ONLINE RESOURCES

Development and characterization of 13 polymorphic microsatellite DNA markers for the pond green frog (*Rana nigromaculata*)

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Introduction

The pond green frog (PGF) (*Rana nigromaculata* Hallowell) is widely distributed in China, far-eastern Russia, Korean Peninsula and Japan (Huang *et al*. 1990). Besides the wide distribution, the PGF has some other characteristics, such as easy sampling, obvious genetic structure due to being philopatric to breeding sites, and easy crossing in the laboratory, making it a good model species for investigating the genetics of wild animal populations (Beebee 2005). On the other hand, vocalization is a striking behavioural feature of many amphibians (Wycherley *et al*. 2002), including the PGF. Compared to birds or mammals, the frogs have lower learning abilities and lower individual mobilities. Therefore, it is generally accepted that all characters in the frogs are inherited directly rather than transferred culturally (Wycherley *et al*. 2002). This feature of the PGF makes it suitable to examine molecular behaviour mechanism and genetic differentiation by using vocalization markers and neutral loci (Wycherley *et al*. 2002; Giordano *et al*. 2007). As a result, the PGF has become a special model species for molecular ecology studies.

Nevertheless, only a few studies have been carried out on the PGF to date. Hirai and Matsui (1999, 2002) investigated feeding habits of this species and Yang *et al*. (2004) examined genetic diversity and phylogeographic structure of eight PGF populations using mtDNA cytochrome *b (cytb)* gene sequences. However, no nuclear markers have been studied in the PGF. In this regard, microsatellites which are highly variable nuclear markers, would be useful tool for studies in molecular ecology and conservation genetics (Jehle and Arntzen 2002; Wan *et al*. 2004) of the PGF. At present, microsatellites have been developed for many frog species, such as common frog (Berlin *et al*. 2000), snouted treefrog (Duryea *et al*. 2008), northern leopard frog (Hoffman *et al*. 2003), wood frog (Newman and Squire 2001; Julian and King 2003), dusky gopher frog (Richter and Broughton 2005).

In this study, we first attempted to isolate microsatellite markers for the PGF by using cross-species amplification of available microsatellites of ranid frogs (Berlin *et al*. 2000; Newman and Squire 2001; Hoffman *et al*. 2003; Julian and King 2003; Richter and Broughton 2005; Duryea *et al*. 2008). However, a very low rate of cross-species microsatellite amplification success was detected in the PGF. Therefore, we redesigned 20 pairs of primers based on some cross-species sequences from ranid frogs (Berlin *et al*. 2000; Newman and Squire 2001; Hoffman *et al*. 2003; Richter and Broughton 2005), but none of them yielded clear amplification products. As a result, it became imperative that we develop PGF specific polymorphic microsatellite loci. Here, we report 13 novel polymorphic microsatellite loci to facilitate analyses of genetic differentiation and population structure of the PGF.

Materials and methods

A total of 35 muscle samples of PGF were collected from Jinhua in Zhejiang, P. R. China, and genomic DNA was extracted using phenol/chloroform method (Sambrook *et al*. 1989). Microsatellites were enriched according to the protocols of He *et al*. (2006) and Wu *et al*. (2008) with the

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[Gong J., Lan H., Fang S.-G. and Wan Q. H. 2010 Development and characterization of 13 polymorphic microsatellite DNA markers for the pond green frog (*Rana nigromaculata*). *J. Genet*. **89**, e7–e10. Online only: http://www.ias.ac.in/jgenet/OnlineResources/89/e7.pdf]

Keywords. microsatellite; heterozygosity; polymorphic; discrimination power; pond green frog.

