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Human Short Tandem Repeat (STR) Markers for Paternity Testing in Pig-Tailed Macaques

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This study investigated the use of human short tandem repeat (STR) or microsatellite loci markers for assessing paternity and genetic structure of pig-tailed macaques (*Macaca nemestrina*) breeding colony. Four human microsatellite primer pairs located at human map position D1S548, D3S1768, D5S820, and D2S1777, were amplified by polymerase chain reaction (PCR) for pig-tailed macaques. Four loci were found to be clearly and reliably amplified, and three loci exhibited high levels of genetic heterogeneity. These loci were sufficiently informative to differentiate discretely between related and unrelated pairs.

Key words: human short tandem repeats (STRs), paternity testing, pig-tailed macaques, *Macaca nemestrina*

INTRODUCTION

The importance of genetic management as a critical component of the overall management of non-human primate colonies is now widely recognized. The current value ascribed to genetic management is due to change in the primate availability and in the research with non-human primates that has occurred over the last 20 years. The availability of wild-caught primates has decreased during this period of time. Therefore, a management that assures the long-term viability of self sustaining captive non-human primate colonies is a need.

Two kinds of approaches are commonly used in genetic management. The first approach is conservation-oriented genetic management techniques that are conceived to achieve the goals of population conservation, particularly in zoo situations and reintroduction of the population into wild. It will supply pedigree information with the goals of maintaining genetic variability and avoiding inbreeding depression. On the other hand, genetic management programs are also important in the research, although the goals may differ from those in conservation-oriented programs. Therefore, the second approach i.e. is used to research-oriented genetic management tries to discover maximum viability of the colony in relation to the production of research subjects, and to produce groups of unrelated animals for use in laboratory. It will result in producing well-characterized animals for experimental requirements (Williams-Blangero *et al.* 2002). Genetic management programs have been applied successfully in the management of rhesus macaques, *Macaca mulatta* (Smith *et al.* 2000; Andrade *et al.* 2004), captive colony of vervets (*Chlorocebus aethiops sabaeus*) (Newman *et al.* 2002), and *Saimiri* breeding colony (Lavergne *et al.* 2003). In these studies, they used genetic variation assessed by microsatellite genotyping to examine the genetic structure, relatedness, and paternity.

The human genome project has stimulated the identification and characterization of thousands of short tandem repeat (STR) loci also known as microsatellites. They have a mutation rate range from 10^{-6} to 10^{-2} per generation (Schlotterer 2000), that made STRs valuable for a wide range of biological studies from linkage mapping to paternity assessment. The results from employing STRs analysis will give information about pedigree relationships of the colony. Pedigree relationships will be taken into account if the researchers would like to have genetically independent animal subjects, or they would like to use shared genetic background animals.

Microsatellite loci consist of a tandemly repeated motif of one to six nucleotides. Furthermore, they are extremely common forms of DNA replication mutation scattered throughout genome. Compare with other markers, microsatellite have many advantages over alternative polymorphic systems, including locus specificity, the small amount of test DNA required, absolute sizing of alleles, and fast screening. Such markers are highly informative for estimating reproductive success (Pope 1990), reconstructing genealogical relationships (Ishibashi *et al*. 1997), characterizing dispersal patterns (Taylor *et al.* 1997) among groups of non-human primates and estimating kinship (Smith *et al.* 2000; Newman *et al.* 2002).

This study presents the results of a pilot study to assess the utility and feasibility of using human STR markers for individuals identification and determining parentage in a colony of pig-tailed macaques (*Macaca nemestrina*).

MATERIALS AND METHODS

Blood samples were supplied by the courtesy of the Primate Research Center, Bogor Agricultural University. The pig-tailed macaques used in this study was originated from Sumatra.

Whole blood was separated into erythrocytes, plasma, and buffy coat by centrifugation at 3,000 rpm for 15 min.

Genomic DNAs were extracted from buffy coat by the method of Kan *et al.* (1977). Four volume of 0.2%-1 mM EDTA were added to the buffy coat. After red blood cell lysis, the buffy coat were collected by centrifugation at 2,000 rpm for 10 min. Hemolysate was removed, then buffy coat was washed with 0.9% NaCl-1 mM EDTA. Centrifugation was performed at 2,000 rpm for 10 min. Supernatant was discarded and pellet was suspended in salted Tris-EDTA (STE), 10% SDS, and digested with proteinase-K (5 mg/ml) for minimum two hrs at 37 ^{o}C. Then genomic DNA was extracted with phenol/chloroform method. Genomic DNA was separated by centrifugation at 2,800 rpm for ten min and removed to sample tube for further analysis.

