Individual identification of Asiatic black bears using extracted DNA from damaged crops

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Abstract: To reduce crop damage by Asiatic black bears (*Ursus thibetanus*), we developed a method to identify individual bears that damaged corn crops based on microsatellite analysis using bear DNA obtained from damaged corn. During summer 2004 in Iwate prefecture, Japan, 99 corn-bite samples were collected, of which 30 (30%) yielded sufficient DNA for 6 complete microsatellite loci. We detected that at least 21 individuals (16 males, 1 female, and 4 of unknown sex) had damaged dent corn in 5 fields. Results enabled individual identification of bears from the samples, but more accurate analysis is needed. Moreover, the sex ratio of nuisance individuals was extremely biased to males compared to that of bears killed through control programs.

Key words: Asiatic black bear, bite marks, corn, crop damage, DNA extraction, Japan, microsatellite, noninvasive sampling, nuisance bear, *Ursus thibetanus*

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Asiatic black bears (*Ursus thibetanus*) damage agricultural crops in the Tohoku region of northern Japan. Local governments permit killing of those bears as a pest control measure, recently using mainly box traps. However, crop damage has not been diminished, because bears are killed indiscriminately without verification of which bears actually damage crops. For real damage abatement, we must investigate the factors that induce bears to harm crops and provide more effective means for damage control. For instance, confirmation of the number of problem bears is the most important information for appropriate management.

Recently, molecular genetics have been used to assess population ecology (Paetkau 2003). Particularly, individual identification has become possible using microsatellite polymorphism (Paetkau and Strobeck 1994). Molecular genetics techniques can be applied to materials collected noninvasively such as feces and hair. They are useful to obtain DNA of source individuals to study genetic diversity and population structure (Taberlet et al. 1997, Wasser et al. 1997, Woods et al. 1999, Mowat and Strobeck 2000, Yamauchi et al. 2000, Murphy et al. 2003).

For this study, we examined bite marks left on crops by bears. We report on our efforts (1) to extract bear DNA from crop-bite samples using an unpublished technique we developed with captive bears and (2) to identify individual nuisance bears from damaged dent corn fields at Shizukuishi town, Iwate prefecture. We report on the minimum number of problem bears and their sex ratio. We attempted to estimate the minimum number of problem bears at each damage site using microsatellite DNA analysis; we then sought to determine the sex of problem bears.

Methods

Sample collection

For the study of nuclear DNA diversity, 153 black bear samples (muscle) were collected from hunted and nuisance-killed animals throughout Iwate prefecture (15,278 km^2) during 2002–04. These samples were mainly collected systematically by the Research Institute for Environmental Science and Public Health of Iwate Prefecture.

The corn-bite samples were collected at five dent corn fields (Nos. 1–5, 0.78 km^2) in Shizukuishi town (39°44′N, 141°00′E), Iwate prefecture, summer 2004. We sampled from fields that were damaged within

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the prior 3 days, based on landowner information. We searched for surrounding feeding signs that are typically left by bears, such as a stalk of corn knocked down in a circle. We took several samples from the freshest corn sample remaining. A total of 99 samples were collected from 5 damage sites during 6 sessions, on 5, 10, 17, and 27 August, and 2 and 9 September.

The surfaces of corn-bite samples were wiped using a sterile cotton swab: cells from various bears' inner mouth cavities were thereby obtained. The swab was inserted into a microtube and suspended in 1 mL of DNA extraction buffer: 0.1% dodecyl sodium sulfate, 150 mM sodium chloride (NaCl), 10 mM Tris aminomethane (Tris-HCl), and 10 mM ethylenediaminetetraacetic acid (EDTA). All samples were stored at 4°C prior to DNA extraction.

DNA extractions

DNA was extracted from muscle and corn samples using proteinase K digestion followed by the phenol/chloroform method. A precipitate was obtained using ethanol (Sambrook et al. 1989).

Polysaccharides were removed from the corn samples by treatment with cetyltrimethylammonium bromide (CTAB; Murray and Thompson 1980) in the following manner: (1) adding CTAB solution (2% CTAB, 0.1 M Tris-HCl [pH 9.5], 1.4 M NaCl, 20 mM EDTA) and incubating at 65°C for an hour; (2) adding a mixture of chloroform and isoamyl alcohol in the ratio of 24 parts to 1; and (3) continuing to obtain DNA by a precipitate with ethanol.

Genetic typing

Extracted DNA was dissolved with purified water and the amount of DNA was determined using a spectrophotometer. The quantity of template DNA of each polymerase chain reaction (PCR) was determined according to the spectrophotometric data. We set the quantities of template DNA at about 50 ng and 100 ng, respectively, for tissue samples and corn samples.

