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ORIGINAL ARTICLE

Genetic Analysis of Multiple Paternity in an Endangered Ovoviviparous Lizard *Shinisaurus crocodilurus*

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Abstract The crocodile lizard (*Shinisaurus crocodilurus*) is an ovoviviparous lizard belonging to a monotypic family that originated during the end of the quaternary ice age. A rare species in the wild, the crocodile lizard was listed in CITES Appendix II . Knowledge of the reproductive biology and mating system of this species is important for designing conservation strategies and improving genetic variation. To investigate the paternity of the crocodile lizards and to interpret their reproductive behaviour, we collected saliva from females, potential fathers and offspring in a semi-natural enclosure experiment and analyzed the paternity of the crocodile lizard using 12 microsatellite genetic loci. The overall observed incidence of multiple paternity was 42.9% (6 of 14 clutches) and *Fis* was 0.089 ± 0.056. These results indicate that the primary mating mode of the crocodile lizard is that males are polygynous while with females are polyandrous, and there is multiple paternity among offspring of the same mother.

Keywords *Shinisaurus crocodilurus*, Mating system, Paternity assessment, Saliva sample, Microsatellite, Polygyny, Polyandry

1. Introduction

Investigations on the mating behaviour of reptiles have advanced substantially over the past decade (Davis *et al*., 2001; Laloi *et al*., 2004; Pearse *et al*., 2001). A number of studies using molecular genetic methods indicate that multiple paternity, mate choice and sperm competition are important components of the reproductive strategies in reptiles (Gullberg *et al*., 1997; Lebas, 2001; Olsson *et al*., 2011). Knowledge of the reproductive biology and mating system of endangered species is important for designing effective conservation strategies and improving genetic variation (Joseph and Shaw, 2011). Promiscuous mating systems and multiple paternity schemes have been reported in major reptile groups. For example, a promiscuous mating system has been observed in the sand lizard, *Lacerta agilis*, and the adder snake, *Vipera berus* (Olsson and Madsen, 2001), whereas multiple paternity has been detected in many species, including for instance the painted turtle, *Chrysemys picta* (Pearse *et al*., 2002), hawksbill turtle, *Eretmochelys imbricata* (Joseph and Shaw, 2011) and the five-lined skink, *Plestiodon fasciatus* (Bateson *et al*., 2011).

The Chinese crocodile lizard, *Shinisaurus crocodilurus*, is an endangered lizard and was listed in CITES Appendix II (Zhang and Tang, 1985). *S. crocodilurus* (adult females snout-vent length(SVL) =147.0 ± 2.3 mm; adult males SVL =143.6 ±1.5 mm, the SVL between the males and females were not significantly different

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(*n* = 69, *P* = 0.193)) thrive in areas between 200 and 1500 m above sea level along densely vegetated karst streams or ponds (He *et al*., 2011;Huang *et al*., 2009) exclusively in the eastern part of the Guangxi (Kwangsi) Zhuang Autonomous Region, the western and northern parts of Guangdong province in southern China and in the mountainous areas of northern Vietnam (Huang *et al*., 2014a). Several aspects of *S. crocodilurus* biology have been investigated in thermal ecology (Wang *et al*., 2008, 2009), the wild population survey (Huang *et al*., 2009), ecology (Wu *et al*., 2007; Zhang, 2002; Zhao *et al*., 2006) and conservation genetics (Huang *et al*., 2014a). Additionally, several studies have evaluated mating behaviour and reproduction (Ysu *et al*., 2006; Yu *et al.,* 2009). Although a previous study that combined behavioural observations and video-camera recordings on individuals in a semi-natural enclosure showed that both sexes are promiscuous, it remains unclear whether multiple matings lead to effective multiple paternity. Such observations have led to the supposition that a promiscuous mating system is employed by *S. crocodilurus* (Yu *et al.,* 2009).

