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ORIGINAL ARTICLE

A Sex-linked Microsatellite Marker Reveals Male Heterogamety in *Quasipaa boulengeri* (Anura: Dicroglossidae)

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Abstract Few amphibians possess morphologically distinguishable heteromorphic sex chromosomes. The classic indirect method is a time-consuming and resource-demanding task to identify the heterogametic sex. Here we have taken advantage of a sex-specific microsatellite marker, by amplifying a large number of samples of known male and female individuals from various populations, to reveal a homogeneous pattern of male heterogamety in *Quasipaa boulengeri*. The identification of the heterogametic sex will help interpreting the evolution of sex determination. Besides relevance for evolutionary studies of sex determination, the sex-linked markers have potential for addressing practical issues in conservation biology because the sex reversal that caused by anthropogenic endocrine disruptors is considered as a reason for amphibian decline.

Keywords microsatellites, sex-specific marker, male heterogamety, *Quasipaa boulengeri*

1. Introduction

Contrasting with birds and mammals, few amphibians possess morphologically distinct sex chromosomes (Eggert, 2004). To identify the sex heterogamety, we often require more means in laboratory, such as sex reversal experiments, or cytogenetic techniques. However, the sex reversal experiments were time-consuming, and the cytological techniques may have suffered from methodology induced artifacts or misinterpretation of chromosome-banding polymorphisms (Schmid *et al.*, 1987; Hillis and Green, 1990; Stöck *et al.*, 2005; Roco *et al.*, 2015). Instead of traditional methods, sex-linked DNA markers are now coming to infer heterogametic patterns in amphibians (Berset-Brändli *et al*., 2006, 2007; Rodrigues *et al*., 2014; Stöck *et al*., 2005, 2011a).

Quasipaa boulengeri, spiny-bellied frog, ranges widely along the margin of the Sichuan Basin in central and southern China. No heteromorphic sex chromosomes had been found so far, although samples were cytogenetically examined at the population level almost covering the whole distribution range of the species (Chen *et al*., 1983; Hu *et al*., 2004; Li *et al*., 1996, 1999; Qing *et al*., 2012; Wang *et al*., 1983; Zhang *et al*., 1997). With a large body size, this frog has been a commercially valuable species for food consumption for several hundred years. In recent years, the natural populations have declined seriously or even gone extinct somewhere, resulted from the over-harvest and exploitation for human consumption, water pollution, habitat destruction and degradation (Xie *et al*., 2007). The frog has already listed as "Endangered" species in China Species Red List (Wang and Xie, 2009) as well as in 2004 IUCN Red List to date (Lau *et al.*, 2004).

Here, we investigated a microsatellite marker potentially located on sex chromosomes, by amplifying totally 376 samples of known male and female individuals from 18 populations, to disclose a homogeneous pattern of male heterogamety in *Quasipaa boulengeri*. Identifying the heterogametic sex will help explaining the evolution of sex determination. Besides relevance for evolutionary studies of sex determination, the sex-linked markers have potential for addressing practical issues in conservation biology, because the sex reversal, that caused by

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anthropogenic endocrine disruptors, has been considered as a reason for amphibian decline (Hayes 2005; Reeder *et al.*, 2005).

2. Materials and Methods

2.1 Samples A total of 376 individuals, including 131 adult males and 245 adult females from 18 populations along the western Sichuan basin of southern China, were sampled during the breeding season from 2006 to 2015 (Table 1). The phenotypic sex was initially determined by their secondary sexual characteristics (i.e. with spiny belly for males or without that for females), and verified by gonadal inspection, following dissection for purposes of other studies (Qing *et al*., 2012). Tissue samples (muscle and liver) were collected from all individuals and preserved in 95% ethanol at –40 °C. DNA extractions were performed following the standard protease K method as described in Sambrook and Russell (2001). The DNA was eluted in a 150 μl volume of ddH_2O and stored at –20 °C.

All animal work in this study has been conducted according to relevant national and international guidelines. All animal care and experimental operations confirmed to the procedures of the Animal Care and Use Committee, Chengdu Institute of Biology, Chinese Academy of Sciences (issued permit number: CIB-20121220A).

