

Social Polyandry and Multiple Paternity in the Omei Treefrog in the Southwest China

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Abstract Group spawning, polyandry reproductive behaviors, and multiple paternity are increasingly reported in anuran species. The Omei treefrog (*Rhacophorus omeimontis*), endemic to subtropical and mountainous forests of central and southwestern China, is a polyandrous lek-patterned breeder commonly showing multiple males-one female matings during the breeding season. To detect the traits of social and genetic polyandry in this species and explore the relationships between these traits, we investigated the breeding behaviors of a population of *R. omeimontis* in the Fengtongzhai National Nature Reserve, Baoxing County, Sichuan, China. We conducted paternity analyses using six microsatellite genetic markers. A total of 30 matings were recorded in the field (four monogamous pairs and 26 spawning groups). Our results revealed high proportions of social polyandry (86.7%) and multiple paternity (70.0%) and the numbers of joining males and genetic fathers among matings ranged from 1 to 8 and from 1 to 4, respectively. There was a significantly positive correlation between the intensities of social and genetic polyandry, indicating that multiple males-one female breeding behaviors could be an important promoter of multiple paternity. We considered the intense social polyandry and multiple paternity as consequences of intense male-male competition under a male-biased sex ratio and genetic benefits pursuing of the females. However, the proportion of genetic fathers in a spawning decreased with the increase of joining male number and most of their offspring belonged to a few males. This might be caused by a “making the best of a bad lot” reproductive strategy of the inferior male individuals.

Keywords Genetic polyandry, multiple paternity, *Rhacophorus omeimontis*, social polyandry

1. Introduction

Recent field observations and empirical studies have revealed increasing evidence of polyandry breeding behavior, also called social polyandry, across animal taxa (Tregenza and Wedell, 2002; Hosken and Stockley, 2003; Neff and Pitcher, 2005). Extra-pair breeding, female individuals mating with several males, or mating group formation, or social polyandry in females are ubiquitous breeding behaviors in insects (e.g., Arnqvist and Nilsson, 2000; Ronkainen *et al.*, 2010) and fish (e.g., Awata *et al.*, 2005; Coleman and Jones, 2011). Reports showed that group mating is also common in amphibians (e.g.,

Roberts and Byrne, 2011; Caspers *et al.*, 2014; Luo *et al.*, 2014; 2015; 2016; Zhao *et al.*, 2016), and that extra-pair mating is broadly observed in reptiles (e.g., Eizaguirre *et al.*, 2007), birds (e.g., Lesobre *et al.*, 2010; Wang and Lu, 2011), and mammals (e.g., Eberle and Kappeler, 2004; Klemme *et al.*, 2008). It is recognized that nearly 5% of known bird species have social polyandry behavior (Emlen *et al.*, 1998; Bennett and Owens, 2002). Social polyandry mating behavior has been observed in a large number of anuran species (Roberts and Byrne, 2011) and many features of life histories in frogs and toads suggest that social polyandry and group mating should be a common phenomenon (Roberts *et al.*, 1999). Other researches pointed out that social polyandry usually lead to multiple paternity during the reproduction of animals (Brante *et al.*, 2010).

With the help of rapid advancing molecular biology

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techniques, paternity analyses have been widely performed to explore genetic mating patterns within animal populations in the recent decades (Field and Wills, 1996; Hancock, 1996; Wang and Lu, 2011; Luo *et al.*, 2016; Zhao *et al.*, 2016). Microsatellite markers and DNA fingerprinting have provided empirical evidence of genetic polyandry in many species exhibiting social polyandry, including several anurans (e.g., D'Orgeix and Turner, 1995; Laurila and Seppä, 1998; Roberts *et al.*, 1999; Byrne and Keogh, 2009). Thus, elucidating the traits of social and genetic polyandry mating patterns and their relationships has become an important issue in understanding reproductive behaviors and the evolutionary mechanisms of animal mating systems (Weber and May, 1989; Coleman and Jones, 2011).

