Journal of Genetics Vol. 97, Online Resources, pp. e147–e151
https://doi.org/10.1007/s12041-018-1029-2

ONLINE RESOURCES

Development and characterization of 33 microsatellite loci for the tiger frog *Hoplobatrachus rugulosus* (Wiegmann 1834) through transcriptome sequencing

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Received 1 May 2018; revised 19 June 2018; accepted 21 June 2018

Abstract. The tiger frog *Hoplobatrachus rugulosus* (Wiegmann 1834) is a large robust dicroglossid frog widely distributed in southern China, Malaysia, Myanmar, Vietnam and Thailand. The escaped bred tiger frog introduced from Thailand hybridized with Chinese native population may have affected the genetic diversity of local Chinese tiger frogs. However, previous microsatellite loci of this species do not offer enough information to construct the genetic map. Here, we reported 33 new microsatellite loci from transcriptome sequencing for *H. rugulosus*. Alleles ranged between 1 and 10 per locus and only one locus (HRT001) was monomorphic. The polymorphic information content, observed and expected heterozygosity were 0–0.794, 0–0.969 and 0–0.831, respectively. None of the loci was observed in linkage disequilibrium and two loci (HRT023 and HRT068) deviated from Hardy–Weinberg equilibrium after Bonferroni correction for multiple tests. These transcriptome-derived microsatellite markers will be used to study the genetic divergence and construct the genetic map in *H. rugulosus*.

Keywords. Dicroglossidae; genetic diversity; microsatellite; transcriptome; *Hoplobatrachus rugulosus*.

Introduction

The tiger frog *Hoplobatrachus rugulosus* (Wiegmann 1834) is a large robust dicroglossid frog, which is widely distributed in southern China, Malaysia, Myanmar, Vietnam and Thailand (Yu *et al.* 2015). The native frog in China is called the Chinese tiger frog, which is listed in Appendix II of CITES as one of Class II national protected species in China (Fei *et al.* 2012). The Chinese native population of this species declined due to habitat destruction and over-hunting (Shao *et al.* 2009). The bred frog introduced from Thailand is called Thailand tiger frog by Chinese, and widely reared by many farmers of China as an edible frog (Yu *et al.* 2015). However, Chinese and Thailand tiger frogs still had been identified as *H. rugulosus* according to Frost's taxonomic methods (Frost 2018). When the escaped Thailand tiger frog from the farms hybridized with Chinese native population, it could affect the genetic diversity of local Chinese tiger frogs (Yu *et al.* 2015).

Microsatellites are usually applied in the studies of species identification, population genetics and genetic maps due to their high variability and abundance. Nine polymorphic microsatellite loci have been isolated for the Chinese tiger frog via magnetic beads enrichment protocol (Shao *et al.* 2009). Further, such a few microsatellite loci cannot offer enough information to understand the level of genetic divergence between native and bred frogs. For a rapid microsatellite isolation, the next-generation sequencing technology has been used by analysing a reference transcriptome *de novo* assembled from RNA-seq data and expressed sequence tags in *H. rugulosus* in this study.

Published online: 14 November 2018

Table 1. Characterization of 33 transcriptome-derived microsatellite loci developed for *H. rugulosus*.

