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Temperature-Dependent Sex Determination Ruled Out in the Chinese Soft-Shelled Turtle *(Pelodiscus sinensis)* via Molecular Cytogenetics and Incubation Experiments across Populations

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Key Words
Comparative genomic hybridization · Genetic sex determination · Sex chromosome · Sex ratio · Turtle

Abstract
The sex determination mechanism for the Chinese soft-shelled turtle *(Pelodiscus sinensis)* is subject to controversy. Some populations have been shown to possess sex chromosomes and thus genotypic sex determination (GSD), while others were reported to exhibit temperature-dependent sex determination (TSD). To test whether TSD and GSD coexist in this species or whether populations differ in their sex-determining system, we conducted egg incubation experiments to investigate how temperature influences hatchling sex in a wide range of populations of this species in China. In parallel, we used comparative genome hybridization (CGH) to study the micro-sex chromosomes of adult *P. sinensis* in the 2 populations that were previously identified to be TSD. The incubation experiments showed that temperature did not affect hatchling sex in any of the studied populations. CGH indicated that turtles have micro-sex chromosomes of the female heterogametic (ZZ/ZW) system in the 2 disputed populations. These results indicate that *P. sinensis* is a GSD rather than a TSD species. Thus, the apparent coexistence of TSD and GSD in this species is the result of previous misdiagnosis in purportedly TSD populations.

The diversity of sex-determining mechanisms in vertebrates has attracted major scientific attention, as they provide suitable systems to study the evolutionary and ecological significances of sex determination in animals [Bull, 1980; Valenzuela and Lance, 2004]. Reptiles are one of the best known groups with highly labile sex-determining systems. The sex of reptiles may be determined by incubation temperature (temperature-dependent sex determination, TSD) [Valenzuela and Lance, 2004; reviewed by Mitchell and Janzen, 2010] or by the genotype of the embryos (genotypic sex determination, GSD) [reviewed by Bogart, 1987; Charlesworth and Mank, 2010; Bachtrog et al., 2014]. All tuataras and crocodilians, most turtles, and some lizards are TSD species. In contrast, all snakes, most lizards, and some turtles are GSD species. At the chromosome level, some GSD reptiles have female

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heterogamety, with ZZ males and ZW females (e.g. *Gekko hokouensis* and the thick-tailed gecko *Underwoodisaurus milii*) [Kawai et al., 2009; Pokorná et al., 2014], while others have male heterogamety, XX females and XY males (e.g. the Australian chelid turtle *Emydura macquarii*) [Martinez et al., 2008].

Transitions from TSD to GSD and vice versa are possible along the continuum of sex determination, and some species possess intermediate mechanisms [Sarre et al., 2011; Valenzuela et al., 2003, 2014], even though these types of sex determinations may be evolutionarily unstable [Bull, 1983]. Recent studies have shown the presence of such intermediate sex-determining systems in some reptiles. For example, the sex of some lizards (e.g. the Australian dragon lizard *Pogona vitticeps* and the eastern 3-lined skink *Bassiana duperreyi*) is not only determined by the genotype of embryos, but may be influenced also by the incubation temperature during embryonic development [Quinn et al., 2007, 2010; Radder et al., 2008]. While a TSD-GSD continuum exists across reptile species, we do not know whether this continuum also occurs among populations within a given species, with GSD in some populations and TSD in other populations, as is the case in some fish (e.g. *Menidia menidia*) [Conover and Heins, 1987].