As primers for amplification of microsatellite loci, we used 6 microsatellite loci to identify individuals: set A was G10B, G10L, and G10C; set B was G10M, G10P, and G10X (Paetkau and Strobeck 1994, Paetkau et al. 1995). Sex identification was performed on samples using primers SE47 and SE48 (Ennis and Gallagher 1994). All forward primers were labeled using fluorescent dye group FAM or VIC (Applied Biosystems, Inc., Foster City, California, USA).

The PCR conditions were as follows: 15 μL reactions containing a concentration of 10 x Ex T*aq* buffer (20 mM Tris-HCl [pH 8.0], 100 mM potassium chloride (KCl); Takara Bio Inc, Otsu, Shiga, Japan), 1.5 mM magnesium chloride ($MgCl_2$), 0.2 mg/mL bovine serum albumin (BSA; Takara Bio Inc.), 0.2 mM deoxyribose-5-phosphate (dNTP), 0.1–0.4 μM each primer, ~5.0 μL template, and 1.25 U Ex Taq polymerase (Takara Bio Inc.).

For microsatellite DNA amplification, PCR was performed using a multiplex PCR of sets A and B (i-cycler; Bio-Rad Laboratories, Inc., Shinagawa, Tokyo, Japan) with the following conditions: initial 3 min at 97°C; 15 cycles, with denaturation for 30 sec at 97°C, annealing for 30 sec at 55°C, and extension for 30 sec at 72°C; 30 cycles with denaturation for 30 sec at 90°C, annealing for 30 sec at 55°C, and extension for 30 sec at 72°C, followed by final extension for 30 min at 60°C.

For sex determination, PCR conditions resembled those of microsatellite amplification with annealing temperature of 63°C. From the amelogenin gene sequence of blood samples for Asiatic black bears, two bands of 191 base pairs (bp) originating from the Y-chromosome and of 245 bp from the X-chromosome were amplified from male bears; a single band of 245 bp was amplified from females (Yamamoto et al. 2002). Three controls were included in each experiment to monitor for contamination: 2 positive controls were of black bear DNA extracted from tissue (1 male and 1 female); 1 negative control was also used.

The PCR products were separated using a genetic analyzer (ABI Prism 310; Applied Biosystems, Inc.) according to protocols described by the manufacturer with Performance Optimized Polymer 4 (POP 4; Applied Biosystems Inc.) run: 1 μL sample, 0.5 μL size standard sample with a LIZ® label (GS 500; Applied Biosystems, Inc.), and 12 μL formamide (Applied Biosystems, Inc.). Data were analyzed using software (GeneScan Analysis version 3.7; Applied Biosystems, Inc.).

Statistical methods

From 153 genotyped data of captured individuals, the number of alleles, expected heterozygosity (H_E), a probability of identity (P_{id}), and P_{id} among siblings ($P_{\mathrm{id\text{-}sib}}$) were calculated using program GENECAP (Wilberg and Dreher 2004). The critical

Table 1. Microsatellite analysis using tissue samples collected in Iwate prefecture (n = 153). Observed number of alleles (A), estimated heterozygosity (H_E), unbiased probability of identity (P_{id}) and P_{id} among siblings ($P_{id\text{-}sib}$), by locus.

Locus	A	He	P_{id}	$P_{id\text{-}sib}$
G10B	4	0.61	0.216	0.501
G10C	7	0.72	0.115	0.417
G10L	3	0.43	0.379	0.629
G10P	12	0.83	0.049	0.346
G10M	7	0.77	0.089	0.388
G10X	5	0.59	0.241	0.517
mean	6.33	0.66	–	–
overall	–	–	9.88×10^{-6}	9.14×10^{-3}

level of match probability was set as $P < 0.05$ according to the criteria reported by Woods et al. (1999).

Quality control

Quality control was undertaken according to the following procedure: (1) about 100 ng of DNA (~1/3 of the sample) was template DNA, as determined by the measured value; (2) results of multiplex PCR performed for individual identification eliminated samples genotyped with fewer than 6 loci; (3) similar genotyped samples (1 mismatch: 1MM and 2MM) were detected using GENECAP. The multiple tubes approach was performed for error checking according to Paetkau (2003). Mismatched markers were re-amplified selectively using the singleplex reaction for confirmation.

Results

Genetic diversity

Genetic diversity of the black bears in Iwate prefecture was measured at 6 microsatellite loci from 153 tissue samples (Table 1). Overall for the 6 loci, the probabilities of identity were 9.88×10^{-6} for P_{id} and 9.14×10^{-3} for $P_{id\text{-}sib}$. The average H_E was 0.66 (Table 1). We compared the genotypes of all 153 samples; no individuals displayed the same genotype. Consequently, the 6 primers were sufficient to identify each bear.

