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Research Article

Sequence Analysis of Mitochondrial DNAs of 12S rRNA, 16S rRNA, and Cytochrome Oxidase Subunit 1(COI) Regions in Slow Lorises (Genus *Nycticebus*) May Contribute to Improved Identification of Confiscated Specimens

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The slow loris (*Nycticebus*) is a prosimian that is popular among exotic pet lovers. In Japan, many slow lorises have been imported illegally. Prosimians that have been confiscated in raids are protected in Japanese zoos, and the number of such animals has increased. In most cases, the country of origin remains unknown and even the species can be difficult to identify from the animal's physical appearance alone. We have attempted to resolve this problem by using DNA analysis. DNA samples of five species, consisting of the Pygmy slow loris (*Nycticebus pygmaeus*), Bengal slow loris (*Nycticebus bengalensis*), Sunda slow loris (*Nycticebus coucang*), Javan slow loris (*Nycticebus javanicus*), and Bornean slow loris (*Nycticebus menagensis*), were extracted, amplified, and the nucleotide sequences of mitochondrial 12S rRNA, 16S rRNA, and the cytochrome oxidase subunit 1(COI) regions were compared. Differences of nucleic acid sequences of representative individuals were demonstrated.

1. Introduction

The slow loris, named after its slow movements in trees, is a small nocturnal arboreal primate that lives in south and south-east Asia, and a part of China. At present, the International Union for Conservation of Nature and Natural Resources (IUCN) classifies these animals into 5 species. Typically, *N. pygmaeus* inhabits Cambodia, Lao PDR, and Vietnam. *N. bengalensis* inhabits Bangladesh, Cambodia, China, India, Myanmar, Thailand, Lao PDR, and Vietnam. *N. coucang* inhabits Thailand, Malaysia (Malay Peninsula, Pulau Island, and Tioman Island), Singapore, and Indonesia. *N. menagensis* inhabits Malaysia (Borneo) and Indonesia (Kalimantan). *N. javanicus* inhabits Indonesia (Java) (Figure 1). The IUCN also reports that *N. bengalensis* inhabits Malaysia and that *N. pygmaeus* can be found in China; moreover, the number of animals is decreasing [1].

The prosimian body has been used in traditional Asian medicines. The animals have also been captured for use as pets. Their large eyes, small body size, and quiet nature are the main reasons behind the high demand for slow lorises as pets. In addition, there has been an increasing loss of habitat due to the development of plantations.

At the 14th CITES meeting held on June 3–15, 2007 in Holland, slow loris species (genus *Nycticebus*) were up listed to CITES appendix I, and international commercial trade of the slow loris was banned. Furthermore, many countries where the various slow loris species are indigenous have their own domestic rules. Following the up listing, Japan's municipal law, the Act on Conservation of Endangered Species

