NOTE *Wildlife Science*

A Genetic Method for Sex Identification of Raccoons (*Procyon lotor*) with Using the *ZFX* and *ZFY* Genes

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ABSTRACT. A genetic method for sex determination in raccoons was developed based on nucleotide differences of the zinc finger protein genes *ZFX* and *ZFY*. Four novel internal primers specific for *ZFX* or *ZFY* were designed. PCR amplification using two primer sets followed by agarose gel electrophoresis enabled sex determination. 141-bp and 447-bp bands were in both sex, and 346-bp band was specific only in male with primer set I. 345-bp and 447-bp bands were in both sex, and 141-bp band was specific only in male with primer set II, which could distinguish raccoon's electrophoresis pattern from three native carnivores in Hokkaido. This method will be useful for conservation genetics studies or biological analyses of raccoons.

KEY WORDS: PCR, raccoons, sex identification, *ZFX* and *ZFY* genes.

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Sex is one of the most important pieces of information about an animal, as it is related to physiology, behavior and reproduction. Thus, developing methods for sex identification are essential in many fields of study, including zoology and ecology. In some mammalian species, the sex of adult individuals can be determined relatively easily based on differences in body size or sexually dimorphic characters [6]. The raccoon (*Procyon lotor*) is a middle-sized carnivore. Although male raccoons have an *os penis*, which can be used in sex determination, it is not easy to identify the sex of a raccoon without a physical examination (i.e., by appearance alone). In such animals, a genetic method for sex determination can be useful; however, thus far, genetic-material-based techniques have not been developed for raccoons. The development of such a genetic method would be useful for conservation genetics studies or biological analyses of raccoons. Genetic sex determination in mammals is based mostly on the specification of the Y chromosome in males. Easy and rapid PCR-based amplification methods have been developed for many mammalian species based on differences in the genes of the X and Y chromosomes, including the amelogenin genes *AMELX* and *AMELY* [2], the sex-determining region Y (*SRY*) gene [3] and the zinc finger protein genes *ZFX* and *ZFY* [1]. *ZFX/ZFY* have been used in rapid amplification methods for sex identification in many mammals, including forest musk deer [5], sika deer [8], American minks [7] and dogs [4]. The aim of this study was to identify differences between *ZFX* and *ZFY* in raccoons and to establish a new genetic method for sex determination of raccoons.

Hair or whisker samples were collected from the carcasses of feral raccoons that were euthanized for eradication control in Hokkaido, Japan. The sex of the animals was determined at the time of sampling by checking for an *os penis*. The samples were kept at −20°C with silica gel until DNA extraction. DNA was extracted from root parts of hair (8 to 10 strands) or whisker (3 to 4 strands) to final volume 30–50 μl using an ISOHAIR^{TM} kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. In four females and four males which were chosen randomly, amplification of *ZFX/ZFY* was performed using the universal primers described by Aasen *et al.* [1], P1-5EZ (5′-ATAATCACATGGAGAGCCACAAGCT-3′) and P2-3EZ (5′-GCACTTCTTTGGTATCTGAGAAAGT-3′), in a 25-μl reaction containing 1 μl of DNA extract, 0.625 U of KAPA*Taq* EXtra DNA polymerase (NIPPON Genetics Co., Ltd., Tokyo, Japan), 5 μl of 5× KAPA*Taq* EXtra buffer (NIPPON Genetics Co., Ltd.), 5 μl of 25 mM $MgCl_2$ (NIPPON Genetics Co., Ltd.), 0.75 μl of dNTP Mix (10 μM each; NIPPON Genetics Co., Ltd.) and 1.25 μl of each of the primers (final concentration; 0.5 μM) described above. The reaction conditions were: 1 cycle of 95°C for 10 min; 35 cycles of denaturation at 95°C for 30 sec, annealing at 53°C for 30 sec and extension at 72°C for 30 sec; and 1 cycle of 72°C for 10 min. To confirm amplification, 5 μl of the product were electrophoresed on a 1.5% agarose gel. The products were purified using a NucleoSpin Gel and PCR Clean-up kit (Takara Bio Inc., Otsu, Japan). The purified male fragment was cloned into pGEM-T easy vector (Promega Co., Madison, WI, U.S.A.) and transformed into competent DH5α *Escherichia coli* cells. The cloned products of *ZFX* and *ZFY* were sequenced using a Big Dye Terminator version 1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, U.S.A.) in both directions with the primers described above. The sequences were analyzed using an ABI

