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TECHNICAL NOTE

Development and characterization of microsatellite markers for the endangered Laotian rock-rat (*Laonastes aenigmamus*) using 454-sequencing technology

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Abstract The Laotian rock rat (*Laonastes aenigmamus*) is the single surviving member of the family Diatomyidae, which has a distribution restricted to the karstic region of Lao-PDR. Here we describe the development of 12 polymorphic microsatellites markers for the endangered Laotian rock rat using 454-sequencing. We successfully tested 12 markers in 30 individuals from 2 populations. Eleven of the 12 loci were polymorphic and the number of alleles detected per locus ranged from 2 to 11. Three of these loci deviated significantly from Hardy–Weinberg equilibrium, which coincides with the detection of possible null alleles. These microsatellite markers are expected to contribute in future research and conservation of *L. aenigmamus*.

Keywords Laotian rock rat · Laonastes aenigmanus · Khanyou · Microsatellite · 454 sequencing · Conservation genetics

Since the discovery of the Laotian rock rat or kha-nyou (*Laonastes aenigmamus*) in 2005 (Jenkins et al. 2005), several studies have described the taxonomy of the species and genus while others using molecular and morphological data have examined its phylogenetic placement into the family Diatomyidae (Jenkins et al. 2005; Dawson et al. 2006; Huchon et al. 2007). Regardless, this enigmatic rodent is the single surviving member of its family and investigations at the population level have not been made in fine scale.

Laonastes has a narrow distribution in a karstic region located primarily in the Lao People's Democratic Republic, in Southeast Asia (Jenkins et al. 2005). Classified as endangered by the UICN, its populations are considered to be declining due to habitat loss and hunting (Aplin and Lunde 2008). Recent molecular data suggest that this species has strong geographical structure (Rivière-dobigny et al. 2011) highlighting the necessity for the development of microsatellite markers to aid in assessing species status definitions and fine-scale genetic differentiation. Here we describe 12 polymorphic microsatellite markers designed for *L. aenigmamus* using 454-sequencing.

Whole genomic DNA was isolated from *Laonastes* liver tissue using standard DNeasy Blood and Tissue kit protocol (QIAGEN). Genomic sample from one individual was submitted, to the University of Florida ICBR, for sequencing on one-eighth of a plate using 454GS-FLX technology (Roche Applied Science).

We obtained 92,433 fragment reads from the 454-sequencing. Their lengths ranged from 40 to 842 bases and 44,331 fragments (47.9 %) were between 301 and 450 bases in length. We screened for di-, tri- and tetra-nucleotide tandem repeats directly from the dataset using MSATCOMMANDER (Jenkins et al. 2005; Faircloth 2008; Rivière-Dobigny et al. 2011). The online version of PRIMER3 (Rozen & Skaletsky 2000) was used to explore the suitability for primer annealing. We arbitrarily tested 96 primer pairs, including di-, tri- or tetra-nucleotide repeats. An *M13-tail* (CACGACGTTGTAAAACGAC) was added in the 5′ end of all forward primers to enable fluorescent-dye labeling (Boutin-Ganache et al. 2001). A *pig-tail* fragment (GTTTCTT) was added to the 5′ end of

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Table 1 PCR primer sequence, repeat motif, fragment size, and NCBI probe accessions for 12 loci from *Laonastes aenigmamus*

Locus	Primer sequence (5′–3′)	Repeat motif	Fragment size range	NCBI probe accession
JLPLaoDi7	F-TCTGTTTTGTGGGTTTGTTTTG	AC	194–202	12852940
	R-TTGGGGGAACTTTTGGTAGA			
JLPLaoDi8	F-GCACTCAGGTTTGTCAGAGAGA	AG	188–194	12852941
	R-GCCAAGCACTCGAAAGAAAG			
JLPLaoDi9	F-TCTGAACGCTTAGGCAGGAG	AG	234–236	12852942
	R-GACATGATGATCCAAATGTCTGA			
JLPLaoDi11	F-GTCTGCTTTGAAACCCGAAA	AT	183–211	12852933
	R-TTTATTCCATAGCCAATATAAGTGC			
JLPLaoDi13	F-GCTCCAGAAAAGAAGCTGGA	AC	195[a]	12852934
	R-AGGCAAGACTGATGCCTGAC			
JLPLaoDi16	F-TTCCTTTCATATCCGTGGAAG	AC	237–241	12852935
	R-GCAAATCTAATTCACTGCATGTTT			
JLPLaoDi17	F-TAACAGGAGCCCTGAAGGAA	AG	211–221	12852936
	R-GCTTTACAAAGCCCGATGTC			
JLPLaoDi20	F-CTTGAATGCCCCAATCACTT	AT	220–228	12852937
	R-CAGTTATTCGCTCATTATCTTCC			
JLPLaoDi51	F-CAACGTATGAACTTCAAGTGTGC	AC	123–131	12852938
	R-GCAGTGTGTATGTTTCTAAGTAGTCTG			
JLPLaoDi57	F-GAAGAGCTGTGTGTTGTTCAGG	AC	229–255	12852939
	R-TGATGGCCAAATTTCTGTGA			
JLPLaoTe3	F-GAAAGTCATTGCCGGGTAGA	CTTT	204–236	12852943
	R-TTGAGCATAGCCTGTGCAAC			
JLPLaoTe4	F-CCATTCCGCAAAACTTCATT	ATCT	241–277	12852944
	R-CCTTCTACCAACCTCCCAAA			

[a] Monomorphic locus

the reverse primers only for dinucleotide loci, to reduce stuttering and improve adenylation during PCR (Brownstein et al. 1996). Primers were discarded when they failed to amplify or when their results were difficult to score.