The Chinese soft-shelled turtle *Pelodiscus sinensis* is a trionychid species that is widely distributed in the central and southern provinces of China and southeastern Asia [Zhao and Adler, 1993]. Complex genetic variation exists in field populations, as well as captive populations, of this species. Consequently, previous studies have identified at least 7 distinct genetic lineages in turtle farms [Fritz et al., 2010; Zhao, 2010]. These lineages are the North Yellow River, Dongting Lake, Poyang Lake, Tai Lake, Southwest Taiwan, and the Japanese lineages, respectively [Zhao, 2010]. The sex-determining mechanism of this species is subject to controversy. Some investigators found that this species has a ZZ/ZW type of sex chromosome in a Japanese population [Kawai et al., 2007; Kawagoshi et al., 2009], employing the GSD sex-determining system in a Singapore population [Choo and Chou, 1985] as well as in an eastern China population [Ji et al., 2003]. *P. sinensis* from an introduced population in Hawaii also exhibits ZZ/ZW sex chromosomes [Badenhorst et al., 2013]. In contrast, other studies have shown that *P. sinensis* employs the TSD sex-determining system in populations located in northern and eastern China [Zhu and Sun, 2000; Nie et al., 2001; Zheng and Zhu, 2006]. The contradictory results of egg incubation experiments raise the question of whether *P. sinensis* is a GSD or TSD species. Possible hypotheses include the following: (1) *P. sinensis* is a GSD species, and the TSD pattern was misidentified in some populations; (2) *P. sinensis* is a GSD species, but the genotypic effect may be overridden by temperature in some populations, as shown in 2 squamate species [Radder et al., 2008; Quinn et al., 2010], or (3) *P. sinensis* possesses distinct sex-determining systems among populations, with GSD and TSD in different populations.

Here, we investigated 2 lines of evidence to resolve this conundrum: (1) the effect of temperature on hatchling sex and (2) the presence of sex chromosomes in different populations of *P. sinensis*. With these data we tested the 3 hypotheses about the mechanism of sex determination in *P. sinensis*. The first hypothesis would be supported if hatchling sex is not affected by the incubation temperature. The second hypothesis would be supported if sex chromosomes exist in all populations and hatchling sex is affected by incubation temperature in some populations, but not in the others. The third hypothesis would be supported if hatchling sex is not affected by incubation temperature in the populations that have sex chromosomes, while it is affected by incubation temperature in other populations lacking sex chromosomes.

We carried out egg incubation experiments to identify the temperature effects on hatchling sex in 6 different populations of this species encompassing most of *P. sinensis'* distribution range in China, including the 2 populations whose sex determination was reported previously [Zhu and Sun, 2000; Nie et al., 2001; Zheng and Zhu, 2006]. To corroborate the sex-determining system in the 2 disputed populations, we studied the sex chromosomes of the 2 populations by using comparative genome hybridization (CGH). This method labels differences in genomes [Ezaz et al., 2005], which is advantageous because sex chromosomes in GSD reptiles may be cryptic and hard to detect by traditional methods, as is the case in several turtles [Ezaz et al., 2006; Kawai et al., 2007; Martinez et al., 2008; Badenhorst et al., 2013].

Materials and Methods

Effect of Incubation Temperature on Hatchling Sex

Egg Collection

We collected freshly laid eggs from private hatcheries at different localities across China. Specifically, the eggs were collected from Shijiazhuang, Jinxian, Changsha, and Guigang counties in 2012 and from Heze and Jiashan counties in 2013 (fig. 1). Turtles from these localities represent different lineages: the North (Shijiazhuang), Yellow River (Heze), Tai Lake (Jiashan), Poyang Lake (Jinxian), Dongting Lake (Changsha), and the Southwest (Guigang) lineages [Zhao, 2010].

