

Male-male competition drives sexual selection and group spawning in the Omei treefrog, *Rhacophorus omeimontis*

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Abstract

Male-male competition and female mate choice based on ornamentation and genetic traits are the main drivers of animal sexual selection and group spawning. If male-male competition is intense, males with specific phenotypes should have advantages in breeding success or occupying superior mating positions. If female choice is important, females should have preferences for mate relatedness or males with good genes or optimal genetic compatibility against themselves. To detect the intensities of male-male competition and female choice and test the good genes and genetic compatibility hypotheses, we observed breeding behaviors, measured individual body lengths and breeding success indicators, and calculated male genetic heterozygosity, male–female relatedness, and genetic dissimilarity in an Omei treefrog (*Rhacophorus omeimontis*) population in Badagongshan, China. Our analyses showed that larger males obtained larger mates, had more mating opportunities, occupied better amplexant positions, and produced more offspring. However, females showed no

inbreeding/outbreeding bias in mate choice, and the good genes and genetic compatibility hypotheses were not supported in female selection on mates and amplexant positions. We considered male-male competition as the main driver of sexual selection and group spawning in this prolonged mating species because the cost of choosing mates with specific genetic traits may be high for females.

Significance statement

Male-male competition and female mate choice are the main drivers of animal sexual selection and mating behaviors. However, their impacts on the evolution of mating system are not yet quite clear. By studying the Omei treefrog, a Chinese endemic anuran species with common group spawning behaviors, this research posed the importance of male-male competition and female mate choice and discussed the mechanisms of sexual selection and multiple mating, which are the most essential and debated issues in evolutionary biology and behavioral ecology. We found that, in this prolonged mating and lek-patterned species, male-male competition is the main driver of sexual selection and group spawning. Larger males can get larger females, have more breeding opportunities, occupy better amplexant positions, and thus, obtain greater numbers of offspring. Whereas, females have no significant preferences on ornamentation and genetic traits of their males or mating positions, and the inferior males “make the best of a bad lot” by joining mating pairs to produce mating groups. Our study provides empirical evidence of reproductive mechanisms of amphibian species and could advance the understandings on the evolution of animals’ sexual selection and mating system.

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Introduction

In general, intra-sexual selection (i.e., members of the same sex, usually males, compete for mates or mating opportunities) and inter-sexual selection (i.e., individuals of one sex, usually females, choose specific members of the opposite sex to obtain high-quality mates and reproductive success) are considered the two main components of animal sexual selection (Emlen and Oring 1977; Bateson 1983; Andersson 1994; Andersson and Simmons 2006; Dale et al. 2007; Nie et al. 2012). In many species, male-male competition based on body size and ornamentation allows larger males or males with better ornamentation to access females as mates, and thus, these males gain additional mating opportunities and higher breeding success (Emlen and Oring 1977; Andersson 1994). However, in other species, sexual selection is primarily controlled by females (female choice or male–female interaction), because they typically have much greater investments than males in gametes and offspring and hence are choosier in reproduction, favoring males with superior phenotypes and genotypes (Williams 1975; Emlen and Oring 1977; Tregenza and Wedell 2000). In essence, across species, diverse relative combinations of male-male competition and female choice determine mating success (Williams 1975; Andersson 1994; Wong and Candolin 2005; Mclean et al. 2012). Studies that simultaneously focus on the intensities of both components of sexual selection can provide empirical evidence for a better understanding of the mechanisms of animal sexual selection.

The development of weaponry and specific ornaments (e.g., larger body size) in males that enhance reproductive success will be favored by evolution through direct combats against other males or through indirect male-male competition (Andersson 1982; Candolin 2003; Bateson and Healy 2005). Body size is considered an overall reliable indicator and fundamental expression of male quality and competitive ability because it was reported to be heritable in some species and could affect the survivability, vitality, and environmental adaptability of the males both phenotypically and genetically (Price 1984; Blanckenhorn et al. 1998; Olsson et al. 2002; Mays and Hill 2004). A number of field- and lab-based studies have revealed that larger males tend to defeat smaller competitors and gain breeding advantages (Trivers 1976; McElligott et al. 2001; Luo et al. 2014). As Devine (1984), Shine et al. (2000), and Cogliati et al. (2014) assumed, if male-male competition based on body size is important in determining outcomes of sexual selection, then there are three plausible results: larger males will mate with larger (more fecund) females, larger males will obtain more numerous mating opportunities, and larger males will produce greater numbers of offspring and have higher breeding success.

To pursue greater reproductive success and fitness, females usually choose their mates according to ornamental and genetic traits in males (Neff and Pitcher 2005). If females mate with

close relatives (inbreeding), the expressions of deleterious recessives in the homozygote state (dominance) or the superiority of certain heterozygotes over homozygotes (overdominance) might reduce offspring fitness over time (Shields 1982; Lynch 1991). By contrast, if females choose distant relatives as mates (outbreeding), genetic segregation and recombination might cause disruptions of local adaptation, underdominance, or epistatic interactions by breaking up co-adapted gene complexes (which are important for the individuals to adapt to local environments), thus allowing deleterious interactions between homozygous loci to become exposed, facilitating expressions of “untested” or even harmful mutations, and thereby reducing offspring fitness over time (Shields 1982; Bateson 1983; Fenster et al. 1997; Turelli et al. 2001). Bateson (1978) considered that females should balance inbreeding and outbreeding during mate choice, and thus, “optimal outcrossing” might be achieved in an animal population (Mays and Hill 2004; Neff and Pitcher 2005). Several empirical studies (e.g., Palmer and Edmands 2000; Du and Lu 2009; Wang and Lu 2011; Luo et al. 2015) found that in certain mammal, bird, amphibian, fish, and invertebrate species, females can discriminate close kin from unrelated males and avoid either extreme inbreeding or outbreeding during mate selection and thus gain advantages in reproductive fitness based on trade-offs between the costs and benefits of avoidance and adaptation via inbreeding and outbreeding (Wilson 1987; Lynch 1991; Futuyma 1998; Mays and Hill 2004; Neff and Pitcher 2005).

For female mate choice, two mechanisms are increasingly recognized and emphasized: the good genes hypothesis and the genetic compatibility hypothesis (Colegrave et al. 2002; Roberts and Gosling 2003; Mays and Hill 2004). Good genes are considered as alleles or allele combinations that increase individual fitness and are linked to the expression of ornamental traits (Mays and Hill 2004; Neff and Pitcher 2005). The good genes hypothesis posits that females prefer males with better ornamentations or genetic traits (e.g., high genetic heterozygosity, large body size, strong claws, loud vocalization) as their mates to obtain genetic benefits for offspring to maximize their fitness (Andersson 1994; Zahavi 1975; Mays and Hill 2004). Previous reports showed that these traits, especially body size and genetic heterozygosity, are mixed and reliable signals of male quality, because body size is closely related to development time, maturity degree, ability to compete and occupy resources, and genetic heterozygosity is closely related to the adaptability to the changing environment (Blanckenhorn et al. 1998; Cotton et al. 2004; Tomkins et al. 2004). Compatible genes are considered as alleles that increase individual fitness when in specific genotypes and paired with specific homologs or alleles at other loci than their own (Neff and Pitcher 2005). The genetic compatibility hypothesis states that because the genetic diversity and fitness of the offspring are affected by combinations of their parental alleles, female mate choice and reproductive success can be determined based on

