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短 報（Note）

Comparison of noninvasive samples as a source of DNA for genetic identification of bark-stripping bears

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Abstract

Bark stripping by Asian black bears (*Ursus thibetanus*) is a management issue in some parts of Japan. To effectively decrease bark stripping, one must identify the individuals causing the damage. We compared the success rates of polymerase chain reaction (PCR) amplification of DNA extracted from two types of noninvasively collected samples left on damaged trees. Specifically, DNA was extracted from saliva and hair samples collected from damaged patches, and five microsatellite loci were examined using PCR. The success rate for fresh hair samples was highest (0.429), while that for old saliva samples was lowest (0.063). No significant difference was detected between the success rates for fresh saliva (0.270) and old hair (0.218) samples. From these results, we recommend the use of hair samples to identify individual bears causing bark-stripping damage.

Key words : hair, microsatellite, saliva, *Ursus thibetanus*

Introduction

Bark stripping of conifer trees by Asian black bears (*Ursus thibetanus*) is a major management issue in some parts of Japan. The price of timber from trees damaged by black bears is dramatically reduced, creating a problem for forest managers as these financial losses lead to a reduction in the number of forest managers employed, ultimately increasing the areas of poorly managed forest. Wrapping plastic tape around trees decreases damage from bark stripping (Yamanaka et al., 1991), but this must be repeated every few years in accordance with the growth of the tree. Some local governments cull bears to decrease the damage, but since the individuals causing the damage are not identified, this may result in the killing of bears not involved in bark stripping. To effectively decrease the damage caused by bark stripping, one must identify the individuals causing the damage and understand the reasons behind this behavior.

Two previous studies have attempted to identify individual bears that participated in bark stripping. Collins et al. (2002) used radio collars on American black bears (*Ursus americanus*) to identify individuals that damaged trees within the bears' home ranges. Another study investigated bark stripping among Asian black bears (Neo Village, 2000) by examining the presence of tissues of conifer trees in the feces of captured bears, which was assumed to indicate that the bears had damaged trees. However, this method was not able to identify with certainty which bears had stripped which trees. Nor could it clarify how many trees had been stripped by any given bear. Given the problems with these methods, we hypothesized that the use of DNA to identify individual bears could shed light on their bark-stripping behavior. Thus, we compared the efficiency of polymerase chain reaction (PCR) amplification of DNA extracted from two types of noninvasively obtained samples, namely, saliva and hairs, left on damaged trees and evaluated the usefulness of these samples for identifying individual bears.

Materials and methods

Field surveys were conducted from late May to early August 2004 at the Ashiu Forest Research Station of Kyoto University (35°16′51″ to 21′20″ N, 135°42′04″ to 47′53″ E) because bark-stripping damage is usually observed in the spring. When a damaged tree was encountered, we assessed the freshness of the damage as follows: fresh—the surface of the damaged patch appears light-yellow ocher and has a high moisture con-

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tent (Photo 1a); old—fungus is present on the damaged surface or the surface is brown or dark brown and dry (Photo 1b). Only the patches that were determined to have been damaged in the current year were used for the analyses. Following the visual assessments, we collected saliva by wiping approximately 100 cm^2 of the damaged area with three sterilized cotton swabs. Each swab was then placed into a microtube containing 1 ml of PBS buffer; the tube was rotated for approximately 10 s and the swab was then discarded. When bear hairs were left around the damaged patches, we used sterilized tweezers to place the hairs into a plastic bag. When obviously separate patches of damage were observed on a single tree, we collected samples from every damaged patch, as these trees were likely to have been stripped at different times or by different bears. Saliva and hair samples were stored at -20°C prior to DNA extraction.