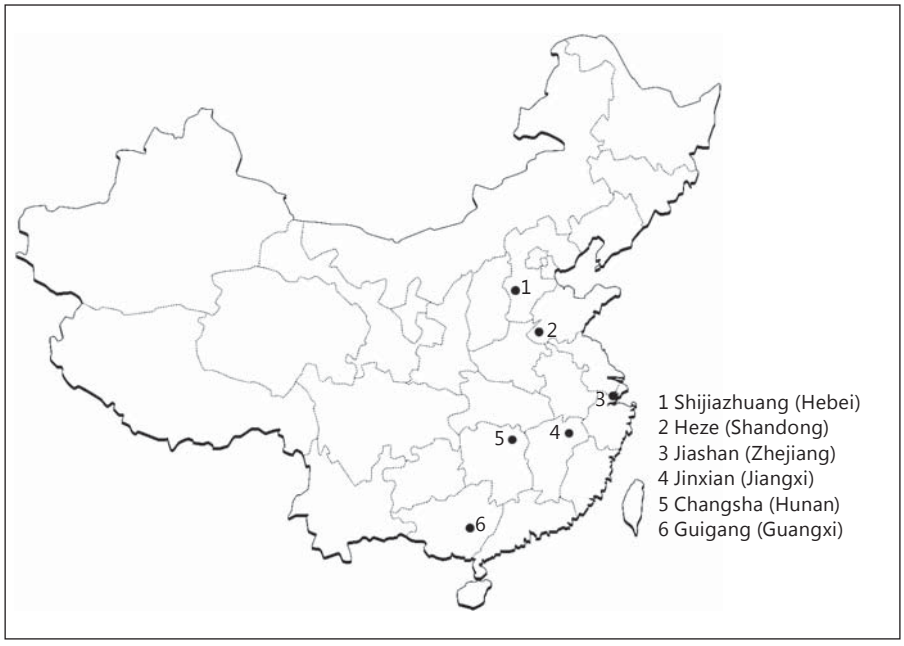

Fig. 1. The sampling sites of *P. sinensis* in mainland China.

Egg Incubation

The collected eggs were transferred to our laboratory and weighed to the nearest 1 mg on an electronic balance (Mettler Toledo AB135-S). The eggs were randomly half-buried in moist vermiculite (–12 kPa) [Du and Zheng, 2004] inside plastic containers (25 × 20 × 10 mm). The containers were then placed into one of 5 incubators (Ningbo Life Science and Technology Ltd, China) set at 26°, 28°, 30°, and 32°C in 2012 and at 24°, 28° and 32°C in 2013, as in previous studies [Zhu and Sun, 2000; Nie et al., 2001; Zheng and Zhu, 2006]. To minimize any effects of thermal gradients inside the incubators, we moved the boxes among shelves twice a week according to a predetermined schedule. To maintain the water potential of the substrate, we added water to the vermiculite every week in the same amount that was lost.

Hatchling Sex

Upon emergence, hatchlings from each temperature treatment were housed in 60 × 40 × 30 cm aquaria (with 15 hatchlings in each aquarium). The aquaria were set up in a temperature-controlled room at 30 ± 1°C, with a 12 h light/12 h dark photoperiod cycle. The turtles were fed an excess amount of commercial food daily for 3 months. The turtles were sexed at the age of 3 months, which is when the sex of *P. sinensis* is easily identified based on the sexual dimorphism of the gonads [Choo and Chou, 1985]. We used the G-test to analyze the effect of temperatures on hatchling sex.

Identification of Sex Chromosomes

Animal Collection

We collected 4 adult *P. sinensis* (2 males and 2 females) from Heze (Yellow River lineage) and Jiashan (Tai Lake lineage). The sex of each turtle was identified by examining the sexual dimorphism in the external morphology and confirmed by the sexual dimorphism of the gonads [Choo and Chou, 1985; Nie et al., 2001].

Blood Leukocyte Culture and Chromosome Preparation. Metaphase spreads of mitotic chromosomes from peripheral blood leukocytes were prepared using the method of Ezaz [Edwards et al., 2005] with some modifications. We collected 0.2 ml blood from each adult turtle using a 1-ml disposable syringe. We used 200 μl of 10,000 U/ml sodium heparin (Sigma, St. Louis, Mo., USA) as anticoagulant when collecting the blood. The blood was then placed into 10 ml of Dulbecco's Modified Eagle Medium (DMEM, GIBCO-Invitrogen, Carlsbad, Calif., USA) with 20% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin (Multicell), and 3% phytohemagglutinin. The mixture was then divided into 2 flasks (Corning). The flasks were incubated at 32°C for 7 days with 5% CO_2. Six hours before harvesting, 75 ng/ml colcemid (Roche, Indianapolis, Ind., USA) and 35 mg/ml 5-bromo-2-deoxyuridine were added to the leukocyte culture. Methanol and acetic acid in a 3:1 ratio was used to fix the metaphases. The fixed cells were centrifuged at 1,000 rpm for 10 min, and the suspension was dropped onto pre-chilled wet slides at 4°C, and were dried at 60°C overnight and stored at –80°C until used for CGH.

