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Non-Homologous Sex Chromosomes in Two Geckos (Gekkonidae: Gekkota) with Female Heterogamety

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Key Words

Chromosome paint · Cytogenetics · FISH · Homology · Reptilia

Abstract

Evaluating homology between the sex chromosomes of different species is an important first step in deducing the origins and evolution of sex-determining mechanisms in a clade. Here, we describe the preparation of Z and W chromosome paints via chromosome microdissection from the Australian marbled gecko *(Christinus marmoratus)* and their subsequent use in evaluating sex chromosome homology with the ZW chromosomes of the Kwangsi gecko *(Gekko hokouensis)* from eastern Asia. We show that the ZW sex chromosomes of *C. marmoratus* and *G. hokouensis* are not homologous and represent independent origins of female heterogamety within the Gekkonidae. We also show that the *C. marmoratus* Z and W chromosomes are genetically similar to each other as revealed by C-banding, comparative genomic hybridization, and the reciprocal painting of Z and W chromosome probes. This implies that sex chromosomes in *C. marmoratus* are at an early stage of differentiation, suggesting a recent origin.

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Transitions between different genetic sex-determining mechanisms involving a change in male and female heterogamety are readily apparent and easy to identify [Chen and Reisman, 1970; Volff and Schartl, 2001; Ogata et al., 2003; Ezaz et al., 2006; Sarre et al., 2011]. On the other hand, the evolution of a new sex chromosome system that does not involve a transition between male and female heterogamety, i.e. a transition from one XY system to a different XY system derived from a different autosomal pair, or a transition from one ZW system to another, can be identified only by assessing homology among the relevant sex chromosomes [Takehana et al., 2007; Tanaka et al., 2007; Cnaani et al., 2008; Henning et al., 2008; Ross et al., 2009; Vicoso and Bachtrog, 2013]. Considered more broadly, failure to incorporate chromosome homology will probably undercount the actual number of transitions among sex-determining mechanisms in a clade when mapping these mechanisms onto a phylogeny [Gamble, 2010]. Therefore, evaluating homology should be a high priority for researchers interested in studying

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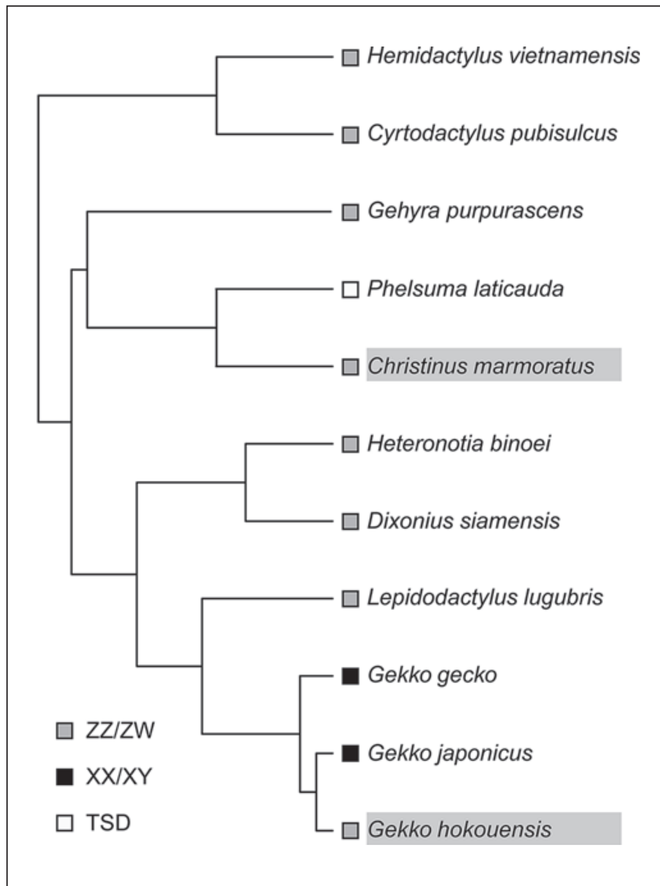

Fig. 1. Phylogenetic relationships among species in the family Gekkonidae with known sex-determining mechanisms. Genera with multiple species that all possess the same sex-determining mechanism are represented by a single species. Phylogeny and sex determination data were taken from Gamble [2010] and Gamble et al. [2012].

the evolution of sex chromosomes and sex-determining mechanisms.