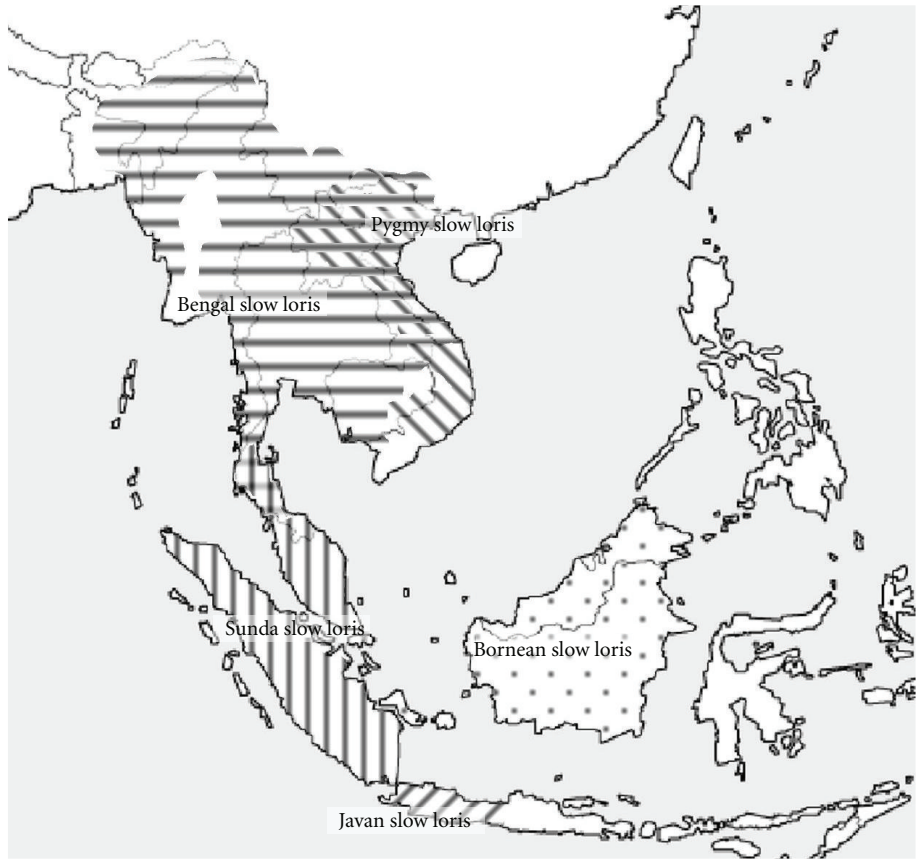


Figure 1: Distribution of Slow Loris species. International Union for Conservation of Nature and Natural Resources (IUCN) classifies the Slow Loris into 5 species. Distribution of Pygmy Slow Loris (*Nycticebus pygmaeus*), Bengal slow loris (*Nycticebus bengalensis*), Sunda Slow Loris (*Nycticebus coucang*), Javan Slow Loris (*Nycticebus javanicus*), and Bornean Slow Loris (*Nycticebus menagensis*) are as demonstrated.

of Wild Fauna and Flora, was implemented on September 13, 2007. However, many slow lorises had already been imported illegally from Asian countries, especially from Indonesia and Thailand [2], before the Act came into effect and the Ministry of Agriculture's Animal Quarantine Office at Narita reported that 448 slow lorises had been confiscated at the international airport in Japan during the period from 2000 to 2007 [3]. Though the Act on Prevention of Infectious Diseases and Medical Care for Patients Suffering Infectious Diseases started to limit the import of primates as pets in Japan, 253 animals were seized in the period from 2005 to 2007. These numbers apply only to confiscated animals, and many more slow lorises are thought to have been imported illegally and escaped detection.

When confiscated, the animals are classified into each species and then distributed to zoos by the Japanese Association of Zoos and Aquariums for further protection. The problem is that many individuals are not easily classified on the basis of appearance alone. Fur coloration, physique and auricular feature are important features in classification, but have yielded misleading results on several occasions. A comparison by craniometry or craniodental parameters was reported [4–7], but *in vivo* measurement of such parameters is practically difficult to perform. Therefore, the possibility of finding a relatively simple and more accurate method of identifying the various species should be explored. DNA analysis is one solution.

Recent advances in genetic analysis based on DNA have highlighted the practical use of these methods for many species. Mitochondrial DNA is suitable at the species level, and various regions have been sequenced [5, 8–12]. One representative method is the barcode system [13]. Hebert et al. identified species from the nucleotide sequences in a small area of the cytochrome oxidase unit 1 (COI), which differs among most of the species [14].

Species of the prosimian, including the slow loris, are the subject of discussion, and the establishment of a clear-cut classification is under development [15]. The present study did not aim for phylogenetic determination of the genus *Nycticebus,* but explored the possibility of classifying unidentified confiscated animals into five known slow loris species based on their mitochondrial DNA. We compared three regions.

2. Materials and Methods

2.1. Animals. Animals used for the study were a *Nycticebus bengalensis* (BS-02) male in the Nasu World Monkey Park, a *Nycticebus coucang* (035) male in the Ueno Zoological

Garden, a *Nycticebus menagensis* (014) female in the Ueno Zoological Garden, a *Nycticebus javanicus* (036) female in the Ueno Zoological Garden, and a *Nycticebus pygmaeus* (PS-02) female in the Nasu World Monkey Park. These were the most characteristic animals of each species as judged by each facility's staff members. Species were confirmed by two of the expert authors (H. S. and H. H.) and further appraised by an outside expert (Dr. K. A. I. Nekaris, Primate Conservation Field Chair, Department of Anthropology, Oxford Brookes University, UK). Animal experiments were approved by the Jikei University School of Medicine Animal Committee.