the genetic dissimilarity between the male mate and the female (Neff and Pitcher 2005). Some studies found genetic similarity enhances fertilization and breeding success (e.g., in frog species *Litoria peronii*; Sherman et al. 2008); however, based on certain empirical data, including those on the major histocompatibility complex (MHC) genes (e.g., Schwensow et al. 2008), females prefer mates with dissimilar genes because the compatibility effect can enhance fertilization capacity and offspring development (Tregenza and Wedell 2000; Evans and Marshall 2005) and also might lead to non-additive genetic variations and produce novel gene combinations enabling their offspring to adapt to the changing environment (Colegrave et al. 2002; Roberts and Gosling 2003; Bernasconi et al. 2004). Although Mays and Hill (2004) stated that both good genes and genetic compatibility can have crucial impacts on animal sexual selection, and also, some research tested and found empirical evidence for both of the two hypotheses in some animal species (e.g., three-spined sticklebacks *Gasterosteus aculeatus*; Lenz et al. 2009), studies that simultaneously consider the two hypotheses are still incomplete.

Anurans generally display mating patterns in which male-male competition and female choice are expressed in various intensities (Wilbur et al. 1978). Because frogs and toads are characterized by concentrated populations during breeding seasons and external fertilization and hatching, all of the individuals in a population can be located during breeding seasons, their body conditions can be conveniently measured, the breeding behaviors can be comprehensively observed, and their eggs can be unambiguously counted in the wild (Wilbur et al. 1978). Thus, anurans provide excellent subjects for the study of animal sexual selection. Furthermore, group spawning (in which multiple males clasp and mate with a single female) has been reported in several anuran species (Roberts and Byrne 2011). Males in different amplexant positions (i.e., dominant vs. subordinate) within a spawning group might achieve discrepant reproductive success, because some studies have considered group mating pattern as a result of male-male competition, i.e., a strategy for inferior males to secure reproductive success by joining with mating pairs and sneaking fertilizations without attracting females through advertisement calls or fighting against dominant males (Fukuyama 1991; Jennions and Passmore 1993; Halliday 1998; Byrne and Roberts 2004). However, female choice in the amplexant positions might also affect male breeding behaviors, their own reproductive success, and the mating pattern of the species. A detailed understanding of the mechanisms and adaptive relevance of group spawning in anurans is lacking. Consequently, direct comparisons of body condition and breeding success among males occupying different amplexant positions are urgently needed. At the same time, analyses of female preference for male relatedness and genetic traits for different amplexant positions remain to be conducted.

The Omei treefrog *Rhacophorus omeimontis* (Anura: Rhacophoridae), is an endemic anuran species of central and

southwestern China (Fei et al. 2012). This species is an arboreal breeder with concentrated populations, lek-patterned breeding behaviors, and limited dispersal ability such that it is distributed in subtropical forests at the elevations of 700–2000 m a.s.l. in the mountainous areas (Liao and Lu 2011a; Fei et al. 2012). During their breeding season from April to July, male frogs gather and produce advertisement calls in a permanent pond (lek) to attract females (Liao and Lu 2010, 2011a, b). No direct fights or aggressive behaviors among males have been observed in the lek. During breeding, a male frog clasps a female, and the amplexant pair climbs up onto a nearby tree and remains on a leaf over the pond for hours before spawning. Frequently, another male or multiple males gradually join the mating before the female lays eggs, thus demonstrating group spawning in which several males amplex with a single female (Liao and Lu 2010, 2011a, b; our field observations).

Based on systematic field observations and molecular analyses of a population of *R. omeimontis*, we expected to detect the relative intensities of male-male competition and female choice within the population and across mating groups for the purpose of explaining the main mechanisms of sexual selection in Omei treefrogs. Our aims were (1) to test whether larger males obtain physically larger female mates, gain more numerous mating opportunities, and achieve greater numbers of offspring; (2) to compare the relatedness of mated partners to the relatedness of random male–female pairs in the population to examine female preference in male relatedness (inbreeding vs. outbreeding); (3) to assess the relationships among reproductive success, genetic heterozygosity of males, and genetic dissimilarity between mating partners so as to test the good genes hypothesis and the genetic compatibility hypothesis; and (4) to compare the body size and offspring number among males in different mating positions within each multiple mating group so as to test male-male competition on amplexant positions and compare genetic heterozygosity among males in different mating positions and compare their relatedness and genetic compatibility against their female mates so as to test female choice on amplexant positions. This study provides empirical evidence and has important implications for anuran breeding ecology and sexual selection theory because it provides an insight into the relative importance of intra- and inter-sexual selection and into the evolution of a largely uninvestigated group spawning mating system.

Methods

Field methods

Our field observations were performed in a pond (a constant lek of *R. omeimontis* chosen based on our long-term surveys) near the Tianpingshan field station (29°47'02" N, 110°05'27" E, elevation: 1413 m; Fig. 1) of the Badagongshan National