In anuran species, reproduction generally involves external fertilization of eggs and a weak or inconsistent parental care (Halliday and Tejedo, 1995). The promiscuity of males chorusing in breeding congregations and the male-biased sex ratio could result in multiple males amplexing a single female (Reading *et al.*, 1991; Halliday and Tejedo, 1995; Lodé and Lesbarrères, 2004). During the reproduction of frogs and toads, reproducing males usually arrive precociously and stay for a longer time than females in the breeding ponds. This reproductive behavior could increase male-male competitive interactions for mates and lead to social polyandry (Fukuyama, 1991; Jennions *et al.*, 1992; Halliday and Tejedo, 1995). Although multiple amplexus has been widely observed in anurans, genetic evidence for polyandry breeding (i.e., multiple paternity) remains scarce (Lodé and Lesbarrères, 2004; Roberts and Byrne, 2011). For example, in *Crinia georgiana*, *Pseudophryne bibronii*, *Rana dalmatina*, and *R. temporaria*, social polyandry related mating behaviors, such as group spawning and repeated mating of females, caused multiple paternity within single egg clutch, demonstrating that reproductive patterns of these species involve simultaneously social and genetic polyandry (Laurila and Seppä, 1998; Roberts *et al.*, 1999; Byrne and Keogh, 2009). Simultaneous social and genetic polyandry were also revealed in some treefrog species such as *Agalychnys callidryas* (D'Orgeix and Turner, 1995), *Chiromantis xerampelina* (Byrne and Whiting, 2011), and *Rhacophorus schlegelli* (Fukuyama, 1991). Since a single male can provide enough sperms to fertilize all eggs produced by a mated female and multiple mating could lead to higher costs (e.g., energy, time) and/or risks (e.g., predator, injury, disease, mortality) for females during reproduction (Thornhill and Alcock, 1983; Rice, 1996;

Luo *et al.*, 2016), the reasons and evolutionary drivers of polyandry mating behavior are highly controversial.

The Omei treefrog *Rhacophorus omeimontis* is endemic to subtropical and mountainous forests at 700–2000 m a.s.l. in central and southwestern China (Fei and Ye, 2001; Fei *et al.*, 2012). It is an arboreal breeder with lek-patterned mating behaviors whose breeding season lasts from April to July (Liao and Lu, 2010; 2011; Fei *et al.*, 2012; Luo *et al.*, 2016). During the breeding season, aggregated in leks and with no physical combat, adult males gather in permanent or temporary ponds and competitively emit advertisement calls to attract mates (Liao and Lu, 2010, 2011; Luo *et al.*, 2016). When a gravid female enters a lek, she is clasped by a male and the amplexant pair climbs up a nearby tree reaching the spawning site: a selected leaf overhanging the pond water. The mated female lays her eggs that are progressively fertilized by the amplexant male. Frequently, other males subsequently join the mating pair before the mated female laying her eggs, resulting in the formation of a one female-multiple males mating group (Liao and Lu, 2010, 2011; Luo *et al.*, 2016). The mated male provides no parental care and leaves his lek immediately after spawning, while his mate mixes her eggs with the coating foamy substance totally secreted by her resulting in a foam nest that she covers with surrounding tree leaves. After the female leaves, her foam-nest will drop into the water below, hatch and develop in the pond. Within *R. omeimontis* populations, each reproducing female produces a single egg-clutch per breeding season (Liao and Lu, 2010, 2011; Luo *et al.*, 2016).

In this study, using field surveys and molecular parentage analyses, we explored the traits of social and genetic polyandry mating patterns in *R. omeimontis* and the relationships between these types of polyandry. Our aim was to determine whether social polyandry leads to multiple paternity in the Omei treefrog?

2. Materials and Methods

2.1 Study site Based on our previous long-term surveys, in 2011, we chose two permanent natural ponds (two leks of *R. omeimontis*; 30°33.231' N, 102°56.655' E, 1658 m a.s.l.) regularly used by *R. omeimontis* for reproduction in the Fengtongzhai National Nature Reserve (30°19'–30°47' N, 102°48'–103°00' E, with a total area of 39039 ha), Baoxing County, Sichuan Province, China.

