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Isolation and Characterization of Polymorphic Microsatellite Markers for the Masked Palm Civet (*Paguma larvata*)

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Abstract The masked palm civet (*Paguma larvata*) has been suspected to be the host of a SARS-like CoV virus that causes severe acute respiratory syndrome in humans. In China, the palm civet lives wild and is farmed, but even though the species is a potential carrier of the virus, its geographic distribution and genetic diversity have never been studied. We report the isolation and characterization of six polymorphic microsatellite markers for *P. larvata*. To characterize each locus, two farmed masked palm civet populations from Shanxi and Guangxi provinces in China were genotyped. The number of alleles per locus ranged from 3 to 15, and the observed heterozygosity for these populations was 47.1 and 68.7%, respectively.

Keywords Masked palm civet · *Paguma larvata* · Microsatellite marker

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Introduction

The masked palm civet (*Paguma larvata*) is widely distributed in tropical and subtropical Asia, throughout the Himalayas from north Pakistan, Nepal, Bhutan, Jammu, Kashmir, and Assam southward to Myanmar, Thailand, Laos, Vietnam, the Malay Peninsula, Borneo, Sumatra, and the Andaman Islands (Pocock 1939; Roberts 1977; Corbet and Hill 1992; Choudhury 1997a, b, 1999; Jha 1999). In China, *P. larvata* lives mainly in the southern regions, but it can also be found in Hebei, Shanxi, Shaanxi, and Xizang provinces (Gao et al. 1987; Yin and Liu 1993). Due to habitat destruction, fragmentation, and hunting, the masked palm civet is threatened by local extinctions in the wild; it has become an endangered species and is included in the Chinese Protected Animal List (Tan 1989). In south China, *P. larvata* is reproduced in captivity and traded for meat production. The associated coronavirus (SARS-like CoV), which causes severe acute respiratory syndrome in humans, has been isolated from wild and captive masked palm civets, suspected to be the hosts of SARS virus (Nicastri et al. 2003). However, the intraspecific taxonomy of *P. larvata* is poorly known, and there are no species-specific markers available for population genetic studies. In this paper, we report the isolation and characterization of six polymorphic microsatellite loci from a *P. larvata* DNA library enriched with $(CA)_n$ repeats, and their polymorphism in two farmed populations in China.

Methods and Materials

A dinucleotide-enriched library for *P. larvata* was obtained according to Kandpal et al. (1994) and Karp et al. (1998) with some modifications. DNA was extracted from a single individual using a modified phenol–chloroform extraction method (Sambrook and Russell 2002; Chen et al. 2005). Genomic DNA was digested with *Mbo*I, and the fragments between 350 and 600 bp were cut out from an agarose gel and purified with an agarose gel DNA Purification Kit (TaKaRa). The selected fragments were ligated to linker sequences and amplified using linker-specific primers on a PE9700 thermal cycler (Armour et al. 1994; Hammond et al. 1998). The PCR product was denatured and hybridized to a biotin-labeled dinucleotide repeat oligonucleotide $(CA)_{15}$ probe, and hybridization was carried out at 50°C for 18 h. The hybridization mixture was mixed with 50-mg Vectrex Avidin D (Vector Laboratories). After a first wash with 0.1× buffer A (15 mM NaCl/10 mM Tris, pH 7.5) at 55°C for 30 min, and two washes with ddH_2O at 65°C for 30 min to remove unbound fragments, the matrix was washed again with ddH_2O to elute the bound fragments, and the supernatant was retained. Another PCR was performed to amplify the target genomic fragments, this PCR condition being similar to the first PCR (Armour et al. 1994; Hammond et al. 1998), and the products were ligated to PMD19-T vector (TaKaRa). *Escherichia coli* DH-5α competent cells were transformed with the ligation product to construct the $(CA)_n$-enriched DNA library. Out of 140 recombinant colonies, 120 clones were screened by amplification using linker primers. A total of 57 clones were sequenced on an ABI377 sequencer with

Table 1 Six microsatellite loci in *Paguma larvata*

Locus	Primer sequences (5′—3′)	Repeat motif	Tm (°C)	*N* alleles	Allele size (bp)	H_o/H_e		GeneBank acc. no.
						Shanxi farm $N = 12$	Guangxi farm $N = 28$	
PC14	F: CCTCCTACGTGTAAGATT	$(TG)_{14}$	50	8	196–210	0.4167/0.5174	0.8214/0.8151	DQ423385
	R: AGAGCAGCCCTGATGAAG							
PC55	F: TAAGTAGCCACTTGAAA	$(CA)_4N_6(CA)_{13}$	50	4	180–190	0.6667/0.7257	0.5714/0.5912	DQ423383
	R: GCTGACGACATAGATAA							
PC119	F: GACAGGGACACGGATACA	$(AC)_6N_4(AC)_4N_{26}(CA)_7$	55	3	111–115	0.0000/0.0000	0.4286/0.3591	DQ423381
	R: ACTGGAATAGAAGGACGG							
PC29	F: GCCAAACGGAAATCAAAG	$(GT)_4N_{24}(GT)_{12}$	54	6	192–226	0.4167/0.3437	0.5714/0.6358	DQ423384
	R: AACCTCCTGCCACAGTCA							
PC58	F: GTCAGGGCAGAAGAGTT	$(GT)_6N_{15}(GT)_{11}$	52	12	139–159	0.5455/0.6529	0.8889/0.8471	DQ423382
	R: GTTAGCAAAGCCTACGTCCATGGAA							
PC11	F: CTTGCATTATATTCATATT	$(TC)_{14}(AC)_{30}$	52	15	141–199	0.7778/0.8086	0.8421/0.8366	EF373533
	R: AGAACAAAGTTACGAGACA							