Allozyme, DNA fingerprinting, amplified fragment length polymorphism and microsatellites are currently available to investigate relatedness of individuals in wild populations (Parker *et al*., 1998). Since Bei *et al*. (2012) have described polymorphic microsatellite loci in *S. crocodilurus*, high-resolution assays can now be performed to answer questions regarding genetic relatedness in this lizard. To identify evidence for multiple paternity in *S. crocodilurus*, the current study employed 12 microsatellite DNA loci in assessing 14 clutches from from two experimental populations maintained in semi-natural enclosures, established in 2011 and 2013.

2. Materials and Methods

2.1 Collection of samples in the semi-natural enclosed population A semi-natural enclosure experiment for the Chinese crocodile lizard was performed at the Luokeng Nature Reserve in Guangdong Province, China. The reserve is located north of Guangdong province (24°36′–24°9′N, 113°13′–113°22′E) (Wan, 2009), at an altitude ranging from 200 to 1587 m. From May to July in 2010 and 2012, prior to the beginning of the mating season, 44 adults (28 males and 16 females) were randomly distributed into a pond with damp soil and supplied with water and *Pyralis* larvae. The animals were allowed to move freely around the pond. To obtain clutches, gravid females were captured from March to May in 2011 and 2013 and maintained in the laboratory until they gave birth. Rearing conditions were similar to those applied for the study of wild populations(water temperature = 20.1 ± 0.5°C) (Wang *et al*., 2008). Regular monitoring allowed us to collect oral swab samples from all individuals, including the parents and offspring. The method of collecting samples was similar to that previously described, the cotton swabs used to collect saliva samples were stored in 1.5 ml centrifugal tubes containing 100% ethanol. After sampling, individuals were immediately returned to the pond where they were captured (Huang *et al*., 2014). A total of 129 individuals were collected, including the 44 introduced adults (28 males and 16 females that had just given birth) and 85 offspring.

Sampling was approved by the Forestry Administration of Guangdong province, Luokeng Nature Reserve. All lizards were immediately released after the saliva was collected. Buccal swabbing is a noninvasive method. The Committee on the Ethics of Animal Experiments of the Guangxi Normal University and the Guangdong Entomological Institute Administrative Panel on Laboratory Animal Care approved the protocol.

2.2 Genomic DNA extraction Total genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Germany), according to the instructions with slight modifications. In brief, samples were dried with water-filter paper to allow ethanol to evaporate. Furthermore, the samples were transferred into a 1.5 mL Eppendorf tube, thoroughly mixed with 480 μL buffer solution and 20 μL proteinase K and then incubated at 56°C for 3 h. The mixture was isolated after centrifuging at 8000 rpm for 1 min, following which 500 μL of buffer solution AW1 was added to the supernatant. After discarding the waste liquid, 500 μL of buffer solution AW2 was added, and the mixture was centrifuged at 14000 rpm for 3 min. Finally, genomic DNA was dissolved in 200 μL of TE buffer and was stored at −20°C until analysis.

2.3 Microsatellite genotyping We amplified 12 microsatellite loci (GenBank Accession Numbers JQ411749–JQ411760) from nuclear DNA using 5′-fluoro-labelled forward primers (Bei *et al*., 2012). Polymerase chain reaction (PCR) amplifications were performed using the following conditions: an initial denaturing step of 3 min at 94°C, followed by 35 cycles of 35 s at 94°C and a Ta (an annealing step) (55°C–61°C) for 35 s (Bei *et al*., 2012), 30 s at 72°C and a final extension step at 72°C for 10 min. The total volume of the PCR reaction mixture was 15 μL, which consisted of 1 μL of template

DNA, 1 μL of forward primers and 1 μL of reverse primers, 7.5 μL of premixed Taq DNA and 4.5 μL of H_2O. Fragment analysis was conducted on an ABI3700 sequencer (Applied Biosystems), and alleles were sized using the programs GENESCAN version 2.1 and GENOTYPER version 2.5 (Applied Biosystems).