Locus (GenBank #)	Primer sequences (5′ – 3′)	Repeat motif	T_a (°C)	N	N_A	Size range (bp)	H_O	H_E	PIC	P_{HWE}
HRT004	F: CACGACGTTGTAAAACGAC TGGAGTTGCGGGACAAAGTT	$(GAG)_7$	60	32	3	277–283	0.469	0.502	0.444	0.7667
MG912875	R: CCCTGTGGGCACTAAGCTAC									
HRT009	F: CACGACGTTGTAAAACGAC TCAGCTGCTGGCCACATTAA	$(TGA)_5$-A-$(GAG)_5$	60	32	2	224–227	0.313	0.268	0.229	0.4470
MG912876	R: CCTGGGGCAATTTCTCCAGT									
HRT014	F: CACGACGTTGTAAAACGAC GCTTCAAGCACACTACAACTCA	$(CA)_{13}$	60	32	8	141–160	0.313	0.492	0.443	1.0000
MG912877	R: AGCCGAGCTGGTATGTGATT									
HRT021	F: CACGACGTTGTAAAACGAC GGAACACCATGGATTTCGATGT	$(TTGA)_5$	60	32	4	280–290	0.625	0.666	0.599	0.7652
MG912878	R: ATCGGCGGCTCATTGTGTAA									
HRT005	F: CACGACGTTGTAAAACGAC GCAAAAGGTATGCCACAAAGC	$(GAG)_5$-G-$(GA)_5$-ACGAC-$(AG)_7$	60	32	5	232–236	0.281	0.581	0.528	1.0000
MG912879	R: AGGCTTCCACGCAGTCATAC									
HRT041	F: CACGACGTTGTAAAACGAC GTCCAAGTGTTGTGCTGTGC	$(AC)_{15}$	60	31	9	239–285	0.710	0.798	0.752	0.9197
MG912880	R: GTCAATTGGCCAGCATGGTG									
HRT011	F: CACGACGTTGTAAAACGAC AACTTGCTGTGTCCTCCTCG	$(TCCA)_5$	60	30	6	117–170	0.600	0.697	0.629	0.9273
MG912881	R: GCCGACACTCCGATACAGAG									
HRT042	F: CACGACGTTGTAAAACGAC CCACGGTCCCTGTGTTGTAA	$(TG)_{14}$	60	32	10	225–259	0.531	0.831	0.794	1.0000
MG912882	R: TATTCCCCCATGGTCCCTGT									
HRT047	F: CACGACGTTGTAAAACGAC CACCCTGAACTGAGACCGAA	$(GT)_{10}$	60	32	9	262–286	0.781	0.752	0.703	0.4303
MG912883	R: AGTGTTGCGTGACAAACTGA									
HRT030	F: CACGACGTTGTAAAACGAC CAGAAGGGGGACAACACCAA	$(CAT)_7$	60	32	4	206–233	0.219	0.642	0.563	1.0000
MG912884	R: GTCTGCGCATTGTTCCCATC									
HRT033	F: CACGACGTTGTAAAACGAC TGCACTTAGCTGAGTATGCCA	$(TAA)_7$	60	27	7	263–278	0.593	0.746	0.693	0.9803
MG912885	R: TAGGGGGCCATTTCTGCTTG									
HRT019	F: CACGACGTTGTAAAACGAC TCACAACAGCCCAGCGTTAT	$(AC)_{10}$	60	32	5	193–201	0.719	0.780	0.732	0.8606
MG912886	R: CAATGGATGGCAGGTGTCCT									
HRT035	F: CACGACGTTGTAAAACGAC GTAGTGCTGGCAGAAGGGAG	$(AC)_{14}$	60	31	7	249–275	0.484	0.809	0.767	1.0000
MG912887	R: GGGATGGGGATTTCTTGGCA									
HRT006	F: CACGACGTTGTAAAACGAC GTGGGTTTTTGTCTGGCCAC	$(ACCG)_5$	60	32	2	180–184	0.375	0.437	0.337	0.8894
MG912888	R: ACCAGCTAAAACGTGGCCTT									
HRT037	F: CACGACGTTGTAAAACGAC TGCGTGTGGTCACTCTCTTC	$(GCT)_7$	60	32	3	267–276	0.469	0.557	0.461	0.9061
MG912889	R: TCAAGGTGAGCTCCGGAAAC									

Table 1 (*contd*)

