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MICROSATELLITE LETTERS

Development of novel microsatellite DNA markers for toad-headed agama *Phrynocephalus vlangalii* using next generation sequencing

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Abstract The ecosystem of the Tibetan Plateau is extremely fragile, and a careful and long-term monitoring program is essential with the global climate changes and rapid social development. *Phrynocephalus vlangalii* is an excellent environmental indicator species, and therefore we developed twenty-five novel microsatellite DNA makers for it. All loci were polymorphic, with number of alleles ranging from 2 to 9. The observed and expected heterozygosity of these loci ranged from 0.00 to 0.85 and from 0.13 to 0.80, respectively. Fifteen loci were found to be significantly deviated from Hardy–Weinberg equilibrium and all loci were in linkage equilibrium. Seven closely-related *Phrynocephalus* species were tested for cross-species amplifiability of those loci; all loci had higher cross-amplification rate, with 17 being successfully amplified in more than three species. These markers will provide useful tools for monitoring the influence of social development in the Tibetan Plateau.

Keywords Microsatellite loci · *Phrynocephalus vlangalii* · Cross-species amplification · Ecological monitoring

The Tibetan Plateau hosts an unparalleled fauna and flora; however, its ecosystem is extremely fragile and easily threatened by global climatic changes and social development (Yu and Lu 2011). The recent completion of the Qinghai-Tibet Railway (QTR) represents one of the largest man-made landscapes on the Plateau and its impacts on the local ecosystem have been carefully monitored. Potential barrier effect of the railway and other roads on some large mammals was anticipated and migratory corridors for these mammals were constructed (Yin et al. 2006). Nevertheless, its impacts on small terrestrial species, such as lizards, have largely been ignored. The toad-headed agama (*Phrynocephalus vlangalii*) is a common reptile species on the Plateau and may serve as an excellent environmental indicator species. We initiated a monitoring program on the impact of the QTR on *P. vlangalii* in 2010. Our early results showed that QTR did not have obvious barrier effect to gene flow of this lizard (Hu et al. 2012). Nevertheless, limited genetic markers might have restricted our detecting power. Therefore, developing additional highly variable genetic markers is of great importance to long term monitoring of this species on the Tibetan Plateau.

We used next-generation sequencing (NGS) technology to isolate microsatellite DNA loci. Compared to more traditional isolation methods (e.g. FIASCO), NGS allows the identification of a large number of microsatellite DNA loci for non-model organisms in a timely fashion and at an

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Table 1 Characterization of 25 novel microsatellite markers for *Phrynocephalus vlangalii* and cross-amplification of those makers for seven other *Phrynocephalus* species

Locus	Primer sequence (5′–3′)	Repeat motif	T_a (°C)	N_A	Size (*bp*)	*P*	H_o	H_e	PFR	PFO	PAX	*PHE*	*PGU*	*PMY*	*PTH*
SSR01	F:AACCTGTCAGCTGCCTGAAC R:ACTTGCCCTGCCTACCTTTC	$(AACCCT)_8$	60	4	110–130	0.09	0.44	0.54	√	√	√	√	√	√	√
SSR02	F:TGTCTGGACTGGAGGAAGGA R:AGATCGCCCTCTCTGGCTAC	$(AAGAG)_{10}$	60	7	212–234	0.18	0.85	0.8	×	×	×	×	×	×	√
SSR03	F:GAGGCTCTGCTCTCACTTTCC R:GCCTCGAGATTCTATCCGGT	$(AATAG)_{10}$	60	8	285–314	<0.0004	0.44	0.8	√	√	√	√	×	×	√
SSR04	F:TGGAAGGGAAGAACAGGACA R:TCCTTGCTTATGACAAATTTCATTC	$(AAAAG)_{10}$	55	4	150–160	0.06	0.41	0.46	×	×	√	√	√	√	√
SSR05	F:TGAGAGCTATCATCTGAAAGAAAGG R:GATCTCACAGGCATGGAAGG	$(AAGA)_{23}$	63	7	210–232	<0.0004	0.19	0.79	×	√	√	√	√	×	√
SSR06	F:GGTGCCTTCCAGATGATGTT R:TTGAACACATGGAGTGCTGG	$(CATA)_{15}$	63	6	154–172	0.69	0.74	0.74	√	√	√	√	×	×	√
SSR07	F:TTAGCCCTCCCTCCATCTTC R:CACGCTGAGACACTTGAAGG	$(TTCT)_{14}$	58	9	270–294	0.08	0.56	0.68	√	√	√	√	√	×	√
SSR08	F:CTTCACTCCCAGAAGAGCCA R:ATGGCACGTCATCAGGAAAC	$(TCA)_{34}$	60	3	244–250	1	0.33	0.34	√	√	√	√	√	√	√
SSR09	F:GTGTAAACAGGCGGAGGATG R:CGGACAACGGAATTACTGGA	$(ATC)_{17}$	60	5	170–185	<0.0004	0.3	0.75	√	√	×	√	√	√	√
SSR10	F:ATGAAATGCCCATCCCTAGC R:GAGAAGGCCAACTCCAGCTT	$(TCA)_{29}$	60	3	280–286	<0.0004	0.07	0.64	√	√	√	√	√	√	√
SSR11	F:CTTCTCCTTCTCGACAGCCA R:GTTTCTCCTCGCCTCCTCTT	$(GAG)_{17}$	60	2	100–103	<0.0004	0	0.14	√	√	√	√	√	√	√
SSR12	F:TTGAGGATCCAGGCACAGAG R:GCGAATGTATGGGTGGAGAG	$(AC)_{50}$	63	2	170–172	0.02	0.26	0.48	×	√	√	√	×	×	√
SSR13	F:CTGGTGGGCAGCAGTTGTAT R:CAGCAAGACGGAGCAACAC	$(GT)_{54}$	63	3	170–176	<0.0004	0.07	0.5	√	√	×	×	×	×	×
SSR14	F:TGTTGAGAGCCACTCTTGAGG R:AAGCAGCACCTTCCATCTCA	$(AC)_{50}$	62	3	237–241	<0.0004	0.15	0.57	√	√	√	×	√	√	√
SSR15	F:AAAGGCAGAGGTGGAAGAGA R:TCAGCGCATCAGACACAAGT	$(GT)_{44}$	62	6	208–220	0.01	0.81	0.8	×	√	×	×	×	×	√
SSR16	F:GCATATTTCAGCAGGTTGGC R:TCTTGCAAAGGGAACTCAGC	$(AG)_{44}$	62	9	198–214	<0.0004	0.52	0.86	×	×	√	√	×	×	×
SSR17	F:TTGTTCCCATGGTTACAGGG R:GTGAAAGCCATGCTTGATCC	$(AC)_{32}$	62	4	288–308	<0.0004	0.22	0.58	×	√	×	×	×	×	√
SSR18	F:TACCATGCCTTGGTGGAGAT R:AATTGAAGGAAGGCGGTCTC	$(AG)_{28}$	60	3	124–130	<0.0004	0.07	0.41	×	√	√	√	×	√	×