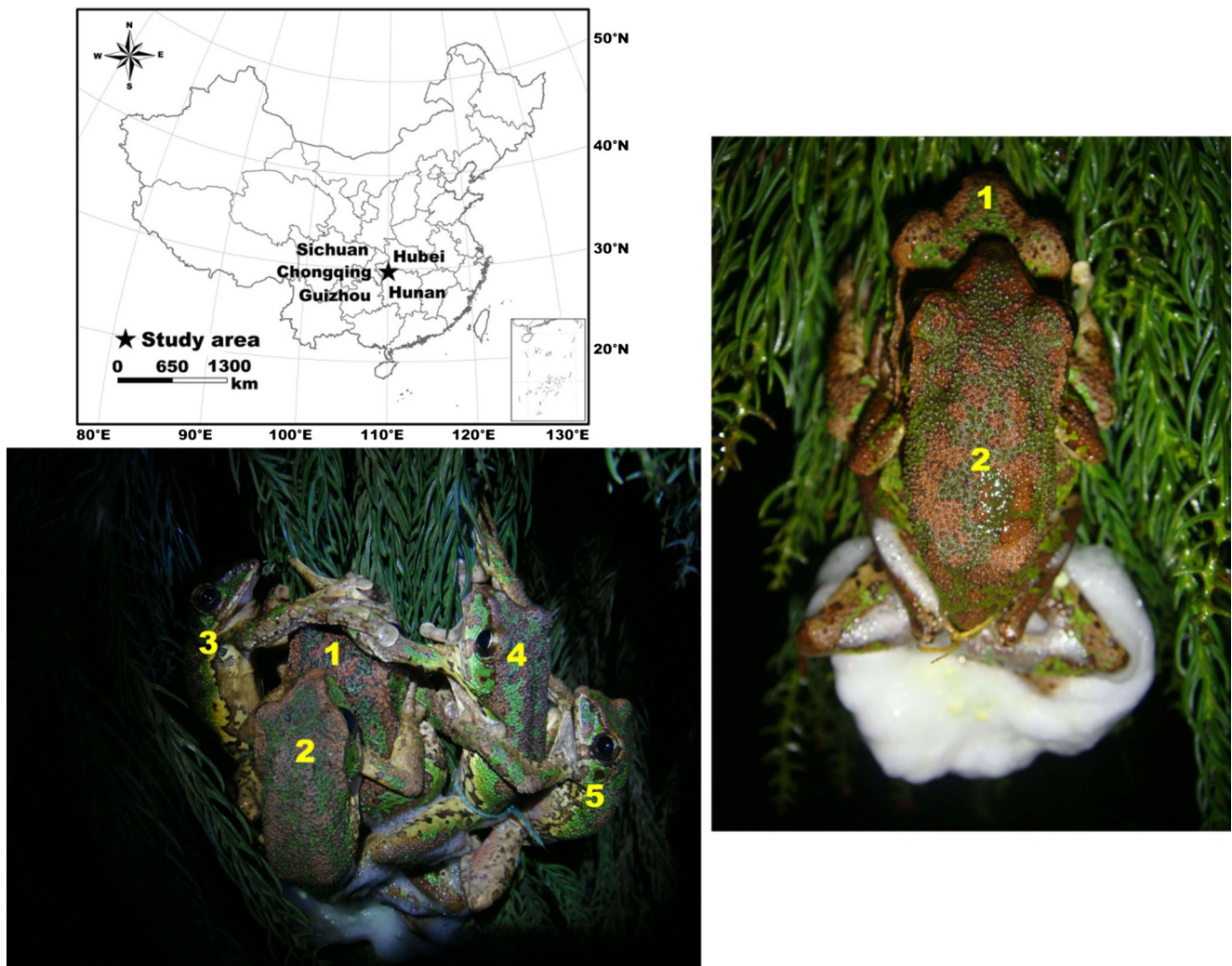


Fig. 1 Study area (top-left panel) and amplexus of the Omei treefrog *Rhacophorus omeimontis*: Multiple males-one female amplexus (group spawning) (left-bottom panel: 1 female, 2 dominant male, 3, 4 1st

subordinate males, 5 2nd subordinate male) and one male-one female amplexus (right panel: 1 female, 2 male)

Nature Reserve (29°38'–29°49' N, 109°41'–110°10' E) in Hunan Province of central China during the *R. omeimontis* breeding seasons in 2011–2013 (18 April to 4 June 2011, 14 April to 27 May 2012, and 22 April to 27 May 2013). Each night, we moved around the pond and the nearby trees searching for individuals of *R. omeimontis* using a 12-V flashlight. We observed and recorded the behaviors of the frogs and determined the sexes based on their positions within the amplexus (the clasping individuals are the males) and the presence (male) or absence (female) of vocal sacs. Because one male-one female amplexus and multiple males-one female amplexus (group spawning) both occurred in our observations, we applied the following definitions. In a spawning group, the male clasped at the dorsal line on the female was the dominant male, whereas the males clasped on the female's left/right body sides and on the periphery of the group were the 1st and 2nd subordinate males (Fig. 1). After the amplexant pairs or groups laid eggs, we arbitrarily captured

the frogs using a string bag, gently but firmly held them in hand until they became docile, measured their dorsal lengths three times from the tip of the nose to the anus (snout-vent length, SVL) to the nearest 0.1 mm using a vernier caliper, and used the mean values as their body length data. Next, we clipped approximately 1 mm × 1 mm of tissues at the ends of the toes using a pair of operating scissors to mark the individuals and collect samples (stored in 95 % ethanol) for molecular analyses. All individuals were released at the capture sites as soon as possible (within 30 min) upon completion of these procedures. Any recaptured individuals were released immediately without measurements or sampling. We collected all of the clutches of eggs from the trees and hatched each clutch in a plastic bucket (with a 10–20 cm depth of water) by the side of the pond. The eggs were counted in each bucket (for data on the number of eggs), and after the hatching process was finished, we counted the tadpoles, unhatched but fertilized eggs, and unfertilized eggs based on our field observational

experiences. The fertility rate and hatchability for each clutch were calculated as follows: fertility rate = (number of eggs – number of unfertilized eggs)/number of eggs and hatchability = number of tadpoles/(number of tadpoles + number of fertilized but unhatched eggs). For molecular analyses of the offspring, we randomly selected 24 tadpoles within each bucket, clipped approximately 1 mm × 1 mm of tissues from the ends of their tails, and stored the samples in 95 % ethanol. Finally, all tadpoles were released back to the pond.

Molecular analyses

We extracted the total DNA of each frog and tadpole from the toe and tail tissue samples using the TIANamp Genomic DNA Kit (TIANGEN Biotech Co. Ltd, Beijing, China) and dissolved them in ddH₂O at a temperature of –20 °C. Because genes of the major histocompatibility complex (MHC) encode cell surface glycoproteins that present foreign (MHC class I genes) and self (MHC class II genes) peptides into T lymphocytes, they can reveal the molecular mechanisms underlying the evolution of immunity-related adaptation (Klein 1986; Hughes and Yeager 1998). Thus, MHC gene diversity might be closely related to the fitness of the treefrogs and their offspring and should have important effects on sexual selection in *R. omeimontis*. In this study, eight microsatellite loci (*OMTF1*, *OMTF2*, *OMTF4*, *OMTF6*, *OMTF7*, *OMTF9*, *OMTF10*, and *OMTF11* with GenBank accession numbers JQ031742, JQ031743, JQ031745, JQ031747, JQ031748, JQ031750, JQ031751, and JQ031752, respectively) and one MHC gene marker (MHC class II B genes exon 2 with the GenBank accession numbers KR232021–KR232080) were chosen based on our previous studies (Zhao et al. 2012; Chen 2013). Detailed descriptions of the primer designs, polymerase chain reactions (PCRs), and genotyping of the microsatellite markers are presented by Zhao et al. (2012), and those for the primer design, PCR, cloning, and sequencing of the MHC gene marker can be found in Chen (2013). We estimated the numbers of alleles for the eight microsatellite loci and the MHC gene markers in FSTAT 2.9.3.2 (Goudet 2002). The observed heterozygosity (H_o), expected heterozygosity (H_e), and polymorphic information content (PIC) per locus were calculated in Cervus 3.0 (Kalinowski et al. 2007). We also evaluated the departure from Hardy-Weinberg equilibrium (HWE) for each locus over the treefrog population using the software packages GenAlEx 6.5 (Peakall and Smouse 2012) and FSTAT 2.9.3.2 (Goudet 2002).

Based on our genotype data from the microsatellite loci, we calculated the genetic relatedness of all individual pairs using the method of Lynch and Ritland (1999) in Coancestry 1.0 software (Wang 2011). That is, the relatedness of mated pairs and the relatedness of all possible male–female pairs in the frog population were obtained. To test the offspring numbers for each mated male, we performed parentage analyses with

Cervus 3.0 software (Kalinowski et al. 2007) using microsatellite genotype data from the adult males and 24 randomly selected tadpoles (detailed procedures are presented in Wang (2014)). The overall exclusionary powers for the eight microsatellite loci were 0.984 for the 1st father and 0.999 for the 2nd father, and only 1.5 % (30 of 2016) of the tadpoles could not be assigned to a genetic father. The offspring number for a given male within a given mating pair/group was calculated as follows: (the number of tadpoles assigned to this male in the 24 randomly selected tadpoles / 24) × the total number of tadpoles from the egg clutch of this mating pair/group.

