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# Restriction Site-Associated DNA Sequencing (RAD-seq) Reveals an Extraordinary Number of Transitions among Gecko Sex-Determining Systems

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## Abstract

Sex chromosomes have evolved many times in animals and studying these replicate evolutionary “experiments” can help broaden our understanding of the general forces driving the origin and evolution of sex chromosomes. However this plan of study has been hindered by the inability to identify the sex chromosome systems in the large number of species with cryptic, homomorphic sex chromosomes. Restriction site-associated DNA sequencing (RAD-seq) is a critical enabling technology that can identify the sex chromosome systems in many species where traditional cytogenetic methods have failed. Using newly generated RAD-seq data from 12 gecko species, along with data from the literature, we reinterpret the evolution of sex-determining systems in lizards and snakes and test the hypothesis that sex chromosomes can routinely act as evolutionary traps. We uncovered between 17 and 25 transitions among gecko sex-determining systems. This is approximately one-half to two-thirds of the total number of transitions observed among all lizards and snakes. We find support for the hypothesis that sex chromosome systems can readily become trap-like and show that adding even a small number of species from understudied clades can greatly enhance hypothesis testing in a model-based phylogenetic framework. RAD-seq will undoubtedly prove useful in evaluating other species for male or female heterogamety, particularly the majority of fish, amphibian, and reptile species that lack visibly heteromorphic sex chromosomes, and will significantly accelerate the pace of biological discovery.

## Keywords

evolution, lizard, phylogeny, reptile, sex chromosome

## Introduction

Sex chromosomes, while best known for their role in sex determination, also have important evolutionary consequences and play a role in speciation, genetic conflict, sexual dimorphism, and sexual antagonism (Rice 1984, 1992; Werren and Beukeboom 1998; Presgraves 2008; Rice et al. 2008; Meiklejohn and Tao 2010; Gamble and Zarkower 2012; Dean and Mank 2014). Despite their biological importance, the structure, function, and genetic content of sex chromosomes are poorly known in all but a handful of model species. Even as an increasing number of genomes are sequenced, sex chromosomes remain understudied, due to difficulties assembling their repeat-rich and palindromic sequences, which accumulate due to suppressed recombination near the sex-determining locus (Rice 1987; Charlesworth 1991; Skaletsky et al. 2003). Indeed, most genome projects choose to sequence individuals of the homogametic sex (XX or ZZ individuals) to avoid assembly problems (Carvalho and Clark 2013; Clark 2014). The result is that knowledge on the origin and evolution of differentiated sex chromosomes (e.g., Y or W) continues to lag far behind our knowledge of other genomic regions.

Much of what we know about sex chromosomes and their evolution comes from studying mammals and *Drosophila*, which have extremely degenerate Y chromosomes that are atypical of the sex chromosomes found in other species (Bachtrog et al. 2014). Many animal species, for example, have morphologically similar, homomorphic, sex chromosomes with limited Y (or W) chromosome degeneration (Gilchrist and Haldane 1947; Matsubara et al. 2006; Stöck et al. 2011; Vicoso, Kaiser, et al. 2013; Otto 2014). If we wish to understand fully the evolutionary patterns and processes affecting sex chromosomes, additional model clades are needed that better represent the diversity of sex chromosomes found across the animal kingdom. However, a major

impediment to this research has been the difficulty of simply identifying the sex chromosome systems in large numbers of animal species. This is because homomorphic sex chromosomes, such as those found in many species of fish, amphibians, and reptiles (Hillis and Green 1990; Hayes 1998; Devlin and Nagahama 2002; Ezaz et al. 2005; Ezaz, Sarre, et al. 2009), cannot be easily diagnosed through traditional karyotyping with light microscopy. Poor knowledge of species' sex-determining systems across huge swaths of the metazoan phylogeny ultimately restricts our understanding of how sex chromosomes arise and evolve and particularly limits our ability to find undifferentiated, newly evolved sex chromosomes.

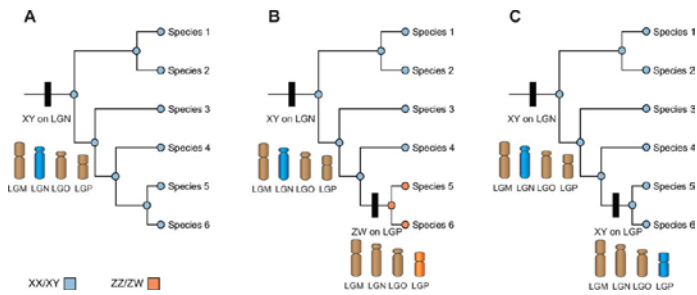
Restriction site-associated DNA sequencing (RAD-seq) is an especially promising technique to efficiently and accurately identify male or female heterogamety in species with cryptic, homomorphic sex chromosomes (Gamble and Zarkower 2014). RAD-seq techniques sequence the DNA flanking a specific restriction site and can generate tens of thousands of markers for genotyping (Baird et al. 2008; Hohenlohe et al. 2010). With a robust sample size of confidently sexed individuals, species with male-specific markers can be inferred to have male heterogamety (XY), whereas species with female-specific markers have female heterogamety (ZW). With these principles in mind, we developed a simple analytical pipeline that uses RAD-seq data from multiple individuals of each sex to discover and validate sex-specific markers (Gamble and Zarkower 2014). Most importantly, this pipeline identifies sex-specific markers in field-collected animals without having to generate test crosses and build linkage maps. Here, we show that this technique can be scaled up using a newly written python script that automates much of our bioinformatic workflow to identify sex chromosome systems from a large number of species. Furthermore, incorporating these new identifications with phylogenetic comparative analyses can inform the study of sex chromosomes and sex-determining systems.