Comparative Genomic Hybridization. We followed the protocol of CGH described by Martinez et al. [2008] with some modifications. The gDNA of male and female turtles was labeled with SpectrumGreen-dUTP and SpectrumRed-dUTP (Vysis, Abbott, Des Plaines, Ill., USA). In brief, chromosome slides were incubated at 60°C for 2 h, denatured in 70% formamide/2× SSC for 2 min at 70°C, and dehydrated through a 70, 80, and 100% ethanol series and air-dried at 25°C. About 500 ng of SpectrumGreen-labeled male and SpectrumRed-labeled female DNA were co-precipitated along with 5 mg of boiled male DNA, 20 mg of glycogen as the carrier, and 3 volumes of ethanol. We treated the homogametic sex as unknown at the onset of this experiment. Thus, reciprocal experiments were performed using female DNA as the competitor. Then, the mix was centrifuged at 15,000 *g* at 4°C after maintaining it for 12 h at –20°C. The DNA probe pellet was resuspended in a hybrid-

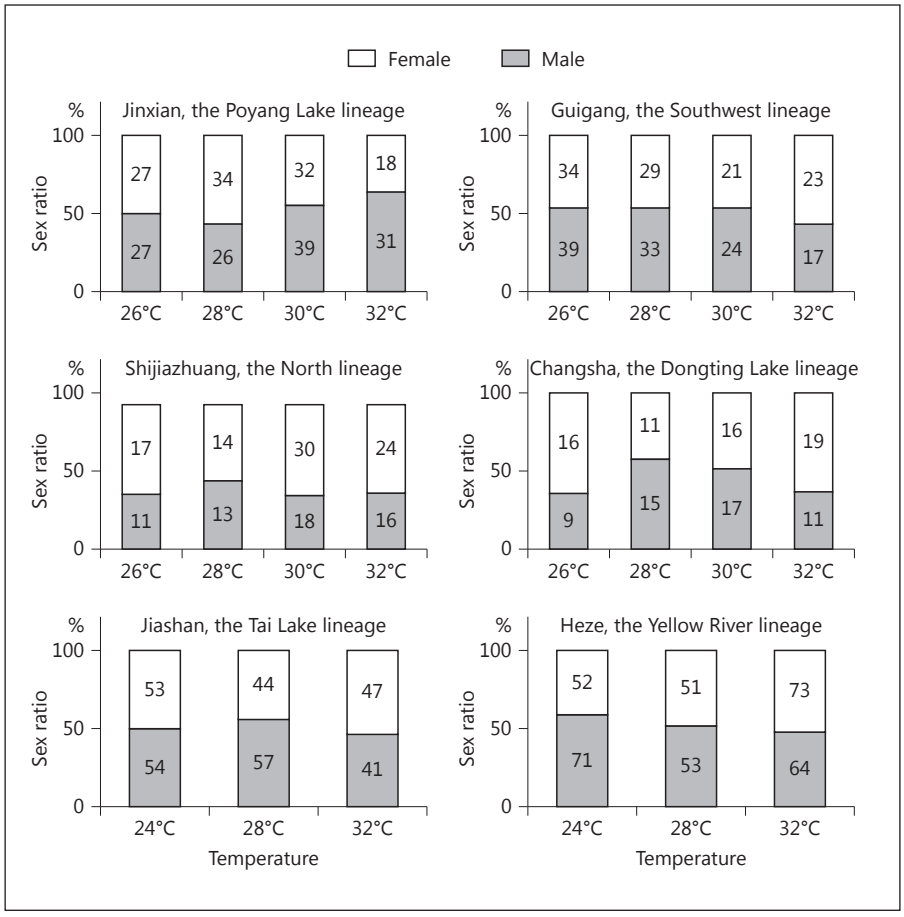

Fig. 2. The effect of incubation temperature on hatchling sex in the 6 different populations of *P. sinensis*. Incubation temperature did not affect hatchling sex in any of the populations.