Table1 Sample sizes from 18 populations of *Quasipaa boulengeri* in western Sichuan basin.

2.2 Microsatellite amplification We initially surveyed the 43 microsatellite loci in four populations including Jinxinxiang, Mt. Qingcheng, Hongkouxiang and Yingxiuzhen (Table 2). These microsatellites were previously developed from the transcriptome and the fast isolation of AFLP of sequences containing repeats in *Quasipaa boulengeri*, respectively (Xia *et al.*, 2012; Yuan *et al.*, 2015). As a result, the locus SSR13-B08 (B08 for short) was suspected as the sex-associated one in the initial populations due to its higher F_{ST} value between males and females. Then, the locus B08 had been detected among a large number of samples from the 18 populations to make sure whether this locus was a sex-linked one or not. For the locus B08, which failed to amplify male alleles in some populations, we had to design a new forward primer.

Forward primers labeled with a fluorescent dye (Sangon Biotech Ltd. Co., Shanghai, China). Polymerase chain reaction (PCR) were performed with a total volume of 20 μl, including 10 μl of 2×Master Mix (TransGen Biotech, Beijing, China), 0.8 μl of each primer, approximately 0.8 ng of extracted DNA and ddH2O. PCR was performed in the Eppendorf PCR Thermocycle Instrument (Eppendorf China Ltd.), according to the following thermal programs: 5 min to initial denaturation at 95°C, followed by 33 cycles of 94 °C for 30 s, annealing temperature at optional primer temperature for 30 s, elongation at 72 °C for 40 s and a final elongation at 72 °C for 8 min. The annealing temperature was based on the protocol described in previous studies (Yuan *et al*.,

Table 1 Sample sizes from 18 populations of *Quasipaa boulengeri* in western Sichuan basin.

Localities	Female	Male	Total
Jinxinxiang, Dayi Co., Sichuan	63	37	100
Daozuoxiang, Qionglai Co., Sichuan	45	8	53
Mt. Tiantai, Qionglai Co., Sichuan	34	22	56
Gaotangsi, Dayi Co., Sichuan	19	9	28
Hemingxiang, Dayi Co., Sichuan	7	-	7
Xilingzhen, Dayi Co., Sichuan	-	2	2
Hepingxiang, Dayi Co., Sichuan	5	3	8
Hongkouxiang, Dujiangyan Co., Sichuan	2	2	4
Longmenshanzhen, Pengzhou Co., Sichuan	18	8	26
Mt. Qingcheng, Sichuan	6	2	8
Cifengzhen, Pengzhou Co., Sichuan	9	22	31
Pinglezhen, Qionglai Co., Sichuan	2	-	2
Shuikouzhen, Qionglai Co., Sichuan	-	1	1
Xuankouzhen, Wenchuan Co., Sichuan	6	1	7
Yingxiuzhen, Wenchuan Co., Sichuan	2	2	4
Mt. Emei, Sichuan	24	12	36
Huatouzhen, Jiajiang Co., Sichuan	1	-	1
Liujiangzhen, Hongya Co., Sichuan	2	-	2
Total	245	131	376

Table 2 Genetic variation for 43 microsatellite markers in two sexes of four populations.