Reptiles have long been of interest for studying sex determination and sex chromosome evolution (Ohno 1967; Bull 1980; Janzen and Paukstis 1991; Wapstra et al. 2007; Ezaz, Sarre, et al. 2009). Their significance is due primarily to the variety of sex-determining systems, including male (XY) and female (ZW) heterogamety, and temperature-dependent sex determination (TSD), with numerous transitions among them (Bull 1983; Viets et al. 1994; Janzen and Krenz 2004; Ezaz, Sarre, et al. 2009; Pokorná and Kratochvíl 2009). Despite the great diversity in sex-determining systems among major lineages of squamate reptiles (lizards and snakes), within many of these lineages sex chromosome systems are remarkably stable (Matsubara et al. 2006; Wapstra et al. 2007; Pokorná and Kratochvíl 2009; Vicoso, Emerson, et al. 2013; Gamble et al. 2014; Rovatsos et al. 2014). The diversity of sex-determining systems found in reptiles contrasts with the single systems found in most mammals and birds. Independently derived sex chromosome systems, such as those found in reptiles, greatly expand the sample size of sex chromosome "experiments" that have evolved in vertebrates and can be used to test many long-standing hypotheses. These include, for example, comparing the processes that govern the origins and evolution of male and female heterogamety (Bachtrog et al. 2011), testing whether some linkage groups are predisposed to becoming sex chromosomes (Graves and Peichel 2010; O'Meally et al. 2012), or illuminating differences in dosage compensation between species with male and female heterogamety (Mank 2013). Therefore, studying these independently derived systems should improve our understanding of the general principles and processes that govern sex chromosome evolution.

Even within a group as varied as squamates one clade stands out as hyperdiverse and worthy of more detailed study, the geckos. Geckos are a species-rich, globally distributed clade of lizards that have all major vertebrate sex-determining systems, with multiple transitions among them (Moritz 1990; Ezaz, Sarre, et al. 2009; Gamble 2010). This diversity makes geckos an ideal vertebrate model to study the origins and evolution of sex chromosomes. However, as diverse as geckos are, there are still large parts of the gecko phylogeny lacking information about sex-determining systems. This paucity of knowledge has hampered their development as a model clade and subsequently hindered our understanding of squamate sex determination overall (Janzen and Krenz 2004).

Here, we illustrate the utility of RAD-seq to efficiently and accurately identify the sex chromosome systems in a number of gecko species. We use these newly generated RAD-seq data, along with data from the literature, to revisit the evolution of squamate sex-determining systems and test hypotheses related to transitions among these systems. Specifically, we test the hypothesis that sex chromosomes can routinely act as an evolutionary trap (Bull and Charnov 1977; Bull 1983; Pokorná and Kratochvíl 2009; Bachtrog et al. 2014). The evolutionary trap hypothesis posits that differentiated, nonrecombining sex chromosomes preclude transitions to other sex-determining systems (Bull 1983). Transitions are inhibited due to one or more of the following factors: 1) The accumulation of deleterious recessive mutations on the Y (or W) inhibits transitions to a new sex chromosome system if selection against transitional YY (or WW) offspring is stronger than selection for the new sex-determining linkage group, 2) the Y (or W) carries genes necessary for the development or fertility of that sex and cannot be eliminated, or 3) there is tight linkage of sexually antagonistic alleles to the sex-determining region (Bull and Charnov 1977; van Doorn and Kirkpatrick 2010; Bachtrog et al. 2014). These scenarios are all known to occur on heteromorphic sex chromosomes that exhibit reduced recombination around the sex-determining locus and linked loci (Rice 1984, 1987; Charlesworth 1991). Such factors, whether individually or in combination, likely contribute to the long-term stability of nonrecombining sex chromosomes, as seen in mammals and birds.

Under the evolutionary trap hypothesis, transitions are only possible from young or otherwise poorly differentiated sex chromosomes, or else from TSD (lacking sex chromosomes altogether) to XY or ZW systems. However, it is not clear whether stability and trap-like behavior is an inevitable consequence for all sex chromosomes, given enough time, or instead the stability observed in birds, mammals, and *Drosophila* is somehow exceptional. For example, there are several examples of turnover among sex chromosome systems in fish, frogs, and flies (Miura 2007; Takehana et al. 2008; Ross et al. 2009; Vicoso and Bachtrog 2013) although, as with stable, trap-like systems, it is not apparent whether this lability is typical of most clades with sex chromosomes. Consequently, examining transitions among sex-determining systems in a phylogenetic context is essential to evaluate the frequency with which sex chromosomes turn over. If most sex chromosomes become traps we would expect to observe only rare transitions among sex chromosome systems in clades once sex chromosomes evolve (fig. 1A). Alternately, if sex chromosomes do not routinely become trap-like we should see relatively frequent transitions among sex chromosome systems, for example, between male and female heterogamety (fig. 1B) or from XY and ZW systems to TSD. Phylogenetic tests of the evolutionary trap hypothesis are constrained by the available data, and limited knowledge of sex chromosome homology can reduce the effectiveness of such tests. In particular, transitions involving recruitment of a new linkage group into a sex-determining role without a concomitant change in heterogamety (fig. 1C) will be overlooked if the sex-determining systems in a clade are coded simply as XY, ZW, and TSD. Nevertheless, this trait-coding scheme will still identify a large subset of potential transitions, and documenting turnovers in the patterns of heterogamety (fig. 1B) can provide important insights into whether sex chromosomes routinely become trap-like even when information on homology is limited. In particular, finding repeated instances of changes in heterogamety (fig. 1B) would falsify the evolutionary trap hypothesis. The diverse sex chromosomes of fish, amphibians, and reptiles, along with an abundance of species with TSD, make all of these clades ideal for testing the generality of the evolutionary trap hypothesis.



**Figure 1** Testing the evolutionary trap hypothesis in a phylogenetic framework. Three identical phylogenies illustrating alternative scenarios either supporting or rejecting the evolutionary trap hypothesis. Vertical black bars on branches indicate transitions in sex-determining systems. (A) Sex chromosome evolution supporting the evolutionary trap scenario. An XY system evolved in the most recent common ancestor (MRCA) of species 1 through 6 on linkage group N (LGN—in blue). There are no transitions away from this sex-determining system. (B) Sex chromosome evolution illustrating nontrap-like behavior. An XY system evolved in the MRCA of species 1 through 6. However, a change in heterogamety occurred in the MRCA of species 5 and 6 with the evolution of a ZW system on linkage group P (LGP—in orange). This nontrap-like scenario would be identified in our phylogenetic comparative analyses. (C) Sex chromosome evolution illustrating nontrap-like behavior. An XY system evolved in the MRCA of species 1 through 6 on LGN. LGP (in blue) was subsequently recruited into a sex-determining role in the MRCA of species 5 and 6 but with no change in heterogamety. This scenario would not be identified with our current analyses, which detect changes in heterogamety but do not assess homology.