2.2. DNA Extraction. A swab of buccal mucosa, small pieces of skin, and hair roots from the hair coat were the sources of DNA. Extractions were performed by the DNA easy Blood and Tissue Kit (Quiagen KK, Tokyo Japan), MagExractor-Genome (Toyobo Co., Osaka, Japan) or Proteinase K/Phenol/chloroform method [16].

2.3. DNA Amplification. Primers used for the polymerase chain reaction (PCR) were as follows: mitochondria 12S rRNA: forward, GTTAATGTAGCTTAGAAATTTAAAGCGAAG, reverse, AGTTCTAGTAATTTCAAAGTGGTCATTTAG, 16S rRNA: forward, GTGCTTGGAATCACCAAAGTGTAGCTTAAG, reverse, GAGTTGAACCTCTGAGTATAAAGTTTTAAG, and COI: forward, AGGCCTGGTAAAAAGGGGATTTGAC, reverse, AATTCAACCTATAATTTAACTTTGAC. These primers were originally designed on the referential mitochondrial sequence of *Nycticebus coucang* (GenBank accession no.: NC 002765). PCR was performed with the GeneAmp PCR System 9700 (Applied Biosystems Life Technologies Japan, Tokyo, Japan) with an initial denaturation at 98°C for 30 s with 35 cycles of denaturation at 98°C for 10 s, annealing at 60–62°C for 10 s, and elongation at 72°C for 1 min (12S,16S) or 1 min and 20 s (COI). The PCR products were purified through 1% low-melting agarose followed by digestion with GELase (Epicentre Technologies, Madison, WI, USA). Sequencings were performed by the BigDye Terminator Cycle Sequencing Kit (ABI system) with the ABI3100-Avant capillary sequencer.

2.4. Similarity. Similarity was compared by analyzing evolutionary distance using the Unweighted Pair-Group Method with Arithmetic Mean (UPGMA).

3. Results

The DNAs of the 12S rRNA, 16S rRNA, or COI area of each species were sequenced. The length of the nucleotides corresponding to CO1 was 1542 and that corresponding to 12S rRNA was 971 except for *N. pygmaeus* (970). The length of the nucleotides corresponding to 16S rRNA was 1587 (*N. coucang, bengalensis*), 1588 (*N. javanicus*), 1590 (*N. menagensis*), and 1595 (*N. pygmaeus*), respectively.

Differences in the nucleic acids among the species were demonstrated. Although each difference was not directly species-specific, there was a tendency for species-specific findings. The typical differences between the species are listed in Table 1. Since full-length sequences were determined, the results were deposited in the GenBank of the National Center for Biotechnology Information (NCBI). Accession numbers are listed in Table 2. Sampling of hairs was less stressful for the animals than the other methods used, but the DNA obtained by all of the three methods was amplified.