Amplification of Microsatellite. A sample of 42 pig-tailed macaques was screened for each of the four tetranucleotide microsatellite loci (Table 1). The loci were chosen based on the results of previous study on long-tailed macaques (Perwitasari-Farajallah *et al*. 2004). The PCR mixture contained 25 mM $MgCl_2$, 0.83 U/µl Taq polymerase (PROMEGA), 10 x PCR buffer, 2.5 mM dNTP, and 25 pM of each forward and reverse primer. The total volume of each PCR was 12.5 µl. Initial thermal cycle routines utilized PCR methods as described by Perwitasari-Farajallah *et al.* (2004).

Amplification was performed in 30 cycles using the following conditions: denaturation at 94 ^{o}C for 40 sec, annealing at 48-57 ^{o}C for 40-60 sec, and extension at 70-71 ^{o}C for 50-60 sec, followed by a 5 min final extension at 72 ^{o}C. PCR fragments were separated in a 5 and 10% polyacrylamide gel and silver-stained following the technique of Tegelström (1986). DNA size marker of 20 bp ladder (BIORAD) was loaded on each gel to provide standards for sizing microsatellite.

Data Analysis. The amount of variation was measured by average heterozygosity (H_o: observed heterozygosity). Gene diversity for microsatellite data was estimated by Nei (1987) equation as follows:

$$\overline{H} = 1 - \sum_{i=1}^{m} X_i^2$$

where X_i is frequency of the i-th allele and m is the number of alleles and average proportion of heterozygosity ($\overline{H}$) is the average over all loci.

Parentage Analysis. Paternity was determined using a simple exclusionary method (Jones & Ardren 2003), whereby genotypes from mother/offspring pairs were compared in order to confirm maternity and to determine which of the alleles of the offspring had inherited from its father. If a potential sire did not posses this allele, he was counted as excluded at that locus. In cases where mother/offspring pairs had identical genotypes, a potential sire was excluded only if he shared no alleles with an offspring at a locus. I minimized the influence of typing errors on the results of exclusions by retyping any putative male-offspring pairs that mismatched at only 1-2 loci to ensure that exclusions reliably identified a single candidate male. Given the number of loci used and their high levels of polymorphism, it is unlikely that other males in the population would match the offspring at all loci by chance. Furthermore probabilities both single-parent (PE2) and non-parent PE1 exclusion (Jamieson & Taylor 1997) were computed for each locus which assume Hardy Weinberg equilibrium conditions and identical gene frequencies in males and females.

RESULTS

Microsatellite Variations. Of the four loci screened, all loci of the pig-tailed samples were successfully amplified and could be sufficiently resolved to unambiguous quantity allele size. The number of alleles detected in the 42 pig-tailed macaques and heterozygosity for each locus was given in Table 2 and 3. All loci were polymorphic. The number of alleles varied between 2 and 11. Furthermore, heterozygosity of the loci ranged 0.0465 to 0.8244. The most polymorphic loci, D3S1768 (11 alleles) showed heterozygosity of 0.8244.

Allele size ranged from 166-200, 126-134, 174-236, and 152-200 bp for markers D1S548, D2S1777, D3S1768, and D5S820, respectively. The STR markers appear to be as variable in pig-tailed macaques as in human without any general tendency to shorter or longer alleles in pig-tailed macaques.

Paternity Discrimination. I selected two of four polymorphic loci for further paternity discrimination based on PE1 values exceeding 0.5 (Table 3). Genotypes of the parent/offspring triads were determined in two loci, D1S548 and D3S1768 (Table 4 & Figure 1). The results showed that two individuals in colony were presumably descendants of male 55.

DISCUSSION

Microsatellite Variations. Previous study in long-tailed macaques, *M. fascicularis* (Perwitasari-Farajallah *et al.* 2004) and rhesus macaques, *M. mulatta* (Morin *et al.* 1997) indicated that three microsatellite loci (D1S548, D3S1768, and D5S820) revealed high levels of average heterozygosity (Table 5).

Table 1. Primer name, repeat type, primer sequences, size in base pairs for the four microsatellite loci used in this study

Locus	Accession ID	Repeat motif	Primer sequences(5'→ 3')	Fragment size (bp) in human
D1S548	GDB:228890	tetra	F : GAACTCATTGGCAAAAGGAA	212
			R : GCCTCTTTGTTGCAGTGATT	
D2S1777	GDB:693873	tetra	F : TCCCCAAGTAAAGCATTGAG	242
			R : GTATGTAGGTAGGGAGGCAGG	
D3S1768	GDB:228929	tetra	F : GGTTGCTGCCAAAGATTAGA	197
			R : CACTGTGATTTGCTGTTGGA	
D5S820	GDB:228910	tetra	F : ATTGCATGGCAACTCTTCTC	199
			R : GTTCTTCAGGGAAACAGAACC	

