-1.41 [35] to demultiplex, filter, and trim raw Illumina reads. RADtools 1.2.4 [36] was used to generate candidate alleles for each individual and candidate loci across all individuals from the forward reads. All species were analyzed separately. Settings for the RADtags script included a cluster distance of 10, minimum quality score of 20, and read threshold of 5. Settings for the RADmarkers script, which generates candidate loci and alleles across individuals using output from the RADtags script, included a tag count threshold of 4 and the maximum number of mismatches set at 2. The RADtools output includes the presence/absence of

each locus and allele for every sampled individual, enabling the identification of sex-specific RAD markers. We used a python script [21] to identify putative sex-specific markers from the RADtools output. This script also produces a second list, a subset of the initial set of sex-specific RAD markers, that excludes from further consideration any sex-specific markers that also appear in the original reads files from the opposite sex, we call these "confirmed sex-specific RAD markers" following Gamble et al. [17]. This removes rare, but potentially inaccurate RAD markers that may arise due to problems with multiplex sequencing on the Illumina platform [47]. Forward and reverse reads from the confirmed sex-specific markers are subsequently assembled into contigs using Geneious R9 [37].

Using the preceding methods, species with an excess of male-specific RAD markers have an XY sex chromosome system while species with an excess of female-specific RAD markers have a ZW sex chromosome system. However, we cannot rule out that some number of sex-specific markers may be identified by chance, particularly when sample size is small, e.g., our python dataset with only three males and four females. False positives may be present because there exists some probability that a RAD marker could exhibit a sex-specific pattern simply by chance. This chance is higher when the number of sampled individuals is small and decreases as the number of individuals increases. The chance of false positives should also increase as the number of RAD markers increases. We addressed this by permuting the sex labels among sampled individuals for each dataset to create a null distribution of the number of sex-specific markers that could be expected solely by chance. We then determined whether the observed number of sex-specific markers is a plausible sample of this null distribution, e.g., contained within the 95% confidence interval of the null distribution, or whether the observed number of sex-specific RAD markers is larger than expected by chance alone. We did this for each species, calculating null distributions using the same number of males and females as our original dataset (Table 1) using 100 permutations. We performed these permutations using the total number of sex-specific RAD markers identified in each dataset not the number of "confirmed sex-specific RAD markers." Evaluating the number of confirmed sex-specific RAD markers would have also involved permuting the raw read data, which was computationally burdensome. However, since the number of sex-specific markers in each dataset is proportional to the number of confirmed sex-specific markers (Table 1) [17, 21] we feel that this is an acceptable means of assessing the significance of our RAD-seq results. It should be noted that previous work has shown that sex-specific markers can still be identified when sample sizes are small [17]. However, the true sex-specific markers will be contained within an increasingly larger sample of false positives as sample size decreases.

Previous studies have recommended RAD-seq experiments involving small sample sizes validate sex-specific markers via PCR to confirm sex-specificity [17, 21, 22, 48]. We used PCR to validate the sex-specificity of a subset of the confirmed sex-specific markers in both boa and python (Table 1). We designed primers using Primer 3 [39, 40]. We conducted PCR validations of the boa TCBoa_2918 RAD marker (Key Resources Table: Oligonucleotides) using 19 male and 22 female *Boa imperator* and the python M3 RAD marker (Key Resources Table: Oligonucleotides) with twelve male and twelve female *Python bivittatus* (Figure S1). Most of the individuals used for PCR were not used to generate the original RAD-seq data (30 of the 41 *Boa imperator* and all of the 24 *Python bivittatus* were new samples, not used in the RAD-seq experiment – see Figure S1, Table S4) so these PCR results represent an independent validation of male heterogamety in these species. We visualized a subset of these PCR amplicons (six males and six females of both boa and python) using an Agilent Bioanalyzer (Figure 1). We attempted to amplify these primers in related boa and python species and were successful in producing male-biased amplification using the boa TCBoa_2918 primers in the South American Boa, *Boa constrictor* (three male and three female samples, Figure S1), but the python primers did not amplify in a sex-specific manner in either the Carpet Python (*Morelia spilota*) or Ball Python (*Python regius*).

Primers designed for several putative sex-specific markers amplified in both males and females (data not shown). This is likely due to a sex-specific restriction site with conserved sequences on either side [22, 48]. These fragments are identified bioinformatically because the restriction sites, and thus RAD markers, are sex-specific. However, PCR of the conserved flanking region amplifies in both sexes. In these situations PCR validation is an overly conservative test of sex-specificity [22]. Because we had so few individuals for the python RAD-seq we wanted to validate a second marker to confirm the XY sex chromosome system. To test this we designed primers that would create a PCR amplicon that spanned the sex-specific restriction site and then restriction digest these PCR amplicons. In an XY species this should result in multiple bands in males (the uncut X allele and the restriction digested Y allele) and a single band in females (the uncut X allele) when run on a gel. We designed PCR primers to amplify across the putative restriction site after mapping several of the male-specific python RAD markers to the python genome. We observed sex-specific digestion in one of these markers (M10 locus; Figure S1). Restriction digest of the M10 PCR amplicon (PCR-RFLP) using *SpeI* leaves the 381 bp X allele unaffected. However, the putative Y allele was digested into two fragments of 220 and 161 bp (Figure S1). Thus, males had three bands consisting of the uncut X allele and the two smaller Y fragments, while females had just a single band consisting of the uncut X allele (Figure S1).