2.4 Detection of microsatellite DNA polymorphism The microsatellite data were analysed using web-based Genepop software, with Markov chain parameters of 1000 dememorisation, 100 batches and 1000 iterations per batch to determine whether each locus deviated from the Hardy-Weinberg equilibrium. The GeneALEx software was employed to calculate the number of alleles (*Na*), average number of alleles (*A*), observed heterozygosity (*Ho*), expected heterozygosity (*He*), and Fis of each locus.

2.5 Paternity analysis Paternity analysis was performed (95% confidence) using the software package CERVUS 3.0.3 (Marshall *et al*., 1998). For each considered pair of individuals, the average number of shared alleles at each microsatellite locus was calculated. To prevent false identification of the father due to genotyping or reading errors, any individuals without a specific genotype as generated by GeneMapper were subjected to another round of PCR amplification.

3. Results

3.1 Genetic variation of parents and offspring The genetic diversity of the 28 males, 16 females and 85 offspring based on the 12 microsatellite loci are listed in Table 1. The *A* of the offspring was higher than that of their parents. The *He* of both parent and offspring exceeded *Ho*.

3.2 Paternity relationships In the present study, a total of 16 clutches were analysed, which included 11 clutches collected during 2011 and five gathered during 2013 (Table 2). Females collected during 2011 and 2013 had broods that ranged in size from 2 to 7 offspring (Table 2); one clutch with only two hatchlings was excluded from the estimation of multiple paternity because it was not possible to detect more than two paternal alleles in such a situation (Laloi *et al*., 2004). Thus, the observed incidence of multiple paternity was 40.0% (4 of 10 clutches). Among the five clutches collected during 2013, multiple paternity was detected in five clutches (50.0%, two of four clutches), which were sired by at least three males. The overall observed incidence of multiple paternity was 42.9% (6 of 14 clutches; Table 2), and the average number of genotypes per clutch was 5.2 (range: 2–7).

4. Discussion

Our data represent the first genetic tests of paternity in *S. crocodilurus*. The incidence of multiple paternity was high in *S. crocodilurus* (42.9% of clutches). Although the number of clutches tested is small and from a limited semi-natural enclosed population, which limits the conclusions that can be drawn with regard to the species as a whole, some important initial observations on the mating system of *S. crocodilurus* can be made. The genetic data supported the assumption generated

Table 1 Genetic diversity of studied parents and offspring at 12 microsatellite loci.

Population	Individual	*N*	*He*	*Ho*	*Fis*
FM	44	5.083 ± 0.69	0.622 ± 0.035	0.570 ± 0.050	0.091 ± 0.479
ZD	85	5.75 ± 0.82	0.635 ± 0.040	0.568 ± 0.046	0.087 ± 0.063
Total	129	5.417 ± 0.528	0.628 ± 0.026	0.569 ± 0.033	0.0.089 ± 0.056

FM = Candidate father and mother; ZD = Offspring; *A* = mean number of alleles per locus; *Ho* and *He* = observed and expected heterozygosities, respectively. Values of the fixation index *Fis* are reported with their 95% confidence, as estimated using GENEPOP

Table 2 Summary of the characteristics of each clutch.

	Clutch size				% Multiple -sired clutches*
	n	Mean ± SE	Min	Max	
Clutches from the 2011 experimental population	11	5.1 ± 1.9	3	7	40.0% (4 of 10)
Clutches from the 2013 experimental population	5	5.4 ± 1.6	3	7	50.0% (2 of 4)
All clutches	14	5.2 ± 1.8	3	7	42.9%

*Clutches with only two juveniles were excluded from the estimation of multiple paternity.

from behavioural observations that at least some female *S. crocodilurus* mate with and produce offspring clutches sired by multiple males (Yu *et al*., 2009). This is a much higher incidence of multiple paternity than found in *Ameiva exsul* (9.1%) and *Vipera berus* (16.7%) (Höggren, 1995; Lewis *et al.,* 2000) and lower than that reported for *Eulamprus heatwolei* (65%–82%) (Morrison *et al*., 2002) but similar to that of the Grand skink, *Oligosoma grande* (46.7%) (Berry, 2006).