DNA analysis using corn-bite samples

We collected 99 corn-bite samples from damaged fields in Shizukuishi and PCR-amplified 99 samples (100%). Among these, 30 (30%) provided sufficient DNA for complete genetic typing at 6 loci. Among them, no evidence of 1MM samples was observed, but 17 2MM samples were found. We reanalyzed the mismatched markers selectively using the singleplex reaction, thereby identifying genotypes of 2 samples. Another 15 samples yielded the same result with primary genotypes. Of the remaining 69 samples, 56 (81%) provided data for at least 4 of the 6 loci, but no more were obtainable because the extract was exhausted. The amplification rate of locus G10M was 13%. Genotyping error was observed for 3 samples (false amplification).

Of 21 identified bears, 16 were males and 1 was female (Fig. 1). Four samples yielded insufficient DNA to determine sex.

Six individuals (F, J, K, L, P, and S) were observed several times. Of these 6 bears, we detected individual K at the same field on 10 and 17 August, and L at the same field on 17 August and 2 September. Because all samples were collected within 3 days after the bear had caused feeding damage, these 2 bears were assumed to have committed multiple offenses at the same field. Another bear, S, moved into a field on 5 August and damaged a different field again about 2 km distant on 17 August.

Discussion

Genetic diversity

The H_E value of 0.66 was slightly lower than those of the defined standards (6 markers would be used when $0.7 < H_E < 0.8$) by Paetkau (2003), but no 2 individuals had identical genotypes. The probability of identity was reasonably low, considering the range of 0.001–0.0001, as suggested by Waits et al. (2001). These data provided reliable genotypes using these 6 amplified microsatellite markers.

DNA analysis using corn-bite samples

DNA from our field-collected corn-bite samples enabled us to estimate the minimum number of problem bears that invaded these farmlands. Because the amount of DNA extracted from crop samples was more limited than that from other non-invasive samples, we set criteria for analyses in the following manner. For DNA amplification, a nanogram to a microgram of genomic DNA is commonly used (Navidi et al. 1992, Taberlet et al. 1996, Waits et al. 2001). In the unpublished pilot study, the extraction method and the amplification method of DNA were examined: DNA extraction was performed using apple samples (n = 48), which were collected from