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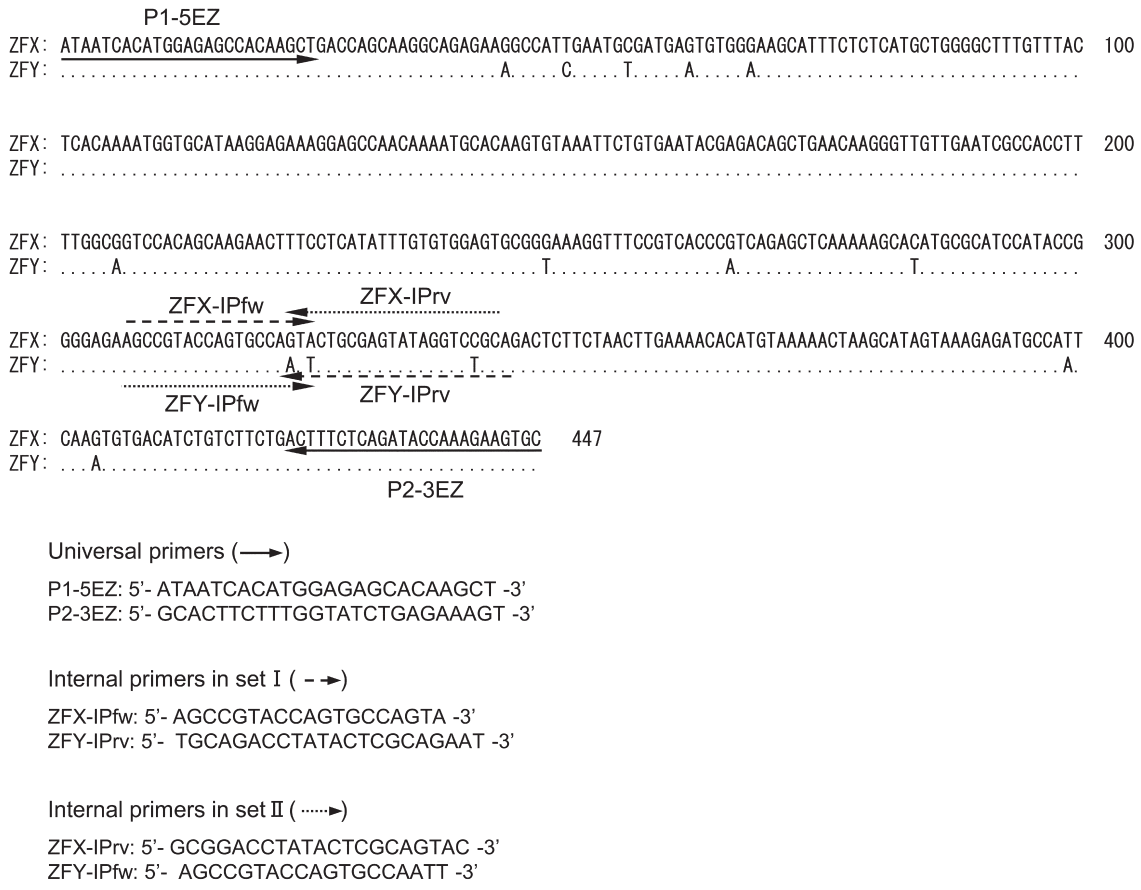

Fig. 1. Partial sequence of the *ZFX* and *ZFY* genes of raccoons and the position of 2 universal primers and 4 internal primers.

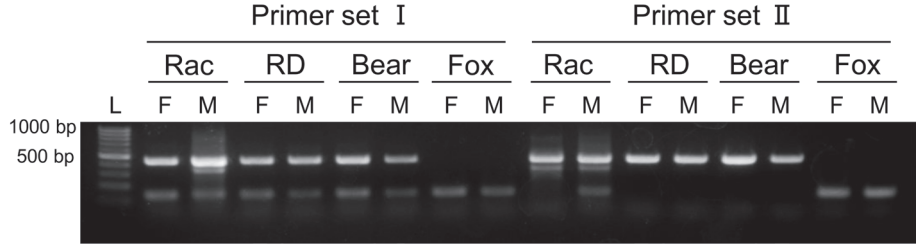

Fig. 2. PCR amplification results of fragments of *ZFX* and *ZFY* genes of four carnivore species in 1.5% agarose gel. L: 1,000 bp ladder marker. F: females, M: males. Rac: Raccoons. RD: Raccoon dogs. Bear: Hokkaido brown bears. Fox: Red foxes. The samples from the same individuals were shown with both primer sets.

