Identification of the species, origin and sex of smuggled douc langur (Pygathrix sp.) remains

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Summary

Information of illegal wildlife trade routes provides trustworthy evidence of the smuggling activities of endangered wildlife. Therefore, identifying the species and origin of confiscated samples provide important data. In this study, mitochondrial control region sequences, compared to the Indochinese Primate Genetics Project database at the German Primate Center, identified 28 dried samples smuggled to China as black-shanked douc langurs (Pygathrix nigripes). Among them, 20 samples most likely originated from Khanh Hoa Province in Vietnam. Another three came from Binh Phuoc Province in Vietnam or Cambodian Mondulkiri Province, whereas one sample came from Dak Lak or Lam Dong Provinces, Vietnam. The origin of four further samples was not clearly traceable. Based on sex-typing PCRs, 17 samples are from females and 11 from males.

Những nhận dạng về loài, nguồn gốc và giới tính từ những mẫu vật buôn lậu là bộ phận vô cùng chìm trong (Pygathrix sp.)

Tóm tắt

Thông tin về tình trạng mua bán động vật hoang dã bất hợp pháp đã cung cấp nhiều bằng chứng xác thực cho quá trình điều tra hoạt động buôn lậu động vật hoang dã gây tổn hại đến giới tự nhiên. Vì vậy, thông qua những nghiên cứu về những loài và nguồn gốc từ các mẫu vật được tích trữ, chúng tôi đã thu được những số liệu quan trọng. Nghiên cứu này sử dụng phương pháp giám định gen di truyền liền thể (mtDNA D-loop), đối chiếu với số liệu gen di truyền của Dự án nghiên cứu về gen di truyền thủy linh trường Đông Dương tại Trung tâm Nghiên cứu Linh trưởng, CHLB Đức, nhận dạng chính xác 28 mẫu vật buôn lậu đến Trung Quốc là bộ phận của loài voọc chà và chân đen (Pygathrix nigripes). 20 mẫu trong số đó có xuất xứ ở tỉnh Khánh Hòa (Việt Nam), 3 mẫu khác có thể ở tỉnh Bình Phước (Việt Nam) hoặc tỉnh Mondulkiri thuộc quốc đảo Campuchia. Ngoài ra, một mẫu vật có thể ở tỉnh Đà Lạt hoặc Lâm Đồng (Việt Nam). Bốn mẫu vật còn lại còn chưa được xác định rõ nguồn gốc. Dựa vào phương pháp giám định giới tính đặc biệt (PCRs), 17 mẫu đã được xác định là của giới cái và 11 mẫu còn lại là của giới đực.
Introduction

Illegal wildlife trade has become increasingly serious in recent years (Yang Guang et al., 1999; Traffic, 2006). Accordingly, populations of a large number of species around the world have been reduced (Robert, 2000; Karlsson & Holmlund, 2007), resulting in many species now being endangered or even close to extinction (Zhou Zhihua & Jiang Zhigang, 2004).

Governments and NGOs dealing with wildlife conservation pay more and more attention to the illegal wildlife trade and conduct measurements to efficiently control it (Martin, 1997; Wright & Kumar, 1997; Wang & Li, 1998). These illegal trades particularly occur in less developed countries with an abundance of natural wildlife resources. Illegal trade often exists in border regions between two countries with one having high abundance of wildlife resources and the other, a high consumption demand (Li Yiming et al., 2000). For example, local forestry policemen in China frequently confiscate smuggled wildlife coming from the neighbouring countries Vietnam, Lao or Myanmar.

To control illegal trans-border activities, knowledge about traderoutes and networks is required, which then can be used to establish efficient strategies to reduce or even stop them. Therefore, the species identity of confiscated specimens or their remains as well as their origin must be correctly identified. However, policemen are not well trained to identify species, and it is mainly specimen fragments, which are difficult to identify morphologically, that are traded. To overcome this problem, DNA analyses represent a useful alternative. Mitochondrial sequence data are especially easy to generate from low-quality DNAs extracted from tissue, feces, hairs, etc. Moreover, a large number of mitochondrial sequence data are available in various databases, and comparisons with such databases allow a rapid identification of specimens and/or their origin. Accordingly, genetic methods are already widely used to study the species identity of e.g. freshwater fishes, white abalones, tigers and primates (Yang Guyang et al., 2002; Wan Qiuhong & Fang Shengguo, 2003; Gruenthal & Burton, 2005; Liu Hui & Wu Xiaobing, 2006; Kyle & Wilson, 2007), or to trace the original place of cetaceans (Baker & Palumbi, 1994; Phipps et al., 1998).