following modifications. About 3-μg DNA was digested with *Sau3*AI, and fragments ranging from 300 to 1000 bp were recovered with a gel extraction column kit (Axygen, Union City, USA). The linkers Sau3AIF (5′-GGC CAG AGA CCC CAA GCT TCG-3′) and Sau3AIR (5′-GAT CCG AAG CTT GGG GTC TCT GGC-3′) were first annealed to form linker pairs. Then, the annealed linkers were ligated to those retrieved fragments and DNA concentration was elevated through PCR amplification using Sau3AIF as the primer. This PCR product was denatured and hybridized to 5′-biotinylated $(AC)_{12}$, $(AAC)_8$ or $(AGC)_8$ probes. Single-stranded DNA fragments containing microsatellites were captured by streptavidin magnetic beads (Roche, Basel, Switzerland) and the enriched fragments were made double-stranded by PCR using Sau3AIF as the primer. The PCR products were ligated into pMD18-T vector (TaKaRa, Dalian, China) and transformed into DH5α competent cells (TaKaRa, Dalian, China). The Sau3AIF was used to amplify and screen positive clones, whose PCR products showed two or three bands. The positive clones presenting similar DNA banding patterns were loaded on the same agarose gel to identify repetitious clones. The clones showing different PCR profiles were sequenced on an automated ABI 3700 DNA sequencer and primers (Applied Biosystems, Foster City, USA) were designed by primer Premier 5.0 software (Singh *et al*. 1998).

A 5′-M13 tail (5′-CAC GAC GTT GTA AAA CGA C) was added to the forward primer of each primer pair to allow fluorescent labelling during the amplification reaction. PCR amplification was performed in 10 μL reactions for all primers, containing 1 μL of 20 mM dNTPs, 1 μL of 10× PCR buffer (TaKaRa, Dalian, China), 1 μL (10 ng/μL) of template of DNA, 0.8 μL of 25 mM $MgCl_2$, 0.5 U of Taq DNA polymerase (TaKaRa, Dalian, China), 0.3 μL of each 10 μM primer and 1 μL of 1 μM IRD labelled M13 primer (LI-COR, Lincolin, USA). PCR amplification was conducted with an initial denaturation at 95°C for 5 min, 33 cycles consisting of 95°C for 30 s, 30 s at optimized primer-specific annealing temperature (table 1), 72°C for 30 s, and followed by a final 10 min extension at 72°C. The PCR products were loaded on a LI-COR 4200 automated DNA Sequencer together with a size standard (50–350 bp) and genotyping was subsequently carried out using SAGA^{GT} software (LI-COR, Lincolin, USA).

Deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were analysed using GENEPOP online (http://genepop.curtin.edu.au/). Cervus version 2.0 (Marshall *et al*. 1998) was adopted to

Table 1. Characterization of *Rana nigromaculata* microsatellite loci. Thirty-five individuals were genotyped for each locus.

Locus ID	Primer sequences (5′ – 3′)	Repeat motif	T_a	Allele size	H_O/H_E
Rnh-1	F:TGAAGTATTCAGGTACAACAGGT R:GGGCCAAAAGAGAGGGT	$(TGC)_7$	58	269–281	0.400/0.422
Rnh-2	F:GCTTCGGGCTATAAATCAAACA R:GCCTGGCCGACTACACG	$(TGC)_4TGTG(GTT)_3TT(TGC)_6$	62	235–250	0.571/0.558
Rnh-3	F:CCGGAAGGCAGTGGAGGACA R:ATGGACATGCGGTGGGGTAGG	$(AAC)_7$	52	221–254	0.600/0.704
Rnh-4	F:CGCTTACTATGGGGGGATA R:GCCTGAGAAGGGTGGTGCT	$(GA)_5$	62	154–166	0.686/0.725
*Rnh-5**	F:GAAGTATGTGAACAGCCCTCC R:TGGGTCCAGACTGGCAAC	$(CA)_{19}$	62	314–342	0.686/0.873
Rnh-6	F:TCTCGGGAGGAAAGCAATGG R:AAGGAGCCTGGGACTATGGTAAAC	$(AAC)_5$	62	208–214	0.257/0.426
*Rnh-7**	F:CTCCATACAGCCGCAGC R:CCATCAGAAGTAAAGAATCCT	$(CGA)_5G(AGC)_8$	56	217–262	0.364/0.883
*Rnh-8**	F:AATACACTTTGCTCCCTAG R:ACAATCGGTGGTTTCA	$(GT)_{16}CT(GT)_4$	51	267–285	0.171/0.858
Rnh-9	F:GCACAGTTAGCGAGATGGA R:CTCACTAGAGCTGGGTGGTAT	$(GCA)_7$	59	155–185	0.657/0.782
Rnh-10	F:AGTGCAACATCAACTTGGGTG R:GCAGAGTCGCTGTCGGGA	$(GCT)_6$	62	165–246	0.743/0.852
*Rnh-11**	F:CGGCTTTCCTATTTACAC R:AGCACTGACGCTCTGG	$(AT)_3(AC)_4AT(AC)_{14}$	60	263–301	0.571/0.912
Rnh-12	F:ATGTTATTGAGCCCAGAG R:GGTCAGCAGCAGGTAA	$(AC)_{20}$	58	174–196	0.914/0.899
Rnh-13	F:GATACGGGAGGCAAACG R:TCCACAGCCCAGCACTC	$(GCA)_5$	56	144–150	0.400/0.441

