4-2018

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Journal of Evolutionary Biology, Vol. 31, No. 4 (April 2018) : 484-490. DOI.

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Abstract

Much of our current state of knowledge concerning sex chromosome evolution is based on a handful of 'exceptional' taxa with heteromorphic sex chromosomes. However, classifying the sex chromosome systems of additional species lacking easily identifiable, heteromorphic sex chromosomes is indispensable if we wish to fully understand the genesis, degeneration and turnover of vertebrate sex chromosomes. Squamate reptiles (lizards and snakes) are a potential model clade for studying sex chromosome evolution as they exhibit a suite of sex-determining modes yet most species lack heteromorphic sex chromosomes. Only three (of 203) chameleon species have identified sex chromosome systems (all with female heterogamety, ZZ/ZW). This study uses a recently developed method to identify sex-specific genetic markers from restriction site-associated DNA sequence (RADseq) data, which enables the identification of sex chromosome systems in species lacking heteromorphic sex chromosomes. We used RADseq and subsequent PCR validation to identify an XX/XY sex chromosome system in the veiled chameleon (*Chamaeleo calyptratus*), revealing a novel transition in sex chromosome systems within the Chamaeleonidae. The sex-specific genetic markers identified here will be essential in research focused on sex-specific, comparative, functional and developmental evolutionary questions, further promoting *C. calyptratus'* utility as an emerging model organism.

Introduction

It should not be surprising that we still lack basic knowledge of sex-determining mechanisms for many species given the extensive variety observed among multicellular organisms (Bachtrog *et al*., **2014**). Until recently, cytogenetic methods to identify morphologically dissimilar, or heteromorphic, pairs of chromosomes that occur in one sex but not the other were the predominant means of determining an organism's sex chromosome system. Species where males are the heterogametic sex are said to have an XX/XY sex chromosome system, and the inverse, female heterogamety, is called ZZ/ZW (Bull, **1983**; Marshall Graves, **2008**). The bulk of what we know about sex chromosomes is mostly based on the handful of 'exceptional' taxa that exhibit heteromorphic sex chromosomes [such as mammals and *Drosophila* (XX/XY) or birds (ZZ/ZW)]. Yet most vertebrate species lack heteromorphic sex chromosomes having instead morphologically similar, or homomorphic, sex chromosomes, or lacking sex chromosomes altogether, e.g. environmental sex determination (Devlin & Nagahama, **2002**; Matsubara *et al*., **2006**; Stöck *et al*., **2011**; Gamble & Zarkower, **2014**; Otto, **2014**). Because standard cytogenetic techniques cannot identify these homomorphic sex chromosomes, the sex chromosome systems of huge swaths of the tree of life remain unknown and unstudied. However, recent advances in both cytogenetics and DNA sequencing techniques have enabled the identification of sex chromosome systems in species with homomorphic sex chromosomes, generating a renewed interest in discovering and cataloging the sex chromosome systems of previously intractable taxa. These data are indispensable if we wish to fully understand the evolutionary patterns and processes affecting the origins, degeneration and turnover of sex chromosomes.

Squamates (~10 000 species of lizards, snakes and amphisbaenians; Uetz *et al*., **2017**) are an excellent model for studying sex chromosome evolution as they exhibit a suite of sex-determining modes, including temperature-dependent (TSD) and genetic (GSD) sex determination, with both female (ZZ/ZW) and male (XX/XY) heterogamety, and numerous independent transitions among them (Bull, **1980**; Wapstra *et al*., **2007**; Ezaz *et al*., **2009**; Pokorná & Kratochvíl, **2009**; Gamble, **2010**; Gamble *et al*., **2015**). However, the sex-determining systems of many squamate clades are poorly known, with knowledge limited to just one or two species (Pokorná & Kratochvíl, **2009**; Gamble *et al*., **2015**). Indeed, many squamate families do not have any species with known sex-determining systems, for example Anguidae (glass and alligator lizards), Cordylidae (girdled lizards), Corytophanidae (basilisks and casque-headed lizards), Gerrhosauridae (plated lizards), Shinisauridae (crocodile lizards), Xantusiidae (night lizards) and Xenosauridae (knob-scaled lizards) (Gamble *et al*., **2015**). This restricts the usefulness of squamates as a model to study the pattern and process of sex chromosome evolution.

Therefore, a concentrated effort to identify sex chromosome systems in these clades should be undertaken to fill gaps in our knowledge.

