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A set of microsatellite markers for population genetics of leopard cat (*Prionailurus bengalensis*) and cross-species amplification in other felids

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ABSTRACT

We describe the isolation and characterization of novel microsatellite loci from the leopard cat, *Prionailurus bengalensis* Kerr, 1792 (Family Felidae). Using Illumina HiSeq2500 sequencing technology, we sequenced the leopard cat genome and identified 1.5 million loci of simple sequence repeats with di- to deca-nucleotide motifs. We developed twelve polymorphic markers with tetra-nucleotide motif types after screening 35 loci for amplification and polymorphism. The observed and expected heterozygosities of the markers were 0.438 and 0.423, respectively. The number of alleles per locus ranged from 2 to 7, with a mean polymorphism information content of 0.383. Eleven loci were at Hardy-Weinberg equilibrium and no linkage disequilibrium was detected among any pairs of loci. We tested cross-species amplification of these markers across five other felids (*Panthera tigris*, *P. pardus*, *P. onca*, *Acinonyx jubatus*, and *Felis catus*). All loci were transferable to at least one other feline species and four amplified all five species. The microsatellite markers developed in this study will be valuable for estimating ecological parameters of populations and to establish conservation and management strategies for feline species.

1. Introduction

The leopard cat, *Prionailurus bengalensis* Kerr, 1792 (Family Felidae), has a widespread distribution that extends from South Asia to East Asia, including Afghanistan, India, Indonesia, China, the Russian Far East, the Korean Peninsula, and Japan (Wilson and Reeder, 2005). Occurring across a broad spectrum of habitats such as forests, shrub lands, creeks, and streams (Sunquist and Sunquist, 2002), the species plays an important role in terrestrial ecosystem as a top predator, particularly in South Korea (Lee et al., 2008). The global population trend of leopard cats is recognized as stable (classified as Least Concern in the IUCN), with an abundant population and widespread distribution (Ross et al., 2015). However, urbanization, hunting, and habitat loss

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are causing species decline in some countries such as China, India, and Viet Nam (Yu, 2010; Seto et al., 2012; Willcox et al., 2014). The species is currently listed in Appendix II of the Convention on International Trade of Endangered Species of Wild Fauna and Flora (CITES). In South Korea, the species is protected at the national level as Class II Endangered Wildlife by the Wildlife Protection and Management Act (Act No. 10977).

Despite the significance of ecology, conservation, and management of the leopard cat, there is limited information on the genetic diversity and population structure of the species (Tamada et al., 2008; Mukherjee et al., 2010; Luo et al., 2014). Mukherjee et al. (2010) investigated Indian populations of leopard cat and discovered ecological variables relating to species distribution that drive genetic variation in the species. Luo et al. (2014) collected leopard cat samples from Southeast Asia and revealed an Indochinese-Sundaic phylogeographic divergence of populations. For South Korean populations of the species, Lee et al. (2008) and Tamada et al. (2008) evaluated genetic diversity and geographic variation. All of these studies applied unisexually inherited mitochondrial DNA or sex-chromosomal markers. To our knowledge, to date, no species-specific genome-wide nuclear markers have been developed for the leopard cat.

Microsatellites or simple sequence repeats (SSR) are one of the genome-wide nuclear markers of choice for ecology and conservation because of their high polymorphism, codominant inheritance, ubiquitous abundance, and rapid mutation rate (Goldstein and Schlotterer, 1999; Selkoe and Toonen, 2006). Here, we isolated and characterized twelve microsatellite loci with tetra-nucleotide repeat motifs for population genetic analysis of the leopard cat, using next generation sequencing technology. We also report the utility of these markers by conducting cross-amplification across additional species in the Felidae: *Panthera tigris*, *P. pardus*, *P. onca*, *Acinonyx jubatus* and *Felis catus*.

2. Materials and methods

2.1. Genomic library construction, next generation sequencing and sequence assembly

Genomic DNA of a leopard cat collected in Hwaseong, Gyeonggi, South Korea was isolated using DNeasy Blood & Tissue Kit (Qiagen Korea Ltd., Seoul, South Korea). To identify microsatellite loci in the leopard cat genome, we conducted next generation sequencing. Briefly, genomic DNA fragmented by sonication was end-repaired by the Paired-End DNA Sample Prep Kit (Illumina, San Diego, USA), followed by the addition of 3′-A overhang, and ligation of the adapters. Size-selection for DNA fragments of approximately 500 bp was performed using a gel extraction kit (Qiagen Korea Ltd., Seoul, South Korea). The constructed DNA libraries were quantified using the Quant-iT™ dsDNA High Sensitivity Assay Kit (Invitrogen, Carlsbad, USA) on an Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, USA). After qPCR validation, the resulting libraries were subjected to paired-end sequencing with a 100 bp read length using the Illumina HiSeq 2500 platform. After error-correction using SOAPec, short reads were assembled using SOAPdenovo 2 (v.2.04) implementing the de Brujin graph algorithm (Luo et al., 2012).

2.2. Microsatellite identification and primer design

Microsatellites were identified using SSR Finder (ftp://ftp.gramene.org/pub/gramene/archives/software/scripts/) with the following parameters: (1) microsatellites consist of tandem repeats of between 2 and 10 bp with a minimum of five repeats; (2) no variation (mutation) in repeat motifs was permitted. Primers were designed from the flanking sequences of microsatellite loci using Primer3 (Rozen and Skaletsky, 2000), an integral part of the MSATCOMMANDER v0.8.2 software (Faircloth, 2008), with a product size range of 120–350 bp. To collect primers derived from unique genomic regions, those derived from repetitive DNA sequences as well as duplicated sequences were excluded. The M13 universal forward sequence tag (M13F: 5′-CACGACGTTGTAAAACGAC-3′) (Steffens et al., 1993) was added to the 5'-end of one primer from each pair to allow fluorescent labeling with the 6-carboxy-fluoresceine (6-FAM) dye during amplification (Schuelke, 2000).

2.3. Sample collection, PCR amplification and genotyping

We collected 30 muscle or blood samples from leopard cats, which had been road-killed or rescued from various natural sites of South Korea from 2005 to 2015. Genomic DNA was extracted from these samples and primers were tested for amplification and polymorphism using 30 DNA samples. PCR was performed following protocols used by Eo et al. (2016). Briefly, PCR reactions were performed in a total volume of 20 μL containing 1X PCR buffer, 2.5 mM of $MgCl_2$, 100 μM of dNTPs, 0.04 μM of sequence-specific forward or reverse primers with M13F tag at their 5′-ends, 0.2 μM of reverse or forward primer, 0.2 μM of 6-FAM labeled M13F, 1 U of DiaStar™ *Taq* DNA polymerase, and ~50 ng of genomic DNA. Amplification conditions were as follows: 1 cycle of step 1 (95 °C for 15 min) + 6 cycles of step 2 (94 °C for 1 min, 64 °C–66 °C (varying by locus) for 1 min, 72 °C for 2 min) + 35 cycles of step 3 (94 °C for 30 s, 52 °C for 30 s, 72 °C for 60 s) + 1 cycle of step 4 (72 °C for 5 min). Genotyping was conducted using an ABI 3730xl genetic analyzer (Applied Biosystems) with GeneScan™ 500 LIZ™ fluorescent size standard (Applied Biosystems) and a Peak Scanner™ v1.0 (Applied Biosystems).