Genes Genet. Syst. (2014) **89**, p. 137–141

Development and characterization of 27 new microsatellite markers for the Indian bullfrog *Hoplobatrachus tigerinus* and its congeneric species

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(Received 30 May 2014, accepted 3 August 2014)

The Indian bullfrog *Hoplobatrachus tigerinus* and its four congeneric species are common frog species and distributed throughout South Asia. Due to recent human activity, they are facing a changing environment and reduction in natural population size. For effective conservation and molecular ecological studies, we therefore isolated and characterized microsatellite loci for these frogs. We obtained genomic data using an Ion Torrent PGM sequencer and designed 54 primer sets for candidate loci. By screening for polymorphic loci in individuals of *H. tigerinus* and its congeneric species, we isolated 27 loci as highly polymorphic microsatellite loci. Eight of these loci were commonly applicable for all species except *H. chinensis*. Within two populations of *H. tigerinus*, the total number of alleles per locus and expected heterozygosity ranged from 2 to 18 and 0.271 to 0.938, respectively. No significant linkage disequilibrium was observed across all loci, and five showed a significant deviation from Hardy-Weinberg equilibrium in some populations after Bonferroni correction. Consequently, our findings suggest that these novel markers will be applicable for conservation genetic studies across varying scales from inter-population to inter-individual.

Key words: amphibian, Anura, microsatellite markers, next-generation sequencing

MAINBODY

The Indian bullfrog, *Hoplobatrachus tigerinus* (Daudin, 1802), is a large, robust-looking semi-aquatic frog belonging to the family Dicroglossidae and inhabiting mostly freshwater wetlands and flooded agricultural paddy fields. This frog is widely distributed in South and Southeast Asian countries including Bangladesh, India, Pakistan, Nepal, Bhutan, Myanmar, Thailand, Malaysia and Indonesia (Hussain and Rahman, 1978). Although the conservation status of *H. tigerinus* is "Least Concern" according to the IUCN Red List of Threatened Species (Padhye et al., 2004), some local populations are now decreasing due to anthropological effects and rapid environmental changes, such as over-exploitation for food, development of infrastructure, and water pollution by pesticides and agrochemicals (Islam et al., 2012). Thus, for future conservation purposes, the intra-specific genetic diversity of this species reflecting its natural evolutionary history needs to be understood before local populations become extinct. Alam et al. (2008) studied the phylogeography of this species and clarified broad intra-

Edited by Yoko Satta
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specific genetic diversity using mitochondrial genes, but finer-scale population structure and genetic variation within populations remain unknown. Furthermore, *H. tigerinus* could be a key species for monitoring ecosystem status, because of its multiple roles as a primary and secondary consumer and detritivore in the food chain and its semi-permeable skin, which, in common with other amphibian species, is sensitive to environmental changes. Notably, this species mainly inhabits agricultural fields such as paddy, where environmental pollution is being caused by agrochemicals. Therefore, demographic dynamics inferred from the finer-scale population genetics of *H. tigerinus* should provide important information for conservation and management of this species and also of the natural environment in the South Asian region.

Microsatellites are highly polymorphic neutral molecular markers that can be used for such finer-scale genetic research in evolution and ecology. However, obtaining nucleotide sequence data for microsatellites and their flanking regions of a target species using traditional methods requires cost and time, especially for screening, cloning and sequencing of microsatellite regions. To overcome these obstacles, next-generation sequencing has achieved faster and higher throughput and enabled researchers to obtain a huge amount of nucleotide sequence data at once. Indeed, hundreds of microsatellite loci were isolated without any screening steps using the Roche 454 sequencing platform (e.g., Clay et al., 2010; Csencsics et al., 2010). In this study, we applied this strategy using another new sequencer, the Ion Torrent PGM, for isolation of microsatellite loci of *H. tigerinus*. In addition, we also validated cross-amplification in four congeneric species.

