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 \(F_{\text{is}}\) 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
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 H2O. 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.

<table>
<thead>
<tr>
<th>Population</th>
<th>Individual</th>
<th>N</th>
<th>He</th>
<th>Ho</th>
<th>Fis</th>
</tr>
</thead>
<tbody>
<tr>
<td>FM</td>
<td>44</td>
<td>5.083 ± 0.69</td>
<td>0.622 ± 0.035</td>
<td>0.570 ± 0.050</td>
<td>0.091 ± 0.479</td>
</tr>
<tr>
<td>ZD</td>
<td>85</td>
<td>5.75 ± 0.82</td>
<td>0.635 ± 0.040</td>
<td>0.568 ± 0.046</td>
<td>0.087 ± 0.063</td>
</tr>
<tr>
<td>Total</td>
<td>129</td>
<td>5.417 ± 0.528</td>
<td>0.628 ± 0.026</td>
<td>0.569 ± 0.033</td>
<td>0.089 ± 0.056</td>
</tr>
</tbody>
</table>

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.

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

*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
can reproduce with sperm from previous 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 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.
Alternatively, 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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References