In the present study, we sequenced the hypervariable region I of the mitochondrial control regions of confiscated douc langur remains and compared obtained data with sequence information deposited at the Indochinese Primate Conservation Genetics Project database. We traced the origin of study specimens and determined whether they are males or females. With this study, we highlight the power of molecular techniques in elucidating the species identity of confiscated primate remains, their geographic origin and sex.

Materials and Methods

Samples

The 28 dried skeleton samples including muscle fragments were smuggled from Vietnam to China and confiscated by Chinese customs in Guangxi Province in 2005. We collected some samples (hairs, bones, muscle) from each body and stored them at 4°C before further processing. A preliminary species identification of the specimens based on photographs was conducted by T. Nadler and U. Streicher (both Endangered Primate Rescue Center, Vietnam).

Laboratory methods

Genomic DNA from dried muscle samples was extracted with a standard proteinase K/SDS/phenol/chloroform method (Sambrook & Russel, 2001). To identify the species and the origin
of samples, a ~450 bp long fragment of the hypervariable region I (HVI) of the mitochondrial control region was amplified via PCR using the oligonucleotide primers 5’-ATTGATTTCACGGAGGATGGT-3’ and 5’-AACCTGGAATTCTATTTAAACTAC-3’. PCR based sex-typing was conducted by amplifying fragments of the amelogenin X (AMELX) and the Y-linked sex-determining region (SRY) gene. The primate-specific primers AMEL-F1 and AMEL-R1 amplify a ~200bp fragment, while SRY-F1 and SRY-R1 amplify a ~165bp fragment (Fiore, 2005). Each PCR reaction contained at least 25ng DNA template, 4µL 10x reaction buffer, each 0.4 µmol/L forward and reverse primer, 0.25 mmol/L mixed dNTPs, 0.75 mg/ml BSA, 2.5 unit of Taq DNA polymerase, and sterile distilled water add to 40µL. Amplifications were performed in an ABI Gene Amp PCR System 9700. Cycling conditions include an initial denaturation at 94° for 2min, 40 cycles each with 94° for 1 min, 60° for 1 min and 72° for 1 min, and a final extension at 72° for 5 min.

All reactions were run with negative controls. PCR products were checked by agarose gel electrophoresis. Mitochondrial products were further purified and sequenced by BGI LifeTech Co Ltd (Beijing, China). Sequences were checked and edited using MEGA3 (Kumar et al., 2004) and aligned with Clustalx1.83 (Thompson et al., 1997).

Statistical analysis

The species identity and local origin of specimens was traced by comparing respective sequences with orthologous sequences from all douc langur species and various localities deposited at the database of the Indochinese Primate Conservation Genetics Project. Pairwise differences between confiscated specimens and those deposited in the database were estimated in MEGA. Phylogenetic relationships based on the neighbor-joining algorithm and uncorrected distances were estimated in PAUP* v4.0b10 (Swofford, 2002).

Results and Discussion

All 28 confiscated langur remains were morphologically identified as douc langurs. However, due to the pure condition of samples and the lack of detailed information, no further identification of specimens concerning species, sex or origin was possible. Hence, various genetic methods were applied. We successfully extracted DNA from all 28 individuals and amplified the HVI of the mitochondrial control region. Pairwise differences between all three douc langur species range from 6.6 to 13.7%. The herein analyzed samples show lowest similarities to Pygathrix nigripes (0.0-6.4%), whereas differences to P. nemaeus and P. cinerea range from 10.9 to 13.7%. Accordingly, all confiscated specimens can be identified as P. nigripes. Among all tested P. nigripes individuals, the confiscated specimens are most similar to samples from Khanh Hoa (individuals W1, W2, W3, W4, W5, W7, W8, W9, W10, W11, W12, W14, W15, W16, W17, W19, W21, W22, W23, W25; 0.5-2.0%), Binh Phuoc-Mondulkiri (individuals W6, W24, W27; 1.0-2.3%) and Dak Lak-Lam Dong (individual W18; 1.0-1.5%). Individuals W13, W20, W26 and W28 show lowest differences to samples from Dak Lak, Lam Dong, Dong Nai, Binh Phuoc and Mondulkiri (3.3-5.5%), but due to the relative large differences, the exact origin is not traceable. Based on phylogenetic tree reconstructions, all confiscated animals cluster together with P. nigripes, and are clearly separated from P. nemaeus and P. cinerea (Fig. 1).

The close affiliation of the confiscated specimens to P. nigripes, as indicated by the relative low pairwise differences, is also supported by the obtained tree topology (Fig. 1) and clearly confirms that the confiscated remains are from black-shanked douc langurs. Further tree reconstructions including P. nigripes samples from various locations provide a more detailed view on the
A geographic distribution of black-shanked douc langur haplotypes (Fig. 2).