Genomic DNA was extracted from toe-clippings of 32 individuals of *H. tigerinus* from Sunamganj and Vola, Bangladesh, 16 of *H. litoralis* from Ukhia, Bangladesh, 16 of *H. chinensis* from Thailand, four of *H. crassus* from Bangladesh and three of *H. occipitalis* from Tanzania, using a DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. We also extracted genomic DNA of an individual *H. tigerinus* from Bangladesh Agricultural University campus and used it for sequencing. The genomic DNA was resuspended in 20 μg/ml RNase A. A genomic DNA library was then constructed using NEBNext Fast DNA Fragmentation & Library Prep Set for Ion Torrent (New England Biolabs, Ipswich, MA). Because we conducted a single sequencing run multiplexing with two individuals of other frog species (*Odorrana narina* and *Buergeria japonica*), the genomic DNA library was attached to adaptor oligonucleotides using an Ion Express Barcode Adaptors 1-16 Kit (Life Technologies, Foster City, CA). The library was amplified and refined using an Ion OneTouch Template OT2 200 Kit (Life Technologies), and sequenced on an Ion Torrent PGM sequencer with an Ion 318 chip and Ion PGM Sequencing 200 Kit ver. 2 (Life Technologies).

The sequencing yielded 537,649 reads longer than 150 bp. From these raw sequence reads, we found 1,344 di-, 463 tri-, 589 tetra-, 161 penta- and three hexanucleotide repeats using the default setting of MSATCOMMANDER ver. 1.0.8 (Faircloth, 2008). Of these, we selected a total of 54 microsatellite loci from reads having the largest number of repeats for each of three repeat types (22 loci for di-, 16 for tri- and 16 for tetranucleotide repeats) and tested amplification and polymorphism. Primer pairs for each locus were designed by Primer3 ver. 2.2.3 (Rozen and Skaletsky, 2000). We employed a post-labeling method for visualizing PCR fragments in subsequent genotyping on a Genetic Analyzer. In brief, this method uses universal primers labeled with fluorescent dyes and nested PCR of amplified products obtained from a specific primer pair for each locus. For the nested PCR, one of the specific primers for each locus needs to be attached to an oligonucleotide that is complementary to the universal primers. Therefore, we used two different universal primers, M13tag (Schuelke, 2000) and BStag (Shimizu and Yano, 2011), and attached their complementary oligonucleotides to the 5′ ends of forward primers of 30 (10 each of di-, tri- and tetranucleotide repeats) and 24 (12 di-, six tri- and six tetranucleotide repeats) loci, respectively. We first tested PCR amplification of all loci, using eight samples comprising one or two representatives of each of the five *Hoplobatrachus* species. We then tested polymorphism of stably amplified loci using four samples from the Vola population of *H. tigerinus*. The PCR for amplification confirmation was carried out in a 10-μl volume containing 5 μl of Emerald Amp MAX PCR Master Mix (TaKaRa Bio, Otsu, Japan), 1 μl of 10 μM primer pairs, and 50 ng of genomic DNA. Thermal cycling was performed under the following conditions: 94°C for 5 min; 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 30 s. PCR products were electrophoresed on a 2% agarose gel and visualized by UV light after staining with ethidium bromide.

Of these 54 loci, 48 (26 loci of M13tag and 22 loci of BStag) were amplified, judging by the presence of clear bands on the gel in most of the samples, and were then genotyped by post-labeling PCR and electrophoresis on a 3130xl Genetic Analyzer (Life Technologies). M13 and BStag universal primers were labeled with FAM, HEX, NED or PET fluorescent dyes. For validation of polymorphisms and genotyping, we used two reagents, Emerald Amp PCR Master Mix (Takara Bio) and KOD FX Neo (TOYOBO, Osaka, Japan) for M13tag and BStag primers, respectively. For M13tag primers, PCR was carried out in a 10-μl volume containing 5.0 μl of 10 μM 2 × Emerald Amp PCR Master Mix, 0.1 μl of 5 μM tagged forward primer, 0.2 μl of 10 μM reverse primer, 0.1 μl of 5 μM M13 fluorescent primer, 1.0 μl of template DNA and ddH_2O.