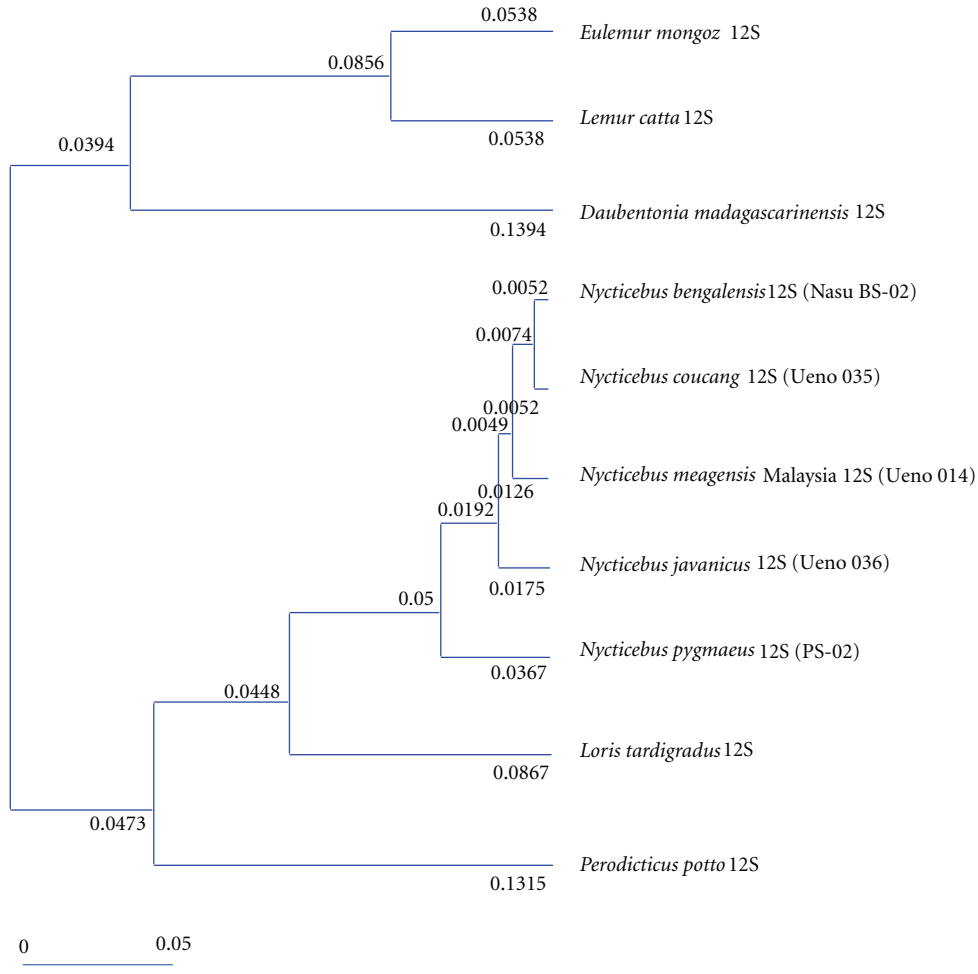
In order to elucidate the characteristics of the species, similarities were estimated. The distance matrix for each of 12S rRNA, 16S rRNA, and the COI barcode region was calculated by UPGMA based on Kimura's two-parameter model (Figure 2). Registered sequences of near prosimians from GenBank (*Loris tardigradus*, accession no.: NC_012763; *Perodicticus potto*, AB371095; *Lemur catta*, AJ421451; *Eulemur mongoz*, AM905040; *Daubentonia madagascariesis*, AM905039) were also included as references. Sequences of *N. pygmaeus* differed from those of the other four species. *N. javanicus* and *N. menagensis* were closer to *N. pygmaeus. N. bengalensis* and *N. coucang* demonstrated the most similarity. No difference between *N. bengalensis* and *N. coucang* was observed in the 16S region; however, differences between these two species were observed in the COI barcode area (Figure 2(d)). All the matrices were similar in general.