*significant deviation from HWE ($P < 0.01$); T_a, annealing temperature (°C); H_O and H_E, observed and expected heterozygosities, respectively. GenBank accession numbers for *Rnh-1–Rnh-13* were FJ555250–FJ555262, respectively.

calculate the possibility of null alleles, the number of alleles, the observed and expected heterozygosities, mean polymorphic information content (PIC) and probability of exclusion (PE). The discrimination power (DP) of each microsatellite locus and the cumulative DP (CDP) of a set of microsatellite loci were calculated as described by Kloosterman *et al*. (1993).

Results and discussion

A total of 140 recombinants that potentially contained microsatellite sequences were obtained. Eighty-seven clones were chosen for sequencing and 56 contained repeat sequences. Thirty-nine primer pairs were designed successfully, and 13 of these yielded clear polymorphic PCR products (table 1).

The loci *Rnh-5, 7, 8* and *11* showed significant deviation from HWE ($P < 0.01$), possibly due to null alleles, inbreeding or population structure in sampled individuals (Duryea *et al*. 2008). LD was only detected between the *Rnh-8* and *Rnh-10* loci ($0.01 < P < 0.05$). Except the four loci deviated from HWE (*Rnh-5, 7, 8* and *11*), whose possible null alleles probably mislead results of parental testing, other nine markers were subjected to the calculation of resolution power. The number of alleles per locus ranged from 3 to 12 with an average of 6.33, presenting moderate PIC value of 0.594 (table 2). The observed and expected heterozygosities of these loci were 0.171–0.941 and 0.422–0.912, respectively (table 1), indicating a relatively high level of genetic diversity in the PGF. These loci showed their overall values of DP, PE-1 (for parentage testing) and PE-2 (for paternity testing) were 0.99997, 0.965 and 0.997, respectively (table 2), indicating high-resolution power of these microsatellite loci. As a result, this suite of microsatellite markers provides a powerful tool to resolve issues of molecular ecology and conservation genetics for the PGF.

Table 2. The values of polymorphic information content (PIC), discrimination power (DP) and probability of exclusion (PE-1 and PE-2) for the nine microsatellite loci in accordance with HWE.

Locus ID	Na	PIC	DP	PE-1	PE-2
Rnh-1	3	0.341	0.416	0.086	0.177
Rnh-2	5	0.505	0.55	0.161	0.319
Rnh-3	8	0.663	0.694	0.302	0.487
Rnh-4	6	0.66	0.714	0.29	0.458
Rnh-6	3	0.367	0.42	0.088	0.205
Rnh-9	5	0.735	0.771	0.376	0.556
Rnh-10	12	0.82	0.84	0.514	0.682
Rnh-12	12	0.876	0.887	0.627	0.772
Rnh-13	3	0.377	0.434	0.094	0.211
Average	6.33	0.594			
Overall			0.99997	0.965	0.997

Na, number of alleles per locus.

Acknowledgements

This work was supported by a grant from the National Basic Research Program of China (973 programme) (No. 2007CB411600).

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Received 4 June 2009, in final revised form 18 October 2009; accepted 22 October 2009

Published on the Web: 20 April 2010