Genetic Variation of Mitochondrial Cytochrome *b* Genes among the Subspecies of Koala, *Phascolarctos cinereus*

Kazutoshi TAKAMI*, Mitsutoshi YOSHIDA[1)], Yoshihiro YAMAMOTO[2)], Masashi HARADA[3)] and Jun-ichi FURUYAMA[2)]

Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, Shizuoka 422–8529, [1)]Laboratory of Animal Reproduction, Faculty of Agriculture, Kagoshima University, Kagoshima 890–0065, [2)]Department of Genetics, Hyogo College of Medicine, Nishinomiya, Hyogo 663–8131, and [3)]Laboratory Animal Center, Osaka City University Medical School, Abeno, Osaka 545–0051, Japan

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ABSTRACT. A conserved DNA region among the subspecies of koala, of mitochondrial cytochrome *b* gene, was employed to analyze the genetic variation among 3 available subspecies of koala. This conserved sequence, 307 bp DNA, was sequenced using polymerase chain reaction and direct DNA sequencing technique. Substitutions in the nucleotide sequences were observed, with which koalas can be divided into 3 DNA haplotypes subspecies, but the molecular data provided inconsistency with current classification of the 3 subspecies of koala. — KEY WORDS: koala, mitochondrial cytochrome *b* gene, subspecies.

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Koala is the sole member of the *Phascolarctidae* family and has an extensive but disjunct distribution from northern Queensland to southern Victoria in Australia [8]. Koala is classified into 3 subspecies based on their morphological and geographical characters: *Phascolarctos cinereus (P. c.) victor* in Victoria, *P. c. cinereus* in New South Wales and *P. c. adustus* in Queensland [15]. Early of this century, the koalas have almost vanished in Victoria and New South Wales as a result of habitat reduction, epidemic disease and hunting for fur. There still was a large population in Queensland. Following a near-extinction, south-eastern Australian koala's populations were reestablished mainly since the French Island colony which has been founded with a few individuals [2]. This history is believed to affect the level of genetic diversity among the populations of the koala's subspecies. In captivity, the number of koalas has been increasing due to success in captive breeding. In the koala preserves, the excess animals are being relocated to other areas. Hence, elucidation of the genetic differentiation in the koala subspecies is crucially needed the proper management of both the wild and captivity animals.

The mitochondrial gene is a useful marker to resolve divergences between different species or subspecies [1, 6, 7]. In this respect, the mitochondrial cytochrome *b* region has been reported to possess phylogenetic information extending from the intraspecific to the intergeneric level [6]. However, there is no information on the nucleotide sequence of mitochondrial cytochrome *b* gene in the 3 subspecies of koala.

The objective of this study is to examine a partial nucleotide sequence of mitochondrial cytochrome *b* gene among the 3 subspecies of koala using polymerase chain reaction (PCR) and direct sequencing method.

Samples were collected from 27 koalas from five zoos in Japan (Saitama Children's, Tokyo Tama, Osaka Tennoji, Kobe Oji and Awaji Farm Park). All of them were pedigreed by local and/or global studbook [4, 5] determining the area where the founder individuals of captive population were captured. Furthermore, they bear a their characteristic appearance of subspecies: the body size increases, ears become shorter and fur color changes from light gray to brownish gray with the difference of their geographical distribution from Queensland to Victoria. DNAs were extracted from hair roots (20 individuals), peripheral blood mononuclear cells (6 individuals) or liver tissue (one individual). Peripheral blood mononuclear cells were isolated from the whole blood by gradient centrifugation (Lymphoprep™, NYCOMED, Oslo, Norway). Then, the hair roots or blood mononuclear cells were incubated in cell lysis buffer for 3 hr at 55°C, subsequently heated at 99°C for 10 min, then cooled at 4°C [12]. After the treatment, the samples were centrifuged and the supernatants were collected and used as DNA templates. The liver tissue was incubated with extraction buffer [25 mM Tris-HCl (pH 7.5), 5 mM EDTA, 100 mM NaCl, 1% sodium dodecyl sulphate and 0.4 mg/m*l* proteinase K] for 3 hr at 50°C, DNA was extracted twice with phenol, once with phenol-chloroform [1:1(v/v)] followed by ethanol precipitation.

Design of the cytochrome *b* primers used in this study was based on those described by Kocher *et al*. [6]: A forward primer, 5'-TTACCAGCACCCTCCAACATTT CAGCTTGATGGAA-3', with the modification of L14841 primer by comparison of the known sequences for 11 marsupial species [7, 9], and the reverse primer, the same as H15149 (5'-AAACTGCAGCCCCTCAGAATGATA TTTGTCCTCA-3'). PCR amplification was carried out using a programmable thermal cycler (Atto, Tokyo, Japan). The PCR mixture (50 μl) contained 500 *n*g of template DNA, 50 pmoles of each primer, 0.2 mM of each dNTP, 2.5 units of Taq DNA polymerase (Nippon Gene, Toyama, Japan), 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$ and 0.1%

* PRESENT ADDRESS: TAKAMI, K., Osaka Municipal Tennoji Zoo, Tennoji, Osaka 543–0063, Japan.

Table 1. The relationship between subspecies and DNA haplotypes of koala

Subspecies	No. of individuals examined	DNA haplotype#
P. c. victor	9	Pcv
P. c. cinereus	3	Pcv
	3	Pca 1
P. c. adustus	2	Pca 1
	10	Pca 2

See the explanation of Fig. 1.