Chameleons (Chamaeleonidae) are one of these poorly known squamates clades. Previous (including anecdotal) evidence has suggested TSD in at least one chameleon species (Schmidt *et al*., 1994) but GSD in others (Viets *et al*., 1994), with extensive variation in karyotypic formula (2*n* = 20–62) (Olmo & Signorino, 2005; Rovatsos *et al*., 2017). Of the approximately 50 chameleon species that have been karyotyped (of ~203 sp. total) (Olmo & Signorino, 2005; Rovatsos *et al*., 2015a, 2017), only three species in two genera have clearly diagnosable heteromorphic sex chromosomes. In the late 1980s, eleven species of South African *Bradypodion* were karyotyped and in one, *Bradypodion ventrale* , the chromosomal complement differed between sexes (2*n* = 34 in males and 35 in females), leading the investigator to assume a ZZ/ZW system [Gordon, pers. comm. to Olmo (in Olmo & Signorino, 2005)]. Unfortunately, these results were never published and any subsequent conclusions based on these data should be treated with caution. Recently, however, Rovatsos *et al*. (2015a) combined modern and classic cytogenetic techniques to provide two additional, well-supported cases of female heterogamety in two species of Malagasy chameleons in the genus *Furcifer*.

Although species in two chameleon genera have ZZ/ZW sex chromosomes, it is unclear whether a ZZ/ZW system is universal across Chamaeleonidae. Some vertebrate clades, including birds and mammals, show extreme conservatism in sex-determining mechanisms (Ohno, 1967; Smith *et al*., 1999; Marshall Graves, 2006; Ellegren, 2010). The prevailing theory within squamate reptiles is that sex chromosome systems are highly labile (Ezaz *et al*., 2009), although this was recently challenged based on evidence from a few squamate clades with relatively stable systems (Gamble *et al*., 2014; Rovatsos *et al*., 2014, 2015b, 2016). Yet, in general, we know very little about sex chromosome systems across most squamate groups. As we gather more data from multiple lineages within diverse clades, we are likely to make surprising discoveries, particularly in groups for which stability has long been inferred (Gamble *et al*., 2017). Therefore, gathering data for a diverse subset of taxa within major squamate clades is indispensable before we can accurately evaluate general hypotheses of squamate sex chromosome evolution.

The goal of this study was to resolve the sex chromosome system in the veiled chameleon, *Chamaeleo calyptratus*. This hardy species is endemic to the south-western Arabian Peninsula (Necas, 1999) but is now exotic and invasive in Florida (Krysko *et al*., 2004) and Hawaii (Kraus & Duvall, 2004), likely due to its popularity in the pet trade. Whether sex is determined in this species via TSD or GSD has been controversial, and two independent, controlled breeding experiments came to conflicting conclusions regarding the effect of incubation temperature on hatchling sex ratios (Andrews, 2005; Ballen *et al*., 2016). Published *C. calyptratus* karyotypes failed to identify heteromorphic sex chromosomes, even using advanced cytogenetic techniques such as comparative genomic hybridization (CGH) (Pokorná *et al*., 2011; Rovatsos *et al*., 2017).

Recent research has extolled *C. calyptratus'* utility as a model for studying the comparative and functional development of the vertebrate body plan, while more specifically being the only currently identified squamate taxon (of the ~10 000 recognized species) available at a significantly early pregastrula stage at the day eggs are laid (Diaz & Trainor, 2015; Diaz *et al*., 2015, 2017; Stower *et al*., 2015). Thus, identifying their sex chromosome system fills an essential knowledge gap in chameleon reproductive life history and may provide insight into the evolution of sexual dimorphism. Using a recently developed RADseq methodology combined with PCR validation (Gamble & Zarkower, 2014; Gamble *et al*., 2015, 2017), we here report that unlike other members of the Chamaeleonidae, *C. calyptratus* possesses an XX/XY sex chromosome system, revealing a novel transition in sex chromosome systems within this family.

Materials and methods

We constructed single-digest, restriction site-associated DNA sequencing (RADseq) libraries for 12 male and 12 female samples of captive bred *C. calyptratus* (Table S1). This species is sexually dimorphic throughout life; thus, sexing is a simple process. At birth, all males have a conspicuous bump or spur on each heel that grows larger as the animal ages and can be later combined with a suite of additional, secondary male sexual characteristics (Andrews, 2005; Diaz *et al*., 2015). Additionally, all animals were dissected post-euthanasia and the gonadal sex corroborated our external diagnosis. Most of the chameleons (32 of 37) used for RADseq and PCR validations were siblings, labelled 'Trainor Lab' in Table S1. The Trainor Lab outcrosses chameleons by regularly introducing new males to prevent inbreeding. Nevertheless, we obtained five additional animals from introduced populations in South Florida, USA, labelled 'Pet Trade' in Table S1, to ensure our results were representative of the species as a whole and not an anomaly limited to a single inbred family.

