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Isolation, characterization and PCR multiplexing of polymorphic microsatellite markers in the threatened murine rodent, *Leopoldamys neilli*

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Abstract

Leopoldamys neilli is a threatened murine rodent species endemic to limestone karsts of Thailand. Twelve microsatellite loci were identified using the method of microsatellite-enriched libraries. Polymorphism was assessed in samples (N=62) from four geographically distinct populations in Thailand. Number of alleles per locus ranged from 9 to 15 (average 11.6). Observed and expected heterozygosities varied from 0.28 to 1.0 and from 0.44 to 0.91, respectively. There was no evidence for linkage disequilibrium, however, four loci showed evidence of departure from Hardy–Weinberg equilibrium in one population. Presence of null alleles was not detected in all the 12 loci. These first microsatellites primers developed for *L. neilli* will provide information on the fine-scale genetic structure of this threatened species and will help in the development of future conservation policies.

Keywords: *Leopoldamys neilli*, murine rodent, microsatellite, multiplex, genetic structure, Southeast Asia

The Neill's Rat, *Leopoldamys neilli* (Marshall, 1976), is a murine rodent species endemic to limestone karsts of Thailand, a strongly threatened habitat patchy distributed within Thailand. *L. neilli* was discovered in 1973 in Saraburi province, Central Thailand (Lekagul and McNeely 1988) and has also been recorded in a few locations in northern and western Thailand (Lekagul and McNeely 1988; Waengsothorn et al. 2007). However, the limits of its geographical range are not clearly resolved. This species was previously classified as "Endangered" on the IUCN Red List, but is now listed as "Data deficient" since very little information is available about its biology and ecology (Lunde and Aplin 2008). Acquisition of information about its geographic range, ecological requirements and the genetic structure of its populations are thus urgently needed to assess the conservation status of *L. neilli*. With the use of microsatellites we aim to describe the levels of genetic differentiation within and between populations of *L. neilli* and to estimate population dynamics between isolated karst regions. A better understanding of these processes is essential for creating effective conservation policies.

Novel microsatellite loci for *L. neilli* were isolated from a microsatellite-enriched library, following the protocol of Billote et al. (1999). Genomic DNA was extracted from skin samples using the DNeasy Tissue Kit (Qiagen Inc., Valencia, California) following the manufacturer's instructions. DNA was restricted by *HaeIII* and the fragments were ligated to *RSA*21 and *RSA*25 self-complementary primers. The ligated fragments were amplified by polymerase chain reaction (PCR), and products were allowed to hybridize to biotinylated $I_5(CT)_8$ and $I_5(GT)_8$ probes, which were later recovered by Streptavidin MagneSphere Paramagnetic Particles (Promega, Madison). After amplification, the microsatellite-enriched fragments were cloned into pGEM-T (Promega) and transformed into XL1-Blue electroporation-competent cells (Stratagene). One hundred ninety-two recombinants were randomly picked and PCR-amplified with *RSA*21 primer. PCR fragments were transferred on Hybon N+ membranes, which were hybridized with [γ32P] dATP end-labelled $(GA)_{15}$ and $(GT)_{15}$ probes. One hundred clones with hybridization signal were sequenced. Sequences were analysed and fifty-two primers pairs were designed using SAT program (Dereeper et al. 2007).

Polymorphism of 20 loci was first tested using monolocus PCRs. From these 20 loci, 12 were selected for further analysis for displaying good quality and polymorphic amplification. The forward primer of each locus was 5'-end labeled with a fluorescent dye (FAM, HEX, NED).

Based on size limitations and amplification specificity, the 12 microsatellites were distributed in four sets: set A (mLn01, mLn02, mLn03), set B (mLn04, mLn05, mLn06), set C (mLn07, mLn08, mLn09) and set D (mLn10, mLn11, mLn12). PCRs were carried out in 10 µl volume containing 0.2 µl of each 10 µM primers, 5 µl of Multiplex PCR kit (QIAGEN) and 2.5 µl of DNA. Amplifications were performed in thermal cycler VWR Unocycler using one activation step (95°C/15 min) followed by 30 cycles (denaturation at 94°C for 30 s, annealing at 57°C (sets A, D) or 61°C (sets B, C) for 90 s, extension at 72°C for 90 s) and final extension step at 72°C for 30 min. PCR products were detected on an ABI 3700 sequencer using 2 µl of amplified DNA, 13.5 µl of formamide and 0.5 µl of ROX 350 size standard (QIAGEN).

The four microsatellite sets were tested on 62 *L. neilli* live-trapped in four localities of Thailand (Loei (north-eastern Thailand): N=17; Nan (northern Thailand): N=16; Nakhon Ratchasima (central Thailand): N=14; Kanchanaburi (western Thailand): N=15) throughout the geographical range of this species. All loci were highly polymorphic in all populations. The observed number of alleles per locus ranged from 9 to 15 (average 11.6) (Table 1). Observed and expected heterozygosities and deviations from Hardy-Weinberg equilibrium were calculated with Arlequin 3.11 (Excoffier et al. 2005). Observed heterozygosity ranged from 0.28 to 1.0 and expected heterozygosity varied from 0.44 to 0.91. Exact tests performed with Arlequin 3.11 revealed significant deviations from Hardy-Weinberg equilibrium for loci mLn01, mLn03 and mLn07 in Kanchanaburi population and for locus mLn06 in Nakhon Ratchasima population. These loci exhibited a deficit of heterozygotes, indicating the possibility of null alleles. However, the values of Null allele frequency (NAF) determined in FreeNA (Chapuis and Estoup 2007) were always ≤ 0.2, indicating that null alleles are not expected to cause significant problems in the analysis. After Bonferroni correction, no significant linkage disequilibrium was detected using Fisher's exact test performed in Genepop 4.0 (Rousset 2008).