Samples W1, W2, W3, W4, W5, W7, W8, W9, W10, W11, W12, W14, W15, W16, W17, W19, W21, W22, W23 and W25 from the confiscated pool cluster together with samples from Khanh Hoa, whereas samples W6, W24 and W27 form a clade together with individuals from Binh Phuoc and Mondulkiri. Sample W18 clusters with samples from Dak Lak and Lam Dong. The four samples W13, W20, W26 and W28 are either distantly related to animals from Dong Nai or branched off first in a clade consisting of animals from Dak Lak, Lam Dong, Dong Nai, Binh Phuoc and Mondulkiri. Accordingly, their exact origin is not traceable. Based on pairwise differences and phylogenetic tree reconstructions and due to the female philopatric nature of douc langurs, samples W1, W2, W3, W4, W5, W7, W8, W9, W10, W11, W12, W14, W15, W16, W17, W19, W21, W22, W23 and W25 most likely originated from animals from Khanh Hoa, whereas samples W18 and W6, W24 and W27 are derived from individuals from Dak Lak-Lam Dong and Binh Phuoc-Mondulkiri, respectively (Fig. 3).

Sex typing was performed by amplifying a region of the Y chromosomal SRY gene. To exclude PCR artifacts, a control PCR amplifying a fragment of the X chromosomal AMELX gene was selected. In all 28 samples, a successful amplification of the AMELX product was generated, indicating the reliability of the data (Fig. 4).

In 17 samples, the SRY product is absent, indicating that these individuals are females, whereas in the other eleven animals a SRY product is present, indicating that these animals are males.

Douc langurs comprise three species, which are distributed in Vietnam, Laos and Cambodia (Roos & Nadler, 2001; Roos et al., 2007). All of them are endangered to different degrees (Nadler et al., 2003), mainly due to hunting and habitat loss throughout their range. Black-shanked douc langurs are found in southern Vietnam and south-east Cambodia, only east of the Mekong. The species occurs from Dak Lak Province in the north southwards to Dong Nai Province. There is no actual population estimate for Vietnam (Nadler et al., 2007), but hunting is the major threat to the species. It seems that species of this genus can adapt to relatively heavily disturbed forests (Nadler et al., 2003), which increases also the contact between langurs and humans. Accordingly, douc langurs can easily be discovered and hunted. The species is believed to have undergone a decline.
of more than 50% in the last three generations (35 years, based on a generation length of 10-12 years) due to forest loss and hunting (Southeast Asia Mammal Data Bank, 2006). It is protected in Vietnam on the highest level under the wildlife protection law (Government of Vietnam, 2006), and listed in the Red Data Book of Vietnam (Ministry of Science and Technology & Vietnamese Academy of Science and Technology, 2007) as "Endangered". The IUCN Red List lists the species also as "Endangered" (EN) under criteria "A2cd" and CITES in appendix 1.

Besides a high demand for douc langurs as food or medicine or even as pet monkeys in Vietnam, Lao or Cambodia, the consumption of wildlife including douc langurs in neighbouring countries is steadily increasing, especially in China. The presented case of confiscated douc langurs in Guangxi Province, China is only one example therefore. Due to ongoing wildlife trade including douc langurs, methods are required to follow such trades by identifying species, their origin and sex. Mitochondrial DNA has become a powerful tool in forensic identification because of the high copy number per cell and the lack of recombination (Balitzki-Korte et al., 2005). The present study successfully used mitochondrial control region sequences for the identification of confiscated douc langur samples. This also shows that mitochondrial DNA sequencing is a quick, reliable and simple approach for species and original identification.

Based on the confirmed origin of most of the confiscated samples, smuggling routes can be traced. Looking at the map of Vietnam and neighbouring countries (Fig. 3), it becomes obvious that the original places of the confiscated samples are

![Map of Vietnam and neighbouring countries. Circles indicate the putative origin of confiscated douc langurs.](image-url)
all well connected by a good transport infrastructure to trading centers such as Ho Chi Minh City or directly to the South China Sea. Accordingly, an efficient and large-scale wildlife trade is possible.

Our results suggest that more attention has to be paid by national and international organizations to prevent wildlife trade across borders. In Vietnam, the province is the most critical implementation unit for strategies, polices and plans of the government, and the conservation in provincial scales is the most efficient level for magnifying the impact of conservation activities (Long et al., 2004). Hence, customs and law enforcement agencies in provinces need to uphold national laws (Polet et al., 2004).

Cross-border cooperation is urgently required to prevent wildlife trade, but also to improve the management of protected areas. Especially sharing experiences and information would be helpful to strengthen conservation efforts and to establish concerted actions against the illegal wildlife trade (Bleisch & Zhang Yingyi, 2004).

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References


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