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PRIMER NOTE

Isolation and characterization of dinucleotide microsatellite loci in the Asian elephant (*Elephas maximus*)

C. KONGRIT,*§ C. SIRIPUNKAW,*§ W. Y. BROCKELMAN,*† V. AKKARAPATUMWONG,‡ T. F. WRIGHT¶ and L. S. EGGERT§

*Department of Biology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand, †Institute of Science and Technology for Research and Development, Mahidol University, Salaya, Nakhornpathom 73170, Thailand, ‡Institute of Molecular Biology and Genetics, Mahidol University, Salaya, Nakhornpathom 73170, Thailand, §Division of Biological Sciences, University of Missouri, Columbia, MO 65211, USA, ¶Department of Biology, New Mexico State University, Las Cruces, NM 88003, USA

Abstract

The endangered Asian elephant is found today primarily in protected areas. We characterized 18 dinucleotide microsatellite loci in this species. Allelic diversity ranged from three to eight per locus, and observed heterozygosity ranged from 0.200 to 0.842 in a wild population. All loci were in Hardy–Weinberg equilibrium, but linkage disequilibrium was detected between two loci in the wild, but not in the zoo elephants. These loci will be useful for the population-level studies of this species.

Keywords: Asian elephant, *Elephas maximus*, microsatellites, noninvasive, population studies

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Populations of Asian elephants are threatened by poaching, habitat loss and conflicts with humans. Almost all populations are fragmented and restricted to protected areas. For these populations to remain viable, effective management will be needed, which will require information about population sizes, age structure and genetic diversity.

Noninvasive genotyping can be used to obtain these data from free-ranging animals in forested habitats. Although microsatellite loci developed for African elephants can be used in studies of Asian elephants (Eggert *et al*. 2000; Siripunkaw 2003) and five tri- and tetranucleotide loci have been characterized in Asian elephants (Fernando *et al*. 2001), additional polymorphic loci are needed for population studies. We developed dinucleotide microsatellite loci using an enrichment protocol (Hamilton *et al*. 1999) along with a polymerase chain reaction (PCR)-based detection technique.

Genomic DNA was extracted from blood samples of two elephants from the Zoological Society of San Diego's Frozen Zoo using the DNeasy Blood and Tissue Kit (QIAGEN). DNAs were digested with *Nhe*I and *Xmn*I, producing fragments of 200–1000 bp, which were ligated to double stranded SNX linkers. After enrichment for GT repeats using the method of Hamilton *et al*. (1999), the DNA was ligated into pBluescript II KS and recombinant plasmids were transformed into *Escherichia coli* XL1-Blue supercompetent cells (Stratagene) and grown on plates containing ampicillin (Hamilton *et al*. 1999).

Colonies containing recombinant plasmids ($n = 576$) were selected and boiled for 10 min in TE buffer. To detect those containing microsatellites, 1 μL of the colony boil was used as template in 2 PCRs. The first used redesigned versions of primers T3 (AATTAACCCTCACTAAAGGG) and T7 (GTAATACGACTCACTATAGGGC) (Stratagene) in a 25-μL volume containing 1× PCR Gold Buffer (Applied Biosystems), 0.2 mM each dNTP, 0.6 μM each primer, 1.5 mM $MgCl_2$, and 0.5 U Ampli*Taq* Gold DNA polymerase (Applied Biosystems). The second contained these reagents and DNA as well as 0.6 μM $(GT)_{10}$ primer. The PCR was performed in an Eppendorf Mastercycler ep and reactions consisted of denaturation for 10 min at 95 °C, followed by 35 cycles of denaturation at 95 °C for 25 s, primer annealing at 55 °C for 45 s, and primer extension at 72 °C for 2 min, and one final 10-min extension cycle at 72 °C. Products were visualized and compared in a 2% agarose gel stained with Gel Star (Cambrex). Reactions containing two primers (T3/T7) produced single bands, while those containing three primers ($T3/T7/GT_{10}$) produced multiple products with or without a smear (indicating products of different

