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Identification of Sex Chromosomes by Means of Comparative Genomic Hybridization in a Lizard, *Eremias multiocellata*

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Eremias multiocellata **is a viviparous lizard that is known to exhibit temperature-dependent sex determination (TSD). Conventional Giemsa staining under light microscope examination has identified the karyotype of this species to be 2 n = 36 l + 2 m, with no detectable heteromorphic sex chromosomes. However, a highly differentiated female-specific chromosome, W, which is homomorphic with the Z chromosome, is found in the present study by the high-resolution cytogenetic method of comparative genomic hybridization (CGH). The results show that *E. multiocellata* is a viviparous lizard with both TSD and ZW heterogametic sex chromosomes. Despite the fact that a different sex ratio of male offspring was found in two populations (separated by an altitude of 1400 m) in previous incubation experiments, we demonstrate, using genomic in situ hybridization (GISH), that there is no significant chromosomal loss or acquisition between the two populations. This suggests that temperature may play a more important role in lowland populations. These results most likely indicate that *E. multiocellata* is transitioning between the evolutionary processes of TSD and genotypic sex determination (GSD) systems, and also give clues to the effect of TSD versus GSD in this process.**

Key words: viviparity, TSD, sex chromosome, comparative genomic hybridization, reptile

INTRODUCTION

A large degree of variability in sex determination systems is thought to exist among reptiles, typically being either genotypic sex determination (GSD) or temperature-dependent sex determination (TSD) (Bull, 1985). In GSD systems, offspring sex is determined by genes or sex chromosomes, while in TSD species the offspring's sex is determined by incubation temperatures during critical embryonic periods. TSD occurs in all crocodilians, most turtles, and many lizards (Sarre et al., 2004; Ezaz et al., 2006a). GSD species often have heteromorphic sex chromosomes that are highly differentiated in gene content and morphology, either as X and Y (for example, in mammals) or as Z and W (for example, in birds). However, other GSD species may have homomorphic sex chromosomes that are identical in morphology but genetically differentiated (Sarre et al., 2004).

Previously it was thought that TSD and GSD are incompatible alternatives in the same species (Janzen and Paukstis, 1991). However, different sex determination modes can be found in species at the intraspecific level, implying certain evolutionary transitions between GSD and TSD systems (Shine et al., 2002; Radder et al., 2008). For example, the live-bearing lizard, *Niveoscincus ocellatus*, has different sex-determining mechanisms depending on the climatic conditions of their distribution, with TSD in lowlands and GSD in highlands (Wapstra et al., 2008). Furthermore, sex reversal has been demonstrated in two oviparous lizards *Pogona vitticeps* and *Bassiana duperreyi* under extreme incubation temperatures (Shine et al., 2002; Ezaz et al., 2005; Quinn et al., 2007; Quinn et al., 2011). This indicates that in some cases there may be an interaction between genotype and environmental factors in sex determination (Conover and Kynard, 1981; Quinn et al., 2007; Radder et al., 2008). Effects of TSD prevailing over an underlying GSD system have been found in fish (Conover and Kynard, 1981; Baroiller et al., 1995; Goto et al., 1999; Devlin and Nagahama, 2002; Luckenbach et al., 2003), urodeles (Dorazi et al., 1995; Chardard and Dournon, 1999), snakes (Dunlap and Lang, 1990), and lizards (Shine et al., 2002). Female heterogamety may even transition into male heterogamety or vice versa, with TSD as an intermediate stage. This has been demonstrated in a variety of fishes and amphibians (Ezaz et al., 2006a). Some studies in reptiles provide evidence for transitions between TSD and GSD, but to date no unequivocal cases between XY and ZW systems have been documented (Ezaz et al., 2006a; Grossen et al., 2011; Quinn et al., 2011).

