Genetic diversity of slow loris (*Nycticebus coucang*) based on mitochondrial DNA

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ABSTRACT Genetic Diversity of the Slow Loris (*Nycticebus coucang*) based on mitochondrial 12S rRNA genes. Research on the genetic diversity of the slow loris *Nycticebus coucang* (kukang) was conducted using DNA collected from the blood and tissue of 12 individuals collected from three locations (Sumedang and Jember in Java, and Lampung in Sumatra).

The mitochondrial 12S rRNA nucleotide sequences were determined to investigate the genetic diversity of this species. The regions were amplified by PCR using L1091 and H1478 primers. As a result of the 386bp sequence analysis, five haplotypes were found, two from Java and three from Sumatra.

Key words : genetic diversity, mitochondrial DNA, *Nycticebus coucang*, slow loris.

The Slow loris (*Nycticebus coucang*) from the genus *Nycticebus*, is distributed from South to Southeast Asia (Lekagul & Mcneely, 1977). Within Indonesia, this species can be found in Sumatra, Java and Kalimantan. The wild population has been in significant decline due to poaching, capturing for selling as pets and habitat degradation (IUCN, 1987). The species currently enjoys protected status based under both "Undang-undang dan Peraturan Perlindungan Binatang Liar" 1931 and Appendix II of the CITES convention (Anonymous, 1996).

Taxonomically, *N. coucang* is known to have three subspecies in Indonesia, which are spread across Kalimantan (*N.c. menangensis*), Java (*N.c. javanicus*) and Sumatra (*N.c. coucang*) (Groves, 1971; PHPA, 1978). However, some morphological reviews have classified the animal into as many as six subspecies (Chasen, 1940; Strein, 1986; Corbet & Hill, 1992). Such similar morphological characters make it extremely difficult to establish the identity (i.e., the subspecies name), and therefore the original location of individual animals taken by the animal trade. That is to say, that until permanent characteristics are established for each subspecies, the conservation of this species in the wild will remain extremely difficult. As such, it was important to explore particular markers so as to determine the genetic characteristics of each subspecies with molecular DNA. For this reason we conducted a genetic analysis on *N. coucang* based on mitochondria 12S rRNA genes to determine genetic diversity and nucleotide differences between populations.

MATERIALS AND METHOD

Sampling locations: Samples representing the three subspecies of *Nycticebus coucang* were collected from five locations: Sumedang (6), Jember (2), Lampung (4), Jambi (2), and Kalimantan (1).

DNA extraction: Total DNA was extracted from blood and tissue (liver, kidney, pulmonary and heart) following standard procedures (Sambrook *et al.*, 1989) using phenol-chloroform.

Amplification and sequencing: Amplification using PCR was performed in 50 ul reactions, each containing buffer 10x 5 ul, 10mM dNTP 4 ul, 2pm Primer (L&H) 5 ul, Taq polymerase 1.25 U 1 ul, DNA template 1-2 ul and distilled water 29 ul. The PCR amplification was performed by denaturation in a 9600 Perkin Elmer machine using the following thermal cycles; 95°C for 30 sec., annealing 55°C for 30 sec, and extension 72°C for 30 sec for 40 cycles, with H1478 and L1091 primers as described by Kocher *et al.* (1989). Amplification products were resolved by electrophoresis in a 1% Seakem agarose gel in 1X Tris-Acetate EDTA (1xTAE) buffer and stained with ethidium bromide to visualize the DNA.

Sequencing: Sequences were determined using PCR products and an automatic sequencer. PCR products were purified using a spin column (Amersham-Pharmacia), following the manufacture's protocol. Direct sequencing was performed using a Thermosequenase dye primer cycle sequencing kit (Amersham-Pharmacia) with an ALFred DNA sequencer. The sequencing reactions were run on 50% stock Long Ranger™ gel solution Acrylamide for 12 hours (over night).

Data analysis: 386 base-pair sequence nucleotides were used for the analysis. Alignment sequences were created using Clustal X (Jeanmougin *et al.*, 1998). Intra and inter population haplotype diversity (h) and nucleotide diversity (π) were estimated following Nei (1987). A phylogenetic tree was constructed using Neighbor in the PHYLIP program (Felstein, 1995).