Historically, chromosome homology was established using chromosomal morphology combined with G-, C-, or R-banding [Stock et al., 1974; Patton and Baker, 1978]. However, these features lack sufficient resolution and are susceptible to convergence, so similarities in morphology and banding patterns do not imply orthology of DNA sequences [Kluge, 1994; Stanyon et al., 1995]. Methods that either directly or indirectly compare DNA sequences between species are now widely used, and homology between the sex chromosomes of different species can be properly evaluated using several techniques. These include: FISH mapping of BACs, fosmids, or cDNAs [Matsubara et al., 2006; Takehana et al., 2007; Ezaz et al., 2009a, b; Ross et al., 2009; Gamble et al., 2014]; PCR amplification of sex-specific markers [Takehana et al., 2008; Gamble and Zarkower, 2014]; qPCR of sex-linked markers [Gamble et al., 2014; Rovatsos et al., 2014]; whole genome sequencing [Chen et al., 2014]; and chromosome painting using flow-sorted or microdissected chromosomes [Phillips et al., 2001; Grützner et al., 2004; Henning et al., 2008; Pokorná et al., 2011].

Here, we describe the preparation of Z and W chromosome paints via microdissection from the Australian marbled gecko *(Christinus marmoratus)* [Gray, 1845] and their subsequent use to evaluate sex chromosome homology with the ZW chromosomes of the Kwangsi gecko *(Gekko hokouensis)* [Pope, 1928]. Both species are in the family Gekkonidae (fig. 1). Although the sex-determining mechanisms have not been yet identified in most gekkonids, female heterogametic sex chromosomes (ZW system) are prevalent in Gekkonidae (fig. 1). Evaluating homology of *G. hokouensis* and *C. marmoratus* sex chromosomes provides an important step in accurately estimating the number of transitions among sex-determining mechanisms that have occurred across geckos. The sex chromosomes of geckos are also of particular interest because the *G. hokouensis* ZW pair is homologous with the avian ZW system [Kawai et al., 2009], supporting the hypothesis that the bird ZW system may be ancestral to reptiles and mammals [Graves, 2009]. However, non-homology of ZW pairs in closely related species [Pokorná et al., 2011] favored the alternate hypothesis that they were independently derived from a particularly propitious autosome [Graves and Peichel, 2010; O'Meally et al., 2012]. Thus studies of sex chromosome homology in geckos can help answer this deep question of amniotes' sex chromosome ancestry and evolution.

Four karyomorphs (2n = 32, 2n = 34, 2n = 36, and 2n = 36 with heteromorphic ZZ/ZW) have been identified in the widely distributed southern Australian *C. marmoratus* [King and Rofe, 1976; King and King, 1977]. The distribution of these karyomorphs' chromosomal forms is discrete and each karyomorph is allopatrically distributed [King and Rofe, 1976; King and King, 1977]. A subset of the 2n = 36 chromosomal form that lacks heteromorphic sex chromosomes has since been described as a separate species, *Christinus alexanderi* [Storr, 1987]. This cytogenetic diversity, coupled with molecular genetic diversity across its range, suggests that *C. marmoratus* is a species complex [Heinicke et al., 2014]. We examined *C. marmoratus* from the vicinity of Canberra (Australian Capital Territory), which are part of the 2n = 36 (ZZ/ZW) group. The heteromorphic Z and W are distinguishable

from each other and most other chromosomes [King and Rofe, 1976] and present an ideal opportunity to microdissect sex chromosomes from a ZW gecko species to generate a gekkonid sex chromosome paint.