Evolutionary correlations between sex-determining systems and reproductive modes are documented in reptiles (Organ et al., 2009; Pontarotti, 2010). For example, viviparity usually occurs in association with GSD in an extant amniote species (Organ et al., 2009). Only two or three viviparous species, *Eulamprus tympanum, Eulamprus*

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heatwolei and possibly *Niveoscincus ocellatus*, are known to have TSD systems (Organ et al., 2009; Pontarotti, 2010). However, the viviparous species, *E. Multiocellata* has recently been shown to also have a TSD rather than GSD system (Zhang et al., 2010). During constant gestation temperature experiments, pregnant lizards from a low-elevation population (Minqin) produced a higher number of male offspring with increasing incubation temperatures (Zhang et al., 2010). Recent work has suggested that the skewed sex ratio of offspring in skink *Bassiana duperreyi* could be a response to the influences of genes, temperature, and hormones (Radder et al., 2008; Radder et al., 2009). Furthermore, genotype involvement in sex determination remains a possibility in *E. multiocellata* since a 100% biased offspring sex ratio was not observed, although no heteromorphic sex chromosomes have been detected thus far with traditional Giemsa staining methods (Guo, 2000; Dai et al., 2004). This leads to the hypothesis that homomorphic sex chromosomes exist in *E. multiocellata*, but remain undetected by traditional staining methods.

Interestingly, a high-elevation population of *E. multiocellata* exhibited a different sex ratio of offspring under the same temperature conditions: the male sex ratio bias of offspring ranges from 40.9% (when incubation temperature is 25°C) to 66.7% (when incubation temperature is 35°C). In contrast, the male sex ratio bias of offspring ranges from 15.4% (when incubation temperature is 25°C) to 83.0% (when incubation temperature is 35°C) in a lowland population Minqin (Tang et al., 2012). Previous studies suggest that the key factors that cause discrepancies of skewed sex ratio between Tianzhu and Minqin populations may be local mean temperature (Tang et al., 2012). However, genetic factors may play a role in promoting offspring sex ratio bias between the populations in this species.

Classical techniques like standard banding and staining often fail to discriminate homomorphic sex chromosomes because they identify chromosome pairs based on morphological differences (Ezaz et al., 2006b). However, in some cases sex chromosomes can be morphologically identical or can both be indistinguishable microchromosomes (Quinn et al., 2011; Badenhorst et al., 2013). In these situations, a high-resolution cytogenetic technique of comparative genomic hybridization (CGH), which distinguishes sex chromosomes through the non-homologous region, is required to identify such chromosomes (Traut et al., 2001). By using this technique researchers have confirmed the XY system in *Drosophila* (Traut, 1999), zebrafish, platyfish and guppy (Traut and Winking, 2001), the ZW system in Lepidoptera (Traut, 1999; Traut et al., 1999; Traut et al., 2001), as well as some microchromosome systems in lizard and turtles (Ezaz et al., 2005; Ezaz et al., 2006b; Martinez et al., 2008).

In the present study, we used both staining methods and CGH techniques to further investigate the karyotype of *E. multiocellata*, and identified a strongly differentiated sex chromosome. We explored potential genetic differences between the two populations at the chromosomal level, such as chromosome loss or acquisition, to give clues to offspring sex ratio bias between different populations, and to provide a basic reference for future studies. We performed genomic in situ hybridization (GISH) between two populations of Minqin and Tianzhu, and propose a new perspective on the role that TSD and GSD play in this species.

MATERIALS AND METHODS

Animals

Eremias multiocellata is a small-sized terrestrial lizard (mean adult snout vent length ≈ 65 mm). Adult individuals were collected from Minqin (38°38′N, 103°05′E) and Tianzhu (37°6′N, 103°9′E) in Gansu province, China (Supplementary material: Figure S1). Minqin is a semi-arid region on the southwest of the Tengeri Desert, with a mean ambient temperature in breeding season of 18.3°C, and mean elevation of 1378.5 m (Zhang et al., 2010; Tang et al., 2012). Tianzhu is a mountainous area located in the eastern Qilian Mountains, with a mean ambient temperature in breeding season of 7.1°C and an altitude of approximately 2800–2900 m (Yan et al., 2011). This population exists in the highest distribution of *E. multiocellata*, and the cold climates in this region are different from that in Minqin, where the climate is warm and dry (Tang et al., 2012). Collected lizards were held in the laboratory in Lanzhou University. Individual sex was identified by hemipene eversion (Zhang et al., 2010). When an adult is sacrificed before metaphase preparations, sex can be further confirmed by dissection of gonads. Animal collection, sampling, anaesthetization, and other relevant procedures were performed following the guidelines of the wildlife conservation law of the People's Republic of China.