Our data revealed a remarkable number of transitions among gecko sex-determining systems compared with other lizards and snakes. We also show how adding even a small number of species from understudied clades can greatly enhance hypothesis testing in a model-based phylogenetic framework and that current data support the hypothesis that sex chromosomes can routinely become evolutionary traps. Finally, we show that the RAD-seq method implemented here can significantly accelerate the pace of sex chromosome discovery, facilitating the study of sex chromosome origins and evolution.

## Results

### Identifying Gecko Sex Chromosome Systems

Sex-specific polymerase chain reaction (PCR) amplification of putative sex-specific markers from the RAD-seq analyses identified both XY and ZW sex chromosome systems among 12 gecko species (fig. 2; table 1). Sex chromosome data for eight of these species are completely new, whereas RAD-seq data in the four remaining species, *Lialis burtonis*, *Heteronotia binoei*, *Thecadactylus rapicauda*, and *Christinus marmoratus*, confirm previous cytogenetic findings (Gorman and Gress 1970; King and Rofe 1976; Moritz 1984; Schmid et al. 2014). Furthermore, of the eight new species, four have been karyotyped previously and showed no evidence of heteromorphic sex chromosomes (table 1). Read depth was variable and typically ranged from about 10× to 60× coverage per individual ([supplementary table S1](#), [Supplementary Material](#) online). Many more putative sex-specific markers were identified bioinformatically than were actually validated (table 1). Many of these are likely false positives (variation between individuals rather than between sexes), which are associated with sampling a limited number of individuals from each sex (Gamble and Zarkower 2014). This is particularly noticeable in a species such as *Lialis burtonis*, which had the fewest samples. Gamble and Zarkower (2014) showed that using a limited number of samples will accurately identify sex-specific markers although they will be contained within an increasingly larger sample of false positives as sample size decreases. Unequal sample size in *Lialis* is likely responsible for the larger number of female-specific false positives and suggests that the number of false positives may be independent of the number of sampled individuals of the opposite sex. Images of PCR gels illustrate validated sex-specific RAD-seq markers and are included in figure 2 and [supplementary figure S1](#), [Supplementary Material](#) online. In most cases, only a single PCR product was amplified in the heterogametic sex. However, some markers, as seen in *Hemidactylus mabouia* and *Sphaerodactylus nicholsi* (fig. 2), produced

two bands in one (the heterogametic) sex and a single band in the other (homogametic) sex. The double band indicates amplification of both X and Y (or Z and W) alleles, and shows that the two alleles are different sizes, whereas the single band indicates amplification of the single X (or Z) allele. Both kinds of markers were observed in the same species in some cases, for example, *Correlophus ciliatus* ([supplementary fig. S1, Supplementary Material](#) online).

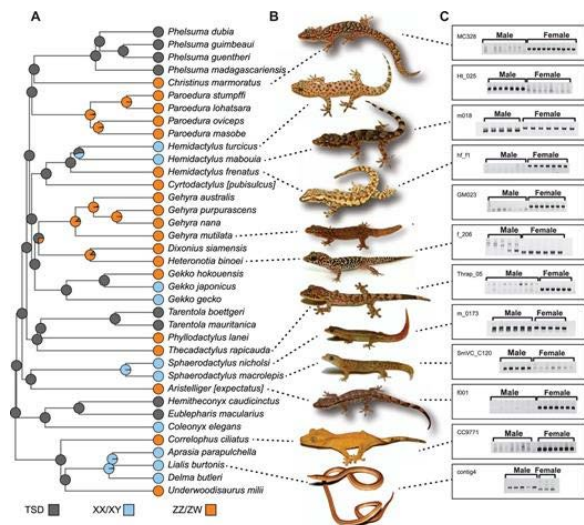


Figure 2 (A) Evolution of sex-determining systems in geckos. Colored circles at the tips of the phylogenetic tree indicate sex-determining systems of sampled species. Circles at internal nodes indicate reconstructions from 1,000 stochastic mapping simulations using the TRAP model. B. Illustrations of the 12 gecko species used for RAD-seq. (C) Gels showing the sex-specific PCR amplification of a representative RAD-seq marker from each species. The locus ID is indicated to the right. Names enclosed by brackets [ ] indicate species with known sex-determining mode that were represented on the original phylogeny by a different, closely related taxon.

Table 1. Summary of RAD-seq Analyses in Gecko Species.

Species	No. of Males	No. of Females	No. of Male-Specific Markers	No. of Female-Specific Markers	Sex-Determining System (RAD-seq)	No. of PCR Markers Tried	No. of PCR Validated Markers	Karyotype	Citation
<i>Lialis burtonis</i>	5	3	21	345	XY	4	1	XY	Gorman and Gress (1970)
<i>Correlophus ciliatus</i>	6	7	0	57	ZW	22	4	H	Pokorná et al. (2011)
<i>Aristelliger expectatus</i>	7	7	0	10	ZW	9	2	NA	NA
<i>Sphaerodactylus nicholsi</i>	7	7	158	7	XY	14	1	NA	NA
<i>Sphaerodactylus macrolepis</i>	7	9	157	0	XY	16	2	NA	NA
<i>Thecadactylus rapicauda</i>	9	5	2	11	ZW	11	3	ZW	Schmid et al. (2014)
<i>Gehyra mutilata</i>	7	7	2	41	ZW	20	3	NA	NA
<i>Hemidactylus mabouia</i>	6	6	14	0	XY	13	1	H	McBee et al. (1987)
<i>Hemidactylus turcicus</i>	7	7	33	2	XY	21	4	H	Branch (1980); Trifonov et al. (2011)
<i>Hemidactylus frenatus</i>	9	7	0	6	ZW	6	1	H	King (1978); Trifonov et al. (2011)
<i>Heteronotia binoei</i>	5	7	2	113	ZW	17	1	ZW	Moritz (1984)
<i>Christinus marmoratus</i>	6	6	32	855	ZW	13	2	ZW	King and Rofe (1976)

NOTE.—The number of samples analyzed, the number of male- and female-specific RAD markers identified bioinformatically, the sex-determining system identified through RAD-seq, the number of RAD-seq markers that were attempted for validation through PCR, the number of validated sex-specific markers using PCR, published sex chromosome complement with citation are listed in this table. XY, male heterogamety; ZW, female heterogamety; H, homomorphic; NA, no published karyotype.