PRISM™ 310 Genetic Analyzer (Applied Biosystems). The fragment lengths were both 447 bp for *ZFX* and *ZFY*(Fig. 1). The sequences of the *ZFX* fragment from four females and four males were identical, while those of the *ZFY* fragment from four males were also identical. Between the sequences of *ZFX* and *ZFY*, 14 base differences were identified (96.9% homology). Nucleotide sequence data reported are available in the DNA Data Bank of Japan databases under accession numbers *ZFX*: AB856034 and *ZFY*: AB856035.

According to differences between the sequences of *ZFX* and *ZFY* based on positions 323 and 325, specific internal primers for sex determination were designed; ZFX-IPfw (5′-AGCCGTACCAGTGCCAGTA-3′) and ZFX-IPrv (5′-GCGGACCTATACTCGCAGTAC-3′) were complementary to *ZFX*. ZFY-IPrv (5′-TGCAGACCTATACTCGCAGAAT-3′) and ZFY-IPfw (5′- AGCCGTACCAGTGCCAATT-3′) were complementary to *ZFY* (Fig. 1). For sex identification, two set of four primers (set I: P1-5EZ, P2-3EZ, ZFX-IPfw and

ZFY-IPrv; set II: P1-5EZ, P2-3EZ, ZFX-IPrv and ZFY-IPfw) were applied. The sizes of the expected products in set I were 141 and 447 bp for females and 141, 346 and 447 bp for males. On the other hand, those in set II were 345 and 447 bp for females and 141, 345 and 447 bp for males. To confirm the amplification pattern, 5 μl of the product were electrophoresed on a 1.5% agarose gel with 20 samples of both sex, respectively. As the result, a 141-bp fragment from *ZFX* and a 346-bp fragment from *ZFY* were successfully amplified with primer set I, and a 345-bp fragment from *ZFX* and a 141-bp fragment from *ZFY* were successfully amplified with primer set II. In electrophoretic profiles, results of PCR tests were obtained as expected (Fig. 2).

In the present study, a PCR test for sex determination in raccoons using newly designed primers was developed based on nucleotide differences between *ZFX* and *ZFY*. The new primer ZFX-IPfw and ZFY-IPfw were designed based on two different sites, while ZFY-IPrv and ZFX-IPrv were designed based on three sites; of these, ZFY-IPrv was considered to be highly specific for *ZFY* due to the absence of a 346-bp fragment in the female samples with primer set I. And, ZFY-IPfw was also considered to be highly specific for *ZFY* due to the absence of a 141-bp fragment in the female samples with primer set II. Therefore, the sex of the animals could be determined rapidly by agarose gel electrophoresis based on the appearance of band position with using primer set I or II. No amplification or a single band was taken to indicate a technical failure. To know a detection sensitivity limit, amplification was done with 8 dilution step template DNA concentrations (100, 10, 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} $ng/\mu l$) in 4 females and 4 males. As the result, different pattern of electrophoresis image based on sex could be clearly observed until 10^{-1} $ng/\mu l$ with primer set I and 1 $ng/\mu l$ with primer set II. When PCR test was done with a variety of annealing temperatures (51°C, 53°C, 55°C, 57°C, 59°C or 61°C) in 4 females and 4 males, similar results were obtained regardless of temperature differences.

We tried to apply these primer sets in three native carnivores in Hokkaido, raccoon dogs (*Nyctereutes procyonides albus*), Hokkaido brown bears (*Ursus arctos yesoensis*) and red foxes (*Vulpes vulpes*). DNA samples from 4 females and 4 males in each animal were used to compare with electrophoresis image of raccoon. Both primer sets could not distinguish sex of other three species (Fig. 2). With primer set I, foxes showed only the smallest molecular weight band, however, raccoon dogs and brown bears showed the same band pattern of raccoon female. On the other hand, with primer set II, foxes showed only the smallest molecular weight band, and raccoon dogs and brown bears showed only the largest molecular weight band (Fig. 2). From these results, applying primer set II could determine sex of raccoons specifically and could distinguish raccoon's electrophoresis pattern from other three native species in Hokkaido.

In summary, in this study, a genetic method for sex determination in raccoons was developed using two sets of novel internal primers based on nucleotide differences between the zinc finger protein-encoding genes *ZFX* and *ZFY*. Our genetic method enables sex determination in the laboratory using a small amount of extracted DNA.

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