Metaphase chromosome preparation

Four female and four male adults of *E. multiocellata* from each population were used for mitotic metaphase preparation. Metaphase chromosome spreads were prepared by fix solution steaming technique of medulla cells (Wu, 1982). Phytohaemagglutinin M (PHA M; Sigma) was injected twice at an interval of 24 hours at a dosage of 40 mg/kg. Colcemid (Sangon Biotech, Shanghai) was injected at a dosage of 8 mg/kg four hours prior to metaphase preparation (except in winter, in which case it was 10 hours, since cell proliferation may be slow in this season). The animal was anaesthetized by diethyl ether, and its tibia was obtained to harvest bone marrow cells. The cells were incubated in 0.4% KCl at 45°C for 40 minutes, and fixed in steamed Carnoy's solution (3:1 methanol: acetic acid) at 45°C for two hours, then in 100% ethanol steam at 45°C for 20 minutes. Finally the slides were washed by Carnoy's solution at a slope of 30°, and air-dried.

DNA extraction and labeling

Whole genomic DNA was extracted from the liver following the phenol-chloroform method (Sambrook and Russell, 2001). Genomic DNA was labeled by nick translation reaction following the manufacturer's instructions. Nick translation was conducted in 0.2 ml Eppendorf tubes in an Authorized Thermal Cycler (Eppendorf, German). For the Minqin population, the purified male DNA was labeled with biotin-11-dUTP (Roche, Shanghai, China), and female total DNA was labeled with digoxin-11-dUTP (Roche, Shanghai, China). Reverse labeling experiments were done for Tianzhu population. The digoxin labeled probes were detected by anti-digoxin antibody combined with Rhodamine (Roche, Shanghai, China), while biotin labeled probes were detected by anti-avidin antibody joint with Fluorescein isothiocyanate conjugated avidin (FITC-avidin) (Vector Laboratories, Burlingame, CA, USA). Slides were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI) in an antifade solution Vectashield (Vector Laboratories).

CGH and statistics analysis

Two sets of experiments were carried out for the Minqin and Tianzhu populations of *E. multiocellata*. Four males and four females from each population were used for CGH analysis, which was performed following the FISH protocol (Jiang et al., 1995) with a few modifications. Briefly, slides from each population were incubated at 65°C for one hour and denatured at 95°C for 90 s in 70%

deionized formamide in 2 × SSC, and then dehydrated through ethanol series (70%, 90%, 100% ehanol; 5 min each) at –20°C, and air-dried. The probe mixture containing 40 μg salmon sperm DNA, 50 ng female probe, and 50 ng male probe in 50% formamide, 10% dextran sulfate in 2 × SSC, was denatured at 94°C for 10 min, placed directly into ice and cooled for at least 20 min. 20 μl of the hybridization mixture was applied onto each slide, and sealed with a coverslip. After an overnight incubation in a humid chamber at 37°C, the coverslip was removed and slides were rinsed twice in rocking 2 × SSC (5 min each), followed by one wash at 42°C in 2 × SSC for 10 min. After another rinse in rocking 2 × SSC for 5 min at room temperature, the slides were placed in 1 × PBS at room temperature for 5 min. FITC-avidin (Vector Laboratories) was used to detect biotin-labeled probes and a Rhodamine-conjugated anti-dig antibody (Roche, Shanghai, China) was used to identify digoxin-labeled probes. The slides with both antibodies went through incubation at 37°C for one hour and were then rinsed twice in 1 × PBS for 5 min, then in ddH_2O for 5 min. After being air dried, the slides were painted with 15 μl DAPI (1.5 μg/ml, Vector, with antifade) for counterstain.

Photographs of chromosomes were taken using a Sensys CCD camera (QIMAGING, RETIGA-SRV, FAST 1394) attached to an Olympus BX51 epifluorescence microscope (Tokyo, Japan). Gray-scale images were captured for each color channel and then merged through the Image Pro Plus 6.0 software (Media Cybernetics).

Genomic In Situ Hybridization (GISH)

Two males and two females from the Tianzhu population as well as one male and one female from the Minqin population of *E. multiocellata* were used to prepare mitotic metaphase slides in the GISH procedure. During female GISH, the biotin-labeled female total genomic DNA from Tianzhu was mixed with the digoxin-labeled female total genomic DNA from Minqin, and hybridized with female slides of the two populations. Slide incubation, denaturation and dehydration were the same as in the CGH method. For each slide, 50 ng of Minqin female probe and 50 ng of Tianzhu female DNA with 40 μg salmon sperm DNA was added in 50% formamide, 10% dextran sulfate in 2 × SSC as hybridization solution. After denaturation at 94°C for 10 min, the hybridization solution was put immediately onto ice for at least 20 min. This was then transferred onto the denatured slides, and sealed with coverslips. Incubation of hybridization and washing procedure was the same as CGH. Male GISH employed the same procedure as females except with the reverse labeled probes. Image acquisition and analysis was the same as CGH.