RESULTS AND DISCUSSION

Sequences as long as 386 base-pair nucleotides could be obtained from only 12 of the 15 individuals sampled. The remaining three individuals could not be either sequenced or analyzed. Thus, the genetic variety of one individual from Kalimantan (*N.c. menangensis*) and both individuals from Jambi (*N.c. concang*) could not be calculated.

Variation of DNA Sequences:

The sequence alignments of 386 bp of gene 12S rRNA DNA mitochondria from the 12 *Nycticebus concang* individuals can be seen in Fig. 1. There are 9 varied (polymorphic) sites. All site variations indicate transitional events and each of the positions underwent one or more base change. This is in accordance with Greenberg *et al.* (1983), who stated that in closely connected populations, transitional events are expected to dominate sequence variation.

CLUSTAL X (1.8) multiple sequence alignment

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SM4       -TTGCCCTATTCAATTAAGCTCTCTATTCTTAATTTACTTCTAAATCCGCCTTAATCACT
SM1       ATTGCCCTATTCAATTAAGCTCTCTATTCTTAATTTACTTCTAAATCCGCCTTAATCACT
SM2       ---GCCCTATTCAATTAAGCTCTCTATTCTTAATTTACTTCTAAATCCGCCTTAATCACT
JM1       ---GCCCTATTCAATTAAGCTCTCTATTCTTAATTTACTTCTAAATCCGCCTTAATCACT
SM3       ------------------------------------TTACTTCTAAATCCGCCTTAATCACT
LP3       ---------TTCAATTAAGCTCTCTATTCTTAATTTACTTCTAAATCCGCCTTAATCACT
SM5       --------ATTCAATTAAGCTCTCTATTCTTAATTTACTTCTAAATCCGCCTTAATCACT
JM2       -------TATTCAATTAAGCTCTCTATTCTTAATTTACTTCTAAATCCGCCTTAATCACT
SM6       -----CCTATTCAATTAAGCTCTCTATTCTTAATTTACTTCTAAATCCGCCTTAATCACT
LP2       ------------------------------------TTACTTCTAAATCCGCCTTAACCACT
LP1       ---GCCCTATTCAATTAAGCTCTCTATTCTTAATTTACTTCTAAATCCGCCTTAACCACT
LP4       --TGCCCTATTCAATTAAGCTCTCTATTCTTAATTTACTTCTAAATCCGCCTTAACCACT
                                                                  *

SM4       TTTTTCATAAGGGGTGGCGTTAATTGTTCTGTGAAGATAGAAAATGTAGCCCATTTCTTC
SM1       TTTTTCATAAGGGGTGGCGTTAATTGTTCTGTGAAGATAGAAAATGTAGCCCATTTCTTC
SM2       TTTTTCATAAGGGGTGGCGTTAATTGTTCTGTGAAGATAGAAAATGTAGCCCATTTCTTC
JM1       TTTTTCATAAGGGGTGGCGTTAATTGTTCTGTGAAGATAGAAAATGTAGCCCATTTCTTC
SM3       TTTTTCATAAGGGGTGGCGTTAATTGTTCTGTGAAGATAGAAAATGTAGCCCATTTCTTC
LP3       TTTTTCATAAGGGGTGGCGTTAATTGTTCTGTGAGGATAGAAAATGTAGCCCATTTCTTC
SM5       TTTTTCATAAGGGGTGGCGTTAATTGTTCTGTGAAGATAGAAAATGTAGCCCATTTCTTC
JM2       TTTTTCATAAGGGGTGGCGTTAATTGTTCTGTGAAGATAGAAAATGTAGCCCATTTCTTC
SM6       TTTTTCATAAGGGGTGGCGTTAATTGTTCTGTGAAGATAGAAAATGTAGCCCATTTCTTC
LP2       TTTTTCATAAGGGGTGGCGTTAGTTGTTCTGTGAGGATAGAAAATGTAGCCCATTTCTTC
LP1       TTTTTCATAAGGGGTGGCGTTAGTTGTTCTGTGAGGATAGAAAATGTAGCCCATTTCTTC
LP4       TTTTTCATAAGGGGTGGCGTTAGTTGTTCTGTGAGGATAGAAAATGTAGCCCATTTCTTC
                              *           *