TritonX-100. The amplification reaction profile comprised 30 cycles: at 93°C for 1 min, 55°C for 2 min and 72°C for 3 min. The final cycle included an additional 7 min at 72°C for complete strand extension. The PCR products were then purified on 1.5% NuSieve GTG agarose gels (FMC, Rockland, ME) and directly sequenced using the dideoxy chain termination method using Dye Terminator Cycle Sequencing FS Ready Reaction Kit (PE Applied Biosystems, Foster city, CA) and an automated DNA sequencer ABI 373S (Applied Biosystems Inc.). The nucleotide sequences, of the both strands of PCR products using the same primers were determined. The sequencing data obtained from *P. c. victor* is registered to appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession number AB001491.

A 307 base pair (bp) segment of the mitochondrial cytochrome *b* gene was successfully sequenced from all individuals of the 3 subspecies of koala. This sequence was conserved within subspecies, but 4 variations in the nucleotide sequences of the 307 bp PCR products were observed and divided 27 individuals into 3 DNA haplotypes: *P.c.victor* type (Pcv), *P.c. adustus* 1 type (Pca 1) and *P.c. adustus* 2 type (Pca 2). Pcv included all 9 individuals of *P. c. victor* and 3 individuals of *P. c. cinereus*, Pca 1 included 3 individuals of *P. c. cinereus* and 2 individuals of *P. c.*

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                F  G  S  L  L  G  M  C  L  M  I  Q  I  I  T  G  L  F  L  A
Pcv          1:CTTTGGATCCCTACTAGGCATATGCCTAATAATTCAAATCATTACAGGTCTTTTCTTAGC
Pca1         1:.....................................................C.....
Pca2         1:.....................................................C.....

                M  H  Y  T  P  D  T  S  T  A  F  S  S  V  A  H  I  C  R  E
Pcv         61:CATACACTACACCCCAGACACCTCAACTGCCTTCTCATCAGTAGCCCACATCTGCCGAGA
Pca1        61:.........................C.................................
Pca2        61:.........................C.................................

                V  N  Y  G  W  L  I  R  N  L  H  A  N  G  A  S  V* F  F  M
Pcv        121:AGTAAACTATGGCTGACTTATCCGCAACCTACATGCTAACGGAGCATCAGTCTTCTTCAT
Pca1       121:.....................................................A.....
Pca2       121:.....................................................A.....

                C  L  Y  L  H  I  G  R  G  I  Y  Y  G  S  Y  L  Y  K  S  T
Pcv        181:ATGCCTTTATCTCCACATCGGCCGAGGAATTTATTATGGCTCATACCTATATAAAAGCAC
Pca1       181:.....................................................C.....
Pca2       181:.....................................................C.....

                W  N  I  G  V  I  L  L  L  M  T  M  M  T  A  F  M  G  Y  V
Pcv        241:ATGAAATATCGGAGTAATTCTACTACTAATAACTATAATAACTGCTTTCATAGGATATGT
Pca1       241:.....................................................C.....
Pca2       241:.....................................................C.....

                L  P
Pcv        301:ACTCCCA
Pca1       301:.......
Pca2       301:T......
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Fig. 1. Nucleotide sequences of 307 bp segment of the mitochondrial cytochrome *b* gene (light-strand) in 3 DNA haplotypes (Pcv, Pca 1 and Pca 2) of koala. Dots indicate identity with the koala Pcv type sequence. Amino acid sequence for the koala Pcv type is shown above the nucleotide sequence; the asterisk indicates a variable amino acid position.

adustus, and Pca 2 included 10 individuals of *P. c. adustus* (Table 1). Between Pca 1 and Pca 2, one nucleotide substitution was observed at the 301st position without change in the encoded amino acid sequences. The sequences of Pca 1 and Pca 2 differed from that of Pcv in 3 or 4 nucleotides: one of them located at the 170th where the first nucleotide of the 57th codon was substituted resulting in mutation of valine (Pcv) to isoleucine (Pca) in both types, but the other substitution were at the third one and thus silent (Fig. 1).

These results show that koala can be divided into 3 DNA haplotypes with 2 amino acid sequence types based on the mitochondrial cytochrome *b*, but they are inconsistent with the current classification of subspecies.

In this study we sequenced a part of the mitochondrial cytochrome *b* gene because this region reported to possess phylogenetic information extending from the intraspecific to the intergeneric level [6] and has widely been applied to the phylogenetic analysis as an appropriate marker in several species [1, 6, 10].

Although 3 subspecies have been described, Strahan [11] inferred that they may represent the arbitrary selections from a cline. The significant low level of genetic variation between south-eastern Australian koala populations have been observed by several analyses except mitochondrial DNA sequencing [3, 13, 14]. These results are likely to be due to the history of near-extinction and reestablishment mainly from the colony founded with a few individuals. The fact that there was no sequence diversity in mitochondrial cytochrome *b* gene among 9 individuals of *P. c. victor* examined in this study is consistent with earlier reports [3, 13, 14]. In addition, a genetic distance among the 3 DNA haplotypes in koala was 0.3–1.3% (1–4/307 bp). This value was less than that measured among the intraspecific but allopatric populations of 4 marsupial species classified *Didelphidae* (*Caluromys lanatus, Marmosops impavidus, Monodelphis adusta and Micoureus demerarae*) [9]. It suggests that the divergences of this region in koalas are low by comparison with other marsupial species.

Conservation efforts in the wild and in captivity need to account for the genetic diversity. Our finding that there are 3 DNA haplotypes and 2 amino acid sequence types of the mitochondrial cytochrome *b* in koala would provide reliable way for quantifying genetic diversity within the species and for monitoring genetic erosion in both the wild and captivity populations.

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