RESULTS

Karyotype of *Eremias multiocellata*

Eighty Giemsa-stained and 20 DAPI-stained metaphase cells of four males and four females of each population were selected for karyotype examination. The Tianzhu population of *E. multiocellata* shares the same karyotype with the Minqin population. Karyotype of female (Fig. 1, Fig. S2) is identical to male (Fig. S3). A total of 38 mitotic metaphase chromosomes were counted, and the karyotype can be described as 2 n = 36 l + 2 m. Chromosomes can be divided into 19 pairs, of which 18 pairs contain 36 macrochromosomes, and the last pair contains two microchromosomes. No heteromorphic sex chromosomes were detected in either male or female metaphases and all the macrochromosomes are telocentric.

CGH of *Eremias multiocellata*

Four male and four female specimens from each population were used for CGH analyses. For each individual, we examined ten metaphases and got concordant results. Unlike the karyotype obtained from traditional Giemsa staining, fluorescence signals of FITC and Rhodamine imply *E. multiocellata* has female specific sex chromosomes. A remarkable red macrochromosome was observed in female individuals of Minqin population (Fig. 2h), and a green macrochromosome was observed in female metaphase of Tianzhu population (Fig. 3h, Fig. S6). Female specific DNA is labeled as red in Minqin population and green in Tianzhu population. This indicates that *E. multiocellata* possess the ZZ/ZW sex-determining mechanism. The Z and W sex chromosomes are morphologically identical, and difficult to identify through traditional approaches. By pairing chromosomes of the CGH results, we can approximately assign the sex chromosomes to the 10th pair (Fig. S4A–C). The entire W chromosome was fully covered by female specific probes, indicating Z and W chromosomes are highly differentiated with each other.

GISH between different populations

There is evidence that both populations have female-

Fig. 1. Giemsa-stained female metaphase karyotype of *E. multiocellata* (2 n = 36 l + 2 m).

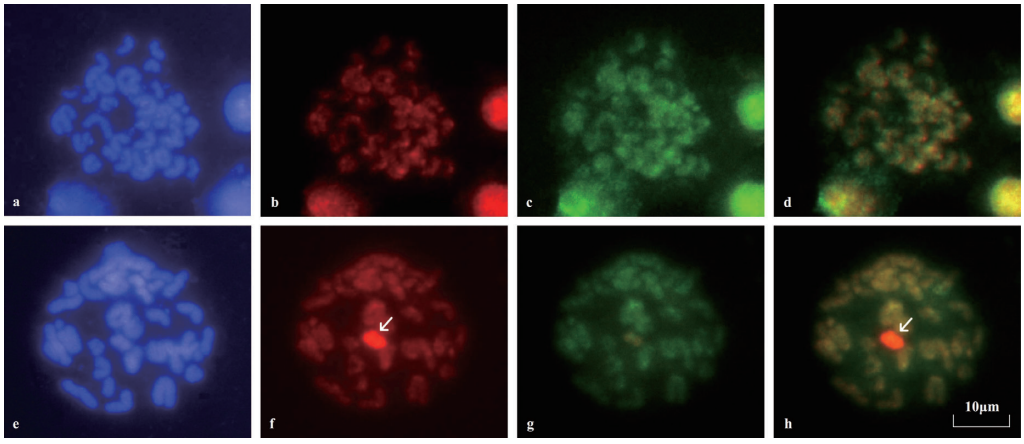

Fig. 2. CGH results in male (up) and female (down) metaphase of Minqin population of *E. multiocellata*. **(A, E)** DAPI stained metaphase chromosome spread; **(B, F)** metaphase hybridized with digoxin-labeled female whole genomic DNA; **(C, G)** metaphase hybridized with biotin-labeled male whole genomic DNA; **(D, H)** Merged images of metaphase hybridized with male and female whole genomic DNA. Scale bar indicates 10 μm. Arrow indicates W chromosome.