Table 1 continued

Locus	Primer sequence (5′–3′)	Repeat motif	T_a (°C)	N_A	Size (*bp*)	*P*	H_o	H_e	PFR	PFO	PAX	*PHE*	*PGU*	*PMY*	*PTH*
SSR19	F:CACCACTGCGACCATAAACA R:GCCATCCAAACAACTTCCAG	$(AG)_{28}$	60	2	138–142	0.01	0.04	0.17	√	√	√	√	√	×	×
SSR20	F:AATAGGGAAGGAGCTCGCAG R:ATCCTCATTCCTCTCCTGGC	$(AG)_{26}$	60	3	126–130	<0.0004	0.07	0.54	×	√	×	×	√	×	×
SSR21	F:TTTGGGAAACATGAGCAGC R:TGCTGAACCTTTCAAATGCAC	$(ACT)_{13}$	58	2	130–136	<0.0004	0.11	0.46	×	√	×	×	×	×	×
SSR22	F:ATCATCCTCGCCATCATCCT R:ACGTGAAGTCGGTGAACTCG	$(CCG)_{13}$	58	3	179–184	<0.0004	0.26	0.5	√	√	√	√	√	√	√
SSR23	F:TCCAGCTAGACGGACCTTTG R:AGAGAGCTTTCTTCCGCCG	$(CCG)_{13}$	58	3	209–218	<0.0004	0.11	0.42	×	√	√	√	×	√	√
SSR24	F:GCTGAAGGTGACGGAGCTT R:CTCCTCTTCCTCTTCCTCGC	$(AGG)_{12}$	58	2	274–277	<0.0004	0.07	0.39	×	√	×	×	×	×	√
SSR25	F:TTTAGTCTGGGCACATGCAG R:TCCTTCACAAAGATGGCAGC	$(TTC)_{12}$	58	4	174–186	1	0.56	0.51	√	√	√	√	√	√	√

T_a, optimal annealing temperature; N_A, number of alleles; *P*, exact tests for Hardy–Weinberg equilibrium; H_o, observed heterozygosity; H_e, expected heterozygosity; F, forward; R, reverse; √, succeeded in amplification; ×, failed in amplification; PFR, *P. frontalis*; PFO, *P. forsythii*; PAX, *P. axillaris*; PHE, *P. helioscopus*; PGU, *P. guttatus*; PMY, *P. mystaceus*; PTH, *P. theobaldi*

affordable cost (Guichoux et al. 2011). As part of a large comparative genome study, we first acquired the transcriptome sequence of *P. vlangalii*. Microsatellite DNA loci were then identified and primers were designed to amplify these regions. Standard PCR amplification with optimized annealing temperature was conducted. PCR products were visualized on 8 % denaturing polyacrylamide gels with silver staining, and the polymorphism of each locus was assessed. After multiple tests, 25 loci were consistently amplifiable and polymorphic.

We used 27 individuals of *P. vlangalii* to characterize these microsatellite DNA loci. All samples were collected from Zoige county, northeastern corner of the Tibetan Plateau (E102°48543′, N33°71389′, with elevation of 3,475 m a.s.l). PCRs were conducted with the same parameter as above, and polyacrylamide gel electrophoresis and silver staining was used to estimate the allele sizes. Hardy–Weinberg equilibrium (HWE), linkage disequilibrium (LD), and several other population genetic parameters were estimated using Genepop version 4.2 (Raymond and Rousset 1995). A sequential Bonferroni correction was applied for multiple tests. The results were summarized in Table 1 and the raw microsatellite DNA data was provided in Online Resource 1. Fifteen loci showed significant deviation from HWE after Bonferroni correction for multiple tests ($P < 0.0004$). All pairs of loci were in linkage equilibrium after Bonferroni correction. A Wahlund effect may cause a large number of loci deviating from HWE, because these 27 individuals were collected from two subpopulations nearby. For all these loci, *Ho* was much smaller than *He*.

Seven closely-related *Phrynocephalus* species were tested for cross-species amplifiability of these microsatellite DNA loci. The result was summarized in Table 1. Seventeen out of 25 microsatellite DNA loci were successfully amplified in more than three species, suggesting high cross-species amplifiability. These high polymorphic microsatellite loci will provide useful tools for long term monitoring programs and promote conservation efforts for the Tibetan Plateau ecosystem.

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