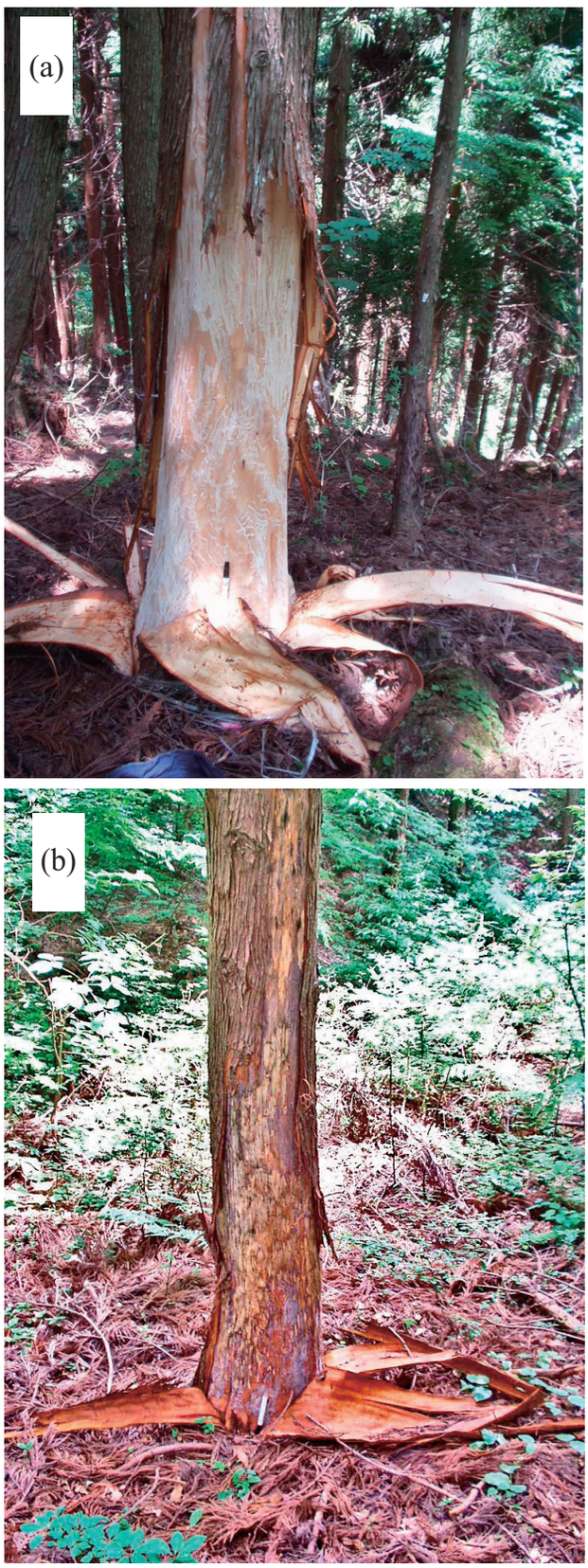

Photo 1. Patches of (a) fresh and (b) old bark-stripping damage.

DNA was extracted with a QIAamp DNA mini kit (QIAGEN). Specifically, 200µl of the saliva-laden PBS was transferred to another tube, and 15µl of proteinase K and 300µl of buffer AL from the kit were added. The tube was then incubated at 55°C for 15 min. To extract DNA from the hairs, four to six hairs were cut into a buffer (192µl of 100 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl, and 2% SDS), and 8µl of 1 M DTT and 20 µl of proteinase K were added to the buffer, which was then incubated at 55°C for 1 h. Following the incubation, we followed the kit's standard protocol for both types of sample.

Five microsatellite loci (G1A, G10B, G10L, G10M, and MSUT-7; Paetkau et al., 1995; Kitahara et al., 2000) were amplified by PCR. Amplification was performed in a total volume of 10µl containing 50 mM KCl, 2 mM $MgCl_2$, 10 mM Tris-HCl (pH8.0), 0.2 mM dNTP, 0.5µM of each primer, 0.3 units of Ex Taq (TaKaRa), and 0.1-0.5 ng of DNA. PCR amplification was performed with a GeneAmp PCR9700 thermal cycler (Applied Biosystems). After denaturation at 94°C for 5 min, cycling was performed for 20 cycles of 10 s at 94°C, 30 s at 53°C, and 15 s at 72°C, then 20 more cycles of 10 s at 94°C, 30 s at 48°C, and 15 s at 72°C, with a final extension at 72°C for 5 min. Genotypes were determined with an ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems) and scored with GeneMapper version 3.7 (Applied Biosystems). We performed PCR three times for each locus, and determined the PCR to be successful when at least three loci showed amplification.