To calculate the genetic heterozygosity of the microsatellite loci of a frog or a tadpole, we used Coltman's index and the HL (homozygosity by loci) index based on Coltman et al. (1999) and Aparicio et al. (2006). Coltman's index defines a standardized individual heterozygosity as the proportion of heterozygous typed loci in an individual divided by the mean observed heterozygosity of all typed loci (Coltman et al. 1999). Because this method assumes a linear relationship between locus-specific heterozygosity and allele numbers and assigns equal weight for all loci regardless of the allelic frequencies, it might underestimate the effect of variable loci in the standardization (Aparicio et al. 2006). Thus, to estimate the homozygosity and heterozygosity weighting for the contribution of each locus to the genetic heterozygosity index, the HL index (values range from 0 to 1) defines a weighted homozygosity by loci for a given individual as the summation of the expected heterozygosities for all the homozygous loci divided by the summation of expected heterozygosities for all the loci (including homozygous loci and heterozygous loci) (Aparicio et al. 2006). Individual heterozygosity could be estimated using the 1-HL index, and both indices were calculated for all mated male frogs and sampled offspring. Next, we counted the number of alleles per male as the indicator of heterozygosity of the MHC class II B genes exon 2 marker. To obtain the microsatellite dissimilarity scores between paired males and females, the pair-wise identity index was computed in the IDENTIX 1.1 software (Belkhir et al. 2002) following the method of Forsberg et al. (2007) because this method is an integrative estimator that considers several dissimilarity coefficients, including the number of shared alleles and allelic distance (Forsberg et al. 2007). For the dissimilarity of the MHC class II B genes exon 2 marker, the Poisson-corrected pair-wise amino acid distance of a mated male and female was calculated in MEGA 5 (Tamura et al. 2011), and the dissimilarity scores were computed using the summation method and maximal distance method following the approach of Landry et al. (2001) and Forsberg et al. (2007).

Statistical analyses

We assessed the normality of all data (body length, relatedness, egg number, fertility rate, hatchability, tadpole number,

heterozygosity indices, and genetic dissimilarity indices) using Kolmogorov-Smirnov (K-S) tests and the homogeneity of variance of their residual distributions using Levene's tests. All of the variables were normally distributed ($P > 0.05$) with homoscedasticity ($P > 0.05$). To assess the sexual dimorphism of body size in our *R. omeimontis* population, we used one-way ANOVA between male and female SVLs.

Three steps were conducted to analyze male-male competition in this study. First, we plotted and linearly fit the relationships between the SVLs of mated males and females. Because one male-one female mating pairs and multiple males-one female amplexus both occurred in the population, we performed the analyses for one male-one female mating pairs, multiple males-one female mating groups, and all mating pairs/groups, respectively, using the individual SVLs as parameters. To avoid pseudoreplication in the analysis, we only used SVL data of the dominant males for the multiple males-one female groups. Next, to explore the relationship between body size and breeding opportunity for male frogs, we counted the number of matings per male within each breeding season and detected its correlation to the male SVL using linear regressions. Finally, to explore the relationship between male body size and breeding success, and considering that the offsprings from the female could be shared by several males in multiple males-one female mating groups, and group spawning could have impacts on the number of offspring each male obtained, we calculated Pearson's correlation coefficients and conducted linear regressions among the male SVLs and their tadpole numbers (for one male-one female mating pairs, all the males were analyzed; for multiple males-one female mating groups, only the dominant males were analyzed; for all mating pairs/groups, all the males in one male-one female mating pairs and the dominant males in multiple males-one female mating groups were analyzed). As female body condition could have effects on breeding success, we also calculated partial correlation coefficients and carried out partial regressions using female SVL as controlling factors.

To examine the female preference in male mate relatedness, we calculated the genetic relatedness frequencies for one male-one female mating pairs, multiple males-one female mating groups (only the dominant males were included), all mating pairs/groups (only the dominant males were included), and all of the possible male-female pairs (based on a random mating assumption) in the population for 2011–2013 and each breeding season, respectively, and employed paired-samples *t* tests to test for differences in their means and variances (all the possible male-female pairs vs. one male-one female mating pairs, all the possible male-female pairs vs. multiple males-one female mating groups, all the possible male-female pairs vs. all mating pairs/groups). Next, linear regressions were performed among the relatedness of mating partners (for the mating groups, only the dominant males were included) and their egg numbers, fertility rates, and hatchability (for one male-one

female mating pairs, multiple males-one female mating groups, and all mating pairs/groups, respectively). To test the good genes hypothesis, we counted the total number of tadpoles per male per breeding season and detected its relationships to the genetic heterozygosity indicators (microsatellite loci: Coltman's index and HL index; MHC class II B genes exon 2 marker: number of alleles) of the male using linear regressions (for 2011, 2012, and 2013, respectively). Then, we used male genetic heterozygosity of each mating pair and genetic heterozygosity of the dominant male per spawning group as an indicator of good genes and applied generalized linear models (GLMs) among this indicator and breeding success parameters (i.e., egg number, fertility rate, and hatchability) for one male-one female mating pairs, multiple males-one female mating groups, and all mating pairs/groups, respectively. We also used paired-samples *t* tests to compare the means of these genetic heterozygosity indicators between all males vs. males from mating pairs, all males vs. dominant males from mating groups, and all males vs. males from mating pairs and dominant males from mating groups. For the genetic compatibility hypothesis, we used GLMs to show the relationships among the male-female genetic compatibility indexes (for the mating groups, only the dominant males were included; microsatellite loci: identity index; MHC class II B genes exon 2 marker: dissimilarity scores by the summation and maximal distance methods) and breeding success parameters for one male-one female mating pairs, multiple males-one female mating groups, and all mating pairs/groups, respectively. We also used paired-samples *t* tests to compare the means of these genetic compatibility indicators between all possible male-female pairs (based on a random mating assumption) vs. males from mating pairs against their females, all possible male-female pairs vs. dominant males from mating groups against their females, and all possible male-female pairs vs. males from mating pairs and dominant males from mating groups against their females. Furthermore, we processed GLMs between microsatellite genetic heterozygosity for the males and their tadpoles (Coltman's index and HL index, respectively; for tadpoles, the means were used) and between parental microsatellite genetic compatibility (identity index) and their tadpole genetic heterozygosity (means of Coltman's index and HL index, respectively).

To detect male-male competition in amplexant position, we used one-way ANOVAs to compare the body lengths (SVL), offspring proportions within the clutch, and offspring numbers among dominant, 1st subordinate, and 2nd subordinate males for multiple males-one female mating groups. For female choice of amplexant position in genetic relatedness and male good genes, one-way ANOVAs were performed based on the relatedness, microsatellite heterozygosity (Coltman's index and HL index), and MHC gene heterozygosity (allele number) among the dominant, 1st subordinate, and 2nd subordinate males for mating groups. Additionally,

the female choice of amplexant position in genetic compatibility was tested using one-way ANOVAs of the identity index (microsatellite loci) and MHC genetic dissimilarity indices (the summation and maximal distance methods) among the dominant, 1st subordinate, and 2nd subordinate males.

To minimize observer bias, blinded methods were used when all behavioral data were recorded and analyzed.