SM4       CCACCTCATAGACTACACCTTGACCTAACGTTTTAATGTGTGGTTCTCCTGCTTACTATG
SM1       CCACCTCATAGACTACACCTTGACCTAACGTTTTAATGTGTGGTTCTCCTGCTTACTATG
SM2       CCACCTCATAGACTACACCTTGACCTAACGTTTTAATGTGTGGTTCTCCTGCTTACTATG
JM1       CCACCTCATAGACTACACCTTGACCTAACGTTTTAATGTGTGGTTCTCCTGCTTACTATG
SM3       CCACCTCATAGACTACACCTTGACCTAACGTTTTAATGTGTGGTTCTCCTGCTTACTATG
LP3       CCACCTCATAGACTACACCTTGACCTAACGTTTTAATGTGTAGTTCTCCTGCTTACTATG
SM5       CCACCTCATAGACTACACCTTGACCTAACGTTTTAATGTGTGGTTCTCCTGCTTACTATG
JM2       CCACCTCATAGACTACACCTTGACCTAACGTTTTAATGTGTGGTTCTCCTGCTTACTATG
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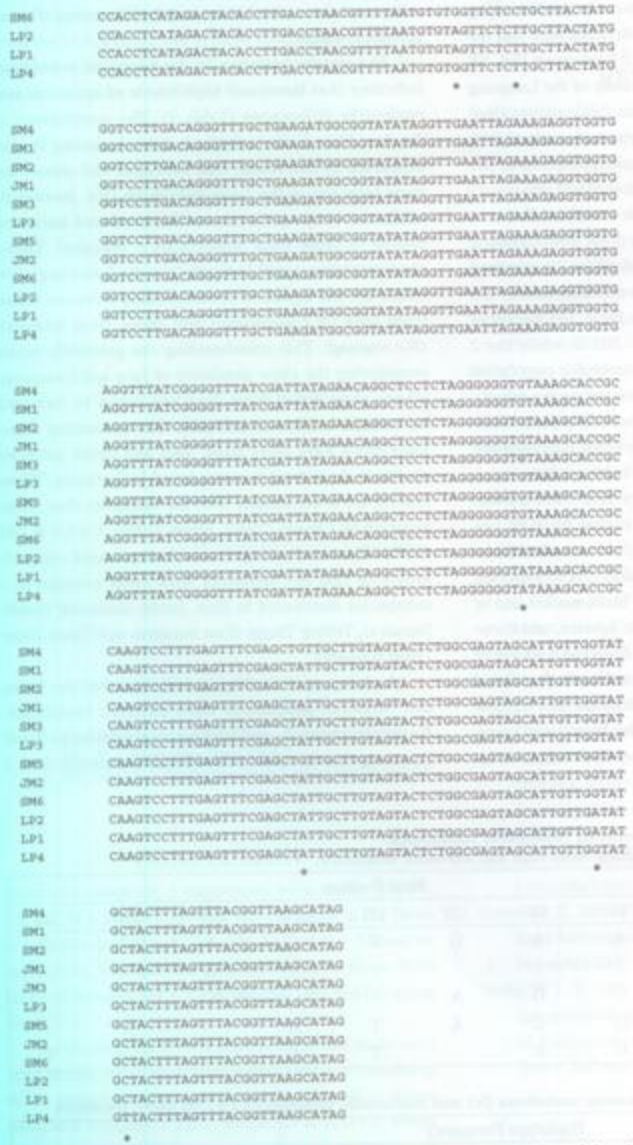

Fig. 1. Sequence alignment 386 bp of 12S rRNA mtDNA gene. Asterisks show the positions of the 9 polymorphic sites.

Genetic Variation:

From the nine polymorphic sites, 5 haplotypes were found (JA, JB, SA, SB, and SC) (Table 1). The frequency and genetic variation of the haplotypes can be seen in Table

2. The JA and JB haplotypes were found in the Sumedang individuals, and the two Jember individuals both had the same haplotype (JB). Meanwhile, 3 haplotypes (SA, SB and SC) were found in the 4 individuals of the Lampung population. The haplotype frequency calculation (Nei, 1987) indicates that the JB haplotype frequency (0,500) is higher than the other four haplotypes, and is the dominant characteristic of the Javanese Slow Loris.