F: forward, R: reverse

D1S548 and D5S820 were polymorphic loci in Taiwanese macaques, *M. cyclopis* (Chu *et al.* 1999), and rhesus macaques (Nurnberg *et al.* 1998). In addition, D1S548 and D3S1768 were found to be polymorphic in rhesus macaques (Kanthaswamy *et al.* 2006). Present study depicted that D2S1777 was polymorphic locus. It has not been detected before in other macaques. Nevertheless Ely *et al.* (1998) reported that this locus showed high polymorphism in chimpanzees. The present result suggests that these four loci will be useful for estimating genetic variability in the pig-tailed macaques, *M. nemestrina.*

Genetic variability in non-human primates is important in biomedical research. Genetic diversity among individuals of the same species may partially account for the differences in susceptibility to infection and to the progression of viral diseases (Cohen 1992). Furthermore, the maintenance of genetic variability is a fundamental goal in the genetic management of any captive breeding colony (Ely *et al.* 1994).

Paternity Discrimination. For the samples studied here, microsatellite loci were informative to virtually ensure paternity exclusion in multimale/multifemale groups even when the female parent of the animal's genotype is not known (Morin *et al.* 1997). In addition, the ability to characterize any given pig-tailed macaques uniquely and genetically warrants that any suspected case of mistaken identity can be easily resolved. Given the cost and effort required to characterize genotypes for all loci, the three informative loci (D1S548, D3S1768, and D5S820) should be enough for routine screening.

Table 2. Allele frequencies and fragment size (bp) of microsatellite loci observed in pig-tailed macaques

Locus	Allele	Fragment size (bp)	Frequency ± SE
D1S548	A	200	0.1786 ± 0.0418
	B	192	0.1071 ± 0.0337
	C	180	0.2500 ± 0.0472
	D	172	0.2738 ± 0.0487
	E	168	0.0833 ± 0.0091
	F	164	0.1072 ± 0.0338
D2S1777	A	134	0.9762 ± 0.0166
	B	126	0.0238 ± 0.0166
D3S1768	A	236	0.0769 ± 0.0302
	B	232	0.0897 ± 0.0324
	C	228	0.0769 ± 0.0302
	D	224	0.2436 ± 0.0486
	E	220	0.0256 ± 0.0179
	F	216	0.2949 ± 0.0516
	G	208	0.0128 ± 0.0127
	H	200	0.0385 ± 0.0218
	I	192	0.0385 ± 0.0218
	J	180	0.0641 ± 0.0277
	K	174	0.0385 ± 0.0218
D5S820	A	200	0.0476 ± 0.0232
	B	188	0.0238 ± 0.0166
	C	180	0.2262 ± 0.0456
	D	176	0.0238 ± 0.0166
	E	172	0.2500 ± 0.0472
	F	168	0.0953 ± 0.0320
	G	160	0.2976 ± 0.0499
	H	156	0.0238 ± 0.0166
	I	152	0.0119 ± 0.0118

Table 4. Allele types detected with two sets of primers

No.		Individual number	Allele type	
			D1S548	D3S1768
1a	Mother	50	C C	D D
b	Offspring	58	C F	D D
	Father	57	B F	D D
2a	Mother	71	A C	D E
b	Offspring	84	A E	D J
	Father	74	A E	C J
3a	Mother	72	C D	D D
b	Offspring	85	A D	D D
	Father	55	A D	D H
4a	Mother	73	C E	A D
b	Offspring	86	A E	D D
	Father	55	A D	D H
5a	Mother	75	A A	C D
b	Offspring	87	A E	B D
	Father	67	A E	B D