We tested the primers in 30 individuals from two geographic localities in Laos, Gnommalath (Population-1) and Maxai (Population-2). Amplification of all loci was performed under the same thermocycling conditions: An initial denaturation at 95 °C (5 min); followed by 15 cycles of 95 °C (0.5 min), 55 °C (1.5 min), 72 °C (0.5 min); and 29 cycles of 95 °C (0.5 min), 48 °C (1.5 min), 72 °C (0.5 min); and a final extension of 72 °C (30 min). Each amplification had a reaction volume of 16 µl. Each reaction contained 7.5 µl of multiplex PCR master mix-2x (QIAGEN), 0.6 µl of forward primer [1 µM], 1.0 µl of reverse primer [10 µM], 4.9 µl of distilled water, 1.0 µl of FAM-labeled M13 primer [10 µM], and 1.0 µl of template DNA [~58.0 ng/µl]. PCR amplicons were diluted and run on an ABI-3730xl 96-capillary sequencer using GeneScan600-LIZ as internal size. Microsatellite genotypes were scored using GeneMarker (SoftGenetics, LLC).

A total of 12 loci were successfully genotyped in individuals from both populations. With combined data from both populations, the number of alleles in polymorphic loci ranged from 2 to 11 with 6 alleles per locus in average. Locus *JLPLaoDi13* was monomorphic among the 30 individuals we tested (Tables 1, 2); however, our surveys of additional populations not reported here detected 4 additional alleles. When the two populations were treated separately, 11 and 9 loci were polymorphic and the number of alleles in those loci ranged from 2 to 9 (average = 5.3) and 2–6 (average = 3.4), in populations 1 and 2 respectively. Results from MICRO-CHEKER (Van Oosterhout et al. 2004) suggest the possibility of null alleles in three loci from population-1. These three loci also deviated significantly from Hardy–Weinberg expectations, which may be associated with the potential of null alleles. We did not detect null alleles in population-2, although two loci showed a significant departure from Hardy–Weinberg equilibrium due to heterozygote deficiency. Genotypic disequilibrium was estimated for 66 pairwise comparisons of loci in each population (2,640 permutations) using FSTAT (Goudet 2001). No linkage disequilibrium was detected after Bonferroni correction (adjusted at the 5 % level; $P < 0.0003790$).

These are the first microsatellite markers developed for the endangered *L. aenigmamus*, which may represent an

Table 2 Characterization of twelve microsatellite loci tested in thirty kha-nyou individuals from Lao-PDR

Locus	$N_{a\ Total}$	Population	N_a	Null alleles	H_O	H_E	HWE
JLPLaoDi7	5	1	3	No	0.6000	0.4529	0.6418
		2	**4**	**No**	**0.73333**	**0.5908**	**1.0000**
JLPLaoDi8	3	1	2	No	0.4000	0.5149	0.6032
		2	**1**	**No**	**–**	**–**	**–**
JLPLaoDi9	2	1	1	No	–	–	–
		2	**2**	**No**	**0.0000**	**0.1287**	**0.0347**
JLPLaoDi11	11	1	9	No	0.6667	0.8069	0.0837
		2	**4**	**No**	**0.4000**	**0.5126**	**0.0501**
JLPLaoDi13	1[a]	1	1	No	–	–	–
		2	**1**	**No**	**–**	**–**	**–**
JLPLaoDi16	3	1	3	Yes	0.0667	0.2460	0.0036
		2	**1**	**No**	**–**	**–**	**–**
JLPLaoDi17	6	1	6	No	0.6667	0.7747	0.3254
		2	**2**	**No**	**0.2667**	**0.3310**	**0.4599**
JLPLaoDi20	5	1	4	Yes	0.2667	0.6230	0.0055
		2	**2**	**No**	**0.3333**	**0.4805**	**0.2967**
JLPLaoDi51	5	1	3	No	0.8000	0.6736	0.1269
		2	**3**	**No**	**0.0667**	**0.1310**	**0.0350**
JLPLaoDi57	10	1	8	No	0.8000	0.8345	0.9197
		2	**3**	**No**	**0.2000**	**0.1908**	**1.0000**
JLPLaoTe3	9	1	8	Yes	0.5333	0.8713	0.0015
		2	**6**	**No**	**0.4667**	**0.6759**	**0.1632**
JLPLaoTe4	7	1	7	No	0.5333	0.6621	0.0735
		2	**5**	**No**	**0.6667**	**0.7977**	**0.4321**

Bold values indicate the results for population 2

[a] Monomorphic locus

$N_{a\ TOTAL}$ number of alleles in both populations combined, N_a number of alleles per population, H_O observed heterozygosity, H_E expected heterozygosity, *HWE* *P* value for deviation from Hardy–Weinberg equilibrium

important resource for future genetic variability, population dynamics and conservation assessments of this enigmatic mammal.

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