This result indicates that genetic variation is lower in Javan Slow Loris' (h = 0,242) than in those from Lampung (Sumatra) (h = 0,714), as characterized by 2 haplotypes from 8 individuals (Table 2). This is also indicated by the nucleotide differences between the Javan and Sumatran (Lampung) populations i.e., π = 0.00205, while the 2 Jember individuals indicate a monomorphic population (it should be noted that this is only a temporary result). The decrease in the genetic variation of the Javan Slow Loris could be due to several factors that including the destruction and exploitation of their habitat for plantation use, which tends to increase every year (Mackinnon, 1987). In addition, uncontrolled illegal hunting can decrease natural populations, which in turn decreases the effective population size. Nursahid (2000) reported that the selling of slow loris on the black market and in malls is the third position of primate species, and these markets are generally found in Java. Based on a survey of slow lories in animal markets, there are more slow lories sold in Java than in Sumatra or Kalimantan. The highest frequency of hunting has been in the West Javan areas of Sumedang and Malimping. Hence, there needs to be some control over the habitat and the harvesting of these animals in nature.

The genetic variation in the Lampung population indicates that there are high levels of variation and nucleotide differences (Table 2). The morphology of the four Slow Loris individuals from Lampung is also supported by this result. One individual had almost the same body size as the Javan individuals (*N.c. javanicus*), while the stripe patterns on the head and back are similar to those from Sumatra (*N.c. coucang*). These characteristics could indicate that the Lampung slow lories are the result of crossbreeding between those from Java (*N.c.javanicus*) and those from Sumatra (*N.c.coucang*). This crossbreeding can potentially occur considering the close proximity of Java and Lampung, where slow lories from Java are taken to Sumatra by animals traders or privately. The remaining three individuals have body sizes, spot and stripe patterns similar to those from Sumatra found in Lampung. These criteria are in accordance with the Sumatran slow lories reported by Chasen (1940), Strein (1986), Corbet & Hill (1992). The results of their morphology-based research indicated that the Sumatran Slow Loris consists of 4 subspecies distributed in Riau, Jambi, Lampung (South Sumatra), Tebing Tinggi (East Sumatra) and Tanah Datar (West Sumatra).

A philogeny tree (Fig. 2) indicates that the Javan and Sumatran populations show different haplotypes. However, there is one ambiguous haplotype from Lampung that is closer to the Javan population than it

Tabel 1. Polymorphic positions between Slow Loris haplotypes.
A period denotes a matching base with the top-most sequence.

Haplotype	Base Position								
	22	49	61	129	135	255	289	323	328
JB	T	A	A	G	C	G	A	G	C
JA	.	.	.	.	.	.	G	.	.
SA	.	.	G	A	.	.	.	.	.
SB	C	G	G	A	T	A	.	A	.
SC	C	G	G	.	T	A	.	.	T

Table 2. Haplotype frequencies, genetic variations (h) and nucleotide differences (π) on three locations

Location	Haplotype Frequency							
	A	B	C	D	E	h	π	n
Lampung	–	–	0.50	0.25	0.25	0.714	0.00129	4
Sumedang	0.333	0.667	–	–	–	0.484	0.00063	6
Jember	–	1	–	–	–	0	0	2
Java-Sumatra	0.167	0.500	0.0183	0.167	0.167	0.709	0.00205	12

is to Lampung. This haplotype differs in two base pairs from the JA haplotype and in three base pairs from the JB (Java). It differs from the SB and SC haplotypes by five and six base pairs respectively (Lampung).

Fig. 2. Phylogenetic tree (Neighbor – Joining Method).
JA, JB = Haplotype from Java
SA, SB, SC = Haplotype from Lampung (Sumatra)

CONCLUSION

From this research, 5 haplotypes were found (JA, JB, SA, SB, SC); two of these (JA, JB) are found in the Javan population and three (SA, SB, SC) in the Sumatran population. The genetic variation between those from Java and Sumatra is $h = 0{,}709$, and that Java and Lampung ($h = 0{,}714$).

As the samples of each subspecies in this study were very limited, there should be further research examining more samples from each location, as well as some additional locations of each subspecies in order to obtain more complete information regarding the existence of slow lories in nature.

ACKNOWLEDGEMENT This study was a part of the research activities of the Biodiversity Conservation Project – JICA (Japan International Cooperation Agency) and APPERI.

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Received 13th Mar. 2006
Accepted 18th May 2006