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

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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 (Pahyke 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 phyllogeography of this species and clarified broad intra-
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 Ukhaia, 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 *Buergersia japonica*), the genomic DNA library was attached to adapter 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 0.130x1 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 ddH₂O.
### 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 |
|---------|---------------|-------------------------------|-------------------------------------------------|-------|-----------------|-----------|
| Htigr225| AB911213      | (AC)10                        | F: F9CCG - TGGGTTGTAATGAGCATGACG                | FAM   | 144–278         | I         |
|         |               |                               | R: CTCTCCCTCTGCAAGTCTCTAG                       |       |                 |           |
| Htigr828| AB911214      | (AC)11                        | F: F9GTC - GTGCACTTATAAGGATACCC                 | HEX   | 161–265         | I         |
|         |               |                               | R: ACAGCATAAAAGTTGGGATGC                       |       |                 |           |
| Htigr646| AB911215      | (AC)10                        | F: F9TAC - ACATGCTGCTGCCATCAAACAC              | NED   | 121–137         | I         |
|         |               |                               | R: CCTGCCATATCCGGCTGATG                       |       |                 |           |
| Htigr901| AB911216      | (TG)10                        | F: F9GCC - TGCACTCTGTACAGGCAAATC               | PET   | 102–152         | I         |
|         |               |                               | R: GGGAGTGTTGTTGTCTGTC                       |       |                 |           |
| Htigr823| AB911217      | (TCTA)5(....(TCTA)2)          | F: F9CCG - TCTCCCTGATGAATAAGCCC               | FAM   | 157–238         | II        |
|         |               |                               | R: AGCCAAACACTACAACAGTG                        |       |                 |           |
| Htigr202| AB911218      | (AGAT)6                       | F: F9GTC - GTGGAGAAATCCGTGAAATG                | HEX   | 123–227         | II        |
|         |               |                               | R: AGCAGTTAAGGGTTAGGTGCT                      |       |                 |           |
| Htigr1284| AB911219    | (TTA)11                       | F: F9TAC - GGTGCCGCTTCTGCATG                  | NED   | 134–173         | II        |
|         |               |                               | R: AGTGCACCGTGACTGCTG                         |       |                 |           |
| Htigr735| AB911220      | (AC)4 (ATAC)6                 | F: F9GCC - GAGATTTACACGGCTTCTGAG              | PET   | 129–150         | II        |
|         |               |                               | R: GGGTTAATGCGATGATG                          |       |                 |           |
| Htigr1984| AB911221    | (AC)11                        | F: F9GCC - TCAAGTTAATCCGGGCGCGC               | PET   | 129–173         | III       |
|         |               |                               | R: GGGGCAGCTGACTGCTG                          |       |                 |           |
| Htigr1409| AB911222    | (AC)5(....(TG)20)             | F: F9AGG - ACACACGACACTGCTGAG                 | PET   | 92–170          | III       |
|         |               |                               | R: AGCATTTGGGCAAGTGGAG                        |       |                 |           |
| Htigr510| AB911223      | (AC)10                        | F: F9GTC - CCACTCCTCTTCTGGCAG                 | PET   | 85–129          | III       |
|         |               |                               | R: TACGGACTAAAGCCCTGGAT                       |       |                 |           |
| Htigr2516| AB911224    | (AGAT)11                      | F: M13 - CTGGGAGGGTTTGGCAG                     | PET   | 121–133         | III       |
|         |               |                               | R: GGAAATTACGTCACAGGCA                        |       |                 |           |
| Htigr2249| AB911225    | (AC)14                        | F: M13 - CTAAGCGTGGGGATGCAG                    | PET   | 151–168         | III       |
|         |               |                               | R: TCCGGGCTGACTGCTG                          |       |                 |           |
| Htigr2250| AB911226    | (TGTA)3                       | F: M13 - CATGGCGTACTAGGTCGAC                  | PET   | 151–220         | III       |
|         |               |                               | R: CGCGCGTGGTGGAGTAC                         |       |                 |           |
| Htigr1262| AB911227    | (AGC)12                       | F: M13 - CACACACGACACTGCTG                   | PET   | 110–124         | III       |
|         |               |                               | R: GAGTTTGGGCAAGTGGAG                        |       |                 |           |
| Htigr303| AB911228      | (TTA)12                       | F: M13 - TTTCAAGCCATTGCTGCTG                  | PET   | 122–152         | III       |
|         |               |                               | R: ACTGGTAGGAGAGGGACTA                        |       |                 |           |
| Htigr2248| AB911229    | (AGAT)6                       | F: M13 - CAAAGACGACGGGCAAAGACG                | HEX   | 169–274         | III       |
|         |               |                               | R: GACGAAATTATGCTACTG                         |       |                 |           |
| Htigr1163| AB911230     | (AGC)12                       | F: M13 - TTTCAAGCAGAAGCAAGCAGG                | PET   | 141–197         | III       |
|         |               |                               | R: AGACTGCGATCAGGCA                          |       |                 |           |
| Htigr156 | AB911231     | (AGC)12                       | F: M13 - CCAGTGGCACCTCTGTCAT                 | PET   | 135–192         | III       |
|         |               |                               | R: GACGGCGGATGCGTTC                          |       |                 |           |
| Htigr635 | AB911232     | (AGAT)6                       | F: M13 - CCTGTGCTGACTGAGG                    | PET   | 141–189         | III       |
|         |               |                               | R: GACGACGAGCTGAGAAGAGG                      |       |                 |           |
| Htigr2609| AB911233    | (AAT)13                       | F: M13 - TGCAGAGCAGGCAGTCC                   | PET   | 145–211         | III       |
|         |               |                               | R: TTGCTAGTGGCTGTCATG                        |       |                 |           |
| Htigr1485| AB911234    | (TG)12                        | F: M13 - CTGGGTGACTTTGAGGAGG                 | NED   | 173–269         | II        |
|         |               |                               | R: ACATTGGTGACCAACATCGAG                     |       |                 |           |
| Htigr243 | AB911235     | (AATG)6                       | F: M13 - GCAGGAAATCTCTCAGGCGATG             | PET   | 148–152         | III       |
|         |               |                               | R: TGGGTATTTCAAGGCGCCAC                      |       |                 |           |
| Htigr1931| AB911236     | (TG)12                        | F: M13 - TGCTGCTGAGGAGG                      | PET   | 123–257         | III       |
|         |               |                               | R: ACCACGACACACACACCTAG                      |       |                 |           |
| Htigr501 | AB911237     | (TTAG)3                       | F: M13 - AGCCAGCTCAGTTGAGGAGTTGAG           | HEX   | 124–183         | III       |
|         |               |                               | R: TTAGGAGAGTGAGTGGCTG                       |       |                 |           |
| Htigr76  | AB911238     | (TC)3(AC)3(....(AAC)7)        | F: M13 - GGTGATGAGAGGCGCTAATAAC              | PET   | 116–202         | III       |
|         |               |                               | R: TTCAGGAGTGGGCTGTCG                        |       |                 |           |
| Htigr409 | AB911239     | (TCTA)7                       | F: M13 - AGGACCGGTTCACATAAACAC              | PET   | 163–242         | III       |
|         |               |                               | R: TGITCAGATCTGTTGCTG                        |       |                 |           |
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 mM of each fluorescent primer (5′-modified with FAM, HEX, NED or PET)), 1.0 μl of template DNA and ddH₂O. 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) 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-
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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*) and observed heterozygosity (*H*) and expected heterozygosity (*H*) in each population using GenAlEx 6.5 (Peakall and Smouse, 2012). Fixation index (*F*) 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* ranged from 2 to 18, and the *H* and *H* 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*, *Htigr269*, *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., 2012). 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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**REFERENCES**