Locus (GenBank #)	Primer sequences (5′ – 3′)	Repeat motif	T_a (°C)	N	N_A	Size range (bp)	H_O	H_E	PIC	P_{HWE}
HRT022	F: CACGACGTTGTAAAACGAC CACCAACTCCCGGCTTAGAG	$(AT)_{10}$	60	32	5	244–252	0.750	0.675	0.602	0.2167
MG912890	R: CCCCTTTCCTGTGCCTCAAT									
HRT003	F: CACGACGTTGTAAAACGAC TGGATGCATGGACAAAGAGAGA	$(ATGG)_5$	60	22	4	107–147	0.318	0.418	0.375	0.9591
MG912891	R: CCTCTGTCTGTCCACCCTCT									
HRT023	F: CACGACGTTGTAAAACGAC CTGAACAGGCAGGGTAAGGG	$(TC)_7$-C-$(CT)_{10}$	60	32	5	282–298	0.906	0.703	0.649	0.0015*
MG912892	R: AGACTTGCTAGAACCGCACC									
HRT015	F: CACGACGTTGTAAAACGAC TCAGGCCCAACAAAGCTTCT	$(AC)_{11}$	60	30	8	229–257	0.833	0.740	0.688	0.1470
MG912893	R: TGAGCTTATAACTCAGAATGGTGGT									
HRT024	F: CACGACGTTGTAAAACGAC AGGCTGCTGTTGAGCTTCAT	$(TCC)_7$	60	32	2	242–245	0.063	0.062	0.059	0.9788
MG912894	R: ACTTAGGGGGACAAGACAACA									
HRT068	F: CACGACGTTGTAAAACGAC ACATTGTGGTGCAGCCTTCT	$(CATA)_8$	60	32	7	159–183	0.969	0.805	0.762	0.0106*
MG912895	R: GGGACAGTGTTCTAGCACGT									
HRT025	F: CACGACGTTGTAAAACGAC CGTTGGTTCCCTCTCTGGAC	$(GT)_{11}$	60	32	7	257–285	0.594	0.767	0.716	0.9939
MG912896	R: AGTGGATGGGGATGGCTTTG									
HRT036	F: CACGACGTTGTAAAACGAC TCAGCACCAACTTAAAGATTCACA	$(AAAC)_5$	60	23	7	164–196	0.565	0.763	0.714	0.9955
MG912897	R: AGAGTTGACTCCGTGTGCAG									
HRT027	F: CACGACGTTGTAAAACGAC GCCGAGCAAATGTCCAATCC	$(CT)_8$-CA-$(CT)_6$	60	29	5	239–247	0.276	0.657	0.576	1.0000
MG912898	R: GTCCCTCCCAGACTCTTCCT									
HRT002	F: CACGACGTTGTAAAACGAC TGCCTTTCTTCCCCCATGTC	$(CTC)_7$	60	32	6	126–144	0.531	0.629	0.587	0.9652
MG912899	R: CTCCCCCTACACCCTACACA									
HRT031	F: CACGACGTTGTAAAACGAC CTGGGAGCCTGGTTACCTTG	$(GT)_{13}$	60	32	8	264–302	0.438	0.750	0.712	1.0000
MG912900	R: TCCCCGCTGTTTACCTTCAC									
HRT001	F: CACGACGTTGTAAAACGAC AGTGTTGTGATTGATGGCCCT	$(ATGC)_5$	60	32	1	223	0	0	0	na
MG912901	R: AGGGTGGTGGTAGTAAGGCA									
HRT049	F: CACGACGTTGTAAAACGAC ATCCTTTATGAGCACCCGGC	$(ATAG)_5$	60	31	3	240–252	0.452	0.531	0.416	0.8606
MG912902	R: TGGCCCACTGTGCATTACAT									
HRT010	F: CACGACGTTGTAAAACGAC GGACGTAGACTGCACACACA	$(AC)_{12}$	60	32	6	123–139	0.625	0.658	0.614	0.8045
MG912903	R: AGTGTGCATGTGACAGCAGA									
HRT050	F: CACGACGTTGTAAAACGAC GTCACTAACAGGCAGATGAAGA	$(TA)_6$-TGT-$(AC)_7$	60	32	3	259–263	0.375	0.419	0.339	0.8273
MG912904	R: ACAGGGTTAGGAACGCACTG									
HRT038	F: CACGACGTTGTAAAACGAC GTCATGTGCAGTCCTCCGAT	$(TG)_{10}$	60	27	4	147–156	0.074	0.660	0.576	1.0000

Table 1 (*contd*)

Locus (GenBank #)	Primer sequences (5′ – 3′)	Repeat motif	T_a (°C)	N	N_A	Size range (bp)	H_O	H_E	PIC	P_{HWE}
MG912905	R: ATTTGGGCAGACAGACAGGG									
HRT066	F: CACGACGTTGTAAAACGAC ACCAAGCCCCAATCTAACAGT	$(TC)_{12}$	60	32	4	114–126	0.594	0.567	0.46	0.4227
MG912906	R: GGGAGAGAGGGAGTGTGAGT									
HRT080	F: CACGACGTTGTAAAACGAC TTCATCGGCTGTGAGGACAC	$(GAG)_7$	60	32	5	164–182	0.719	0.746	0.685	0.7606
MG912907	R: AGGAGTGAAACTCTGCAGGC									

M13 are underlined. T_a, annealing temperature of primer pairs; N, number of individual genotyped; N_A, number of alleles; size range, size range of fragment; bp, base pair; H_O, observed heterozygosity; H_E, expected heterozygosity; PIC, polymorphic information content; P_{HWE}, probability of deviation for the Hardy–Weinberg equilibrium (P value).
*Significant deviation from Hardy–Weinberg equilibrium after Bonferroni correction for multiple tests ($P < 0.05$).

Materials and methods

Chinese tiger frogs were obtained from the froggery of Lishui University. After anaesthetization using 0.05% MS-222 (Sigma), ovary and testis tissues were respectively collected and immediately frozen in liquid nitrogen and stored at −80°C. Ovary or testis tissues from three individual frogs were subjected to RNA extraction using Trizol reagent (TaKaRa, Dalian, China). After total RNA isolation, cDNA library construction and *de novo* assembly of transcriptome, we collected unigene database, constructed a microsatellite library with MISA software, and designed primer pairs with Primer 3 software. Based on ≥2 repeat units as a motif and ≥20 bp of microsatellite sequence, 211 primer pairs could be designed. We randomly chose 50 primer pairs to synthesize and tested in a cultivated native population of *H. rugulosus* (total 32 individuals) from Jiangxi province, China.