The sex chromosomes of *G. hokouensis* represent the first attempt to identify a sex chromosome linkage group in a gecko [Kawai et al., 2009]. This species is widely distributed in southeastern China, Taiwan, some of the Ryukyu islands, and part of Kyushu, Japan. *G. hokouensis*, like *C. marmoratus*, is most likely a species complex [Shibaike et al., 2009]. All populations possess a diploid chromosome number of 2n = 38, although heteromorphic ZW sex chromosomes are present in only some populations [Kawai et al., 2009; Shibaike et al., 2009]. In contrast to a dissimilarity of their karyotypes, *G. hokouensis* and *C. marmoratus* sex chromosomes have a similar morphology: the Z chromosomes are acrocentric with small short arms, and the W chromosomes are distinct bi-armed chromosomes [King and Rofe, 1976; Kawai et al., 2009] (fig. 2). Sex chromosome heteromorphism in both species is likely to be the result of a pericentric inversion in the W, although the *G. hokouensis* W shows evidence of at least 1 additional rearrangement [King and Rofe, 1976; Kawai et al., 2009]. Despite their superficial similarity in morphology, we show that the ZW sex chromosomes of *C. marmoratus* and *G. hokouensis* are not homologous and represent independently derived ZW sex chromosomes within the Gekkonidae.

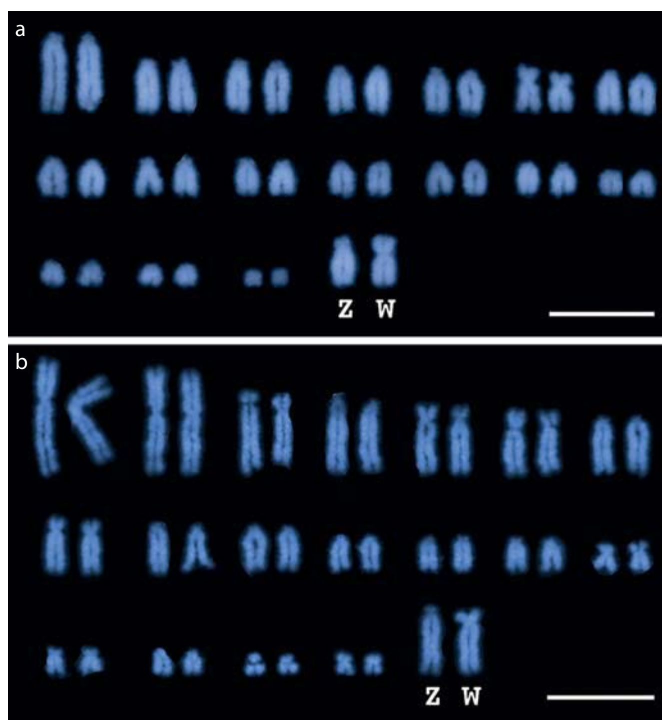

Fig. 2. DAPI-stained karyotypes of females of *C. marmoratus* (**a**) and *G. hokouensis* (**b**). Scale bars = 10 μm.

Materials and Methods

Animals

Tail tips were cut from 4 male and 5 female *C. marmoratus* and used for cell culture. Animal collection, handling, sampling, and all other relevant procedures were performed following the guidelines of the Australian Capital Territory Animal Welfare Act 1992 (Section 40), and conducted under CEAE 11/07 (the Committee for Ethics in Animal Experimentation at the University of Canberra). Fibroblasts of a male and a female *G. hokouensis* originally collected at Nakagusuku, the southern part of Okinawajima Island, the Ryukyu Archipelago, Japan, were used for cell culture [Kawai et al., 2009].

Chromosome Preparation

Metaphase chromosome spreads were prepared from fibroblast cell lines of tail tissues following the protocol described in Ezaz et al. [2008]. Briefly, minced tail tissues were implanted in a T25 culture flask containing AmnioMax medium (Life Technologies, Carlsbad, Calif., USA) and were allowed to propagate under the condition of 28°C and 5% CO_2. Once the fibroblast cells had grown to about 80% confluency, they were split into T75 flasks and subsequently split up to 4 passages before the chromosomes were harvested. Colcemid (Roche, Basel, Switzerland) was added to the culture flask at a final concentration of 75 ng/ml prior to harvesting. Following harvesting, cultured cells were suspended in 0.075 M KCl and fixed in 3:1 methanol:acetic acid, and the cell suspension was dropped onto glass slides, air-dried and stored at –80°C.