Table 1. Primer information for 27 microsatellite loci isolated from *Hoplobatrachus tigerinus*

Locus	Accession no.	Repeat motif	Primer sequence (5′-3′)	Dye	Size range (bp)	Multiplex
Htigr1225	AB911213	$(AC)_{10}$	F: F9CCG - TGGGTGTAATGAGCATGACG R: CCTTTCCCTTGCAAGTCCTATG	FAM	144–278	I
Htigr828	AB911214	$(AC)_{11}$	F: F9GTC - GTGCACTTTAGACCTTCACCC R: ACGGACTAAAGTTGGCATGC	HEX	161–265	I
Htigr646	AB911215	$(AC)_{10}$	F: F9TAC - ACATGTCTGCCATTCAAACAC R: CGTCCCTATCCCGCTGTATG	NED	121–137	I
Htigr901	AB911216	$(TG)_{10}$	F: F9GCC - TGCATCTCTGTACAGCCAATC R: GGGCATGGTGGTGTCTGTC	PET	102–152	I
Htigr823	AB911217	$(TCTA)_5$ ……$(TCTA)_7$	F: F9CCG - TCCTTCCATGAATAACGCCTC R: AGCCAAACACTAACCATGAGTG	FAM	157–238	II
Htigr202	AB911218	$(AGAT)_6$	F: F9GTC - GTGTCACCATGCTTGAAATGC R: CCTGCAGTGAAGTGTAATCTGC	HEX	123–227	II
Htigr1284	AB911219	$(TTA)_{11}$	F: F9TAC - GGTGCGCCTTTCCCTTTATC R: AGTCTCACTACATTGCTGCTG	NED	134–173	II
Htigr735	AB911220	$(AC)_4$ $(ATAC)_6$	F: F9GCC - GGATTCCACCGCCTCTGAAG R: AGTTTCCAGGCCACAGAAGG	PET	129–150	II
Htigr1984	AB911221	$(AC)_{11}$	F: F9CCG - TCAACTTTAACTGTGCCCGC R: GGCTCTTCCTCTTTCCTCTAGG	FAM	129–173	III
Htigr1409	AB911222	$(AC)_4$ ……$(TG)_{10}$	F: F9AGG - ACACACCACCACTGCTCAG R: GGATGTTTGGCGATCGATAGG	HEX	101–124	III
Htigr510	AB911223	$(AC)_{10}$	F: F9GTC - CCCATCCTGTTCTTGTGCAG R: TACGGACTAAGCCTGGGATG	PET	92–170	III
Htigr2516	AB911224	$(AGAT)_{11}$	F: M13 - CTGGGTAGGTTTGCATGGG R: GGAAACTTCATGTGGACCCG	FAM	85–129	