Genomic DNAs of 32 individuals were extracted from toe muscle tissues using the DNeasy Tissue kit (Qiagen). A nested PCR method with M13 tail (5′-CACGAC GTTGTAAAACGAC-3′)-labelled primers was used (Schuelke 2000). M13 tail was added to the 5′ ends of all forward primers, and a FAM fluorescent labelled M13 primer was added to the PCR mix. The reaction volume of each PCR mixture was 25 μL, containing 100 ng genomic DNA, 12 μL Premix Taq (TaKaRa, Japan), 5 μM of each forward, FAM-M13 and reverse primers. Conditions of the PCR amplification are as follows: 95°C (5 min), then 30 cycles at 95°C (30 s)/T_a (the optimal annealing temperatures, see table 1) (30 s)/72°C (30 s), followed by eight cycles 95°C (30 s)/53°C (30 s)/72°C (30 s), and a further extension at 72°C for 10 min. Thirty-three microsatellite loci were successfully amplified. The PCR products were genotyped on an ABI 3730 sequencer (Applied Biosystems) and analysed with GeneMarker v. 1.8 software. Number of alleles (N_A), observed heterozygosity (H_O), expected heterozygosity (H_E) and polymorphic information content (PIC) were calculated using Cervus 2.0 software (Marshall *et al.* 1998). Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium were determined with FSTAT 2.9.3.2 software (Goudet 1995).

Results and discussion

Thirty-three primer pairs were successfully amplified, and all loci except two (HRT001 and HRT024) showed a significant genetic variation ($H_E > 0.1$). All genetic characteristics of the 33 loci for *H. rugulosus* are shown in table 1. The N_A, heterozygosities (H_O and H_E) and PIC ranged from 1 to 10 (mean ± SD = 5.303 ± 2.325), 0 to 0.969 (mean ± SD = 0.502 ± 0.237), 0 to 0.831 (mean ± SD = 0.609 ± 0.201), 0 to 0.794 (mean ± SD = 0.552 ± 0.197), respectively. No significant linkage disequilibrium was observed after Bonferroni correction for multiple tests ($P > 0.05$). Of the 33

loci, two (HRT023 and HRT068) deviated significantly from HWE testing ($P < 0.05$; table 1) after Bonferroni correction for multiple tests. Overall, 22 loci had high polymorphism degree (PIC > 0.5), 10 loci had low polymorphism degree (PIC < 0.5), and one locus (HRT001) was monomorphic (table 1). These transcriptome-derived markers will be used to study genetic divergence and level of hybridization in *H. rugulosus*.

Acknowledgements

Our experimental procedures complied with the current laws on animal welfare and research in China. This study was funded by the National Science Foundation of China (31500308), Zhejiang Provincial National Science Foundation of China (LQ16C040001), China Postdoctoral Science Foundation (2016 M601843) and Key Research Projects of Lishui City (20151206, SH2017001) and Zhejiang Science and Technology Innovation Programme for College Students (2018R434003). We thank Jing-Yi Chen and Hua-Li Hu for their help during the study.

Corresponding editor: INDRAJIT NANDA

References

Fei L., Ye C. Y. and Jiang J. P. 2012 *Colored atlas of Chinese amphibians and their distributions*. Sichuan Science and Technology Publishing House, Chengdu, China.

Frost D. R. 2018 *Amphibian species of the world: an online reference. Version 6.0 (date of access: 31 January, 2018)*. American Museum of Natural History, New York, USA.

Goudet J. 1995 FSTAT: a computer program to calculate F-statistics. *J. Hered.* **86**, 485–486.

Marshall T. C., Slate J., Kruuk L. E. B. and Pemberton J. M. 1998 Statistical confidence for likelihood-based paternity inference in natural populations. *Mol. Ecol.* **7**, 639–655.

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

Shao C., Wang Y. and Qiao N. N. 2009 Isolation and characterization of microsatellite loci in tiger frog (*Hoplobatrachus rugulosus*). *Conserv. Genet.* **10**, 1601–1603.

Yu D. N., Zhang J. Y., Li P., Zheng R. Q. and Shao C. 2015 Do cryptic species exist in *Hoplobatrachus rugulosus*? An examination using four nuclear genes, the cyt b gene and the complete MT genome. *PLoS One* **10**, e0124825.