Correspondence: Chalita Kongrit, Fax: +662 3547161; E-mail: g4536199@student.mahidol.ac.th

Table 1 Characterization of 18 Asian elephant (*Elephas maximus*) microsatellite loci. Repeat motifs, primer sequences, annealing temperature (T_a), allele sizes and numbers (A), expected (H_E) and observed (H_O) heterozygosities for the zoo and Salak-Phra samples and null allele frequency (Nullfreq.); * denotes a significant deviation from HWE (P = 0.003)

				Zoo samples				Salak-Phra samples					
Locus	Repeat motif	Primer sequence (5′–3′)	T_a (°C)	Allele sizes (bp)	A	H_E	H_O	Allele sizes (bp)	A	H_E	H_O	Null freq.	Accession no.
EMU01	$(GT)_{12}$	F: 6-FAM-TTTTCTTTGGTCCCCATGAT R: AGACCTTGGGTCTTGTGCTG	58	78–82	3	0.640	0.769	78–82	3	0.481	0.389	0.058	EF643823
EMU02	$(GT)_4CT(GT)_9$	F: VIC-TGAGGGGAAGGGACGTTAG R: TCCTCAGCCTGCTTTTCACT	58	108–116	2	0.271	0.154	108–116	3	0.528	0.600	–0.118	EF643824
EMU03	$(GT)_6GC(GT)_8$	F: PET-AGAAGCAAAACCCATGAAGC R: TTGAAACTTGCCAGCCTCTT	58	137–143	4	0.579	0.615	137–143	4	0.598	0.467	0.086	EF643825
EMU04	$(TG)_{12}$	F: 6-FAM-TGACTCTCCCTCTTCTGCATC R: GGCTGAGAGGGAAAGAAATTG	58	97–107	6	0.760	0.769	97–105	5	0.760	0.700	0.124	EF643826
EMU05	$(AC)_{10}$	F: VIC-CGAGTGCACGCAAACTGT R: TTATTACGGGGCAGCTGTGT	58	114	1	0.000	0.000	112–118	4	0.233	0.250	–0.129	EF643827
EMU06	$(GT)_9$	F: PET-TTTTTGGGGCTAGAAACTGG R: CCCAGTGTTCAATAGATGCTTT	58	146–158	4	0.403	0.385	148–160	2	0.185	0.200	–0.106	EF643828
EMU07	$(TG)_{15}$	F: 6-FAM-GAGCAGTGCCTTTCGTGAC R: AGCCTGGGAGGTAAGTAGCA	58	102–122	5	0.723	0.615	100–124	7	0.848	0.824	0.002	EF643829
EMU08	$(AC)_9$	F: VIC-TGACTTGGAGGGCATGTTAG R: CGCTCAACCACATGAGTCAG	58	115–127	3	0.342	0.308	115–127	4	0.437	0.353	0.093	EF643830
EMU09	$(TG)_{13}$	F: PET-TCCGTAATTGCACACTTTTAGC R: ATGAGGGGTAATGAGGGTCA	58	163–169	4	0.686	0.846	163–169	4	0.724	0.421	0.200	EF643831
EMU10	$(CA)_{17}$	F: PET-AATCGACTCAGCAGCAACAG R: CCAGTAAATCCATATCACTCGTC	58	94–104	5	0.655	0.539	94–104	4	0.614	0.700	–0.083	EF643832
EMU11	$(TG)_9$	F: 6-FAM-CAATATGGGTGTGGGTTTCC R: GAAATGCAGCATAAATAATATCATGG	58	122–136	5	0.560	0.536	122–130	4	0.529	0.500	0.008	EF643833
EMU12	$(AC)_8$	F: VIC-CCAAAGAAGACCCATGTTCC R: CTGACTATGGGGGAGACTGC	58	120–152	5	0.625	0.615	120–152	5	0.671	0.650	0.014	EF643834
EMU13	$(GT)_{17}$	F: PET-GTATTTGGGCTGGCATGGT R: GTGGGGTCTGTGGTCAAGTG	58	100–110	6	0.812	0.692	102–108	3	0.624	0.429	0.155	EF643835
EMU14	$(GT)_{15}$	F: 6-FAM-GCCTACATGCAGGGTTTGC R: TGAGCCTCTGGCATTTATGA	58	130–138	4	0.665	0.769	130–140	5	0.718	0.842	–0.134	EF643836
EMU15	$(AC)_{14}$	F: VIC-TTCGGGATGTTCTCTTCTGT R: GGGGCTTAACTAATAGGCTTCA	58	142–154	6	0.652	0.539	146–154	5	0.653	0.632	–0.016	EF643837
EMU17	$(GT)_{16}$	F: PET-CACTCAGAGTTCCAAGAAGCAG R: TGCCAGCCATTTCCTCTC	58	119–137	8	0.809	0.769	119–131	6	0.816	0.600	0.102	EF643838
EMU18	$(AC)_{10}$	F: 6-FAM-GGCCCTCATACAGGGATCTT R: GAGAATGGGTTTGGATGAGC	58	138–142	3	0.615	0.308*	138–142	3	0.377	0.375	0.017	EF643839
EMU19	$(GT)_6GC(GT)_6$	F: 6-FAM-GGTGAAAACTGTGGAAGTTGC R: AAAAGCCACCCAGGAGGA	58	99–115	3	0.615	0.692	99–115	3	0.494	0.421	0.041	EF643840