4. Discussion

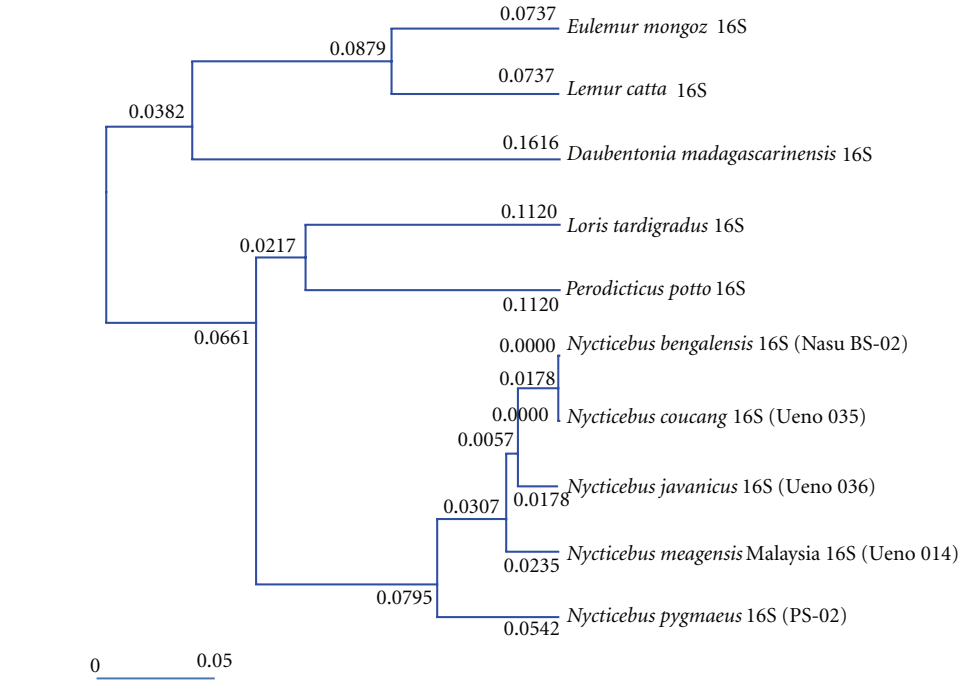
Unfair international trading is one of the most important problems associated with the protection of animals.

The slow loris has been in high demand as a pet in many countries, including Japan. They have been captured indiscriminately, and there has been a considerable amount of illegal trade. For this reason, the slow loris has been up listed to CITES appendix I and commercial trade banned. Obvious trafficking has decreased in Japan, but illicit movement persists and remains unclear. Several issues must be resolved to prevent this trade. Even if smuggling is uncovered and the animals are confiscated, their country of origin remains uncertain. While the shipping site can be determined, the shipping airport does not necessarily reflect the true country of origin of the individual animals [17]. Identification of the species is difficult at inspections, which generally are performed on the basis of physical appearance. Even within the same species, color patterns or schemes of fur can be dissimilar when the habitat differs. Since many variations exist, time, and experience are required to make an accurate evaluation. Moreover, identification of young animals is much more difficult [18].

Even when the species is determined, classification of the slow loris itself remains under discussion, and the relationship between the species and habitat is confusing. During the past 100 years, the most popular classification was the 2 species classification into *Nycticebus coucang* and *N. pygmaeus* [19]. This classification continued to have support [4], with N. coucang being classified into multiple subspecies, until the 2000s, when Groves classified the slow loris into the three species of *N. pygmaeus*, *N. coucang*, and *N. bengalensis* based on their morphological appearances. He also proposed three subspecies of *N. coucang.* These were *javanicus, menagensis, and coucang* [15, 20]. On the other hand, Supriatna stated that the Javan slow loris is a separate



(a)



(b)

Figure 2: Continued.