Identification of the boa and python sex chromosomes

We were also interested in identifying which chromosome was the sex chromosome in both boa and python. We identified putative sex chromosome associated scaffolds in the boa and python genomes (boa assembly SGA and python assembly GCA_000186305.2) [25, 26] using three methods, the first method was used for boa while the second and third methods for python. First, in boa, we identified sex-specific SNPs in the RAD-seq data by mapping male and female RAD-seq reads onto the boa genome using CLC Genomics workbench v.9.0. We did this twice, mapping RAD reads from the ddRAD boa dataset and single digest boa RAD dataset separately. We identified genome scaffolds containing sex-specific SNPs using a Bonferroni-corrected Fisher exact test. We subsequently used BLASTn [38] of python transcripts to identify genes on the putative sex-specific genome scaffolds

and matched them to syntenic regions of the *Anolis* and human genomes from Ensembl v85 [49] (Table S2). This genomic region does not appear in recent *Gallus* and *Taeniopygia* genome assemblies so we were unable to include them in our synteny comparisons. A similar experiment was performed with the python RAD-seq data but it was not used as the small number of individuals used to generate RAD-seq data resulted in the identification of an unreasonably large number of scaffolds, presumably false positives. Thus, the small number of individuals used for the python ddRAD data required different methods to identify the python sex chromosomes. To accomplish that we used BLASTn [38] to match previously mentioned PCR-validated male-specific RAD markers to genome scaffolds in both boa and python. As before, we used BLASTn of python transcripts to identify genes and determine chromosomal synteny with *Anolis* and human. Finally, we searched for sex-specific SNPs in RNA-seq reads from male and female pythons. RNA-seq data for intestinal tissue from six males and two females was generated following Andrew et al. [50]. We mapped RNA-seq reads (SI4; NCBI SRA: PRJNA382362) onto the python transcriptome using CLC Genomics workbench v.9.0 and identified transcripts with sex-specific SNPs using a Bonferroni-corrected Fisher exact test. We matched these transcripts to syntenic regions of the *Anolis* and human genomes using Ensembl v85 [49] (Table S3).

A comment on the number of sex-specific markers

The number of sex-specific genetic markers identified from the analysis of RAD-seq data varies significantly among our four datasets (Table 1) and raises the question as to why such variation exists among datasets and species. While there are many factors that can impact the number of RAD markers that are produced [43, 46, 51] we suggest that the number of sex-specific RAD markers is influenced primarily by two things: 1) the size of the non-recombining portion of the sex chromosome, with species possessing large non-recombining regions (presumably older, more heteromorphic, sex chromosome systems) having more sex-specific markers that species having small non-recombining regions (presumably younger, homomorphic, sex chromosome systems); and 2) the overall number of markers produced from the RAD library, which is dictated by the details of a specific RAD-seq library protocol. The number of genetic markers produced from any given RAD-seq library involves the following variables: genome size, with larger genomes producing more markers than smaller genomes; the restriction enzyme(s) used, with frequent cutters producing more markers than rare cutters; library size selection, with broad size ranges producing more markers than narrow size ranges; and the type of sequencing library used, either single or double digest libraries. Single digest libraries, digest genomic DNA which is randomly sheared, size selected, and then sequenced [43]. Double digest (ddRAD) libraries, on the other hand, digest genomic DNA using two restriction enzymes, which are then size selected and sequenced [44]. ddRAD will typically produce fewer markers than a single digest protocol using one of the same restriction enzymes [44].

Given all of these variables, it is easy to see why our four datasets produced significantly different numbers of markers. Looking at the rattlesnake data, for example, one might predict that because they have heteromorphic sex chromosomes [23] and a presumably large non-recombining region of the sex chromosomes, they should have significantly more sex-specific markers than the boa and python that lack heteromorphic sex chromosomes [3, 52]. However, this is countered by the fact that, in the rattlesnake, we used ddRAD with very tight size selection and a different restriction enzyme, which together produced relatively few RAD markers. Thus, our finding of only three confirmed female-specific markers in the rattlesnake is not unexpected (Table 1).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical tests and software used are described in Method Details (above).

DATA AND SOFTWARE AVAILABILITY

Sequencing reads are available at the NCBI Short Read Archive (*Boa*, NCBI SRA: PRJNA382366, PRJNA387612; *Python*, NCBI SRA: PRJNA382347; *Crotalus*, NCBI SRA: PRJNA269607).