Underlined alleles were inherited from the putative fathers

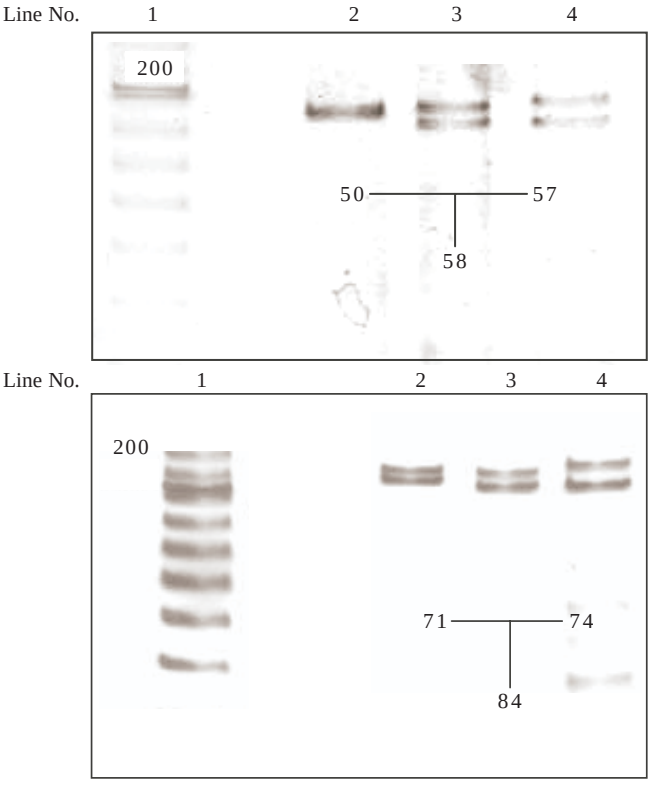

Figure 1. Representative results of electrophoresis of microsatellite fragments. a. Primer sets shown here were D1S548 and b. D3S1768. Males 57 and 74 was putative father of offspring 58 and 84, respectively. a. Lane 1: 20 bp marker, lane 2: female 50 (genotype CC), lane 3: offspring 58 (genotype CF), lane 4: male 57 (genotype BF); b. Lane 1: 20 bp marker, lane 2: male 71 (genotype CJ), lane 3: offspring 84 (genotype DJ), lane 4: female 71 (genotype DE).

Table 3. Screening of four microsatellites loci for 42 pig-tailed macaques

Locus	Number of alleles	Allele size (bp)	Observed heterozygosity (H_o)	Average heterozygosity (H)	PE1	PE2
D1S548	6	166-200	0.8007	0.6141 ± 0.1893	0.635	0.460
D2S1777	2	126-134	0.0465		0.247	0.147
D3S1768	11	174-236	0.8244		0.619	0.441
D5S820	9	152-200	0.7846		0.457	0.285

Table 5. Heterozygosity comparison of polymorphic loci in *M. nemestrina*, *M. mulatta*, *M. cyclopis*, *Pan troglodytes troglodytes*, and *M. fascicularis*

	Species	Aim of study	n	Locus			
				D1S548	D2S1777	D3S1768	D5S820
				h	h	h	h
Present study	*M. nemestrina*	Paternity discrimination	42	0.8007	0.0465	0.8244	0.7846
Morin *et al*. (1997)	*M. mulatta*	Colony management	176	0.7400	-	0.8700	0.8200
Nurnberg *et al*. (1998)	*M. mulatta*	Genetic tracking (conservation)	400 <<	-	-	-	0.7400
Ely *et al*. (1998)	*Pan troglodytes*	Individual identification and paternity determination	41	-	0.8610	-	-
Chu *et al*. (1999)	*M. cyclopis*	Paternity identification	12	-	-	-	0.7500
Goossens *et al*. (2000)	*Pan troglodytes troglodytes*	Population genetics	59	0.7100	-	-	-
Perwitasari-Farajallah *et al*. (2004)	*M. fascicularis*	Collecting basic data	84	0.6391	-	0.8267	0.8525

h: heterozygosity, n: sample size

More than one offspring sired by male 55 appears to have behavioural implications. There are two plausible explanations for this finding, although behavioural observations of this colony have not been conducted.

Dominance rank is the factor correlated with reproductive success and the influence of the mating strategy that is used by an individual. However middle and low-ranking males may have a greater chance of success if they can perform a consortship with a female. Alpha males and high-ranking males in chimpanzees, *P. t. troglodytes* were responsible for 45 and 50% conception, respectively (Constable *et al.* 2001). The importance of high rank to reproductive success has been demonstrated in other primates, including long-tailed macaques, *M. fascicularis* (de Ruiter *et al.* 1994), stump-tailed macaques, *M. arctoides* (Bauers & Hearn 1994), and bonobos, *P. paniscus* (Gerloff *et al.* 1999). In addition, paternity analysis of the Jamaican fruit-eating bat (*Artibeus jamaicensis*) showed that dominant males sired most of the offspring in groups, and they maintained their dominant status from one season to the next (Ortega *et al*. 2003). It indicated that they have the highest lifetime reproductive success among all adult males.

Sexual size dimorphism is common in vertebrate species and males are usually the larger sex. Most studies proposed that sexual dimorphism arises from competition between males for access to females or from female preference/female choice (Balmford & Read 1991). Field study conducted by Kraaijeveld-Smit *et al.* (2003) revealed that larger males were more successful in siring offspring than smaller males in the population of carnivorous marsupial, *Antechinus agilis*. Therefore, male weight is a likely factor that can be taken into account for present study.

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