## Evolution of Squamate Sex-Determining Systems

TRAP model provided the best fit to the data for both data sets (table 2). Maximum-likelihood ancestral state reconstruction in both data sets recovered the root as TSD with repeated switches to either XY or ZW sex chromosome systems (figs. 2 and 3, [supplementary figs. S2–S6, Supplementary Material](#) online). The more permissive all rates different (ARD) model also recovered a TSD root in both data sets ([supplementary figs. S3 and Supplementary Data, Supplementary Material](#) online). Combining XY and ZW modes into a single genotypic sex determination (GSD) category using the ARD model also recovered TSD at the root and in the ancestor of squamates when skinks with putative TSD were included. Excluding skinks with putative TSD resulted in an equivocal reconstruction at the root and GSD as the ancestral squamate character state ([supplementary figs. S7 and Supplementary Data, Supplementary Material](#) online). Most transitions to sex chromosome systems occurred early in the history of the major squamate clades with the exception of Gekkota and Acrodonta, which maintained TSD well after they began to diversify. The reconstruction of the most recent common ancestor of Scincidae is equivocal when the putative TSD skink species are included, being either TSD or XY, but is XY when putative TSD skinks are excluded.

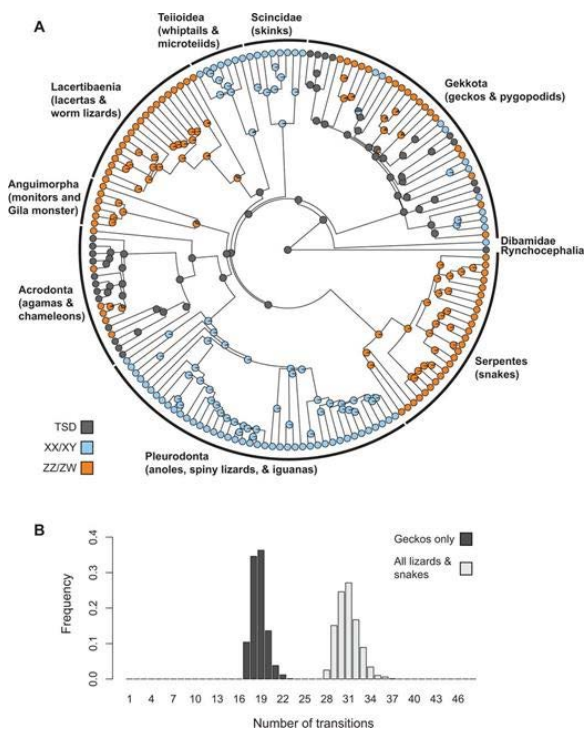


Figure 3 (A) Evolution of sex-determining systems in lizards and snakes (squamates) and tuatara. Colored circles at the tips of the phylogenetic tree indicate sex-determining systems of sampled species. Circles at internal nodes indicate maximum-likelihood ancestral state reconstructions using the TRAP model. (B) The number of transitions among sex-determining systems calculated by 1,000 stochastic mapping simulations under the TRAP model. The count for all lizards and snakes is on the right (gray). The count for just geckos is on the left (black). Data set for (A) and (B) excluded skinks with putative TSD.

Table 2. Comparison among Transition Rates Models Used in the Maximum-Likelihood Ancestral State Reconstruction and Stochastic Mapping.

		TSD Skinks Included		TSD Skinks Excluded	
Model	No. of Parameters	Log Likelihood	AIC	Log Likelihood	AIC
ARD	6	-73.213	158.426	-62.584	137.1684
SYM	3	-81.264	168.527	-68.538	143.0759
TRAP	2	-75.424	154.848	-61.492	126.9831
ER	1	-81.545	165.090	-72.365	146.7296

NOTE.—The number of parameters for each model is listed. The likelihood scores were produced using two data sets, either including or excluding skink species with putative TSD. Models were compared using the AIC and AIC scores of the best-fitting model for each data set are in italics. The following models were compared: ARD, SYM, TRAP, and ER.

Stochastic mapping under the optimal TRAP model counted between 28 and 37 (mean = 30.8) transitions among sex-determining systems in squamates using the data set excluding putative TSD skinks and (table 3). All transitions were from TSD to either XY or ZW systems with approximately twice as many origins of ZW systems as XY systems. The data set including skinks with TSD, using the ARD model, counted 31–45 (mean = 36.2) transitions and included a small number of changes from XY to TSD but was otherwise similar to the TRAP model (table 3). Geckos accounted for between ½ to nearly ⅔ of transitions among squamate sex-determining systems (table 3, fig. 3).

Table 3. The Mean (min, max) Number of Transition among Sex-Determining Systems in Lizards and Snakes (squamates) Estimated from 1,000 Stochastic Mapping Simulations.

Data Set	Clade	Model	Total Transitions (min, max)	TSD to XY	TSD to ZW	XY to ZW	ZW to XY	XY to TSD	ZW to TSD
TSD skinks included	Squamates	ARD	36.2 (31, 45)	12.67	20.14	0	0	3.40	0
		TRAP	35.3 (32, 43)	15.37	19.91	NA	NA	NA	NA
	Geckos	ARD	19.0 (17, 25)	6.64	12.06	0	0	0.31	0
		TRAP	18.6 (17, 24)	6.65	11.96	NA	NA	NA	NA
TSD skinks excluded	Squamates	ARD	30.8 (28, 37)	10.71	20.16	0	0	0	0
		TRAP	30.9 (28, 37)	10.69	20.27	NA	NA	NA	NA
	Geckos	ARD	18.6 (17, 22)	6.57	12.07	0	0	0	0
		TRAP	18.7 (17, 23)	6.55	12.09	NA	NA	NA	NA

NOTE.—Transition counts were produced using one of two data sets, including or excluding skinks with putative TSD, each using two different transition rate models, the six-parameter ARD model or the two-parameter TRAP model. Separate counts are shown for just geckos for both data sets. Additional columns show the mean number of transitions for every possible rate matrix transition. NA, not available.

