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.

KEYWORDS: 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™ 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], 1P-5EZ (5′-ATAATCACATGGAGAGCCACAAGGT-3′) and P2-3EZ (5′-GCACCTTCTTG-GTATCGAGAAAGT-3′), in a 25-µl reaction containing 1 µl of DNA extract, 0.625 U of KAPA EXtra DNA polymerase (NIPPON Genetics Co., Ltd., Tokyo, Japan), 5 µl of 5× KAPA EXtra buffer (NIPPN Genetics Co., Ltd.), 5 µl of 25 mM MgCl₂ (NIPPN Genetics Co., Ltd.), 0.75 µl of dNTP Mix (10 µM each; NIPPN 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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The fragment lengths were both 447 bp for \textit{ZFX} and \textit{ZFY} (Fig. 1). The sequences of the \textit{ZFX} fragment from four females and four males were identical, while those of the \textit{ZFY} fragment from four males were also identical. Between the sequences of \textit{ZFX} and \textit{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 \textit{ZFX}: AB856034 and \textit{ZFY}: AB856035.

According to differences between the sequences of \textit{ZFX} and \textit{ZFY} based on positions 323 and 325, specific internal primers for sex determination were designed; \(5’\)-AGCGTACCCGATCGCAAT-3’\) and \(5’\)-TCGACACCTATCAGTTTTTGGCAAT-3’\) were complementary to \textit{ZFX}. \(5’\)-TGACACCTATCAGTTTTTGGCAAT-3’\) and \(5’\)-AGCGTACCCGATCGCAAT-3’\) were complementary to \textit{ZFY} (Fig. 1). For sex identification, two sets of four primers (set I: P1-5EZ, P2-3EZ, ZFX-IPfw and P2-3EZ) were used. The samples from the same individuals were shown with both primer sets.

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

**Fig. 2.** PCR amplification results of fragments of \textit{ZFX} and \textit{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.
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ent pattern of electrophoresis image based on sex could be

−1

indicate a technical failure. To know a detection sensitivity

set I or II. No amplification or a single band was taken to

based on the appearance of band position with using primer

could be determined rapidly by agarose gel electrophoresis

samples with primer set II. Therefore, the sex of the animals

due to the absence of a 141-bp fragment in the female

ZFY

And, ZFY-IPfw was also considered to be highly specific for

a 346-bp fragment in the female samples with primer set I.

were successfully amplified with

ZFX

ZFY

ZFX

ZFY

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/µl) 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/µl with primer set I and 1 ng/

µl 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 ob-

tained regardless of temperature differences.

We tried to apply these primer sets in three native carni-

vores in Hokkaido, raccoon dogs (Nyctereutes procyonides

alus), 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 electro-

phoresis image of raccoon. Both primer sets could not dis-

tinguish sex of other three species (Fig. 2). With primer set I,

foxes showed only the smallest molecular weight band, how-

ever, 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 deter-

mination 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

genoic method enables sex determination in the laboratory

using a small amount of extracted DNA.

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