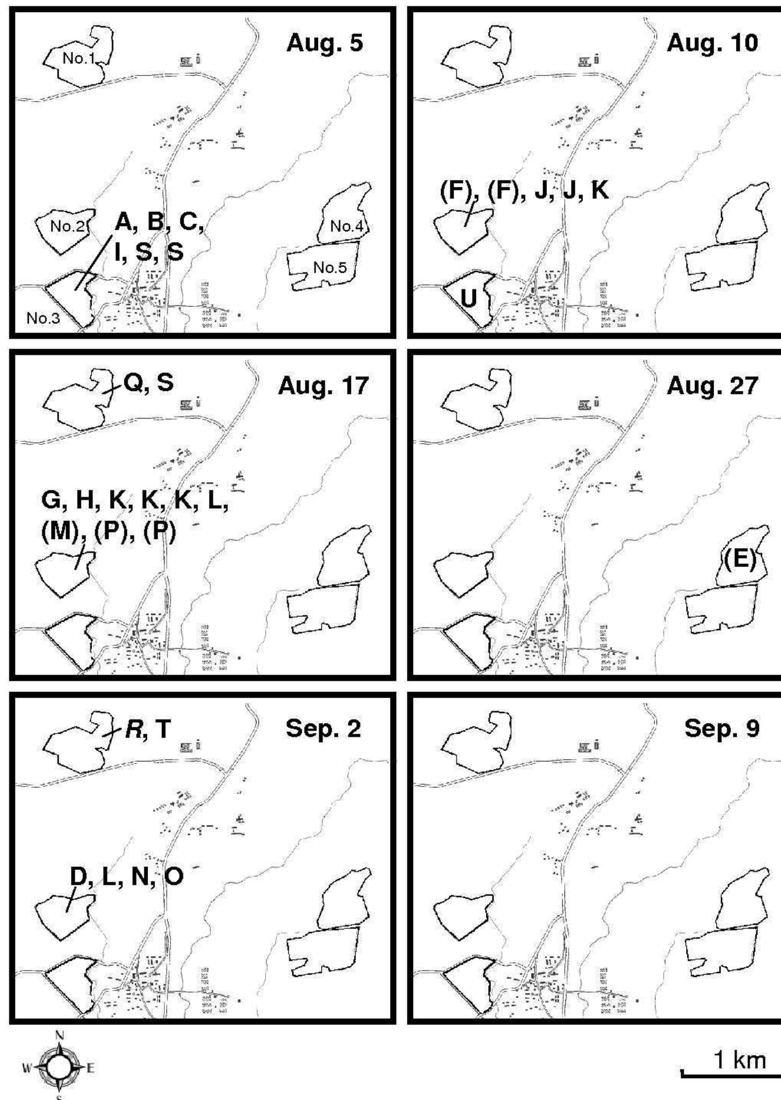

Fig. 1. Temporal distribution of nuisance bears (A–U) from corn-bite sampling and genotyping (*n* = 30) for dent corn fields 1–5 in Shizukuishi town, Iwate prefecture, summer 2004. Gender is shown as follows: male, non-marked; female, only bear R; sex-unknown, parenthesis. Samples from two 2MM pairs of bears (A and B; K and L) were collected from the same field on the same day; these pairs were among 17 confirmed 2MM pairs. Size of fields 1–5 were, respectively, 0.20, 0.11, 0.12, 0.19, and 0.16 km^2.

captive bears (3 bears of each sex). When the average of 97 ng (range: 47–240; corresponding to 1/5 of the sample) of genomic DNA was used, 98% (47/48) of samples were sufficient to amplify the amelogenin gene. Furthermore, for analysis of non-invasive samples containing DNA of low quantity and quality, the multiple-tubes approach is recommended to avoid genotyping errors such as false allele and allelic dropout (Taberlet et al. 1996, Morin et al. 2001, Fernando et al. 2003, Paetkau 2003). Because of this, we also conducted 5 replicate amplifications using apple samples (*n* = 48). In our pilot study, all samples were determined for sex, which suggests that, in cases with bite-mark samples, a reliable genotype was available when using about 100 ng of genomic DNA. For the present study, we also quantified DNA from corn samples using a spectrophotometer. From template DNA, it was estimated that about one-third of the sample was consumed per reaction. The reason for the greater quantity of

template DNA compared to the present study noted above is probably that the DNA degraded with time. Bellemain et al. (2007) showed a negative correlation between the age of fecal samples and amplification success. Samples with very low DNA quantities, such as from fecal samples, suggest that incorrect results are increasingly observed concomitantly with dilution of samples (Morin et al. 2001, Fernando et al. 2003). For analysis at the 99% confidence level, at least 5U template DNA per locus (~35 picogram) is needed (Taberlet et al. 1996). Therefore, for low DNA concentrations such as that on corn samples, dilution of the samples seemed to increase the error risk. For that reason, we performed 3 reactions at the first pass: individual genotyping by the multiplex method (sets A and B), the amelogenin gene, which is the longest target region (245 bp), was amplified using the singleplex reaction. Furthermore, the amount of template DNA was about 100 ng and added up to one-third of the sample to limit the risk of error. We discarded samples for which genotypes of the 6 microsatellite markers did not amplify during the first pass. Samples showing similar genotypes (1MM and 2MM) were reanalyzed for the mismatched markers selectively using the singleplex reaction.

Although the amount of template DNA was 96 ng (range 24–278) on average, the success rate of genotypes according to the criteria described above was only 30% (30 of 99). This result is expected to arise from multiplex PCR; 3 loci were amplified together. In fact, the lower the peak height shown using GeneScan, the longer the base length. Indeed, PCR products tended to show a low peak over 200 bp in this study. Of 69 eliminated samples, primers targeting the longest fragment (locus G10M, 197–211 bp) amplified in only 13% of samples, whereas primers targeting the shortest fragment (locus G10C, 104–122 bp) amplified in 83% of samples. Sefc et al. (2003) indicated that low DNA concentration also caused non-amplification at the longer loci, and proposed amplifying DNA fragment of 200 bp or less for highest accuracy. In this study, G10M (13%), for which the amplification was the worst of dismissed samples, was greater than 200 bp. A shorter primer will improve the accuracy of analysis in the future.

We identified 16 problem male bears and only a single female, whereas the ratio of males to females killed for nuisance control has been 2:1 (Iwate Prefecture 2003). These starkly different ratios suggest that females may be erroneously targeted as nuisance bears. The number of bears killed as nuisances has recently increased in Japan (Oi and Yamazaki 2006). For that reason, it is important to prevent erroneous targeting of bears, not only from the perspective of damage reduction, but also from the viewpoint of management of the bear population. Consequently, gathering individual information must be a part of future work.

We demonstrated that it is possible to identify bears from corn-bite samples collected in agricultural fields using microsatellites, although only 30% of the samples provided sufficient DNA for analyses. For future studies, we raise several issues. The main problem is the low amount of DNA obtained from corn-bite samples. In fact, DNA from crop samples is easily dissolved by the moisture of plants and is degraded by the high temperatures and humidity during summer. Therefore, measures should be taken to collect the samples as soon as possible after bears damage crops. Even with such measures, many samples might be eliminated because of insufficient DNA. We recommend the collection of fresh samples, comprising more than a dozen bitten crop samples, from damaged fields to identify the greatest number of problem bears.

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