## Discussion

### Identifying Gecko Sex Chromosome Systems

Understanding the origins and evolution of sex chromosomes across a range of organisms requires reliable and reproducible techniques to quickly and accurately identify sex-determining systems. RAD-seq has been proposed as one means of achieving this and has been used to identify sex-specific markers and sex-determining regions in the genomes of a variety of species (Anderson et al. 2012; Carmichael et al. 2013; Palaiokostas, Bekaert, Davie, et al. 2013; Palaiokostas, Bekaert, Khan, et al. 2013; Gamble and Zarkower 2014). Our use of RAD-seq in geckos highlights both the effectiveness of this approach and how readily deployable it is to nonmodel systems, confirming that our methods and pipeline can efficiently identify sex-specific markers in multiple species. Furthermore, unlike most previous studies that relied on experimental crosses to identify sex-specific markers from linkage maps, we used adult individuals, most collected from natural populations. This reduces the time and cost needed to generate data and enables study of species that do not readily breed in captivity or have small numbers of offspring. Concordance between RAD-seq and cytogenetics in four gecko species with previously identified sex chromosomes further confirms the accuracy of the RAD-seq method.

The RAD-seq pipeline used here may not identify sex-specific markers in all instances. For example, we generated RAD-seq data for an additional six gecko species (data not shown) that failed to produce sex-specific PCR products. Our RAD-seq workflow will be less effective under several situations, particularly when the

nonrecombining portion of the Y or W chromosomes is extremely small. Sampling individuals from highly structured populations or populations with very large effective population sizes will also be less effective. Biases in the RAD-seq method itself related to GC content, library preparation, and other factors may limit success (Davey et al. 2013). Furthermore, success depends upon sampling an adequate number of individuals from each sex with sufficient read depth (Gamble and Zarkower 2014). All of these factors should be taken under consideration when interpreting results. However, violating one or more of these tenets does not inevitably lead to failure and the RAD-seq workflow seems generally robust. For example, we identified and validated a sex-specific marker in *Lialis burtonis* despite our poor sampling, possibly due to the large nonrecombining portion of the Y chromosome in this species (Gorman and Gress 1970). The PCR validation step requiring presence/absence or a sex-specific size polymorphism makes our workflow particularly conservative, and sex-specific markers that are actually present in the data may be missed. Most of the putative sex-specific RAD-seq markers we tested amplified in both males and females, producing just a single band on a gel (table 1). Under our current workflow we would consider those markers as failing the validation step and they would not be further analyzed. However, we have subsequently identified sex-specific SNPs and short indels by Sanger sequencing the PCR amplicons from a handful of these failed markers, and these were consistent with results from the validated markers in the same species. Indeed, Sanger sequencing a handful of markers in four of the six so-called “failed” species also recovered sex-specific markers. These preliminary data suggest that alternative means of validating sex-specific markers are quite feasible and can provide a high overall success rate in using RAD-seq to assess sex chromosome status.

We identified sex-specific markers in 12 gecko species, 8 of which were previously uncharacterized for sex-determining systems. With these new data, coupled with recent cytogenetic discoveries (Pokorná, Rens, et al. 2014), sex-determining systems are now known in at least one species in each of the seven gecko families and in most cases, several species in each family. This raises the number of putative transitions among sex-determining systems in geckos from 8 or 9 (Gamble 2010) to between 17 and 25 (table 3, fig. 3). Part of this increase may reflect a change in the method used to count transitions, as model-based stochastic mapping used here will typically provide a higher, and more realistic, count of character changes than the parsimony method used previously (Gamble 2010; Revell 2013). However, methodological differences are not the sole reason for the disparity. The largest difference comes from the increased species sampling in previously undersampled parts of the gecko phylogeny, due mostly to the RAD-seq data generated here.

Our results highlight the dynamic nature of gecko sex determination, which is particularly striking given that fewer than 3% of gecko species have a sex-determining system known with high confidence ([supplementary table S5, Supplementary Material](#) online). Compared with other squamates, the number of transitions in geckos is disproportionate to the number of gecko species. Approximately 16% of squamate species are geckos, 1,579 geckos out of 9,671 squamate species ([www.reptile-database.org](http://www.reptile-database.org), September 15, 2014), yet geckos have roughly one-half to two-thirds of observed transitions among sex-determining systems. The dramatic number of transitions among sex-determining systems in geckos can be attributed to the retention of TSD later in the clade’s diversification.

RAD-seq recovered two instances of closely related gecko species with different sex chromosome systems. The first was among *Hemidactylus* where *H. turcicus* and *H. mabouia* have male heterogamety whereas *H. frenatus* has female heterogamety. The second example involves the Sphaerodactylidae where two *Sphaerodactylus* species examined have male heterogamety whereas *Aristelliger* has female heterogamety. *Aristelliger* and *Sphaerodactylus* are the first two genera in Sphaerodactylidae with confirmed sex chromosome systems. Previously, *Gonatodes ceciliae* had been suspected of having male heterogamety due to a heteromorphic chromosome pair observed in two males (McBee et al. 1987). However, these results are suspect as no females were sampled and both males exhibited different karyotypes, suggesting polymorphism in

karyotypes. The European leaf-toed gecko *Euleptes europaea* may also have male heterogamety (Gornung et al. 2013); however, only one individual of each sex was examined and heteromorphism was slight. Further work including additional cytogenetics and/or RAD-seq is necessary to confirm sex chromosome systems in both of these species.

## Evolution of Squamate Sex-Determining Systems

We found that TSD was the most likely sex-determining system in the most recent common ancestor of squamates using both the ARD model and the preferred TRAP model. This result was consistent in data sets that included and excluded skinks with putative TSD. TSD has been recovered as the ancestral squamate sex-determining system in previous comparative phylogenetic reconstructions (Janzen and Krenz 2004; Pokorná and Kratochvíl 2009), whereas additional ancestral state reconstructions have been equivocal (Organ and Janes 2008). However, based on other, noncomparative data, it has been assumed that reptiles are predisposed to evolve TSD from genetic systems rather than the reverse as observed here (Georges et al. 2010). Although the plurality of our analyses recovered TSD in the most recent common ancestor of squamates, this result is sensitive to character coding and taxon sampling ([supplementary fig. S8, Supplementary Material](#) online) and should be interpreted cautiously. The fixation of sex chromosomes early in a clade's history appears to contribute to sex chromosome stability. Sex chromosomes evolved early in most of the major squamate clades, with acrodonts and geckos, and possibly skinks, being the exception. It is also worth noting that our results involve far fewer ambiguous ancestral nodes as compared with previous attempts to reconstruct the evolutionary history of squamate sex-determining systems (Janzen and Krenz 2004; Organ and Janes 2008; Pokorná and Kratochvíl 2009). This is likely due to better phylogenetic resolution and more accurate estimates of branch lengths, avoiding ambiguous/duplicate character states like "GSD," broader taxonomic sampling, and a model-based framework to identify and use the transition model that best fit the data.