2.2 Field observations From 7 April to 18 July, during the night (from 7:00 p.m. to 7:00 a.m. of the next morning), we daily searched for *R. omeimontis*

individuals in the ponds and on the nearby trees using a 12-V flashlight. We caught and sexed them based on their amplexus positions (clasping individuals being presumably considered as males) and the presence (males) or absence (females) of vocal sacs. We observed and recorded all the behaviors of reproducing frogs. After the amplexant pairs or groups laid eggs, we captured the frogs and used the toe-clipping method to collect samples for molecular parentage analyses. About 1 mm³ of tissue was removed from the end of one toe for each individual using a pair of surgical scissors and stored in anhydrous ethanol. Then, as tags, we tied the waists of the frogs with cotton lines in different color combinations to uniquely identify the sampled individuals (Qi *et al.*, 2015). All individuals were released at their respective capture sites within 10 minutes after these manipulations, and recaptured individuals were released immediately without resampling. In addition, we collected all the foam-nests from the trees and hatched each egg clutch near the pond in a plastic container with 15 cm depth of water. When the hatching process finished, 24 tadpoles were randomly selected within each bucket for molecular paternity analyses. We clipped 1 mm³ of tissues from the ends of their tails and stored the samples in anhydrous ethanol. Finally, all tadpoles were immediately released back to their respective ponds.

2.3 Molecular experiments The total genomic DNA was extracted from the toe or tail tissue sample of each sampled frog or tadpole using TIANamp Genomic DNA kit (TIANGEN Biotech Co. Ltd., Beijing, China) and was dissolved in ddH₂O at -20°C of temperature. Six microsatellite loci (OMTF3, OMTF6, OMTF7, OMTF9, OMTF10, and OMTF11; GenBank accession numbers JQ031744, JQ031747, JQ031748, JQ031750, JQ031751, and JQ031752) were used in our experiments. We designed their respective primers following the procedure described in Zhao *et al.* (2012). For each individual, a 80 ng of DNA (0.3 µl) was amplified by PCR (polymerase chain reaction) in a 10 µl reaction volume containing 5 µl of Premix r Taq (TaKaRa Bio Inc., Otsu, Shiga, Japan), 0.4 µl of each primer pair (forward and reverse), and 3.9 µl of ddH₂O. The forward primers were end-labeled with one of the fluorescent dyes (FAM, TAMRA, or HEX). The PCR conditions were: 95 °C for 5 min (initial denaturation), 31 cycles of 94 °C for 30 s, primer-specific annealing temperatures (53–60 °C) for 30 s, 72 °C for 45 s, and 72 °C for 10 min (final elongation). All the PCR process were carried out on Applied Biosystems GeneAmp 9700 (Applied Biosystems, Foster City, CA, USA) and the genotyping was conducted on an ABI

Prism 3730 Genetic Analyser (Applied Biosystems, Foster City, CA, USA). The microsatellite alleles were scored using ABI Gene Mapper 4.0 (Applied Biosystems, Foster City, CA, USA) and analyzed using GeneMarker 1.3 (SoftGenetics, LLC., State College, PA, USA).

2.4 Paternity analyses and statistics We calculated the number of alleles per microsatellite locus in FSTAT 2.9.3.2 (Goudet, 2002) and computed the observed heterozygosity (Ho), expected heterozygosity (He), and polymorphic information content (PIC) using Cervus 3.0 (Marshall *et al.*, 1998; Kalinowski *et al.*, 2007). Departures from Hardy-Weinberg equilibrium (HWE) for all the six microsatellite loci and their linkage disequilibrium were evaluated using the software package Genepop 4.2 (Raymond and Rousset, 1995; Rousset, 2008).