ization buffer that was pre-warmed at 37°C for about 40 min. The hybridization buffer was composed of 50% formamide, 2× SSC, 10% dextran sulfate, 40 mmol/l sodium phosphate (pH 7.0), and 1× Denhardt's solution. The chromosome slides plus hybridization mixture were denatured for 8 min at 70°C and immediately kept on ice for 2 min. Further, a single 16-μl drop of the probe was added to each slide. Following incubation at 37°C for 3 days, the slides were initially washed with 0.4× SSC/0.3% Tween 20 at 55°C for 2 min. Then, the slides were washed a second time with 2× SSC/0.1% Tween 20 at room temperature for 1 min. The slides were then air-dried at 25°C and stained with 0.001 mg/ml DAPI for 30 s. Images of the chromosomes were captured by an epifluorescence microscope (Zeiss).

Results

The sex ratio (female:male) of turtles from different thermal treatments ranged from 0.37 to 0.63 (fig. 2). The incubation temperature did not affect hatchling sex in any of the populations, including the North (G-test = 0.85, df = 4, $p > 0.05$), Yellow River (G-test = 3.18, df = 3, $p > 0.05$), Tai Lake (G-test = 1.89, df = 3, $p > 0.05$), Poyang Lake (G-test = 4.64, df = 4, $p > 0.05$), Dongting Lake (G-test = 3.92, df = 4, $p > 0.05$), and the Southwest (G-test = 1.54, df = 4, $p > 0.05$) lineages.

In the 2 disputed populations (Yellow River and Tai Lake lineages), females exhibited a notable and specific hybridization signal during CGH, whereas males did not (fig. 3), consistent with *P. sinensis* possessing ZZ/ZW sex chromosomes as reported previously [Kawai et al., 2007; Kawagoshi et al., 2009; Badenhorst et al., 2013].

Discussion

Our results demonstrate that *P. sinensis* hatchling sex is not influenced by incubation temperature in any of the geographically separated populations spanning most of the species range. Additionally, we confirmed that this turtle exhibits a GSD system, and it is not a TSD species. These results are consistent with previous studies on the

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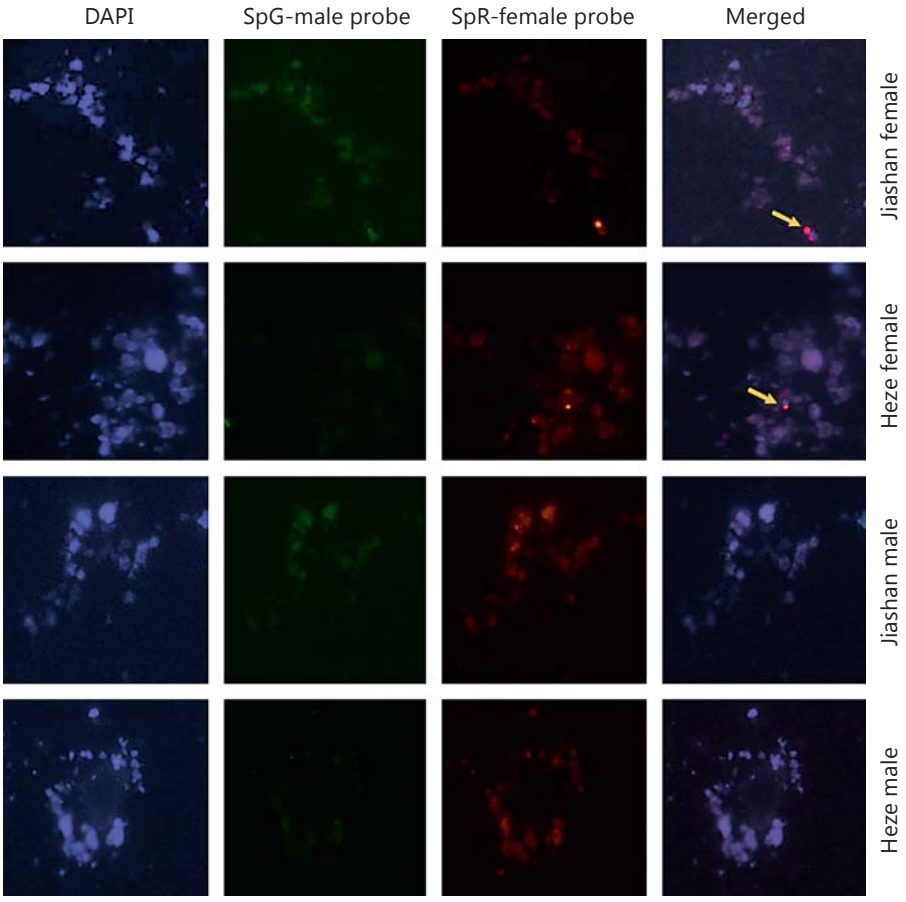