The evolution of sex chromosomes early in a clade's history may provide time for sufficient degeneration between the X and Y (or Z and W) to inhibit transitions, thereby triggering an evolutionary trap. Indeed, the stability of sex chromosomes in mammals and birds has been attributed to the trap model (Bull 1983; van Doorn and Kirkpatrick 2010). Although the data suggest that most, if not all, sex chromosomes in squamates also act as traps, this may not be true in all vertebrate groups. Known exceptions occur in amphibians (Hillis and Green 1990; Miura 2007; Evans et al. 2012) and fish (Mank et al. 2006; Takehana et al. 2007; Ross et al. 2009). These exceptions could reflect the undifferentiated nature of the homomorphic sex chromosomes in most amphibians and many fish species rendering them nontrap-like. It should be pointed out that not all homomorphic sex chromosomes, as defined cytogenetically, are similar to each other at the DNA sequence level. Significant allelic differences and distinct gene content between the X and Y (or Z and W) can evolve without a concomitant change in visible chromosomal morphology (Vicoso, Emerson, et al. 2013; Gamble et al. 2014). Sex chromosomes can avoid degeneration and remain poorly differentiated for several reasons. Frequent turnover of sex-determining systems can inhibit differentiation as newly derived sex chromosomes will not have had time to degenerate (Charlesworth 1991; Volff et al. 2007; Graves 2008; Blaser et al. 2014). Sex chromosome differentiation can also be slowed through persistent recombination between the X and Y (or Z and W), either through occasional sex reversals or by the advantages conferred by maintaining recombination by limiting Hill–Robertson effects and thus preventing degeneration through Muller's ratchet (Perrin 2009; Adolfsson and Ellegren 2013; Otto 2014). Any sex chromosome system that has not yet reached a sufficient level of degeneration may be susceptible to capture by another system. Indeed, theoretical models indicate a variety of mechanisms can lead to transitions (Bull and Charnov 1977; Werren and Beukeboom 1998; van Doorn and Kirkpatrick 2007, 2010; Grossen et al. 2011; Quinn et al. 2011; Blaser et al. 2013). The level of sex chromosome degeneration necessary to prevent turnover is unknown and may vary significantly among lineages. However, comparing sex chromosomes in clades with trap-like systems to clades with high rates of turnover can provide important insights into what

makes some systems inert to transitions whereas others are prone to transitions (Vicoso, Emerson, et al. 2013; Gamble et al. 2014). It is important to note that although the TRAP model provided the best fit to our data, this conclusion could be challenged by the future discovery of additional squamate sex-determining systems or the addition of homology data that could identify cryptic transitions that did not involve a change in heterogamety (e.g., fig. 1C).

Recovery of TSD as the ancestral sex-determining system in squamates, acrodonts, and geckos sets up several predictions that can be tested with additional data. One prediction is that TSD mechanisms among squamates are retained from a common ancestor and will likely have a common molecular mechanism (Pokorná and Kratochvíl 2009). Additionally, if skinks with putative TSD are indeed shown to have sex chromosomes and male heterogamety is the ancestral state for Scincidae, then the temperature sensitivity exhibited by many skink species would most likely be secondarily derived. This scenario would make skinks ideal subjects for examining the evolution of genotype/environment interactions and testing hypotheses concerning the adaptive evolution of environmental sex determination, for example, Charnov/Bull hypothesis (Charnov and Bull 1977; Bull 1981; Shine 1999; Langkilde and Shine 2005). Using a technique like RAD-seq to target a phylogenetically diverse set of skink species would be an extremely productive means to solve this problem and would go a long way toward resolving the current confusion in that clade.

TSD in the most recent common ancestor of squamates generates another prediction; sex chromosomes in different lineages will be independently derived and not homologous. This appears to be true given the limited data in reptiles and there are no known cases of shared homology among sex chromosomes in any of the major squamate lineages (Matsubara et al. 2006; Ezaz, Moritz, et al. 2009; Kawai et al. 2009; Alföldi et al. 2011; O'Meally et al. 2012; Srikulnath et al. 2014). Among lineages that retained TSD fairly late into their history, acrodonts (chameleons and agamas), geckos, and possibly skinks, this also appears true. No formal assessments of skink sex chromosome homology have been performed but Donnellan (1991) suggested that the sex chromosomes in *Saprosaurus czechurai* and *Lampropholis guichenoti* (aka *Lampropholis* sp. C) were different chromosomal linkage groups. Among agamids three phylogenetically distinct groups have female heterogamety and their sex chromosomes appear to be nonhomologous (Ezaz, Quinn, et al. 2009). Although these agamid examples require additional confirmation they appear to confirm that retention of TSD later in a radiation's history can enable repeated, independent recruitment of sex chromosomes from different autosomal linkage groups. Among geckos, comparative chromosome fluorescent in situ hybridization (FISH) painting shows no evidence for homology among sex chromosomes of five species from three different families (Pokorná et al. 2011; Matsubara et al. 2014). Accurately counting the large number of transitions in geckos makes homology assessment a high priority. Knowing sex chromosome homology is necessary to properly count the number of transitions among sex-determining systems more generally. For example, the ZW systems in snakes, lacertas, softshell turtles, and birds are all derived from different autosomal linkage groups (Kawai et al. 2007; Srikulnath et al. 2014). Simply coding these as "ZW" in a comparative analysis fails to describe the actual diversity of sex-determining systems under examination and could undercount the number of transitions that have occurred.