Paternity of all the tadpoles was molecularly determined using maximum likelihood calculations in Cervus 3.0 program (Kalinowski *et al.*, 2007). By comparing the genotypes of the tadpoles to the genotypes of the potential fathers, we assigned paternity to each offspring individual using the highest log-likelihood ratio (LOD). Based on the allelic frequency of the tadpoles within the population, we conducted 10000 iterations of the paternity analysis process (Marshall *et al.*, 1998; Kalinowski *et al.*, 2007).

To identify the social and genetic breeding patterns of *R. omeimontis*, we mapped the frequencies of mating pairs/groups along number gradients of joining males and genetic fathers within the matings, respectively. Then, the relationship between the amounts of joining males and genetic fathers at mating pair/group level was revealed using a linear regression. We calculated the ratio of genetic father number against joining male number for each spawning, and compared the means of the ratio among number gradients of joining males by a one-way ANOVA. Furthermore, proportion of tadpoles obtained by each male in each mating was calculated, and we detected the variations of the proportions among the males with the most, 2nd, 3rd, 4th, and 5th–8th amounts of offspring using a one-way ANOVA.

2.5 Ethical statements Our field studies were conducted under a permission delivered by the Management Bureau of the Fengtongzhai National Nature Reserve. The research was approved by The Animal Ethics Committee of The Central China Normal University. We adhered to the “Guidelines for the Use of Animals in Research” published in *Animal Behaviour* and the laws of China. Our observations, captures, and toe clipping procedures

did not induce substantial stress or suffering in the study animals and caused no obvious disturbance to the mating process, reproductive success, behavioral rhythm, or the ultimate survival of the frogs and tadpoles.

3. Results

Thirty females and 90 males were involved in four monogamous mating pairs and 26 multiple mating groups. Figure 1(a) showed the frequency of matings along the numbers of joining males. We used the tadpoles from the multiple matings for paternity analyses and only 22 clutches of tadpoles were successfully identified. The numbers of alleles and polymorphic parameters (H_o and H_e) for the six microsatellite loci of the population were shown in Table 1. All the loci conformed to the HWE, except for OMTF7, and no linkage disequilibrium was detected among the loci. The multi-locus exclusion probability based on the six loci was 99.9% in the paternity analyses (Table 1). The results revealed that only one of the multiple males-one female mating groups had a single genetic father. In four mating groups, each of the joining male obtained a certain proportion of offspring. The frequency of mating pairs/groups among the numbers of genetic fathers was showed in Figure 1(b).

A significantly positive relationship was found between the numbers of joining males and genetic fathers within each spawning ($P < 0.01$, $R^2 = 0.414$; Figure 2), suggesting that more males participating in a mating might lead to higher level of multiple paternity. However, with the increase of the number of participating males in a mating, the proportion of males that obtained offspring obviously decreased (1–2 joining males: 100% of the males obtained offspring; 3–4 joining males: $79.2\% \pm 12.5\%$ (mean \pm SE, the same below) of the males obtained offspring; 5–6 joining males: $50.0\% \pm 17.5\%$ of the males obtained offspring; 7–8 joining males: $45.2\% \pm 3.4\%$ of the males obtained offspring; One-way ANOVA, $F_{3,24} = 7.435$, $P < 0.01$). Additionally, the numbers of offspring gained by different males in a spawning were significantly various (one-way ANOVA, $F_{4,105} = 76.979$, $P < 0.01$). The majority of the offspring belonged to the 1st male ($76.3\% \pm 22.0\%$ of the tadpoles), followed by the 2nd male ($19.3\% \pm 14.4\%$ of the tadpoles) and the 3rd male ($7.4\% \pm 8.1\%$ of the tadpoles; Figure 3).