Locus	T (°C)	Female					Male					F_{ST}
		N	*Na*	*He*	*Ho*	F_{IS}	*N*	*Na*	*He*	*Ho*	F_{IS}	
QBb26 #	60	33	14	0.8886	0.7273	0.1838*	6	6	0.8636	0.4286	0.6364*	–0.03
QBc7 #	60	33	12	0.8923	0.8788	0.0154*	7	10	0.8901	0.8333	0.04	–0.0151
QBb025 #	60	32	10	0.6935	0.25	0.6432*	7	5	0.7692	0.5	0.4627	0.0126
QBz16 #	60	33	3	0.3184	0.1818	0.4328*	7	4	0.5714	1.3333	0.2653	0.0171
QBy3 #	60	33	3	0.642	0.7273	–0.1353	7	3	0.6703	1	0.3793*	–0.0407
QBb42 #	60	32	10	0.8606	0.875	–0.017	7	6	0.8571	0.6	0.1781	–0.0338
QBb1 #	60	33	10	0.8895	0.9394	–0.057	7	9	0.9451	0.9	–0.0633	–0.0184
QBb5 #	60	33	10	0.7478	0.6061	0.1919*	7	7	0.8901	0.7	0.3766*	0.0135
QBb45 #	60	33	10	0.841	0.8485	–0.0090*	7	7	0.8681	0.7	0.1892	0.036
QBc12 #	58	21	9	0.8223	0.4762	0.4269*	6	5	0.8636	0.5556	0.4444*	0.0083
QBd12 #	58	18	5	0.6302	0.3333	0.4783*	4	5	0.7857	1	0.4	–0.0887
SSR01-C24	63	19	2	0.4908	0.0526	0.8953*	13	2	0.3692	0	1.0000*	0.197
SSR02-D56	64	19	2	0.5007	0.6316	–0.2706	13	2	0.5077	0.3846	0.25	–0.0315
SSR03-D63	56	19	3	0.6671	0.7895	–0.1894	13	3	0.5569	0.7692	–0.4035	0.1324
SSR04-D3	56	19	3	0.5989	0.6316	–0.0562	13	3	0.52	0.6923	–0.35	0.0394
SSR06-D34	56	19	4	0.7269	0.7368	–0.0141*	13	4	0.7354	0.7692	–0.048	0.0155
SSR07-C10	56	19	2	0.4438	0.6316	–0.44	13	2	0.2123	0.2308	–0.0909	0.0867
SSR08-C27	56	19	3	0.4794	0.6316	–0.3292	13	2	0.2123	0.2308	–0.0909	0.0922
SSR09-B20	56	19	3	0.542	0.7895	–0.4754*	13	3	0.4892	0.4615	0.0588	0.0339
SSR10-B16	55	19	2	0.0526	0.0526	na	13	1	0	0	na	–0.0106
SSR11-D20	55	19	3	0.4964	0.6316	–0.2819	13	2	0.2123	0.2308	–0.0909	0.1139
SSR12-A03	55	19	2	0.5007	0.1053	0.7943*	13	2	0.3231	0.0769	0.7692*	0.2198
SSR13-B08	56	19	1	0	0	na	13	2	0.4923	0.7692	–0.6	0.659
SSR14-B11	55	19	2	0.4438	0.6316	–0.44	13	2	0.2123	0.2308	–0.0909	0.0867
SSR15-D60	56	19	2	0.478	0.6316	–0.3333	13	2	0.2123	0.2308	–0.0909	0.131
SSR17-D68	55	19	2	0.4438	0.6316	–0.44	13	2	0.2123	0.2308	–0.0909	0.0867
SSR18-K	56	19	1	0	0	na	13	1	0	0	na	na
SSR19-B07	55	19	2	0.478	0	1.0000*	13	2	0.44308	0	1.0000*	0.1335
SSR20-B10	55	19	1	0	0	na	12	1	0	0	na	na
SSR21-D12	55	19	3	0.5121	0.7368	–0.4566	13	3	0.28	0.3077	–0.1034	0.0772
SSR22-C03	56	19	1	0	0	na	13	1	0	0	na	na
SSR24-X	57	19	3	0.6145	0.7895	–0.295	13	3	0.5262	0.4615	0.1273	0.025
SSR25-B12	56	19	3	0.5989	0.8421	–0.4222	13	3	0.5631	0.4615	0.1864	–0.019
SSR26-B14	55	19	2	0.4438	0.3158	0.2941	13	2	0.5077	0.2308	0.5556	0.089
SSR27-N	58	19	4	0.5078	0.2632	0.4886*	13	2	0.4708	0.2308	0.52	–0.0398
SSR29-C18	56	19	1	0	0	na	13	1	0	0	na	na
SSR31-Y1	56	19	2	0.4438	0.6316	–0.44	13	2	0.2123	0.2308	–0.0909	0.0867
SSR32-Y2	56	19	3	0.5761	0.6316	–0.0992	13	3	0.3354	0.3846	–0.1538	0.1045
SSR33-Y19	56	19	2	0.478	0.5263	–0.1043	13	2	0.3231	0.3846	–0.2	0.0448
SSR34-Y18	56	19	2	0.4438	0.6316	–0.44	13	2	0.2123	0.2308	–0.0909	0.0867
SSR35-Y15	56	19	2	0.4438	0.6316	–0.44	13	2	0.2123	0.2308	–0.0909	0.0867
SSR37-Y12	56	19	3	0.6785	1	–0.4934*	13	3	0.6123	1	–0.6774*	0.0319
SSR38-Y17	54	19	3	0.4794	0.6316	–0.3292	13	3	0.28	0.3077	–0.1034	0.0671