Htigr2249	AB911225	$(AC)_{14}$	F: M13 - CTAAGCATGTGGGAGTGCTG R: TTTGGCCCGTACTGTGTG	HEX	121–133	
Htigr2250	AB911226	$(TGTA)_9$	F: M13 - CATGCGTCATATGCTGCAC R: CTGACCACTGAACTACAAGGG	NED	151–168	
Htigr1262	AB911227	$(AGC)_{12}$	F: M13 - CACGCATCGACCTGGTATTG R: GCAGGCGTTGGAAGTGAC	PET	151–220	
Htigr303	AB911228	$(TTA)_{12}$	F: M13 - TTTCAGCCATGTCTGCTCAG R: ACTGTTGGCGCATATTAAATCC	FAM	122–152	
Htigr2248	AB911229	$(AGAT)_8$	F: M13 - CAAGACAGACCGCAAAGACG R: AGCACCATCAGTTTGTACAGTG	HEX	169–274	
Htigr1163	AB911230	$(AGC)_{12}$	F: M13 -CCTTTCAAGCAAGCAAGGAG R: ACCTGGTGCCAAAGGAAGTC	NED	141–197	
Htigr156	AB911231	$(AGC)_{12}$	F: M13 - CCGGTGCACCTCTCTTATTC R: GCAGGCGTTGGAAGTGAC	PET	135–192	
Htigr635	AB911232	$(AGAT)_8$	F: M13 - CCCTGATCACGGATGGCTG R: GCACACATGACTGAAGTTGC	FAM	141–189	
Htigr2069	AB911233	$(AAT)_{13}$	F: M13 - TGCATAGTGGTAGCAGGTCC R: TTCTGCACTGTGACTATCATCC	HEX	145–211	
Htigr1485	AB911234	$(TG)_{12}$	F: M13 - CTGTGTCATTAGCCAGGGAG R: ACATTGTCAGCACCAATCGAG	NED	173–269	
Htigr243	AB911235	$(AATG)_8$	F: M13 - GCAGGAATCTCTAACCGCATG R: TGGTGATTTACAAGCGGCAC	PET	148–152	
Htigr1931	AB911236	$(TG)_{12}$	F: M13 - TGTTCTTGGTTTGGGTGCTG R: ACCAGACACAACCACCCTAG	FAM	123–257	
Htigr501	AB911237	$(TTAG)_8$	F: M13 - AGCCACTCATTGAAGCTTTGAG R: TTACAGGAGTGAGCTGTGCG	HEX	124–183	
Htigr76	AB911238	$(TC)_3(AC)_3$ ....$(AAAC)_7$	F: M13 - GGTGGCTAAGACGCGTAAAC R: TTCAGGGTTGTTGTGTTTGC	NED	162–208	
Htigr409	AB911239	$(TCTA)_7$	F: M13 - AGGCACCGTTCACATAAACATC R: TGTTCACATCTTGGTACTGTCG	PET	163–242	