Note: Tm, annealing temperature. H_o, observed heterozygosity. H_e, expected heterozygosity

M13 universal primers (Invitrogen), and 38 contained simple sequence repeats. PCR primers for amplifying the found microsatellite loci were designed using the Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA) (Table 1). The PCR conditions were optimized through gradient PCR amplification (MJ Research). Only six primer pairs reliably amplified a single polymorphic locus.

To characterize each locus, we genotyped two farmed masked palm civet populations from China (40 individuals; Shanxi and Guangxi provinces). Genomic DNA was extracted from hair and skin tissues using a modified phenol–chloroform extraction method (Sambrook and Russell 2002; Chen et al. 2005). PCR reactions were carried out in a PE9700 thermal cycler using the following conditions in a total volume of 10 μl: 50–100 ng genomic DNA, 0.25 μM of each primer, and 1× PCR buffer containing 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 U *Taq* DNA polymerase (Premix Taq, TaKaRa). The reactions were performed under the following conditions: denaturation at 95°C for 3 min, followed by 35 cycles of 94°C for 30 s, annealing temperature for 30 s (Table 1), 72°C for 30 s, with a final extension step at 72°C for 10 min. Genotyping of the individuals was performed on an ABI 3100 genetic analyzer using 5′ fluorescent-labeled (MedProbe) forward primers.

Results and Discussion

All six loci were highly polymorphic in *Paguma larvata*; the number of alleles ranged from 3 (PC119) to 15 (PC11), with a mean of 8.0 (Fig. 1), and all loci were

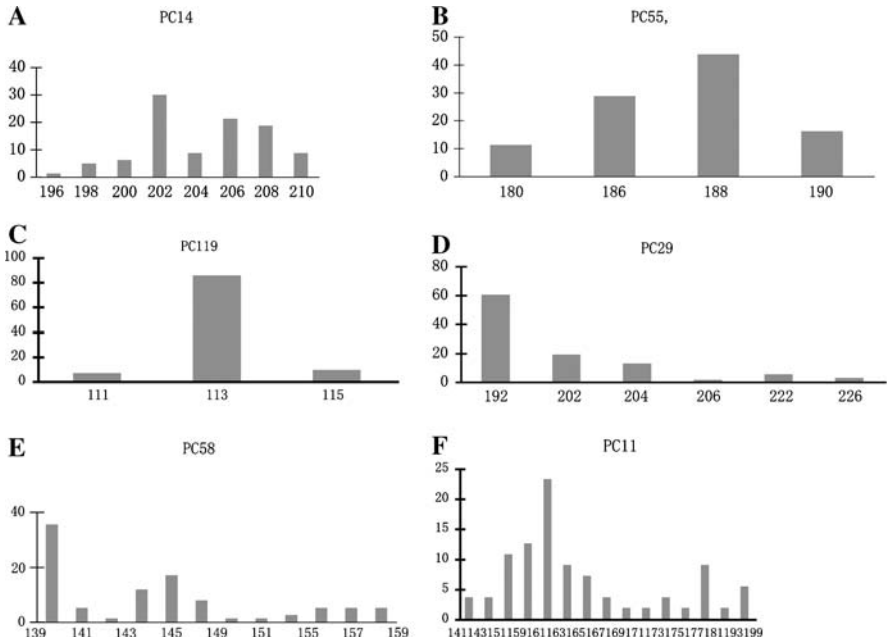

Fig. 1 Allele frequency (on y-axis) and allele base-pair length (on x-axis) for six masked palm civet microsatellite loci (**a–f**) in two farmed populations

dinucleotide repeats. The expected heterozygosity was 0.508 and 0.681 in the two farmed populations. Observed heterozygosity ranged from 0.471 to 0.687 (Table 1). Exact tests for Hardy-Weinberg equilibrium (Guo and Thompson 1992) were computed at each locus using GenePop (Raymond and Rousset 1995); no significant deviations from Hardy-Weinberg equilibrium ($P < 0.01$) were observed in the two populations. Tests for linkage disequilibrium between the polymorphic loci within each species were calculated using FStat version 2.9.3.2 (Goudet 2002). Significant linkage disequilibrium was found only between locus PC55 and locus PC58 in the Guangxi population. Allele frequency distributions showed 15 rare alleles (frequency $< 5\%$) of a total of 48 alleles summed over all loci, for a mean of 31.3% (Fig. 1).

The markers presented in this study will provide a useful tool for monitoring genetic diversity and population structure in the masked palm civet.

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