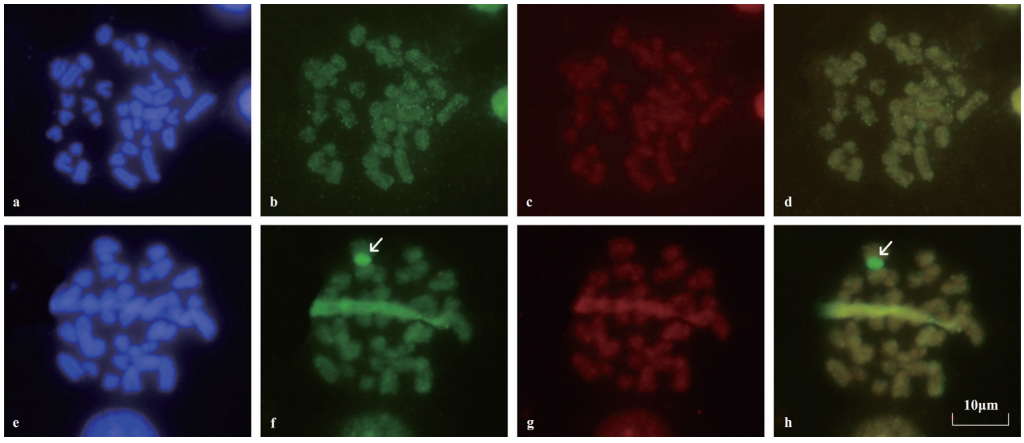

Fig. 3. CGH results in male (up) and female (down) metaphase of Tianzhu population of *E. multiocellata*. **(A, E)** DAPI stained metaphase chromosome spread; **(B, F)** metaphase hybridized with biotin-labeled female whole genomic DNA; **(C, G)** metaphase hybridized with digoxin-labeled male whole genomic DNA; **(D, H)** Merged images of metaphase hybridized with male and female whole genomic DNA. Scale bar indicates 10 μm. Arrow indicates W chromosome.

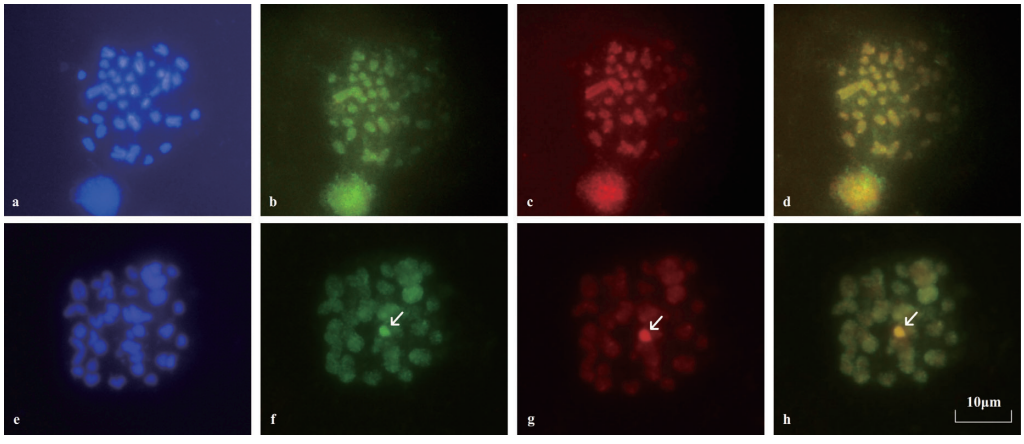

Fig. 4. GISH results between Minqin and Tianzhu population of *E. multiocellata* in male (up) and female (down) metaphase. **(A, E)** DAPI-stained metaphase chromosome spread from Minqin population; **(B)** metaphase hybridized with biotin-labeled male whole genomic DNA of Minqin population; **(C)** metaphase hybridized with digoxin-labeled male whole genomic DNA of Tianzhu population; **(D)** merged images of metaphase hybridized with Minqin and Tianzhu populations male whole genomic DNA; **(F)** metaphase hybridized with biotin-labeled female whole genomic DNA of Tianzhu population; g: metaphase hybridized with digoxin-labeled female whole genomic DNA of Minqin population; h: merged images of metaphase hybridized with Minqin and Tianzhu populations female whole genomic DNA. Scale bar indicates 10 μm. Arrow indicates W chromosome.

specific W chromosomes, and that W chromosomes are highly differentiated from the Z chromosomes. Males are characterized by homogenous Z chromosomes, with no sex-specific signals detected. Mixed probes of the same sex of both populations were put together and put on to metaphase slides of Minqin and Tianzhu population respectively. Neither females nor males of the two populations exhibited distinguishable population specific signals (Fig. 4, Fig. S7). This indicates no detectable chromosome gain or loss has taken place in these two populations.