4. Discussion

Our results indicated high intensities of both social (26 in 30 matings, 86.7%) and genetic (21 in 30 matings, 70.0%;

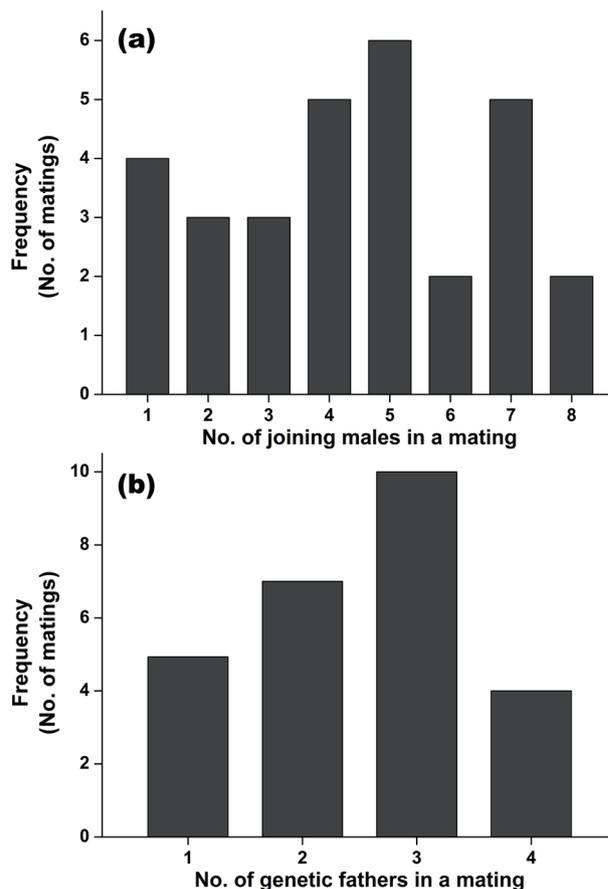


Figure 1 Frequencies of mating pairs/groups along number gradients of joining males (a) and genetic fathers (b) within the matings.

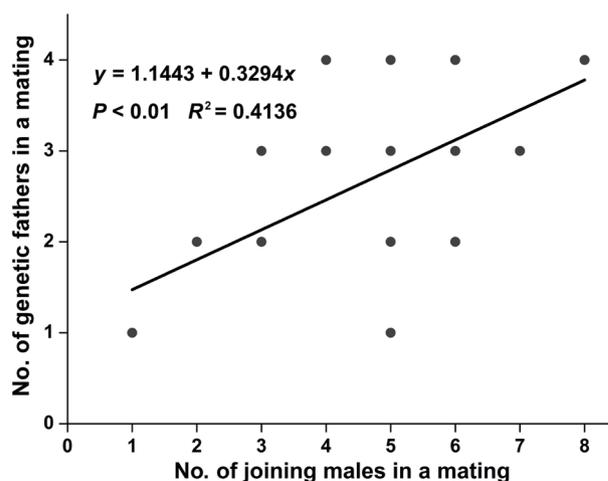


Figure 2 Relationship between the numbers of joining males and genetic fathers within each mating.

21 in 26 social polyandry matings, 80.8%) polyandry in the *R. omeimontis* population. Although not all the males within polyandry matings obtained offspring, more males taking part in a single spawning could cause greater levels of multiple paternity, suggesting that multiple males-

Table 1 Allele numbers, genetic polymorphism, and paternity exclusion probability of the microsatellite loci for the *Rhacophorus omeimontis* population.

Locus	Size (bp)	Number of alleles	Ho	He	Exclusion probability
OMTF3	108–130	14	0.688	0.685	0.7
OMTF6	96–162	8	0.335	0.365	0.823
OMTF7	168–232	14	0.784	0.795	0.933
OMTF9	168–220	21	0.816	0.869	0.421
OMTF10	204–280	13	0.788	0.776	0.578
OMTF11	154–218	10	0.878	0.787	0.599
Multi-locus		16	0.721	0.706	0.999