C-Banding

The C-banded chromosomes were obtained with the CBG method [Sumner, 1972]. Chromosome slides were treated in 0.2 N HCl for 40 min and rinsed by distilled water. Then the slides were denatured in 5% $Ba(OH)_2$ for 5 min at 50°C. Denaturation was stopped by rinsing the slides in 0.2 N HCl and distilled water. The chromosome slides were renatured by incubation in 2× SSC for 60 min at 60°C. Then the slides were rinsed with distilled water and stained with 4% Giemsa for 30 min.

Microdissection of Sex Chromosomes and Preparation of Chromosome Paints

We performed microdissection using an inverted phase-contrast microscope Zeiss Axiovert.A1 (Zeiss, Oberkochen, Germany) equipped with Eppendorf TransferMan NK 2 micromanipulator (Eppendorf, Hamburg, Germany). Glass needles were made from 1.0 mm diameter capillary glass using a glass capillary puller, Sutter P-30 Micropipette Puller (Sutter Instrument, Novato, Calif., USA) and sterilized by ultraviolet irradiation. Z and W chromosomes were scratched from freshly prepared slides of a female *C. marmoratus* with a glass needle using the micromanipulation system and transferred into 0.2-ml PCR tubes. Because the Z chromosome could be confused with several autosomes of similar size and

morphology (fig. 2a), microdissected Z chromosomes from each metaphase were transferred into separate PCR tubes. In contrast to the Z, the W chromosome was easily distinguishable from other chromosomes (fig. 2a). Thus, while we collected some W chromosomes individually, others were collected from multiple metaphases and placed into 1 PCR tube. Chromosome DNAs were amplified using GenomePlex® Single Cell Whole Genome Amplification Kit (Sigma-Aldrich, St. Louis, Mo., USA) according to the manufacturer's protocol with slight modification. The volume for all reaction steps was scaled down to half, and PCR amplification was increased to 30 cycles.

FISH with Chromosome Paints

FISH and CGH were conducted according to our previous study [Ezaz et al., 2005] with slight modification as follows. Chromosome paints were labeled by nick translation incorporating SpectrumGreen-dUTP (Abbott, North Chicago, Ill., USA) or SpectrumOrange-dUTP (Abbott). The labeled paint was precipitated with 20 μg glycogen as carrier, and dissolved in 15 μl hybridization buffer (50% formamide, 10% dextran sulfate, 2× SSC, 40 mM sodium phosphate pH 7.0 and 1× Denhardt's solution). The hybridization mixture was placed on a chromosome slide and sealed with a coverslip and rubber cement. The probe DNA and chromosome DNA were denatured by heating the slide on a heat plate at 68.5°C for 5 min. The slides were hybridized overnight in a humid chamber at 37°C. Hybridization was carried out for 2 days in cross-species chromosome painting. The slides were then washed by the following series: 0.4× SSC, 0.3% IGEPAL (Sigma-Aldrich) at 55°C for 2 min followed by 2× SSC, 0.1% IGEPAL at room temperature for 1 min. The slides were dehydrated by ethanol series and air-dried and then counterstained using 20 μg/ml DAPI, 2× SSC and mounted with anti-fade medium, Vectashield (Vector Laboratories, Burlingame, Calif., USA). FISH images were captured using a Zeiss Axioplan epifluorescence microscope equipped with a CCD camera (Zeiss). ISIS software or AxioVision (Zeiss) was used for microphotography and analyzing images.

CGH

Total genomic DNA was extracted from cultured fibroblasts using the DNeasy kit (Qiagen, Venlo, The Netherlands) and following the manufacturer's protocol. Genomic DNA was labeled by nick translation incorporating SpectrumOrange-dUTP (Abbott) for females and SpectrumGreen-dUTP (Abbott) for males. The labeled male and female DNA was coprecipitated with 20 μg glycogen as carrier, and dissolved in 15 μl hybridization buffer. The hybridization and washes were carried out as above.

Results

C-Banding and CGH

All *C. marmoratus* examined for this study had karyotypes with 2n = 36 chromosomes, and all females had morphologically differentiated ZW chromosomes (fig. 2a). C-bands were detected on the centromeric regions and telomeres of almost all chromosomes (fig. 3a). A faint C-band was also detected on the proximal region of the W chromosome. This C-band pattern indicates that most of the W chromosome is not heterochromatic.