We extracted genomic DNA using the Qiagen DNeasy Blood and Tissue extraction kit from fresh tail or liver samples. We constructed RADseq libraries following a modified protocol from Etter *et al*., 2011 (as described in Gamble *et al*., 2015). In brief, we digested genomic DNA using a high-fidelity *SbfI* restriction enzyme (New England Biolabs), after which we ligated individually barcoded P1 adapters onto each sample's *SbfI* cut site. Next, we pooled samples into multiple libraries, sonicated and size-selected (200- to 500-bp fragments) using magnetic beads in a PEG/NaCl buffer. Afterwards, we blunt-end repaired, dA tailed and ligated a P2 adapter containing unique Illumina barcodes to each of the pooled libraries. We then amplified libraries using NEBNext Ultra II Q5 polymerase (New England Biolabs) for 16 PCR cycles and size-selected a second time (250- to 600 bp fragments), again using magnetic beads in PEG/NaCl buffer. Finally, we pooled and sequenced libraries using paired-end 125 bp reads on an Illumina HiSeq2500 at the Medical College of Wisconsin. Demultiplexed reads are deposited at the NCBI Short Read Archive (SAMN08341003-SAMN08341026).

RADseq data were analysed using a previously described bioinformatics pipeline (Gamble & Zarkower, 2014; Gamble *et al*., 2015, 2017), but in brief, (i) we filtered and demultiplexed raw Illumina reads using the process_radtags script from Stacks (Catchen *et al*., 2011) with forward reads trimmed to 110 bp; (ii) we generated RADtags for each individual and identified candidate loci and alleles across all individuals from the forward reads using RADtools 1.2.4 (Baxter *et al*., 2011); (iii) we utilized a customized python script (Gamble *et al*., 2015) to identify putative sex-specific markers from the RADtools output. These are RAD markers found in one sex but not the other. This script also produces a second list of 'confirmed' sex-specific RAD markers, a subset of the initial list of sex-specific RAD markers; it excludes from further consideration any sex-specific markers that also appear in the original reads files from the opposite sex; and lastly, (iv) we used Geneious R10 (Kearse *et al*., 2012) to assemble forward and reverse reads into contigs for all confirmed sex-specific RAD markers. We presume that these sex-specific loci are unique to the heterogametic chromosome (Y or W), so that species with a surplus of male-specific RAD markers must logically have an XX/XY system, and vice versa for a female heterogametic (ZZ/ZW) system (Gamble & Zarkower, 2014; Gamble *et al*., 2015; Gamble, 2016). Our large sample size (n = 12m, 12f) should reduce the likelihood of false positives (Gamble & Zarkower, 2014; Gamble *et al*., 2017).

We used PCR to validate the sex specificity of the sex-specific RAD loci, as has been performed in previous studies (Gamble & Zarkower, 2014; Gamble *et al*., 2015, 2017; Fowler & Buonaccorsi, 2016; Gamble, 2016). This included PCR in an additional eight males and five females that were not part of the RAD sequencing (Table S1). PCR primers were designed using Geneious R10 (Table 1). Annealing temperatures ranged from 55 to 56°C.

Table 1. PCR primers used to validate sex-specific RADseq markers

Primer ID	Sequence (5'–3')
Cham_M2-F	CTG AAA GAC AAC CAC CAA GCG

Cham_M2-R	TAG GCA TGC CAT TGG TGT GAT
Cham_M3-F	AGG AAC TGT GTG AGT CTC AAT CA
Cham_M3-R	TTG CAC AAA AAG CTC AGA GCC
Cham_M11-F	GGG AAG GCT ATC AGG AAA CCC
Cham_M11-R	GTG GAC TGA GAG TGG TTC AGG
Cham_M12-F	CAA CCT CCT GCC AGG GAT TCT
Cham_M12-R	GAG GTG GAA GGA TTA GCC GAG
Cham_M13-F	ATT TGG GCA TCC TCA GGG AAG
Cham_M13-R	TGC TGT CTT CTT GAG CTG GTT

Results

We recovered 115 703 RAD markers, 91 378 with two or fewer alleles, which included 16 male-specific and two female-specific markers. Of these, 13 were 'confirmed' male-specific RAD markers and two 'confirmed' female-specific RAD markers. This excess of male-specific RAD markers suggests an XX/XY sex chromosome system. Of the 13 putatively Y-specific PCR primer pairs we designed, five amplified in a sex-specific manner (Table 1), producing a single bold band in each of the male samples (Fig. 1).