sizes) in microsatellite-containing plasmids. Colonies in which the secondary band or smear was approximately 200-bp smaller than the T3/T7 product were selected and the T3/T7 product was sequenced in both directions in an ABI PRISM 3730 automated DNA sequencer.

Thirty primer pairs were designed using PRIMER 3 (Rozen & Skaletsky 2000), and screened for polymorphism using 13 samples from the Zoological Society of San Diego's Frozen Zoo. Eighteen loci were screened on 20 dung samples from the population of Salak-Phra Wildlife Sanctuary, Thailand. DNA from dung samples was extracted following Boom *et al*. (1999). Amplifications were performed in 12.5-μL volumes containing 15–20 ng template DNA for zoo samples and 2.0 μL of DNA extracted from dung, 1× PCR Gold Buffer (Applied Biosystems), 0.4 μM fluorescently labelled forward primer, 0.4 μM reverse primer, 0.2 mM each dNTP, 2.0 mM $MgCl_2$ and 0.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems). The PCR profile for the zoo samples consisted of 10-min denaturation at 95 °C, followed by 40 cycles of 45-s denaturation at 95 °C, 45-s primer annealing at 58 °C, 1-min primer extension at 72 °C, followed by a final extension of 10 min at 72 °C. For Salak-Phra samples, cycles were amended as follows: denaturation for 1 min, primer annealing for 1 min, and a final extension time of 15 min. Fragment sizes were determined in an ABI PRISM 3730 Genetic Analyser. Scoring of Salak-Phra samples was confirmed twice for heterozygotes and three times for homozygotes.

All loci were polymorphic with three to eight alleles (Table 1). While EMU05 was monomorphic in zoo samples, it was polymorphic in Salak-Phra samples. Observed heterozygosities ranged from 0.154 to 0.846 in zoo samples (not including EMU05) and 0.200–0.842 in Salak-Phra samples. We tested for departures from Hardy–Weinberg equilibrium (HWE) and for linkage disequilibrium (LD) between loci using GENEPOP 3.4 (Raymond & Rousset 1995). For zoo samples, all loci except EMU18 were in HWE and no LD was found after a Bonferroni correction was applied (Rice 1989). The departure from HWE was not surprising, as zoo samples do not represent a natural population. For Salak-Phra samples, no deviation from HWE was found but LD was detected between EMU04 and EMU09, likely due to small sample size. Null allele frequencies were calculated (Table 1) using MICRO-CHECKER (van Oosterhout *et al*. 2004) and no large allele dropout was detected in Salak-Phra samples.

These loci will be useful in our study of population structure and population genetics of wild Asian elephant populations.

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