In conclusion, these first microsatellites primers developed for *L. neilli* will provide information on the fine-scale genetic structure and dynamics among populations of this threatened species and help in the development of future management and conservation plans.

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Table 1 Characteristics of 12 microsatellite loci from *Leopoldamys neilli* with primer sequences, number of alleles, size range of PCR product, expected (H_E) and observed (H_O) heterozygosities with deviation from Hardy-Weinberg proportions (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) and null allele frequency (NAF)

Locus GenBank	Primer sequence (5'-3')	Repeat motif	No. of alleles	Size range	Population	H_E/H_O	NAF
mLn01	F: FAM-CCCCTTGTGGACAGTTATT	$(GT)_{20}$	9	165-185	Loei	0.83/0.94	0
HQ848952	R: AGCTGAGACTCTGGAAAGC				Nan	0.56/0.44	0.042
					N. Ratchasima	0.60/0.57	0
					Kanchanaburi	0.82/0.73*	0
mLn02	F: HEX-CCCTTGTTGTCATTTGCT	$(GT)_{16}$	11	187-219	Loei	0.78/0.59	0.079
HQ848953	R: TCCTAGTCTGCCTTCTGG				Nan	0.83/0.87	0
					N. Ratchasima	0.44/0.43	0
					Kanchanaburi	0.82/0.87	0
mLn03	F: FAM-TCTGGATGTTCTGAAAGGA	$(TG)_{19}$	15	251-293	Loei	0.82/0.76	0.027
HQ848954	R: TGGCTGACTTGTTAAGAGC				Nan	0.76/0.87	0
					N. Ratchasima	0.67/0.78	0
					Kanchanaburi	0.73/0.53*	0.096
mLn04	F: NED-CGGTGCAAGAAGCAAATA	$(TG)_{23}$	12	186-212	Loei	0.84/0.88	0
HQ848955	R: CGACCTCTGACCTCCATT				Nan	0.72/0.69	0.046
					N. Ratchasima	0.71/0.71	0
					Kanchanaburi	0.60/0.53	0
mLn05	F: FAM-CCCGCGTACATTTGGTAT	$(AG)_{23}$	10	222-248	Loei	0.67/0.76	0
HQ848956	R: CAAATACCCGGCAAAGAC				Nan	0.58/0.56	0
					N. Ratchasima	0.75/0.78	0
					Kanchanaburi	0.74/0.87	0
mLn06	F: HEX-TTCATCTCCTAGAATAGCAACA	$(TG)_{17}$	14	243-275	Loei	0.9/1.0	0
HQ848957	R: CATTAACCCACCATCTGC				Nan	0.82/0.94	0
					N. Ratchasima	0.62/0.28**	0.189
					Kanchanaburi	0.72/0.67	0.021
mLn07	F: HEX-CACATCTTCCCAGTTTGC	$(TC)_{23}(CA)_{21}$	14	174-202	Loei	0.91/0.94	0
HQ848958	R: GCTCCCTTTTCTCCTGTC				Nan	0.71/0.75	0
					N. Ratchasima	0.88/0.93	0
					Kanchanaburi	0.81/0.53*	0.130
mLn08	F: FAM-TGAAACAAAGAAGTCTCAACC	$(GT)_{19}$	9	191-209	Loei	0.86/1.0	0
HQ848959	R: CAAGTTCAGCCCAAAAGA				Nan	0.63/0.69	0
					N. Ratchasima	0.54/0.57	0
					Kanchanaburi	0.58/0.67	0
mLn09	F: NED-TGATTTGATAGGTGTTGTGG	$(GT)_{18}$	14	202-240	Loei	0.86/0.94	0
HQ848960	R: AGCTTGGACAACTTGATTG				Nan	0.71/0.62	0.064
					N. Ratchasima	0.74/0.86	0
					Kanchanaburi	0.70/0.67	0
mLn10	F: FAM-CGATGGACAAACCTGAAG	$(AC)_{18}$	10	174-194	Loei	0.61/0.47	0.04
HQ848961	R: TTTTCTTATTTGCTTGATTGG				Nan	0.72/0.75	0
					N. Ratchasima	0.32/0.36	0
					Kanchanaburi	0.76/0.67	0.025
mLn11	F: HEX-TCCAAGGAGGCTTTTCTT	$(TG)_{23}$	12	191-217	Loei	0.77/0.82	0
HQ848962	R: GGGTCCTGGTCAGTATGTC				Nan	0.80/0.87	0
					N. Ratchasima	0.78/0.93	0
					Kanchanaburi	0.71/0.73	0
mLn12	F: NED-TGCATACTCCTGACTCCTT	$(GT)_{21}$	9	194-230	Loei	0.67/0.59	0.020
HQ848963	R: CATTAGGCAACTACACTCTTG				Nan	0.49/0.44	0.027
					N. Ratchasima	0.57/0.71	0
					Kanchanaburi	0.73/0.80	0