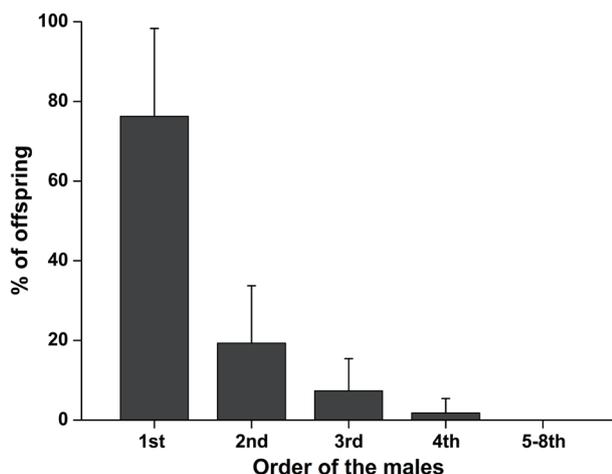


Figure 3 Mean proportions of offspring for the males within each mating. The orders of the males are the males obtained the 1st, 2nd, 3rd, 4th, and 5th–8th amounts of offspring in a mating.

one female breeding behavior (social polyandry) is an important driver of genetic polyandrous reproduction pattern. We considered that this behavior could be a consequence of intense male-male competition of the Omei treefrog (Luo *et al.*, 2016). Because sex ratio in the *R. omeimontis* population was male-biased (male:female = 3:1) and each reproductive female producing a single egg clutch per breeding season (Liao and Lu, 2010), not all the male frogs could get mates independently. In order to increase breeding success, males always do their best in getting mating partners and fertilizing females' eggs. The inferior males might expect to enjoy a certain chances of producing offspring by joining mating pairs as many as possible (convenience polyandry). They would have had forced the female polyandry, by forced copulation, as females are unable to avoid the happening of group spawning (Davies and Halliday, 1977; Rowe, 1992; Byrne and Roberts, 2004; Rice *et al.*, 2006). Furthermore, Liao and Lu (2010) found a higher intensity of social polyandry (100%) in the *R. omeimontis* population at the same site in 2007–2008 than that in our study. We

considered that this difference might be a result of inter-annual variations on the environmental conditions, habitat quality, and body conditions of the breeding individuals (Luo *et al.*, 2016), whose effects on the reproductive patterns of the treefrogs need detailed research in the future.

For females, mating with several males simultaneously could cause superfluous inputs of energy and time and might lead to great risks of wounding, diseases, predation, and death (Hurst *et al.*, 1995; Rice, 1996; Byrne and Roberts, 1999; Fedorka and Mousseau, 2002; Ronkainen *et al.*, 2010). To reduce the unexpected costs in the attempts of resisting males' clasps, female *R. omeimontis* might accept high rates of multiple mating. However, polyandry could enhance reproductive benefits for the females (Yund and McCartney, 1994; Zeh, 1997; Levitan, 1998). Fertilization insurance (i.e., the fertilization guarantee hypothesis) is considered to be one of the possible advantages in group breeding (Gibson and Jewell, 1982), in which more participating males promote fertilization rates of their eggs (e.g., in *Chiromantis xerampelina*, Zeh, 1997; Byrne and Whiting, 2008; in *Heliocidaris erythrogramma*, Evans and Marshall, 2005). Furthermore, based on previous research on amphibians, polyandrous mating could reduce the possibility for the female pairing with a single and close relative (the inbreeding avoidance hypothesis; Tregenza and Wedell, 2002), and also might increase its chances to breed with a male individual that has good genes (the good gene hypothesis; Fisher, 1915), compatible genotypes to its own (the genetic compatibility hypothesis; Neff and Pitcher, 2005), or high genetic diversity (the genetic diversity hypothesis; Yasui, 1998), thus enhance the fitness of its offsprings.

According to this study, when the number of joining males rises in a social polyandrous mating group, the proportion of the males to be genetic fathers generally diminishes, and the offsprings are just belonged to a few fathers. These might be outcomes of the “making the base of a bad lot” reproductive strategy in males (Byrne and Roberts, 2004; Luo *et al.*, 2016). As inferior males usually fail in male-male competition and can not get mates by defeating other ones, the only way to retain the expectations of obtaining breeding success for them is to join the existing mating pairs or groups as many as possible as satellite males.

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