DISCUSSION

In this study we examined the karyotype of *E. multiocellata* and identified highly differentiated ZZ/ZW sex chromosomes using the high-resolution genetic method CGH. GISH experiments between Minqin and Tianzhu populations revealed no chromosome gain or loss between habitats of different elevations. Here, we will discuss these results in light of their implications for the evolution of GSD and TSD in this species.

The existence of heteromorphic sex chromosomes is often the first step to determine whether a species has adopted genetic sex determination (Valenzuela et al., 2003). GSD species usually have heteromorphic sex chromosomes, such as the ZW system in birds, and the XY system in mammals (Ezaz et al., 2006a). However, a lack of heteromorphic sex chromosomes does not always mean the species uses a TSD system, as there may be homomorphic sex chromosomes in effect (Bull, 1985; Valenzuela et al., 2003). In the present study, we were able to confirm that the karyotype of *E. multiocellata* is 2 n = 36 l + 2 m, with no heteromorphic chromosomes identified. In contrast, CGH results verified this species has homomorphic sex chromosomes, and thus should be considered as GSD species. Since previous studies have shown that temperature can bias its offspring sex ratio, *E. multiocellata* may be a species in which offspring sex determination affected by both genetic and environmental factors. In snakes and lizards with a GSD system, heterochromatin of sex chromosomes changes greatly from a small block to a whole chromosome. For example, the W chromosome in *Pogona vitticeps* was shown to be highly differentiated microchromosomes (Ezaz et al., 2005), and the Y chromosome in *Chelodina longicollis* was identified as an early stage of differentiated microchromosomes (Ezaz et al., 2006b). In *Emydura macquarii* a male-specific heterogametic signal was discovered to be on the tip of the Y chromosome (Martinez et al., 2008). The female specific signal was detected on the NORs of the 7th chromosome in the amphibian *Bufo marinus* (Abramyan et al., 2009). In this study, when mixed DNA probes of both

sexes of *E. multiocellata* were put onto female metaphase slides, female specific probes exclusively painted the W chromosome, with rare competition from male DNA probes. This may indicate a high level of differentiation between chromosome Z and W.

A previous study showed that the proportion of male offspring in *E. multiocellata* ranges from 15.4% to 83% when constant incubation temperature varies from 25 to 35°C (Zhang et al., 2010). This suggests that TSD may override the effect of sex chromosomes in this species. Alternatively, in contrast with being a strict TSD species, none of those temperature treatments produced 100% male or female offspring. One possibility is that both GSD and TSD are playing roles in sex determination and cross talking with each other. An interaction between TSD and GSD has been found to occur in oviparous reptile species (Shine et al., 2002; Ezaz et al., 2005; Quinn et al., 2007), and *E. multiocellata* is the first viviparous species discovered to have both sex determination modes. An interesting study on *E. multiocellata* populations of different elevations brings light to the understanding of the interaction between TSD and GSD. Based on former data, Tang et al. (2012) compared the offspring sex ratio bias of Minqin and Tianzhu populations, and obtained a different bias of male offspring in the two populations (83% male of Minqin population and 66.7% of Tianzhu population at the same incubation temperature). This leads us to speculate that for this lizard, even at the intraspecific level, the effect of TSD tends to be a bit stronger in relatively warmer conditions (lowlands 1357 m), but weaker in cooler conditions such as highlands. This is advantageous for populations, which may experience harsher conditions. When temperature varies greatly in the highlands, lizards would select GSD to avoid extra breeding output to equalize sex ratio (Bulmer and Bull, 1982; Dooren and Leimar, 2003; Leimar et al., 2006). Another explanation to the variation might be that genetic natural selection has happened to the Tianzhu population, subsequently caused physiological changes, eventually lead them to be less sensitive to the ambient thermal changes.

Our results highlight that *E. multiocellata* is a viviparous lizard where TSD and ZW sex chromosomes co-occur, and may represent a transitional state in the evolution of sex determination. Nevertheless, there are still a few questions that need to be tackled to verify the sex determination system in this species. For example, sex ratio bias could have been caused by either abortion rate bias or sex reversal (Valenzuela et al., 2003). We could not determine whether the individuals in the TSD experiment had experienced sex reversal under lower or higher temperature or not due to the availability of the samples in previous experiments. Further studies such as generation of sex specific genotypic markers could be helpful to pull back the veil of the sex determination mode in this species.

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