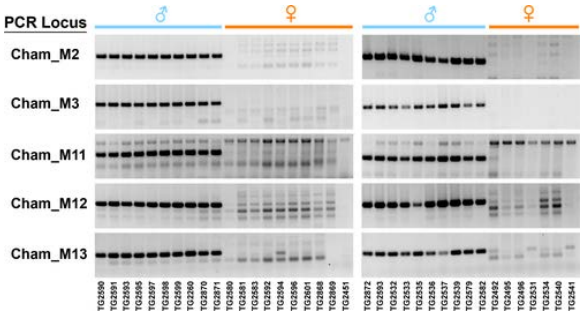

Figure 1 PCR validation of five male-specific RADseq markers in the veiled chameleon, *Chamaeleo calyptratus*. Primers amplified in a male-specific manner in the twenty males and seventeen females examined (see Table S1). Specimen ID numbers are listed below each well.

Discussion

These results provide the first robust evidence of sex chromosomes in the genus *Chamaeleo*, increasing our meagre knowledge concerning the phylogenetic distribution of sex chromosomes in the family Chamaeleonidae (Fig. 2). The RADseq methodology used herein has been pivotal in discovering previously unknown sex chromosome systems (Gamble & Zarkower, **2014**; Gamble *et al*., **2015**, **2017**, In Press; Fowler & Buonaccorsi, **2016**). The discovery of XX/XY sex chromosomes in *C. calyptratus* implies that at least one transition between ZZ/ZW and XX/XY systems has occurred within the Chamaeleonidae (Fig. 2). Our current understanding of vertebrate sex chromosome evolution is biased towards groups such as mammals and birds, which possess highly conserved, heteromorphic sex chromosome systems (e.g. Shetty *et al*., **1999**; Marshall Graves, **2006**; Ellegren, **2010**). Many other vertebrate clades, however, exhibit some degree of plasticity in sex-determining mechanisms (Hillis & Green, **1990**; Devlin & Nagahama, **2002**; Gamble *et al*., **2015**; Pan *et al*., **2016**). In general, reptiles have a high incidence of turnover between sex-determining mechanisms (Sarre *et al*., **2004**; Ezaz *et al*., **2009**; Rovatsos *et al*., **2016**), although recent work has discovered that a few, particularly diverse squamate lineages in fact possess highly conserved sex chromosomes (Vicoso *et al*., **2013**; Gamble *et al*., **2014**; Rovatsos *et al*., **2014**, **2015b**, **2016**). Yet these clades appear to be exceptions to the rule. Recent work on snakes, for example, which were previously assumed to all possess a ZZ/ZW sex chromosome system, revealed that boas and pythons in fact possess independently derived XX/XY sex chromosome systems – overturning 50+ years of perpetuated orthodoxy (Gamble *et al*., **2017**). Such studies illustrate that there is much more yet to

discover and that we still lack basic information as to the distribution of different sex-determining systems across large parts of the squamate phylogeny. As we continue to explore patterns of sex chromosome evolution across reptiles (and in particular, across squamates), the current results support the theory that transitions among squamate sex chromosome systems are more widespread than not (Ezaz *et al*., **2009**; Rovatsos *et al*., **2016**).