Loci derive from FIASCO protocol and the genetic parameters inferred from the population Jinxinxiang; other loci from transcriptome data and the related parameters from the population of Mt. Qingcheng, Hongkouxiang, and Yingxiuzhen. T: annealing temperature; *N*: sample size; *Na*: number of alleles; *He*: expected heterozygosity; *Ho*: observed heterozygosity; F_{IS}: inbreeding coefficient; F_{ST}: pair-wise F_{ST} between female and male populations estimated from the microsatellite loci; na: not applied. *$P < 0.05$.

2015). PCR products with different color labeling were checked on 1.5% agarose gel at 140 V ($m \cdot v^{-1}$), mixed with the internal size standard LIZ500 (ABI) and separated by capillary electrophoresis in the ABI 3130 Genetic Analyzer (Applied Biosystems, ABI Shanghai Ltd. China) in Sangon Biotech Ltd. Co (Shanghai, China).

2.3 Data analysis All data were independently read by two people. Less than 5% of the genotyped readings were found to be inconsistent between the readers, and incompatible genotypes were blanked. In order to ensure the accuracy of the genotyped data, we did additional independent PCRs and sequencing. Allele sizes were

estimated using GeneMarker v.1.95 (Softgenetics, State College, Pennsylvania, USA). The frequencies of null alleles were estimated with Micro-Checker v.2.2.3 (van Oosterhout *et al.*, 2004). To identify X and Y chromosomal alleles, genotypic frequencies and allele size distributions were compared between females and males.

For all populations, we calculated the number of alleles (*Na*), observed heterozygosity (*Ho*), expected heterozygosity (*He*), fixation indices (F_{IS} and F_{ST}) within and between sexes by Genepop v.4.2 (Rousset, 2008; Raymond and Rousset, 1995). It is expected that strong differentiation between male and female group would generate both high F_{ST} values and significantly negative F_{IS} values, due to an excess of heterozygotes relative to Hardy-Weinberg equilibrium (HWE) (Berset–Brändli *et al.*, 2006, 2007).

3. Results

Summary statistics for all 43 loci detected in four populations are provided in Table 2. Except locus B08, similar allelic distributions in males and females were observed in 42 loci pointing to the autosomal localization. In females, allelic number (*Na*) ranged 1–14 (average 3.95), expected heterozygosity (*He*) 0.0526–0.8923 (average 0.50673), and observed heterozygosity (*Ho*) 0.0526–0.9394 (average 0.4470) (Table 2). In males, *Na* ranged 1–10 (average 3.19), *He* 0.2123–0.9451(average 0.51290), and *Ho* 0.2308–1.0000 (average 0.42600) (Table 2). The remaining locus B08 showed highly significant differentiation between sexes with the estimation of fixation indices in all individuals (F_{ST} = 0.6590), as well as strong heterozygosity excess in males (F_{IS} = –0.6000) (Table 2). Of all 43 loci, only the locus B08 revealed sex-specific allelic distributions in the tested populations.

Locus B08 presented five alleles with the range 386–422, including three common alleles of 388, 416 and 422, across the 18 populations in western Sichuan basin (Table 3; Figure 1). Allelic frequencies differed strongly between sexes but not within sexes. Alleles of 408, 416 and 422 were more common in females, with a summed frequency > 95% vs. approximately 50% in males. By contrast, alleles of 386 and 388 had a higher frequency approximately 50% in males vs. < 5% in females. All males were heterozygous at this locus, each males having actually each one copy of allele 386 or 388, plus one copy of 408, 416 or 422. This resulted in a strong deviation from HWE within populations ($P < 0.05$), and exhibited