Fig. 3. CGH in the chromosomes of *P. sinensis* females from 2 different populations: a Jiashan female (first row) and a Heze female (second row). DAPI staining was used to show the metaphase chromosomes. SpectrumRed (SpR)-labeled female total genomic DNA and SpectrumGreen (SpG)-labeled male total genomic DNA were used for in situ hybridization. All 3 channels were merged and shown in the last column. The arrows indicate the W chromosome.

Zhejiang (China) and Singapore populations of this species [Choo and Chou, 1985; Ji et al., 2003], refuting the conclusions drawn from investigations on the Wuhu and Heze populations in China [Zhu and Sun, 2000; Nie et al., 2001; Zheng and Zhu, 2006].

It was previously reported that the Heze and Wuhu populations possess a TSD mechanism when eggs were incubated at the temperature range from 24° to 33°C [Zhu and Sun, 2000; Nie et al., 2001]. We carried out similar incubation experiments on the same population from Heze and a sister population (Jiashan) from the same lineage of the Wuhu population to identify any temperature effects on hatchling sex. The sex ratio data obtained in our experiments revealed that the 2 populations showed a GSD pattern rather than a TSD pattern. In addition to the incubation experiments, the 2 preceding studies were unable to detect the sex chromosome in this turtle, leading to the conclusion that it was a TSD species [Nie et al., 2001]. This conclusion was based on the fact that GSD species mainly have a male/female heterogamety genotype with heteromorphic sex chromosomes, while TSD species generally do not [Ezaz et al., 2009b; Valenzuela et al., 2014]. However, the sex chromosome of some GSD reptiles is extremely cryptic to the extent that it may only be detected using high-resolution cytogenetic techniques, such as CGH [Ezaz et al., 2005, 2006; Kawai et al., 2007; Martinez et al., 2008; Badenhorst et al., 2013]. Thus, in the current study, we sought to identify the sex chromosomes in these 2 populations using the same technique that previously detected cryptic sex chromosomes in this species [Kawai et al., 2007]. CGH indicated that the turtles from both populations had ZZ/ZW sex chromosomes, further verifying the presence of a GSD sex-determining system (fig. 3).

It should be noted that in previous studies the Wuhu population showed the male-female TSD pattern where males are produced at cooler temperatures and females at warmer ones, whereas the Heze population showed the female-male TSD pattern where females are produced at cooler temperatures and males at warmer ones, with a

pivotal temperature around 29°C [Zhu and Sun, 2000; Nie et al., 2001]. Theoretically, the evolutionary and ecological advantages for the presence of 2 opposite TSD determination mechanisms in a single species are difficult to determine. One explanation is that the different TSD pattern was the result of methodological error (e.g. sex identification at hatching is unreliable in this species), or other unknown reasons.

In conclusion, this study provides clear evidence that *P. sinensis* is most likely a GSD species with a ZZ/ZW pattern throughout its distributional range, as all evidence ruled out the presence of TSD including the 2 populations previously reported as TSD (fig. 2). Our finding is also consistent with other species in the family of Trionychidae. To date, all species in this family have been reported to have GSD from incubation experiments or molecular cytogenetics [Bull and Vogt, 1979; Janzen, 1993; Valenzuela, 2004; Badenhorst et al., 2013]. Our study also ruled out the hypothesis that *P. sinensis* is a GSD species susceptible to thermal sex reversal, as is the case of the dragon lizard *P. vitticeps* [Ezaz et al., 2009a; Quinn et al., 2010], because incubation temperature did not override the genotypic effect on sex ratios in any of the populations studied.

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