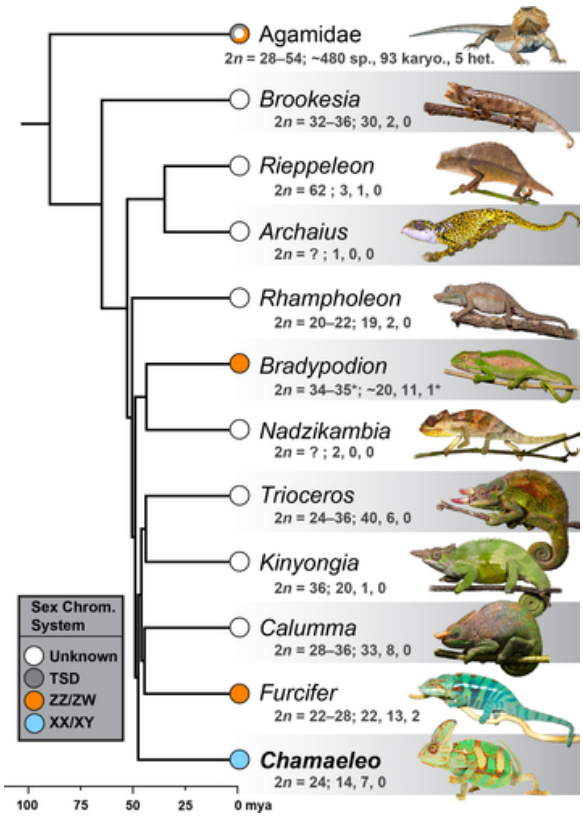

Figure 2 A time-calibrated phylogeny of the Acrodonta (modified from Tolley *et al*., 2013) displaying relationships among chameleon genera. Sex chromosome systems, if known, are indicated by coloured circles to the left of taxon names. Series of numbers under taxon names indicate diploid (2*n*) chromosomal complement (when known), the number of described species within the lineage, the subset that have been karyotyped, and the number that exhibit heteromorphic sex chromosomes (*the karyotypic data for *Bradypodion* remain unpublished). An XX/XY sex chromosome system in the genus *Chamaeleo* (in bold) is reported here for the first time, suggesting a transition between female and male heterogamety within the family.
See Acknowledgments for photograph credits.

The PCR validated male-specific loci (Table **1**) will further promote the use of *C. calyptratus* as a laboratory model for exploring sex-specific, comparative, functional and developmental evolutionary questions (Diaz *et al*., **2015**, **2017**). Sex determination assays provide a way to sex embryos prior to the onset of morphological sex determination or gonadogenesis, thus enabling researchers to study the development of sexually dimorphic phenotypes. Such assays have been crucial in mouse and chicken developmental studies (Hacker *et al*., **1995**; Smith *et al*., **1999**; McClive & Sinclair, **2001**; Clinton, **2009**), but few resources exist for the other amniote taxa. *Chamaeleo calyptratus* is highly fecund with an average clutch size of 40–50 (and up to 90) eggs and is easily bred in captivity, and embryos from early developmental stages such as pregastrulation and preneurulation are easily obtained from recently laid eggs (Diaz *et al*., **2015**, **2017**). The ability to now accurately sex these embryos is thus a powerful resource.

Our PCR-based sex test in *C. calyptratus* will also be useful for studying possible sex reversal in embryos due to environmental or maternal influence. Studies on the agamid, *Pogona vitticeps*, deftly illustrated that being able to diagnose an individual's genotypic sex was crucial for recognizing the co-occurrence of TSD and GSD in the same species, through identification of individuals with mismatched genotypic and phenotypic sex (Quinn *et al*., **2007**; Holleley *et al*., **2015**). Recent findings suggest that embryonic sex in *C. calyptratus* may be influenced by the interaction of incubation temperature and egg size (Ballen *et al*., **2016**). These results conflict with earlier observations of even sex ratios at a variety of incubation temperatures (Andrews, **2005**). It is reasonable to assume that if incubation temperature and egg size interact to influence sex that some proportion of the resulting embryos would be sex reversed. The PCR primers provided herein (Table **1**) provide a tool to accurately identify these mismatched individuals and should help clarify the extent of environmental and/or maternal influence on *C. calyptratus* sex determination.

Acknowledgments

The authors wish to thank Brendan Pinto for assistance in the laboratory; Aaron Griffing, Itzel Matamoros, Nick Schneider and Marley Borham for animal husbandry at Marquette University, Gardy for accommodation in Palmetto Bay, and Diana Baumann, Richard Kupronis, David Jewell, Kristy Winter and Ella Leslie of the Reptile and Aquatic Facility at the Stowers Institute for Medical Research. For the use of their wonderful photos, we thank Tyrone Ping, Werner Conradie, Henrik Bringsøe, Ferdy Timmerman and Susie Hassocks. All experiments were carried out in accordance with animal use protocols at Marquette University (AR279) and the Stowers Institute for Medical Research (2017-0177). This manuscript was improved via discussions with members of the Gamble Lab. This work was supported by Marquette University start-up funds to TG, and the Stowers Institute for Medical Research to PAT. We declare no conflict of interests.

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