Table 2. Number of alleles and heterozygosities of 27 microsatellite loci isolated from *Hoplobatrachus tigerinus* populations and its congeneric species

Locus	All sites			*H. tigerinus*-Sunamganj (N = 16)			*H. tigerinus*-Vola (N = 16)			*H. litoralis* (N = 16)			*H. chinensis* (N = 16)			*H. crassus* (N = 4)			*H. occipitalis* (N = 3)		
	N_a	F_{ST}	PIC	N_a	H_O	H_E	N_a	H_O	H_E	N_a	H_O	H_E	N_a	H_O	H_E	N_a	H_O	H_E	N_a	H_O	H_E
Htigr1225	14	0.494	0.811	6	0.688	0.736	5	0.750	0.689	6	0.875	0.773				4	0.750	0.563	1	0.000	0.000
Htigr828	13	0.608	0.645	4	0.563	0.502	7	0.625	0.609	3	0.250	0.320				2	0.000	0.375	2	0.333	0.278
Htigr646	9	0.730	0.861	6	0.750	0.807	7	0.625	0.730	1	0.000	0.000									
Htigr901	12	0.648	0.812	8	0.813	0.695	7	0.750	0.742							1	0.000	0.000	3	0.667	0.500
Htigr823	18	0.614	0.936	14	0.938	0.916	11	0.813	0.854	5	0.438	0.375				1	0.000	0.000			
Htigr202	16	0.544	0.925	12	0.938	0.895	14	0.938	0.887	7	0.875	0.818				1	0.000	0.000			
Hti1284	8	0.630	0.753	4	0.750	0.686	5	0.375	0.604	7	0.875	0.795									
Htigr735	6	0.760	0.607	3	0.375	0.389	4	0.563	0.639	2	0.313	0.264				1	0.000	0.000			
Htigr1984	13	0.601	0.757	6	0.625	0.631	3	0.375	0.490	2	0.438	0.404				5	0.750	0.688			
Htigr1409	13	0.383	0.687	4	0.375	0.510	8	0.750	0.807	5	0.688	0.727	5	0.750	0.727	2	0.000	0.375	1	0.000	0.000
Htigr510	10	0.622	0.813	5	0.625	0.717	6	0.625	0.721	5	0.500	0.662							1	0.000	0.000
Htigr2516	11	0.241	0.831	8	0.938	0.816	8	0.750	0.834	2	0.125	0.117	2	0.438	0.451	2	0.250	0.219	2	1.000	0.500
Htigr2249	9	0.731	0.830	5	0.688	0.785	9	0.813	0.795												
Htigr2250	6	0.792	0.751	4	0.625	0.631	5	0.750	0.563												
Htigr1262	29	0.545	0.945	16	**0.563**	0.930	17	**0.625**	0.914				11	0.800	0.850						
Htigr303	11	0.733	0.789	7	1.000	0.811	6	0.625	0.721	1	0.000	0.000									
Htigr2248	24	0.557	0.913	15	1.000	0.898	13	0.867	0.904	7	0.750	0.816									
Htigr1163	29	0.560	0.945	16	0.813	0.920	13	**0.688**	0.895				9	**0.438**	0.783						
Htigr156	31	0.505	0.933	18	0.750	0.938	17	**0.500**	0.898							3	0.250	0.406	3	0.500	0.625
Htigr635	22	0.303	0.843	7	0.938	0.793	13	0.875	0.879	5	0.438	0.504	10	**0.429**	0.806	5	0.750	0.750	1	0.000	0.000
Htigr2069	19	0.455	0.841	9	0.750	0.754	10	0.938	0.852	2	0.688	0.482	4	0.571	0.582	1	0.000	0.000	2	0.333	0.278
Htigr1485	8	0.692	0.544	3	0.313	0.271	2	1.000	0.500	2	1.000	0.500				3	0.500	0.406			
Htigr243	9	0.823	0.625	5	0.375	0.375	5	0.688	0.629	1	0.000	0.000									
Htigr1931	10	0.668	0.692	6	0.563	0.504	4	0.688	0.619	2	0.813	0.482				2	0.250	0.219			
Htigr501	15	0.555	0.848	8	0.750	0.822	8	0.750	0.787	3	0.438	0.471				2	0.500	0.375	1	0.000	0.000
Htigr76	6	0.701	0.722	5	0.438	0.537	5	0.563	0.531	3	0.813	0.529									
Htigr409	22	0.398	0.901	13	0.813	0.865	9	**0.500**	0.830	2	0.500	0.492				3	0.500	0.625	3	0.500	0.625
mean	14.6	0.589	0.799	8.0	0.694	0.709	8.2	0.696	0.738	3.5	0.515	0.454	6.8	0.571	0.700	2.4	0.281	0.313	1.8	0.303	0.255

N, number of individuals; N_a, number of alleles; H_O, observed heterozygosity (significant departures from Hardy-Weinberg equilibrium are indicated by boldface type); H_E, expected heterozygosity; F_{ST}, fixation index; PIC, polymorphic information content.