Results and discussion

Even though the observed number of damaged trees was highest in early July, all of the damage observed then was old (Fig. 1). Fresh damage was observed

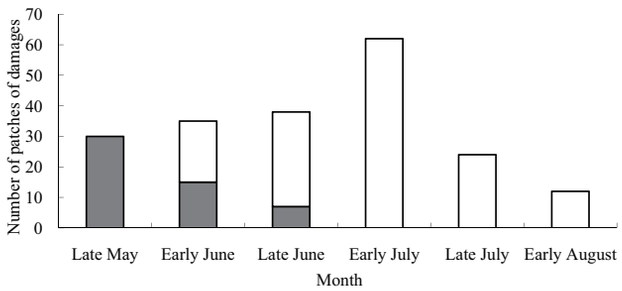

Fig. 1. Number of damaged patches. Shaded bars represent fresh damage; white bars indicate old damage.

most frequently in late May, and continued up to late June. Bear hairs were found significantly more frequently on patches of fresh damage (0.71, 15 of 21 trees) than on patches of old damage (0.40, 60 of 149 trees, $P < 0.01$; Fisher's exact test). The success rate of PCR for saliva samples was significantly higher in saliva from patches of fresh damage than in that from patches of old damage ($P < 0.005$; Fisher's exact test; Table 1). Although the success rate for fresh hair samples was higher than that for old hair and fresh saliva samples, the differences were not significant ($P > 0.05$), which may have been due to the small sample size in the case of fresh hairs ($n = 14$). Significantly higher success rates were achieved for old hair samples than for old saliva samples ($P < 0.01$).

Table 1. Success rates of PCR for each type of sample. Numbers of successfully amplified and analyzed samples are shown in parentheses.

	Saliva	*Hair*
Fresh	0.270 (10 / 37)[a,b]	0.429 (6 / 14)[a,c]
Old	0.063 (6 / 95)	0.218 (12 / 55)[b,c]
Total	0.121 (16 / 132)	0.261 (18 / 69)

Shared letters indicate the lack of a significant difference ($P < 0.05$; Fisher's exact test).

In consideration of sampling efficiency, we recommend collecting bear hairs from damaged trees in May and June for DNA analysis of bark-stripping bears. Fresh hair samples showed the best results, while old hair samples gave results similar to those from fresh saliva samples. Saito et al. (2008) conducted genetic identification using samples from the surface of corn bitten by the Asian black bear, and successfully amplified at least four of six loci in 86.9% of samples (86 of 99 samples)—a much higher success rate than in the present analyses. They used samples that had been bitten within the preceding 3 days, and suggested that freshness was an important factor for PCR success. Freshness has also been suggested to be a key factor in the analysis of fecal samples of the brown bear, *Ursus arctos* (Bellemain et al., 2007). In deep forests, however, finding trees that had been stripped within the past few days would be almost impossible. Moreover, the freshness, which we based on a visual assessment of damage, is affected by estimator bias. In contrast, finding hairs around bark-stripping damage is not particularly difficult and the bias in sampling is not likely to be adverse. Although it is not always possible to find fresh hairs around bark damage, the success rate of PCR for old hair samples is similar to that for fresh saliva samples.

Noninvasive samples (e.g., hairs, saliva, and feces) from bears have recently been used for genetic analysis. When using noninvasive samples for genetic investigation, one must pay attention to misgenotyping caused by factors such as allelic dropout and the presence of false alleles (Taberlet et al., 1996; Gagneux et al., 1997; Paetkau, 2003). Given that our present focus was on the comparison of sampling methods, we did not investigate this issue. However, these previous reports discuss the issue of misgenotyping, and their recommendations should be taken into account when examining samples collected from patches of bark-stripping damage.

Although we investigated five microsatellite DNA loci from samples left on damaged patches in the present study, we successfully amplified 10 microsatellite loci as well as a part of the amelogenin gene for sex identification, and determined the sequence of about 700 bp of a mitochondrial DNA control region from hairs left on damaged patches (Kitamura and Ohnishi, 2011). These molecular techniques enable us to investigate the identification, sex, and genetic relationships of bark-stripping bears, which will provide insight into the reasons behind bark-stripping behavior.

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樹皮剥ぎ加害クマの遺伝的な個体識別にむけた非侵襲的サンプルの比較

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要 旨

ツキノワグマ (*Ursus thibetanus*) による樹皮剥ぎは、日本各地において森林管理上の問題となっている。加害個体を特定することが、樹皮剥ぎ被害の防止に向けて有効である。我々は被害痕跡に残されていた唾液と体毛から DNA を抽出し、マイクロサテライト DNA 領域 5 遺伝子座の PCR 成功率を比較した。PCR 成功率は新しい被害痕跡から採集した体毛を用いた場合で最も高く (0.429)、古い被害痕跡の唾液では最低だった (0.063)。新しい被害痕跡の唾液 (0.270) と古い被害痕跡の体毛 (0.218) の間に有意な差は見られなかった。これらの結果より、樹皮剥ぎ加害個体を特定するためには体毛を使うことが効果的である。

キーワード：体毛、唾液、ツキノワグマ、マイクロサテライト DNA

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