We performed CGH on chromosome spreads from 3 female and 2 male *C. marmoratus* to examine whether the W chromosome contains any female-specific DNA. CGH images showed equal intensity of red signal (female genomic DNA) and green signal (male genomic DNA) on whole chromosomes and detected no accumulation of female-specific DNA (fig. 3b, c). Thus, the sex chromosomes of this species are morphologically differentiated but the DNA sequence of the W chromosome is not prominently divergent from that of the Z chromosome.

Chromosome Painting with Z and W Paints

We prepared 4 W chromosome probes and 10 Z chromosome probes. While 2 of the 4 W chromosome probes were amplified from single W chromosomes, the other 2 were amplified from 5 and 4 W chromosomes, respectively. All 10 Z probes were amplified from single Z chromosomes.

We tested the quality of each probe and the homology between the Z and W by painting each back onto metaphase spreads of female *C. marmoratus*. All 4 W probes successfully produced bright hybridization signals on whole regions of both Z and W chromosomes (fig. 3d). The 2 probes from multiple W chromosomes showed more intense signals than the 2 probes from single chromosomes (data not shown). Three of the 10 Z probes failed to hybridize, probably because of loss of the single chromosome at the microdissection step. The other 7 Z probes produced bright hybridization signals on whole regions of both Z and W chromosomes (fig. 3e). The Z and W painting patterns were similar (fig. 3d, e). The probes of both chromosomes produced intense signals around the C-band-positive centromeric region of the W chromosome, suggesting accumulation of some repetitive sequences in this region. The hybridization of Z and W probes to both Z and W chromosomes provides further evidence that DNA sequences of the 2 chromosomes are not highly differentiated from each other. Both Z and W probes produced hybridization signals on telomeric regions of several autosomes and faint hybridization signals on interstitial regions of a few autosomes. This suggests that some repetitive sequences are shared between sex chromosomes and autosomes.

Cross-Species Chromosome Painting to G. hokouensis Chromosomes

We carried out cross-species chromosome painting using *C. marmoratus* sex chromosome probes to meta-