Thermal cycling was performed under the following conditions: 94°C for 3 min; 30 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 30 s; eight cycles of 94°C for 30 s, 53°C for 30 s and 72°C for 30 s; and a final extension period of 72°C for 10 min. For BStag primers, PCR was carried out in a 10-µl volume containing 4.8 µl of 2 × KOD FX Neo buffer, 1.6 µl of 2 mM dNTP mix, 0.2 µl of 1 unit/µl KOD FX Neo, 1.6 µl primer mix (0.1 µl of 5 mM of each labeled forward primer, 0.2 µl of 5 mM of each unlabeled reverse primer and 0.1 µl of 5 µM of each fluorescent primer (5′-modified with FAM, HEX, NED or PET)), 1.0 µl of template DNA and ddH_2O. The thermal cycling conditions were the same as those for the M13tag amplification except that the elongation temperature was 68°C instead of 72°C and the annealing temperature was 49°C instead of 53°C in the final eight cycles. The PCR products were electrophoresed on a 3130xl Genetic Analyzer (Life Technologies) together with GeneScan LIZ 500 (Life Technologies) as an internal size standard, and genotyped using GeneMapper 4.0 (Life Technologies). Finally, we screened 27 loci (16 and 11 loci with M13tag and BStag primers, respectively) which were polymorphic and stably genotyped (Table 1). Of the different repeat unit lengths, the tetranucleotide repeat was the commonest among the polymorphic loci (10 of di-, 6 of tri- and 11 of tetranucleotide repeats) and had a slightly higher success rate of marker development than the others (45.5% of di-, 37.5% of tri-, and 68.8% of tetranucleotide repeats), although the difference was not significant (P = 0.504, Fisher's exact test).

Using these 27 loci, we genotyped two populations of *H. tigerinus* and validated cross-amplification in the conge-

neric species *H. litoralis*, *H. crassus*, *H. occipitalis* and *H. chinensis* by the same procedure as described above. For genotyping, we checked the combination of loci for multiplex PCR of the BStag loci, and combined eleven loci into three multiplex reactions (Table 1). We first checked the plausible occurrence of null alleles using Micro-Checker 2.2.3 (Oosterhout et al., 2004) and calculated the number of alleles (N_a), observed heterozygosity (H_O) and expected heterozygosity (H_E) in each population using GenAlEx 6.5 (Peakall and Smouse, 2012). Fixation index (F_{ST}) values were also calculated using Arlequin version 3.5.1.2 (Excoffier and Lischer, 2010). Tests for deficiency of Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were performed using GENEPOP version 4.2.1 (Rousset, 2008). Polymorphic information content (PIC) for two populations of *H. tigerinus* was calculated using Cervus 3.0 (Kalinowski et al., 2007). Within these two populations, the observed N_a ranged from 2 to 18, and the H_O and H_E ranged from 0.313 to 1.000 and from 0.271 to 0.938, respectively (Table 2). In cross-amplification tests, we verified the stable amplification of these newly developed loci in related species, and found that 21, 16, 11 and 6 loci were stably amplified and genotyped in *H. litoralis*, *H. crassus*, *H. occipitalis* and *H. chinensis*, respectively. Of these, *Htigr1225*, *Htigr828*, *Htigr1409*, *Htigr2516*, *Htigr635*, *Htigr2069*, *Htigr501* and *Htigr409* were commonly amplified and genotyped in all species except *H. chinensis* (Table 2). The number of cross-amplified loci in each species appeared to correspond to the relatedness on the phylogenetic tree (Alam et al., 2008). Within populations of all species, no evidence of significant LD deviation was observed in any combination of loci after Bonferroni correction, and five loci indicated significant deviation from HWE after Bonferroni correction ($P < 0.0005$): *Htigr1262* in the Sunamganj and Vola populations, *Htigr1163, Htigr156* and *Htigr409* in the Vola population, and *Htigr1163* and *Htigr635* in the *H. chinensis* population.

In conclusion, we developed 27 newly designed microsatellite markers using the Ion Torrent PGM. Eight of these markers were commonly amplified in congeneric species except *H. chinensis*, which suggests their usefulness as promising genetic tools for various kinds of biological studies, especially for population genetics and conservation management of these species and also conservation of the natural environment in South Asia.

We are grateful to the Wildlife Management and Nature Conservation Division, Bangladesh for their permission to collect and transport frogs from Bangladesh to Japan. This research was supported by a Grant-in-Aid for Scientific Research (B) (No. 24310173) to M. S. and a Grant-in-Aid for Young Scientists (B) (No. 23710282) to T. I. from the Japan Society for the Promotion of Science. We also express our gratitude to Chie Iwamoto, Nana Fukuda, Mai Fujimi and Kanako Onizuka for their support in the experiments.

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