Results

A total of 157 adult individuals (78 females, 79 males) were measured and sampled in this study, including 40 one male-one female amplexus and 44 multiple males-one female amplexus (2–7 males clasping with a single female). The one-way ANOVA of male and female SVLs showed significant sexual dimorphism ($F_{1,156} = 621.213$, $P < 0.001$) showing that females (77.77 ± 4.86 mm (mean \pm SE; the same below), ranging from 65.62 to 86.50 mm), are obviously larger than males (64.31 ± 3.76 mm, ranging from 51.31 to 74.97 mm). The egg number per mating pair/group ranged from 237 to 963. The number of tadpoles obtained by each male treefrog in each mating ranged from 0 (the male could not succeed in mating) to 769. The number of matings per male per breeding season ranged from 0 to 5. Our results showed significant positive linear relationships between male SVL and female SVL across mating pairs or groups ($P < 0.05$, Fig. 2), i.e., larger males obtained larger females as their mates for all the mating pairs/groups ($P < 0.05$, Fig. 2a), and for one male-one female mating pairs ($P < 0.05$, Fig. 2b) and multiple males-one female mating groups ($P < 0.05$, Fig. 2c), respectively. The count of number of matings per male frog per breeding season showed that larger males had more numerous mating opportunities on average (Fig. 3), and the positive trends between body length and number of matings were significant for the breeding seasons of 2011, 2012, 2013, and 2011–2013, respectively, according to the one-way ANOVAs ($P < 0.05$) and linear regressions ($P < 0.03$, $R^2 > 0.5$). Using the mating pair/group as the unit, we calculated Pearson's correlation coefficients (r) and conducted linear regressions of the male body lengths against their tadpole numbers, and no positive correlation (males in one male-one female mating pairs $r = -0.094$, $P = 0.441$; dominant males in multiple males-one female mating groups $r = -0.137$, $P = 0.277$; males in mating pairs and dominant males in mating groups $r = -0.125$, $P = 0.375$) was found. Because breeding success can be influenced by female body condition, we performed partial correlation analyses (r_p) using female SVL as the controlling factor and did not find significant relationship (males in one male-one female mating pairs $r_p = -0.221$, $P = 0.627$; dominant males in multiple males-one female mating groups $r_p = -0.298$, $P = 0.426$; males in mating pairs and dominant males in mating groups $r_p = -0.309$, $P = 0.510$).

The genetic diversity of the microsatellite loci, MHC class II B genes exon 2 marker, and their departures from the Hardy-Weinberg equilibrium for the *R. omeimontis* population are shown in Online Resource 1. The pair-wise relatedness (microsatellite loci) of mated males and females ranged from -0.334 to 0.681 in our *R. omeimontis* population, whereas those of random male–female pairs (during the breeding seasons of 2011, 2012, and 2013) ranged from -0.377 to 1.000 . The relatedness frequencies of mated (for mating groups, only the dominant males were included) and random male–female pairs and their paired-samples t tests revealed no significant differences in their means and SE (2011–2013: $\text{relatedness}_{\text{all matings}} = -0.031 \pm 0.143$, $\text{relatedness}_{\text{mating pair}} = -0.033 \pm 0.119$, $\text{relatedness}_{\text{mating group}} = -0.029 \pm 0.141$, $\text{relatedness}_{\text{random}} = -0.028 \pm 0.134$, paired-samples t tests $P > 0.5$; 2011: $\text{relatedness}_{\text{all matings}} = -0.020 \pm 0.140$, $\text{relatedness}_{\text{mating pair}} = -0.022 \pm 0.144$, $\text{relatedness}_{\text{mating group}} = -0.020 \pm 0.137$, $\text{relatedness}_{\text{random}} = -0.027 \pm 0.141$, paired-samples t tests $P > 0.5$; 2012: $\text{relatedness}_{\text{all matings}} = -0.051 \pm 0.140$, $\text{relatedness}_{\text{mating pair}} = -0.057 \pm 0.137$, $\text{relatedness}_{\text{mating group}} = -0.049 \pm 0.141$, $\text{relatedness}_{\text{random}} = -0.024 \pm 0.139$, paired-samples t tests $P > 0.5$; 2013: $\text{relatedness}_{\text{all matings}} = -0.028 \pm 0.130$, $\text{relatedness}_{\text{mating pair}} = -0.030 \pm 0.120$, $\text{relatedness}_{\text{mating group}} = -0.033 \pm 0.131$, $\text{relatedness}_{\text{random}} = -0.036 \pm 0.111$, paired-samples t tests $P > 0.5$), which means no inbreeding or outbreeding bias emerged during female mate choice in this *R. omeimontis* population. The linear regressions of the male–female relatedness against egg number, fertility rate, and hatchability also showed non-significant relationships ($P > 0.05$, $R^2 < 0.07$; Online Resource 2).

The ranges of the microsatellite HL index and Coltman's index were 0.001 – 0.639 and 0.386 – 1.573 , respectively, for adult male frogs, and the ranges of those heterozygosity indices for the offspring were 0.089 – 0.479 and 0.622 – 1.277 (Fig. 4a, b, Online Resource 3). The number of alleles within the MHC class II B genes exon 2 marker ranged from 1 to 4 in adult males (Fig. 4c, Online Resource 3). Our results showed significant and positive relationships of tadpole number per male per breeding season against the genetic heterozygosity indicators (HL index, Coltman's index, MHC allele number) of the males for 2011, 2012, and 2013, separately ($P < 0.05$; $R^2 > 0.05$, Fig. 4). The GLMs among the male genetic heterozygosity (HL index, Coltman's index, MHC allele number) and breeding success indices (egg number, fertility rate, and hatchability) across mating pairs/groups showed limited linear relationships, with most characterized by $P > 0.05$ and low explanatory powers ($R^2 < 0.10$) (Online Resource 3a–f, 3i–k), which means that genetic heterozygosity in males might have quite weak effects on breeding success. Furthermore, genetic heterozygosity indicators among males from mating pairs, dominant males from mating groups, and all male individuals showed non-significant differences (HL_{all}