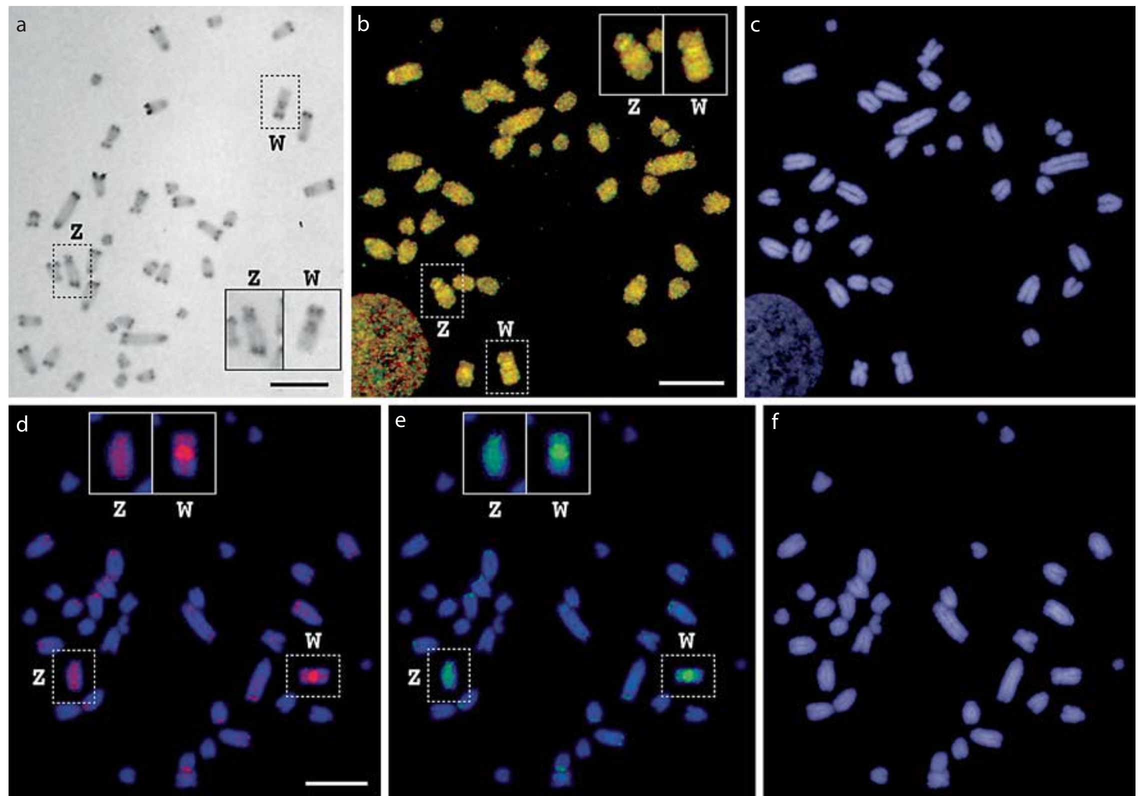

Fig. 3. C-banding and chromosome painting of female *C. marmoratus*. **a** C-banding distinguishes Z and W chromosomes. **b** CGH with female (red) and male (green) genomic DNA shows no obvious sex-specific sequences. **c** DAPI staining of the same metaphase. **d**, **e** Chromosome painting with W (**d**) and Z chromosome probes (**e**) to the same metaphase spread and the DAPI-stained image (**f**). Larger images of Z and W chromosomes are shown in **insets** (**a**, **b**, **d**, **e**). Scale bars = 10 μm.

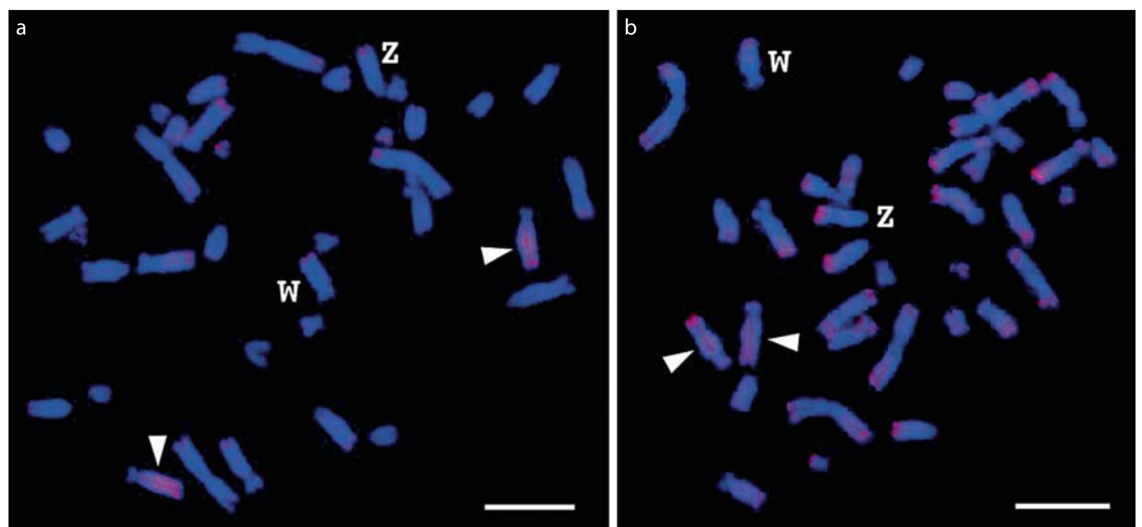

Fig. 4. Cross-species chromosome painting with *C. marmoratus* W (**a**) and Z (**b**) chromosome probes to metaphase spreads of a female *G. hokouensis*. Z and W chromosomes are indicated. Arrowheads point to hybridization signals. Scale bars = 10 μm.

phase spreads of female *G. hokouensis* in order to test for homology of the sex chromosomes between the 2 species. The *C. marmoratus* W probe hybridized to a large region of the fifth-largest autosomal pair of *G. hokouensis* (fig. 4a). The Z probe hybridized to the same autosomal pair, although the hybridization signal was not as strong as with the W probe (fig. 4b). These results indicate that *C. marmoratus* sex chromosomes share homology with *G. hokouensis* autosome number 5, and are not homologous to the sex chromosomes. Hybridization signals were also detected on interstitial and telomeric regions of additional chromosomes. Such signals were also observed in the painting within *C. marmoratus* (fig. 3d, e). These signals were probably caused by hybridization of the repetitive sequences shared among the chromosomes of the 2 species.