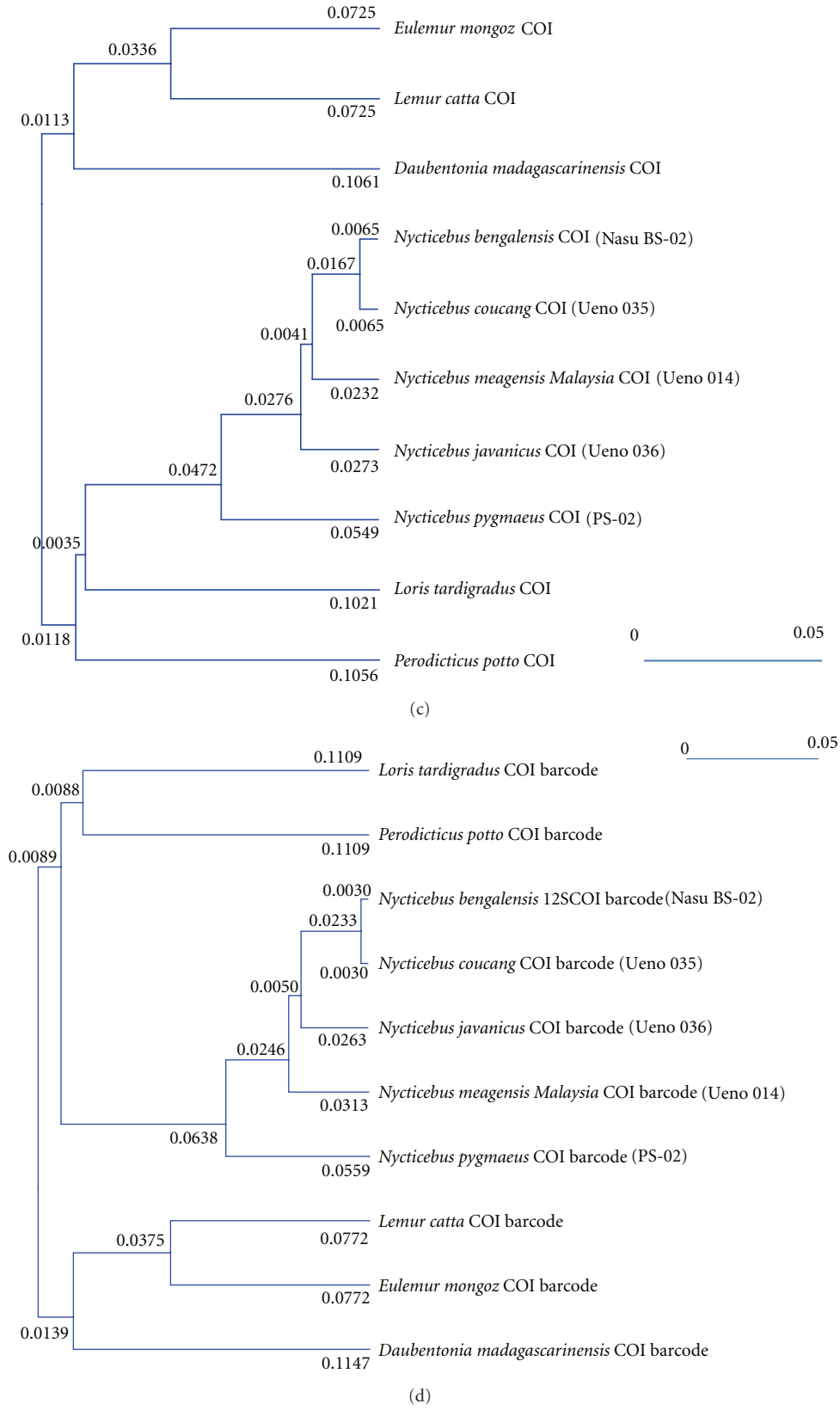


Figure 2: Distance matrixes of 12S rRNA, 16S rRNA, and COI region of loris species. All matrixes were drawn by UPGMA with Kimura's two-parameter model. Distance scale was floating, and tree width was 80. Numbers of nodes represent means of pair-wise evolutionary distances. Data contain sequences of near prosimians. Figures 2(a) 12S rRNA, 2(b) 16S rRNA, 2(c) COI, and 2(d) COI barcode region.

Table 1: Sequence variations. The sequence variations among the 5 species are shown. Differences in the nucleic acids are shadowed in the table. Variance between *N. bengalensis* and *N. coucang* is limited and null in the 16S rRNA region. Upper 12S rRNA, middle 16S rRNA, and lower COI bar-code.