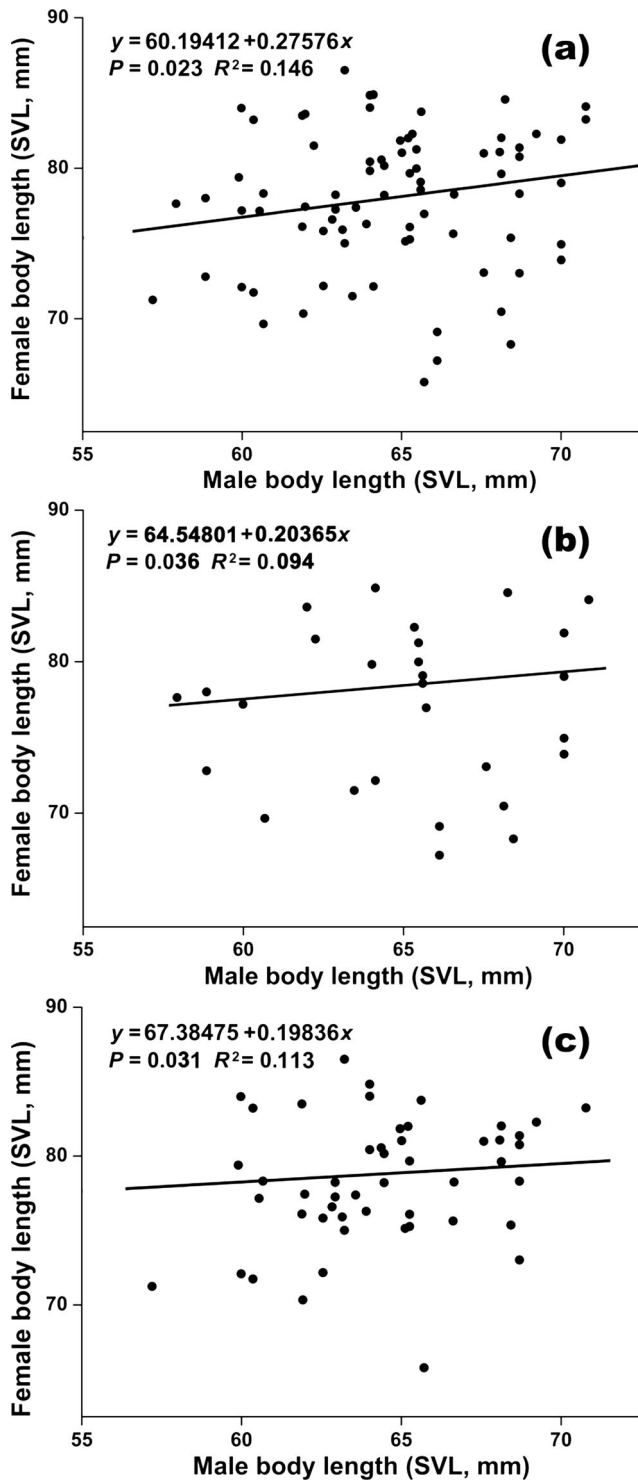


Fig. 2 Relationships of body lengths between mated males and females among mating pairs and groups: **a** male (males in mating pairs and the dominant males in mating groups) SVL vs. female SVL for all the mating pairs and groups; **b** male SVL vs. female SVL for one male-one female mating pairs; **c** dominant male SVL vs. female SVL for multiple males-one female mating groups

$\text{males} = 0.333 \pm 0.131$, $\text{HL}_{\text{males from mating pairs}} = 0.330 \pm 0.141$, $\text{HL}_{\text{dominant males from mating groups}} = 0.336 \pm 0.166$, $\text{HL}_{\text{males from mating pairs and dominant males from mating groups}} = 0.328 \pm 0.161$,

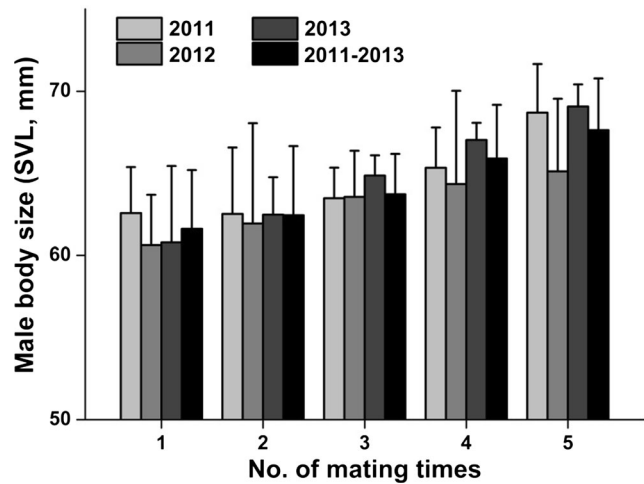


Fig. 3 Relationship between number of mating times and body length of the male Omei treefrog *Rhacophorus omeimontis* in Badagongshan. The columns represent the numbers of mating times per breeding season. The lengths of the bars represent the mean body sizes of the males. The error bars represent the standard errors of the male body sizes

paired-samples t tests $P > 0.5$; Coltman's $\text{index}_{\text{all males}} = 0.959 \pm 0.199$, Coltman's $\text{index}_{\text{males from mating pairs}} = 0.971 \pm 0.212$, Coltman's $\text{index}_{\text{dominant males from mating groups}} = 0.951 \pm 0.222$, Coltman's $\text{index}_{\text{males from mating pairs and dominant males from mating groups}} = 0.966 \pm 0.201$, paired-samples t tests $P > 0.5$; no. of alleles $_{\text{all males}} = 2.256 \pm 0.408$, no. of alleles $_{\text{males from mating pairs}} = 2.305 \pm 0.441$, no. of alleles $_{\text{dominant males from mating groups}} = 2.242 \pm 0.337$, no. of alleles $_{\text{males from mating pairs and dominant males from mating groups}} = 2.274 \pm 0.430$, paired-samples t tests $P > 0.5$). Thus, the good genes hypothesis was not supported by our empirical data. However, the HL and Coltman's indices between males and their tadpoles showed significant positive (monotone increasing) trends ($P < 0.05$, $R^2 > 0.2$; Online Resource 3g, h), which means that male heterozygosity promotes genetic diversity of their offspring.

The male–female pair-wise genetic compatibility of microsatellite loci (identity index) ranged from 0.253 to 0.963 (Online Resource 4a–c) in our *R. omeimontis* population, whereas that of the MHC marker ranged from 0.001 to 6.231 and from 0.001 to 3.155 based on the summation method (Online Resource 4d–f) and maximal distance method (Online Resource 4g–i), respectively. Weak linear relationships among genetic dissimilarity scores (identity index and two MHC dissimilarity indices) and breeding success were detected by the GLMs, with most characterized by $P > 0.05$ and $R^2 < 0.10$ (Online Resource 4a–i). Furthermore, genetic compatibility indicators among males from mating pairs against their females, dominant males from mating groups against their females, and all of the possible female–male pairs showed non-significant differences ($\text{identity}_{\text{all possible male–female pairs}} = 0.591 \pm 0.175$, $\text{identity}_{\text{males from mating pairs against their females}} = 0.595 \pm 0.179$, $\text{identity}_{\text{dominant males from mating groups}}$