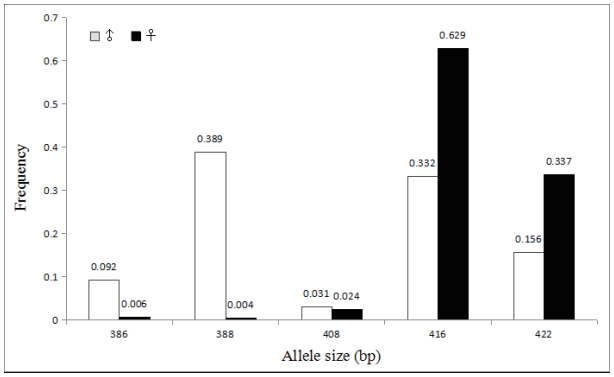

Figure 1 Allelic frequencies at locus B08 for female (black bars) and male (white bars) spiny-bellied frogs.

Table 3 Allelic number, frequencies and genetic variability at locus B08.

Allele	Female	Male
386	0.006	0.092
388	0.004	0.389
408	0.024	0.031
416	0.629	0.332
422	0.337	0.156
Ho	0.7061	1
He	0.4919	0.7071
F_{IS}	–0.4369***	–0.4166***
F_{ST}	0.195	

He and *Ho*: expected and observed heterozygosity; F_{IS}: heterozygote excess; *** $P < 0.001$. The forward primer used here is 5'-GCACGTACATTCATTCTGTG-3' (designed by Y. XIA and S. YUAN).

significantly negative F_{IS} values in males ($P < 0.05$; Table 3). One-third females were homozygous (416/416) and the remaining ones were heterozygous with the genotypes 408/416, 408/422 and 416/422. The above causes also lead to significantly negative F_{IS} values for females ($P < 0.05$; Table 3) and significant departure from HWE ($P < 0.05$).

Allelic distributions of the locus B08 were strikingly different between the sexes. This pattern was consistent throughout the 18 populations (Figure 1). Moreover, it could be parsimoniously interpreted that locus B08 lies on the non-recombining segment. Therefore, the shorter alleles (386 and 388) located on the Y chromosome, whereas the longer ones (408, 416 and 422) segregated on the X chromosome.

4. Discussion

The alleles of locus B08 revealed sex-specific distributions, shown to be sex-linked in a large sample size among various populations along western Sichuan

basin, presenting evidence for male heterogamety (XX/XY) in *Quasipaa boulengeri*. Generally, males could be distinguished by the presence of distinct sex-specific alleles (386 and 388), whereas females characterized by the absence of these alleles. The allelic variation of the locus could be used as a diagnostic tool to identify the heterogametic sex in the samples at juvenile stages, as well as in adults when phenotypic criteria are considered unreliably.

Identifying the heterogametic sex will be help to decipher the evolution of sex chromosome and sex determination. Although genetic sex determination (GSD) prevails in most species of amphibian studies (Hillis and Green, 1990), it could be overridden by the epigenetic factors under some circumstances (Schmid and Steinlein, 2001), even found frequently in nature (Alho *et al*., 2010; Dufresnes *et al*., 2014). Compared with other GSD vertebrates, such as mammals and birds, most amphibians do not exhibit heteromorphic sex chromosomes. In amphibians, the sex-determining genes with great instability, shift their position fast enough to stop the evolutionary decay of sex chromosomes, that is proposed in mammals and birds. The recombination is depressed between the heterogametic sexes. We can estimate the depressed rates of recombination in the heterogametic sex at the sex-linked loci, to detect the evolutionary instability of sex-determining genes and understand if the GSD control exists or not in amphibian groups.

In addition, the sex-specific markers can give the possibility of addressing important issues in conservation biology. Some pesticides may induce sex reversal in amphibians potentially. Atrazine, for instance, acts as a feminizing endocrine disruptor by inducing aromatase that converts androgens to estrogens (Hayes, 2005). Environmental pollutants, such as pesticides and anthropogenic endocrine disruptors, result in the primary sex ratio biased, which was considered as one of the several potential causes of amphibian decline in recent years (Reeder *et al*., 2005). We can identify the heterogametic sex at any developmental stages using sex-specific markers to estimate the sex ratio biased, and to decide if the endocrine disruptor is an explanation for amphibian decline or not.

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