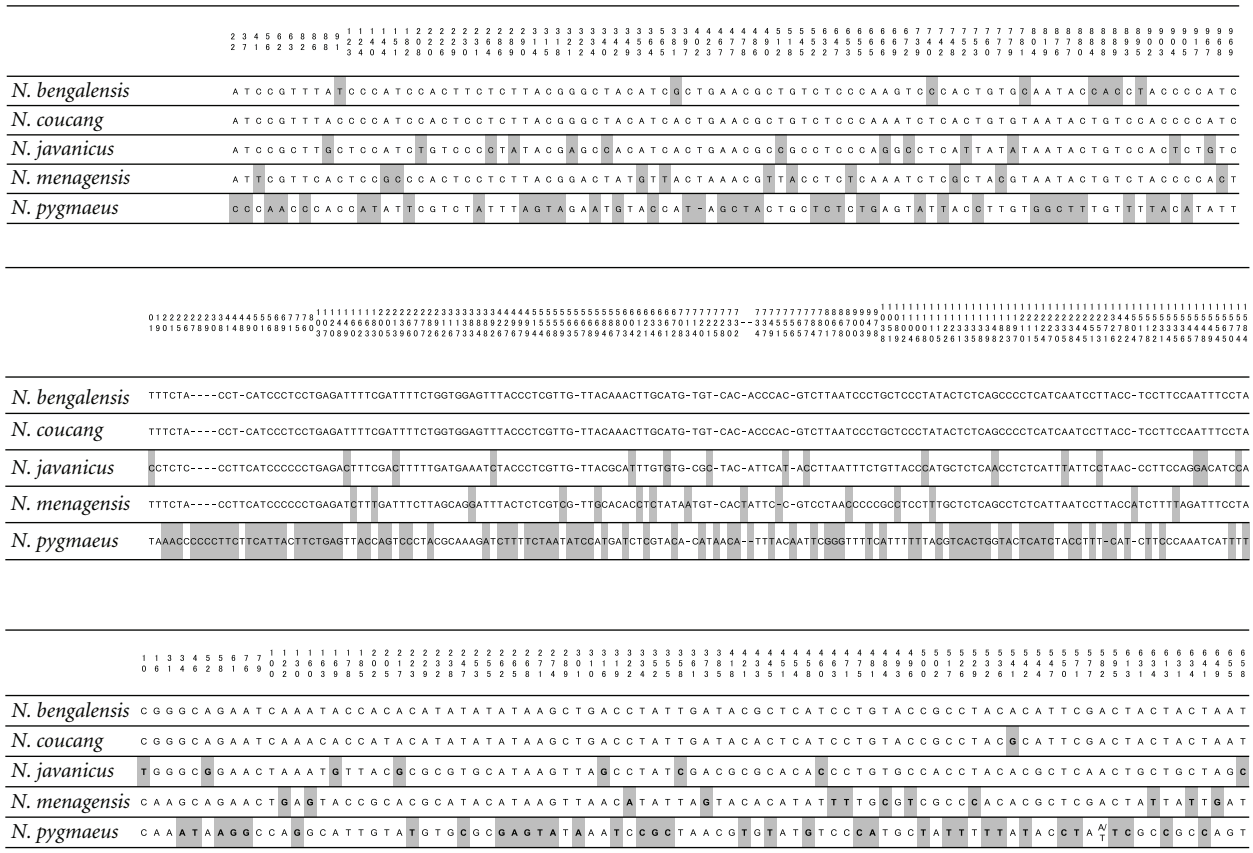


Table 2: List of registration numbers of ncbi GenBank. Registered or accession numbers of sequences in GenBank are listed here.

Accession	EU680968	*Nycticebus bengalensis*	12S ribosomal RNA gene, complete sequence
Accession	FJ640066	*Nycticebus bengalensis*	16S ribosomal RNA gene, complete sequence
Accession	GQ259899	*Nycticebus bengalensis*	Cytochrome oxidase subunit (COXI) gene, complete cds,
Accession	GQ253659	*Nycticebus coucang*	12S ribosomal RNA gene, complete sequence
Accession	GQ253662	*Nycticebus coucang*	16S ribosomal RNA gene, complete sequence
Accession	GQ259900	*Nycticebus coucang*	Cytochrome oxdase subunit (COXI) gene, complete cds,
Accession	GQ253660	*Nycticebus javanicus*	12S ribosomal RNA gene, complete sequence
Accession	GQ253663	*Nycticebus javanicus*	16S ribosomal RNA gene, complete sequence
Accession	GQ259903	*Nycticebus javanicus*	Cytochrome oxdase subunit (COXI) gene, complete cds,
Accession	GQ253661	*Nycticebus menagensis*	12S ribosomal RNA gene, complete sequence
Accession	GQ253664	*Nycticebus menagensis*	16S ribosomal RNA gene, complete sequence
Accession	GQ259901	*Nycticebus menagensis*	Cytochrome oxdase subunit (COXI) gene, complete cds,
Accession	EU680969	*Nycticebus pygmaeus*	12S ribosomal RNA gene, complete sequence
Accession	FJ640067	*Nycticebus pygmaeus*	16S ribosomal RNA gene, complete sequence
Accession	GQ259902	*Nycticebus pygmaeus*	Cytochrome oxdase subunit (COXI) gene, complete cds,