Discussion

Differences between *C. marmoratus* Z and W appear to be small. Evidence for similarity between the Z and W includes limited heterochromatin accumulation on the W based on C-banding, absence of female-specific signal using CGH, and the reciprocal hybridization of Z and W paints along nearly the entire length of both chromosomes. This implies that the sex chromosomes are at an early stage of differentiation, and suggests that they evolved recently. Both CGH and chromosome painting are not able to detect tiny sex-specific chromosomal regions so that finer-scale analyses such as gene mapping and whole chromosome sequencing are necessary to identify differentiated chromosomal regions between the Z and W chromosomes. Since the *C. marmoratus* species complex is relatively young, at most 10 million years old [Heinicke et al., 2014], this might mean that the differentiation of new sex chromosomes occurred after divergence with *C. alexanderi*. However, the sex determination system of *C. alexanderi* is unknown so far and sex chromosomes can remain morphologically undifferentiated over long periods of time [Ogawa et al., 1998; Matsubara et al., 2006; Gamble et al., 2014]; so these results should be interpreted with caution. Further work that includes other members of the species complex will be needed to better understand the origin and evolution of *Christinus* sex chromosomes.

Hybridization of the *C. marmoratus* Z and W paints with *G. hokouensis* chromosomes revealed no homology between the ZW sex chromosomes of the 2 species. This lack of homology suggests independently derived ZW sex-determining mechanisms within Gekkonidae. This means sex chromosome evolution within Gekkonidae, and Gekkota more generally, is more complex than initially thought [Janzen and Krenz, 2004; Janzen and Phillips, 2006; Pokorná and Kratochvíl, 2009; Gamble, 2010], and may indicate substantial cryptic diversity in the ZW mechanisms of sex determination in this group. Evaluating sex chromosome homology among more gecko species should be a high priority for future research. The development of the *C. marmoratus* ZW paints, along with chromosome paints from flow-sorted chromosomes in additional gecko species [Trifonov et al., 2011], will be useful in this regard.

Homology between the ZW sex chromosomes of birds and *G. hokouensis*, along with homology to monotreme sex chromosomes, led to the hypothesis that an avian-like ZW may be the ancestral sex chromosome for all amniotes [Graves, 2009]. Our results are the first to explore sex chromosome homology between gecko species with female heterogamety and suggest that all instances of ZW sex determination in the Gekkonidae cannot be automatically viewed as homologous. Further testing of this hypothesis through sampling additional gecko species is particularly important because geckos are the sister clade to the remaining squamates, exclusive of the limbless dibamids [Townsend et al., 2004; Vidal and Hedges, 2009]; and, of course, one of the species central to generating the hypothesis is a gecko. Examining sex chromosome homology between the avian ZW and additional squamate species, including additional geckos, yielded little support for this hypothesis. Pokorná et al. [2011] examined 2 gecko species with male heterogamety and heteromorphic sex chromosomes, *Lialis burtonis* (Pygopodidae) and *Coleonyx elegans* (Eublepharidae). In neither species were sex chromosomes homologous with the avian Z (and the *G. hokouensis* ZW). The discovery here that sex chromosomes of *C. marmoratus,* an additional gecko species in the same family (Gekkonidae), do not share homology with the *G. hokouensis* and avian ZW further weakens support for the ancestral avian-like ZW hypothesis. Rather, this region of the genome must have been chosen independently at least 3 times, suggesting that it contains genes (perhaps such as *DMRT1*) that are particularly suitable for the job of determining sex [Graves and Peichel, 2010; Matson and Zarkower, 2012; O'Meally et al., 2012].

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