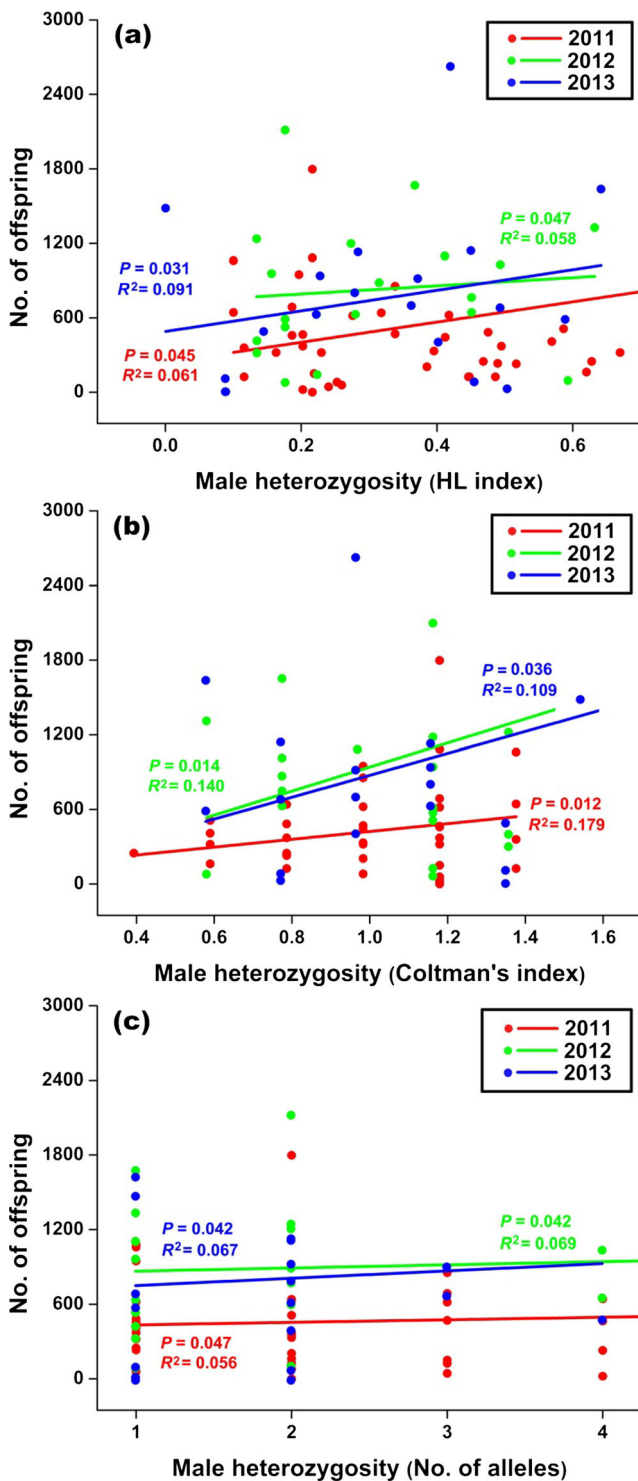


Fig. 4 Linear regressions of genetic heterozygosity indicators (microsatellite and MHC markers) against total tadpole numbers of males for 2011 (red), 2012 (green), and 2013 (blue): **a** HL index vs. total tadpole number, **b** Coltman's index vs. total tadpole number, **c** number of alleles vs. total tadpole number

against their females = 0.588 ± 0.177 , $\text{identity}_{\text{males}}$ from mating pairs and dominant males from mating groups against their females = 0.590 ± 0.167 , paired-samples t tests $P > 0.5$; $\text{summation}_{\text{all possible male-female}}$

pairs = 3.618 ± 0.446 , $\text{summation}_{\text{males}}$ from mating pairs against their females = 3.615 ± 0.365 , $\text{summation}_{\text{dominant males}}$ from mating groups against their females = 3.598 ± 0.455 , $\text{summation}_{\text{males}}$ from mating pairs and dominant males from mating groups against their females = 3.612 ± 0.444 , paired-samples t tests $P > 0.5$; $\text{maximal distance}_{\text{all possible male-female pairs}}$ = 1.742 ± 0.234 , $\text{maximal distance}_{\text{males}}$ from mating pairs against their females = 1.751 ± 0.207 , $\text{maximal distance}_{\text{dominant males}}$ from mating groups against their females = 1.746 ± 0.241 , $\text{maximal distance}_{\text{males}}$ from mating pairs and dominant males from mating groups against their females = 1.741 ± 0.255 , paired-samples t tests $P > 0.5$). However, our results showed obviously positive relationships between parental genetic compatibility (identity index) and offspring genetic heterozygosity (HL index and Coltman's index) ($P < 0.05$, $R^2 > 0.2$; Online Resource 4j, k). In other words, although genetic compatibility might not significantly enhance the breeding success (the genetic compatibility hypothesis was not supported) of the treefrogs, their offspring could obtain benefits or advantages from higher genetic heterozygosity.

The one-way ANOVAs among males from different amplexant positions in multiple males-one female mating groups revealed that dominant males were significantly larger than subordinate males in body size (ANOVA $F_{1,105} = 28.145$, $P = 0.034$; Fig. 5a), obtained significantly higher proportions of offspring within the mating groups (ANOVA $F_{1,94} = 34.498$, $P < 0.001$; Fig. 5b), and thus produced a greater number of tadpoles (ANOVA $F_{1,94} = 60.871$, $P < 0.001$; Fig. 5b). These results suggest that male-male competition for mating positions might be obvious in the *R. omeimontis* population because better amplexant positions could lead to breeding advantages for the male frogs. In contrast, our comparisons among the dominant, 1st, and 2nd subordinate males relative to relatedness (microsatellite loci), genetic heterozygosity (indices of microsatellite and MHC class II B genes exon 2 markers), and compatibility (indices of microsatellite and MHC class II B genes exon 2 markers) showed non-significant differences (ANOVA $P > 0.05$; Table 1), which means that the females might have obvious preferences for these genetic traits in selecting males for different amplexant positions; thus, the good genes hypothesis and the genetic compatibility hypothesis were not supported in female mate choice of amplexant positions in this study.

Discussion

Male-male competition

Male-male competition might be an important driver of sexual selection in our focused population of *R. omeimontis* because two of the intra-sexual selection assumptions (see the Introduction; Devine 1984; Shine et al. 2000; Cogliati et al. 2014) were supported by our empirical data on the spawning

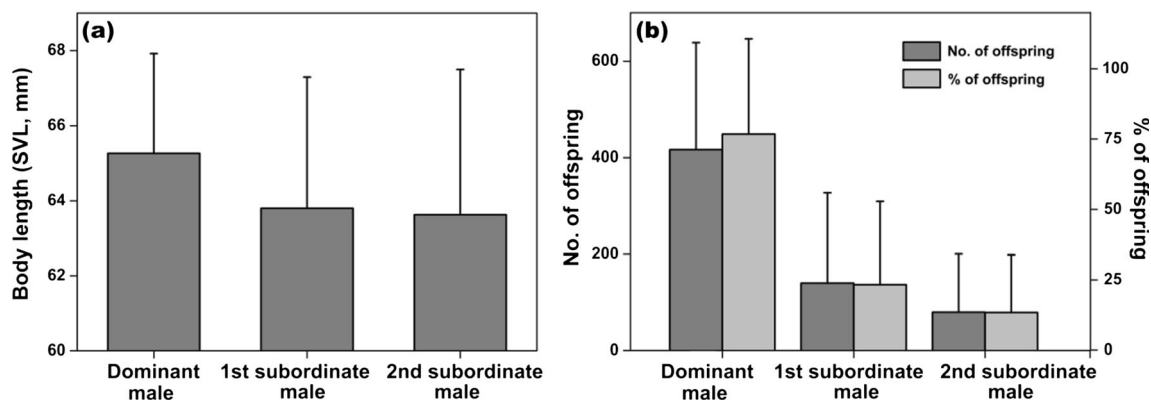


Fig. 5 Comparisons of body length (a), offspring proportion, and offspring number (b) among the dominant, 1st subordinate, and 2nd subordinate males for multiple males-one female mating groups of the Omei treefrog *Rhacophorus omeimontis* in Badagongshan. The columns represent the dominant, 1st, and 2nd subordinate males. The lengths of

the bars represent the mean body sizes of the males (a) and the mean numbers and proportions of offspring the males obtained per mating (b). The error bars represent the standard errors of the male body sizes (a) and the standard errors of the numbers and proportions of offspring the males obtained per mating (b)

groups and mating pairs. Although the exact ways that larger males can defeat the smaller ones during male-male competition to achieve better females are not clear (no direct combats were observed but other ways, e.g., recognition of the variations on vocalization, body size, or skin color by competing males, might contribute; Zahavi 1975; Andersson 1994; Mays and Hill 2004) and need detailed research, the positive relationships between male body length and female body length for the one male-one female mating pairs, multiple males-one female mating groups, and all mating pairs/groups, respectively, and the positive relationship between male body length and male mating times per year, indicated that large males could pair with large females and mate several times in a single breeding season and thus might have advantages in breeding against small males.