species [21]. Until September 2008, IUCN had employed a classification of four species and one subspecies. At present, IUCN categorizes genus *Nycticebus* into 5 species based on research with morphology as well as genetic and geographic morphometrical analysis [22]. Additionally, researchers are concerned about the reports of the presence of hybrids in overlapping habitats [10, 20]. Phylogeny is not a topic of the current study, but we emphasize that our data show DNA similarity between *Nycticebus bengalensis* and *Nycticebus coucang* compared to the other three species.

In the DNA analysis, recent advances in sequence technology enabled us to use noninvasive samples such as the hair root. Amplification and determination of the mitochondrial DNA from the hair root was not difficult in our study. In recent years, it has become possible to use hair shafts or feces for the extraction of DNA [23]. In the present study, skin, buccal mucosa, or hair root were used to avoid DNA contamination from animals living in the same cage or previously hosted individuals. In various species, 12S or 16S rRNA, has been utilized for identification. The recent concept of the DNA barcode uses partial mitochondrial COI for identification of the species [14]. Therefore, we determined and compared the full length of the areas of 12S rRNA, 16S rRNA and COI of *Nycticebus*. Based on this information, we determined the species of unknown individuals by simultaneous comparison of these regions. We believe that the method was reliable. As an example, the classification of one animal as *N. coucang* based on examination by an expert at a Japanese zoo was different from the result of our DNA sequencing, which revealed the species to be *N. menagensis*. Our result was supported through reevaluation by native experts (data not shown). The production of offspring though inadvertent crossbreeding might occur as the result of the inaccurate judgment of species. Hybrids of the Bengal and Sunda slow lorises have been reported in a zoo [6]. Our approach may provide strong evidence in such a case. In fact, we confirmed crossbred offspring of *N. menagensis* and *N. coucang* in a Japanese zoo by our methods. While the father was *N. coucang* judged by both appearance and COXI sequence, two offspring demonstrated the sequence of typical *N. menagensis*. Identification by simple comparison of mitochondrial DNA sequences is especially useful in Japan, since the number of experts is limited, and many confiscated animals are currently located in zoos waiting for classification.

For population genetic analysis, the control region of the D-loop or the partial cytochrome b gene of the mitochondria has been sequenced [8, 10]. We previously sequenced the D-loop of the mitochondria (GenBank accession no.: GQ449387 *Nycticebus bengalensis*, GQ449391 *Nycticebus pygmaeus*, GQ449390 *Nycticebus menagensis*, GQ449389 *Nycticebus javanicus*, and GQ449388 *Nycticebus coucang*). The area varies more than the regions of 12S rRNA, 16S rRNA, or COI. When all the sequence data in each animal's habitat or native area have been accumulated, it might be possible to deduce the area from where the individual has been brought. By the combination of such information and the circumstances of confiscation, tracking of the smuggling route could become easier. Moreover, accurate determination of the species of confiscated animals is valuable for animal care facilities such as zoos. Because endangered or threatened species are listed in CITES appendix I, pedigree registries are mandatory for such animals. They are cared for and bred in *exsitu* conservation under strict administration; hence, the genetic information is useful.

We are currently storing the sequences of native samples and confiscated animals. The data will be useful for estimating the original habitat in order to prevent overhunting in south-eastern Asia and unlawful imports to Japan. As the next step, determination of parentage using nucleic DNA will be important to prevent illegal trading. In Japan, display or domestic trading of the slow loris for commercial purposes is possible only under the registry of captive breeding. However, contraband individuals are sometimes falsely registered by fabricating the parental certification. Paternity or maternity must be proved by DNA analysis. Our approach will contribute the general applicability of laboratory approaches to conservation problems like a good deal of work by Nicolaas van der Merwe's group on the applicability of chemical signatures to the identification of the elephant populations from ivory [24]. We will explore further sequence data for more solid evidence for conservation of the genus *Nycticebus*.

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