Alam, M. S., Igawa, T., Khan, M. M. R., Islam, M. M., Kuramoto, M., Matsui, M., Kurabayashi, A., and Sumida, M. (2008) Genetic divergence and evolutionary relationships in six species of genera *Hoplobatrachus* and *Ruphyctis* (Amphibia: Anura) from Bangladesh and other Asian countries revealed by mitochondrial gene sequences. Mol. Phylogenet. Evol. 48, 515–527.

Clay, C., Gleeson, D., Howitt, R., Lawrence, H., Abdelkrim, J., and Gemmell, N. (2010) Characterisation of microsatellite markers for the primitive New Zealand frog, *Leiopelma hochstetteri*. Conserv. Genet. Resour. 2, 297–303.

Csencsics, D., Brodbbeck, S., and Hölderegger, R. (2010) Cost-effective, species-specific microsatellite development for the endangered dwarf bulrush (*Typha minima*) using next-generation sequencing technology. J. Hered. 101, 789–793.

Daudin, F. M. (1802) (An. XI). Histoire Naturelle des Rainettes, des Grenouilles et des crapauds. Quarto version. Levrault, Paris, France.

Excoffier, L., and Lischer, H. (2010) Arlequin suite ver. 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol. Ecol. Resour. 10, 564–567.

Faircloth, B. C. (2008) MSATCOMMANDER: detection of microsatellite repeat arrays and automated, locus-specific primer design. Mol. Ecol. Resour. 8, 92–94.

Hassain, K. Z., and Rahman, M. M. (1978) The amphibian fauna of Bangladesh. Bangladesh J. Zool. 6, 157–158.

Islam, M. N., Shaikat, A. H., Khan, S. A., Hasan, M. M., and Kriger, K. M. (2012) Concern over destroying frog habitat on the occasion of Save The Frogs Day in Bangladesh. FroLog 20, 28.

Kalinowski, S. T., Taper, M. L., and Marshall, T. C. (2007) Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. Mol. Ecol. 16, 1099–1106.

Oosterhout, C. V., Hutchison, W. F., Wills, D. P. M., and Shipley, P. F. (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Mol. Ecol. Notes 4, 535–538.

Padhye, A., Mananendra-Arachchi, K., De Silva, A., Dutta, S., Kumar Shrestha, T., Bordoloi, S., Papenfuss, T., Anderson, S., Kuzmin, S., Sharif Khan, M., and Nussbaum, R. (2004) *Hoplobatrachus tigerinus*. In: IUCN 2013. IUCN Red List of Threatened Species. Version 2013.2. www.iucnredlist.org. Downloaded on 20 February 2014.

Peakall, R., and Smouse, P. E. (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research – an update. Bioinformatics 28, 3257–3259.

Rousset, F. (2008) GENEPOP’007: a complete re-implementation of the GENEPOP software for Windows and Linux. Mol. Ecol. Resour. 8, 103–106.

Rozen, S., and Skaletsky, H. J. (2000) Primer3 on the WWW for general users and for biologist programmers. In: Bioinformatics Methods and Protocols: Methods in Molecular Biology (eds.: S. Misener and S. A. Krawetz), pp. 355–386. Humana Press Inc., Totowa (NJ).

Schuelke, M. (2000) An economic method for the fluorescent labeling of PCR fragments. Nat. Biotechnol. 18, 233–234.

Shimizu, T., and Yano, K. (2011) A post-labeling method for indel and SNP markers in single tube with bar-coded split tag (BStag). BMC Res. Notes 4, 161.