Number of offspring is important in determining the mate choice patterns and reproductive strategies of amphibians (Andersson and Simmons 2006). A number of studies on frog (e.g., Jennions et al. 1992; Friedl and Klump 2005) and toad (e.g., Davies and Halliday 1977; Bowcock et al. 2013) species concluded that larger males can fertilize a larger number of eggs, achieve higher hatchability, produce more numerous tadpoles, and finally achieve greater breeding success than smaller males during intra-sexual competition. In contrast, this study found no significant increase in such indicators with increasing male body size for the one male-one female mating pairs, multiple males-one female mating groups, and all mating pairs/groups, respectively, i.e., our empirical data do not provide sufficient evidence for the third assumption of intra-sexual competition from Devine (1984), Shine et al. (2000), and Cogliati et al. (2014). We considered this result as a consequence of multiple mating behaviors of large males such that they could mate with several female frogs and produce a mass of offspring during a single breeding season. It might hint that they suffer sperm depletion in given matings or it might be lead

by energy (number of sperms, time, etc.) input strategies among matings across the whole breeding season by the large males. Furthermore, certain other factors, such as sex ratio and environmental conditions, might also contribute to the variations in fertilization, hatchability rates, and offspring amount.

Our study suggests that intra-sexual interactions might have important impacts on amplexant position competition among males within a spawning group because male frogs with larger body sizes generally possessed better positions and produced larger numbers of tadpoles, although the exact ways that larger males can defeat the smaller ones to get dominant amplexus positions are not clear and need more studies. Because superior mating positions can lead to advantages in breeding success (e.g., higher fertility rate), similar situations have been reported in other frog species that exhibit group spawning, such as *R. arboreus* (Kusano et al. 1991), *R. schlegelli* (Fukuyama 1991), *Agalychnis callidryas* (D'Orgeix and Turner 1995), and *Chiromantis xerampelina* (Jennions et al. 1992). For inferior males, as females were limited in the population, the joining of mating pairs may represent a breeding strategy for “making the best of a bad lot.” In other words, by sneakily joining mating pairs, subordinate males may compensate for competitive inferiority due to phenotypic constraints (e.g., body size) and produce a certain number of eggs or offspring (Byrne and Roberts 2004).

Female choice on genetic relatedness

Our study found no significant difference among the relatedness of the observed partners in the one male-one female mating pairs, multiple males-one female mating groups, all mating pairs/groups, and the expected male-female relatedness under a random mating assumption, which suggested that females in the treefrog population display neither inbreeding nor

Table 1 Comparisons of relatedness, genetic heterozygosity, and compatibility indices among the dominant, 1st subordinate, and 2nd subordinate males for multiple males-one female mating groups of the Omei treefrog *Rhacophorus omeimontis* in Badagongshan

	Dominant male (mean ± SE)	1st subordinate male (mean ± SE)	2nd subordinate male (mean ± SE)	ANOVA $F_{1,105}$	ANOVA P
Relatedness	-0.039 ± 0.139	-0.025 ± 0.122	-0.040 ± 0.118	1.054	0.534
Good genes (heterozygosity)					
Microsatellite loci					
HL index	0.315 ± 0.159	0.353 ± 0.171	0.339 ± 0.192	0.196	0.817
Coltman's index	1.012 ± 0.238	0.973 ± 0.266	0.973 ± 0.270	0.994	0.498
MHC class II B gene exon 2					
No. of alleles	1.723 ± 1.016	1.744 ± 0.860	2.292 ± 1.334	1.868	0.375
Genetic compatibility					
Microsatellite loci					
Identity index	0.740 ± 0.157	0.731 ± 0.120	0.743 ± 0.132	-0.076	0.940
MHC class II B gene exon 2					
Summation method	4.437 ± 1.797	4.104 ± 1.916	4.292 ± 1.916	0.071	0.878
Maximal distance method	2.113 ± 0.867	1.954 ± 0.954	2.053 ± 0.868	0.987	0.524

outbreeding bias in selection of mates. Within each single female-multiple males mating group, this study found no significant difference in genetic relatedness of the female against the males occupying different amplexant positions (dominant, 1st subordinate, and 2nd subordinate). Similarly, a random mating pattern in genetic relatedness was reported in a few frog species, such as *Allobates femoralis* (Ringler et al. 2012). We considered that, as a prolonged mating species, Omei treefrogs arrive at the breeding pond randomly and sequentially, and therefore, the females cannot compare all mates simultaneously. Choosing males with specific relatedness traits (inbreeding or outbreeding) means losing all current reproductive benefits by not breeding immediately (Kokko and Ots 2006). The high costs of searching for close or remote relatives might drive females to ignore their preference for genetic relatedness in mate choice and promote a more random mating pattern.

Female choice on good genes and genetic compatibility

The results showed that males with high genetic heterozygosity obtained great breeding success (abundant offspring) during each breeding season, which provided evidences for genetic heterozygosity as an index of good genes. This study generally found no positive relationships between reproductive success (egg number, fertility rate, or hatchability) and male genetic heterozygosity or pair-wise compatibility indicators (both for microsatellite and MHC gene markers) on the mating pair/group unit for the one male-one female mating pairs, multiple males-one female mating groups, and all mating pairs/groups, respectively. In addition, genetic heterozygosity of males from mating pairs or the dominant males from mating groups was not significantly greater than that of all males, while genetic compatibility among males from mating

pairs or the dominant males from mating groups against their females was not significantly greater than that of all the possible male-female pairs. These results provided insufficient support for the good genes or genetic compatibility hypotheses. Furthermore, our results detected limited differences in the indicators of good genes and genetic compatibility among males from various amplexant positions within a mating group and thus might indicate no significant female selection on mate genetic traits across mating ranks. We considered that it might be caused by the multiple mating behaviors of the high heterozygosity males and their energy input strategies among matings across a breeding season and the making the best of a bad lot strategy of the inferior males under intensive male-male competition. Also, within the sequential-mating-patterned Omei treefrog population, the high cost of obtaining males with specific good genes or genetic dissimilarity could drive females to ignore potential benefits from good genes or genetic compatibility and to mate immediately (Kokko and Ots 2006). Furthermore, we only involved the contributions of genetic heterozygosity, allele number, and genetic dissimilarity in female mate choice in this study, although various individual traits (such as body mass, vocalization, claw, movement, color, etc.) also may be important indicators for good genes and genetic compatibility and thus might have effects on sexual selection in the treefrogs (Mays and Hill 2004). However, our results revealed increasing trends in offspring genetic heterozygosity with the increase in paternal genetic heterozygosity or parental genetic dissimilarity for the one male-one female mating pairs, multiple males-one female mating groups, and all mating pairs/groups, respectively. Therefore, to a certain extent, higher individual genetic diversity and male-female genetic compatibility might produce higher genetic diversity and better adaptation to the changing environments among their offspring.

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Compliance with ethical standards Our field studies were conducted under a permit issued by the Management Bureau of the Badagongshan National Nature Reserve. The research was conducted under animal ethics approval granted by 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, measurements, egg counting, 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.

Conflict of interest The authors declare that they have no competing interests.

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