Future study of squamate sex-determining systems should also focus on improving taxon sampling. Methods like RAD-seq will be useful to fill the many remaining sampling gaps in geckos and other squamates. Several squamate clades still lack any information on basic sex-determining systems. These include Xantusiidae (night lizards), Cordylidae (girdled lizards), Gerrhosauridae (plated lizards), Anguillidae (glass lizards and alligator lizards), Xenosauridae (knob-scaled lizards), and Shinisauridae (crocodile lizards). Additional squamate clades have limited sex determination data, only one species per clade in most cases. These include Dibamidae (blind lizards), Amphisbaenia (worm lizards), Teiidae (tegu and whiptails), and Chamaeleonidae (chameleons). Furthermore, it is quite likely that there are many more instances of sex chromosomes yet to be discovered in the two clades with the most transitions, Agamidae (agamids) and Gekkota (geckos). One assumption of

phylogenetic comparative methods is that all the relevant taxa have been sampled (Omland 1999). Consistent with this assumption, we show here that the addition of even a few taxa with alternate sex-determining systems can impact model choice and ancestral state reconstructions. Given how many squamate clades remain poorly known, or completely unknown, regarding sex determination it stands to reason that our current results could change with the addition of data from more squamate taxa. Therefore, filling in sampling gaps should be a top priority. Greater knowledge about squamate sex-determining systems will enhance their utility as a model clade to study the origins and evolution of sex-determining systems and there are likely many more insights to be discovered.

## Conclusions

Squamates, and geckos in particular, provide an excellent set of replicate evolutionary “experiments” to examine the origin and evolution of sex chromosomes and sex-determining systems. Identifying multiple examples of independently derived XY and ZW taxa will be important to distinguish factors common to all XY (or ZW) clades from factors unique to each individual group and help answer the many outstanding questions about the origins and evolution of sex chromosomes (Charlesworth and Mank 2010; Naurin et al. 2010; Bachtrog et al. 2011, 2014; Otto et al. 2011; Adkins-Regan and Reeve 2014). Fully developing these model clades requires identification of sex-determining systems in additional species. RAD-seq is a critical enabling technology that can help fill that sampling gap.

## Materials and Methods

### Identifying Gecko Sex Chromosome System

Sex chromosomes in 12 gecko species (table 1 and [supplementary table S1, Supplementary Material](#) online) were investigated using RAD-seq and a modified version of a previously published analytical pipeline (Gamble and Zarkower 2014). RAD-seq libraries were constructed following a modified protocol from Etter et al. (2011). Briefly, genomic DNA was extracted from tail clips or liver and digested with high-fidelity *SbfI* restriction enzyme (New England Biolabs). Individually barcoded P1 adapters were ligated onto the *SbfI* cut site for each sample. Samples were pooled by sex into separate male and female libraries and sonicated using a Fisher Scientific model 500 Ultrasonic Dismembrator. Libraries were size selected into 200- to 500-bp fragments using magnetic beads in a PEG/NaCl buffer (Rohland and Reich 2012). Libraries were blunt-end-repaired, and a 3'-adenine overhang added to each fragment. We added a P2 adapter containing unique Illumina barcodes for separate male and female libraries. Libraries were amplified through 16 PCR cycles with Phusion high-fidelity DNA polymerase (New England Biolabs) and size-selected a second time into 250- to 550-bp fragments using magnetic beads in polyethylene glycol (PEG)/NaCl buffer. Samples were sequenced on an Illumina HiSeq2000 at the University of Minnesota Genomics Center, using 100-bp paired-end reads. We were able to multiplex between 35 and 41 samples per HiSeq lane. Complete adapter and barcode sequences are listed in [supplementary table S2, Supplementary Material](#) online. Sequences are available at the NCBI Short Read Archive (PRJNA267722).

We used the `process_radtags` script from Stacks-1.01 (Catchen et al. 2011) to demultiplex raw Illumina reads. Forward reads were trimmed to 85 bp, removing low-quality bases at the 5'-end of the read and ensuring all reads were the same length. RADtools 1.2.4 (Baxter et al. 2011) was used to generate candidate RADtags 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 output from the RADtools scripts includes the presence or absence of each locus and allele for every sampled individual, permitting the identification of putative sex-specific markers. However, further steps are necessary to confirm and validate the accuracy of these putative sex-specific markers. We wrote a python script ([supplementary file S1, Supplementary Material](#) online) to automate the confirmation process as described in Gamble and Zarkower (2014). Briefly, the script identifies putative sex-specific markers from the RADtools output and excludes from further consideration any markers that appear in the original reads files from the opposite sex. The script then selects forward and reverse reads from the remaining putative sex-specific markers. These paired reads were subsequently assembled into contigs using either Sequencher 5.0.1 (GeneCodes) or MIRA 3.4 as implemented in Galaxy (Chevreux et al. 1999; Giardine et al. 2005; Goecks et al. 2010). We used PCR to validate the sex specificity of putative sex-specific markers. In most cases, we attempted to validate only a subset of the sex-specific markers identified by RAD-seq (table 1). We prioritized markers for PCR validation using several ad hoc criteria including prioritizing markers from the sex exhibiting the most sex-specific markers; choosing markers that lacked repeat motifs as identified using Repeatmasker (Smit et al. 2014); and choosing markers with lower read depth, indicative of a hemizygous allele. It should be noted that PCR validation only detects the presence/absence or significant size differences among markers. It is possible that some sex-specific alleles identified by our pipeline would not be substantiated by the PCR validation step. Such markers would amplify in both sexes but differ in sequence polymorphisms, such as multiple ( $\geq 3$ ) single nucleotide polymorphisms (SNPs) or short, 1–5 bp indels. Validating these sorts of markers would require further work. PCR primers were designed using Primer3 (Koressaar and Remm 2007; Untergasser et al. 2012) and validated primers are listed in [supplementary table S3, Supplementary Material](#) online. We used the following PCR profile in all reactions with primer-specific annealing temperatures: An initial 5-min denaturation at 94 °C followed by 32 cycles of denaturation (30 s at 94 °C), annealing (45 s at 52–55 °C), and extension (1 min at 72 °C), followed by a final extension of 5 min at 72 °C.