In lacertids, multiple paternity is often related to the coexistence of conflicting male mating strategies (Laloi *et al*., 2004). The question of whether territorial behaviour affects the level of multiple paternity is pertinent. There are two species showing strong territoriality that also have high levels of multiple paternity. One is *Sceloporus virgatus* (62% of clutches are multiple sired) (Abell, 1997), whereas the other is *E. heatwolei* (65%–82%) (Morrison *et al*., 2002). However, some territorial species are associated with notably low incidence of multiple paternity, for example *A. exsul* (9.1%) and *V. berus* (16.7%) (Höggren, 1995; Lewis *et al.,* 2000). Furthermore, the sand lizards are not territorial, but levels of multiple paternity remain high (Gullberg *et al*., 1997). There are currently insufficient data to allow a rigorous phylogenetically controlled test of the relationship between categories, such as pair bonding and territorial and nonterritorial lizards (Uller and Olsson, 2008). In *S. crocodilurus*, adult males show the strongest territoriality (Wan, 2009); however, it remains difficult to determine whether territorial behaviour affects the level of multiple paternity.

Sperm storage plays an important role in reptile reproduction, particularly when male and female cycles do not coincide (Joseph and Shaw, 2011). In addition, sperm storage has been well documented in many snake, lizard and turtle species (Schuett and Gillingham, 1986; Valenzuela, 2000; Villaverde and Zucker, 1998); *S. crocodilurus* anatomy supports the presence of a sperm storage structure (Zhang, 2002). However, many of the arguments for the adaptive significance of sperm storage in these taxa may not apply to *S. crocodilurus*. For example, it has been proposed that in turtles producing multiple clutches per season, eggs moving down the oviduct may 'sweep' away sperm moving upwards that would have been used to fertilise subsequent clutches (Gist and Jones, 1989). However, *S. crocodilurus* produces only a single clutch per season (Yu *et al*., 2006) and may not require stored sperm for subsequent clutches in the same year. Another argument for sperm storage in turtles and some snakes is that many species show asynchrony in gonadal cycles between the sexes (Galbraith 1993; Halpert *et al*. 1982). Studies of *S. crocodilurus* hormonal cycles have shown that male testosterone levels increase significantly during June, while there are no significant differences in female estradiol levels during the breeding season (Huang *et al*., 2014b). According to the previous study, we cannot determine whether the gonadal cycles of *S. crocodilurus* develop asynchronously. Therefore, multiple paternity in *S. crocodilurus*is is most likely due to within season multiple matings, and the utilisation of sperm storage structures remains unknown, further research on the possibility of switching out males in the enclosure to look into the adult female still reproduce with sperm store will be carried out in the future.

In addition, we noticed that some females produce multiply sired clutches, while others do not, and some fathers could not be identified (Table 2). For singly sired clutches, some females may mate either only once or multiple times with the same male. It is also possible that females mate with multiple males, but only one male employing the best timing manages to fertilise the eggs. Alternately, sperm competition and sperm selection may also account for singly sired clutches by females which have mated multiply (Davis *et al*., 2001). The missing fathers may have been dead or may have been released back into the wild.

The average expected heterozygosity (*He* = 0.628) of the semi-natural population is similar to that of the wild groups (*He* = 0.61) (Huang *et al*., 2014a). The previous study showed low genetic diversity in wild population (Huang *et al*., 2014a), whereas the semi-natural population did not show changes in the level of genetic diversity. It is possible that the genetic difference in parents is low, resulting in inbreeding and leading to the deficit in heterozygosity.

Although these data reveal multiple paternity of *S. crocodilurus*, a number of questions remain unanswered. These include whether multiple paternity is a strategy employed by *S. crocodilurus* in wild populations, whether the mating order of males affects offspring genotypes and the degree to which multiple mating affects offspring genotypes. Undoubtedly, further investigations of the *S. crocodilurus* mating system are required to uncover complex interactions as well as genetic and environmental determinants of offspring genotypes.

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