## Evolution of Squamate Sex-Determining Systems

The newly described gecko sex-determining systems, coupled with other recent discoveries about squamate sex determination (Gamble et al. 2014; Pokorná, Rens, et al. 2014; Pokorná, Rovatsos, et al. 2014; Rovatsos et al. 2014) prompted us to re-examine the evolution of sex-determining systems in squamates. We gathered sex-determining system data for lizards and snakes from the literature ([supplementary table S4, Supplementary Material](#) online) as well as newly discovered sex chromosome systems from the RAD-seq (see Results). We considered all sex-determining systems as one of three discrete states: TSD, male heterogamety (XY), or female heterogamety (ZW). Species with multiple sex chromosomes, such as the  $X_1X_1X_2X_2/X_1X_2Y$  system occurring in *Lialis burtonis* and some *Anolis* (Gorman and Atkins 1966; Gorman and Gress 1970) or the  $Z_1Z_1Z_2Z_2/Z_1Z_2W$  seen in some lacertids and elapids (Singh et al. 1970; Odierna et al. 1996), were included as male (XY) or female heterogamety (ZW), respectively. We did not include species that have GSD, as determined through incubation experiments, but lack evidence of either XY or ZW sex chromosomes. These species most certainly have as yet unidentified XY or ZW sex chromosomes and a separate “GSD” category has been used in past comparative analyses for these species (Janzen and Krenz 2004; Pokorná and Kratochvíl 2009). However, inclusion of a fourth category overlapping with the other two character states introduces unnecessary uncertainty into the analyses and may violate basic assumptions about phylogenetic comparative analyses (Omland 1999). We also excluded species with questionable sex-determining systems following Pokorná and Kratochvíl (2009) and Ezaz, Sarre, et al. (2009) for nongecko squamates and we reevaluated geckos here ([supplementary table S5, Supplementary Material](#) online).

Sex chromosomes and TSD have been considered ends of a continuum and both systems appear to coexist in a handful of squamate species (Sarre et al. 2004). In these species, extreme incubation temperatures will create a mismatch between genotypic and phenotypic sex, a phenomenon known as temperature-induced sex reversal

(Yoshida and Itoh 1974; Tokunaga 1985; Quinn et al. 2007, 2009; Radder et al. 2008). Temperature-induced sex reversal is distinct from typical TSD due to the presence of sex chromosomes and the sex reversal is biased to favor only one sex; for example, sex reversal in bearded dragons (*Pogona vitticeps*) turns genotypic males into phenotypic females but never the reverse (Quinn et al. 2007). We considered these species as having either XY or ZW in our analyses as it appears that sex chromosomes are the primary sex determiners in these taxa (Quinn et al. 2007, 2009; Radder et al. 2008). The relative roles of temperature and genotype are more ambiguous in skinks. Temperature has been shown to influence sex ratios in several skink species lacking evidence of heteromorphic sex chromosomes (Langkilde and Shine 2005) and these species have been considered as having TSD in previous analyses (Organ and Janes 2008). However, the literature concerning TSD as a distinct sex-determining system in skinks is far from conclusive (Pokorná and Kratochvíl 2009). It is possible that additional research will show that these skink species do not have proper TSD but instead have homomorphic sex chromosomes with temperature-induced sex reversal. Therefore, to accommodate the current uncertainty in skink TSD we performed our analyses twice, both with and without putative TSD skink species, and examined whether their inclusion had any significant influence on our results.

We used a well-sampled molecular phylogeny of squamates to map sex-determining systems. The original maximum-likelihood tree consisted of 4,161 lizard and snake species using tuatara as an outgroup (Pyron et al. 2013) and was time-calibrated using penalized likelihood (Pyron and Burbrink 2014). We trimmed the tree to include just taxa matching our sex determination data (see [supplementary table S4, Supplementary Material](#) online). This consisted of 163 taxa for the data set that excluded skink species with putative TSD and 166 taxa for the data set that included these species. A few species with sex determination data were not included in the Pyron et al. (2013) phylogeny; in those cases (listed in [supplementary table S4, Supplementary Material](#) online), we used another closely related species instead. These substitutions should have limited influence on the subsequent analyses. Several clades, including snakes, Lacertidae, and the pleurodont genera *Anolis* and *Sceloporus*, have numerous species with described heteromorphic sex chromosomes. We sampled only representative taxa from these clades as sex chromosome systems appear to be invariant within these lineages (Matsubara et al. 2006; Vicoso, Emerson, et al. 2013; Gamble et al. 2014; Rovatsos et al. 2014; Srikulnath et al. 2014) and subsampling should not impact count estimates of the number of transitions among sex-determining systems across squamates as a whole. A few additional species were excluded from analyses because they were not included on the phylogeny and there were no appropriate substitutes, for example, *Pseudemoia* (Hutchinson and Donnellan 1992). Sex determination data and pruned phylogenies are available on DRYAD (doi:10.5061/dryad.n69t3).

We reconstructed the evolution of sex-determining systems in lizards and snakes through maximum likelihood using the ace command in the R package Ape 3.1-4 (Paradis et al. 2004). We identified the transition rate matrix that best fit the data by comparing likelihood scores among alternate transition rate models using Akaike Information Criterion (AIC). Four transition rate models were considered: A six-parameter model that had different rates for every transition type, the ARD model; a three-parameter model that had equal forward and reverse rates between states, the symmetrical rates (SYM) model; a two-parameter model that assumes once sex chromosomes evolve there are no transitions away from them, the TRAP model (Bull and Charnov 1977; Bull 1983; Pokorná and Kratochvíl 2009); and a single-parameter model with equal rates (ER) among all transitions. We explored the robustness of our root state estimates to different character coding schemes by rerunning the maximum-likelihood analysis with XY and ZW character states combined into a single GSD category.

We counted the number of transitions among sex-determining systems in lizards and snakes with stochastic mapping (Nielsen 2002; Huelsenbeck et al. 2003) implemented in the R package phytools 0.4-31 (Revell 2012). Stochastic mapping summarizes the results of multiple trait mappings onto a phylogeny using a continuous-time Markov process. We used both the ARD model and the transition rate matrix that best fit the data, as



determined previously. We ran 1,000 simulations using the `make.simmap` command and summarized results using `describe.simmap`. We compared the number of transitions in geckos with the total number of transitions estimated across all lizards and snakes using the `extract.clade.simmap` command.

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## Author notes

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