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Gender Determination by PCR Assay for The Sex-Determining Region Y(*SRY*) Gene Amplification in Linnaeus's Two-Toed Sloth (*Choloepus Didactylus*)

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Abstract

In Linnaeus's two-toed sloths (*Choloepus didactylus*), there is no distinct sexual dimorphism. It is an obstacle for gender determination from the external genitalia, especially in newborns or young sloths. Hence, easy, rapid, and reliable genetics-based methods for gender identification of the sloths are needed to continue captive breeding more successfully. In this study, a PCR-based technique that allows gender determination of two-toed sloths by using a sex-determining region Y (*SRY*) gene marker was described. The hair samples from young (suspect gender) and adult sloths (known gender) were used in genetic analysis. Initially, genomic DNA was isolated from hair root samples using Roche high pure PCR template preparation kit. The *SRY* primers were specifically designed based on the NCBI and Ensembl databases, and they were verified with the BLAST program concerning the two-toed sloth genome. PCR amplification with the *SRY*-specific primers was carried out by a programmable thermal cycler device using FastStart High Fidelity PCR System, Roche dNTPack. The samples were then electrophoresed on 2% agarose gels and were visualized by a gel documentation and analysis system. A specific band in the electrophoresis pattern is diagnostic for a male individual with a partial *SRY* region. Hence, the analysis demonstrated that the samples belonged to a male two-toed sloth. Two-toed sloth species are commonly preferred animals in zoos. Gender determination is inevitable for these animals in captivity to be raised successfully and healthily. Molecular genetic techniques allow high efficiency in taxonomic evaluations and gender identification in species that do not display sexual dimorphism. The PCR assay described here may be helpful for a rapid genetic analysis that can be widely used in gender determination for two-toed sloths.

Keywords: Two-toed sloth, PCR, Sex identification, *SRY* gene

Introduction

The Linnaeus's two-toed sloths (*Choloepus didactylus*) belonging to Xenarthra supraorder (Edenta) are nocturnal and arboreal animals living in the low and upland tropical rainforests of Central and South America.[1, 2] Xenarthra is one of the most basal mammalian clades among Placentalia. The sloths can be investigated through two genera: two-toed (*Choloepus*, Megalonychidae) and three-toed (*Bradypus*, Bradypodidae) sloths.[3] Sloths are in the Pilosa order, which also includes anteaters. Furthermore, two-toed sloths are categorized into two species, *Choloepus hoffmanni* (Hoffman's two-toed sloth) and *Choloepus didactylus* (Linnaeus's two-toed sloth).[1, 3] They belong to the family Choloepodidae.

Choloepus didactylus is larger than three-toed sloths. Large eyes and long fur are characteristic of them (Figure 1). In general, individuals of *Choloepus didactylus* have a larger head, arms, ears, and hind feet but a shorter tail compared to the Bradypodidae.[4] They are arboreal and herbivorous mammals active for 1–2 h.[5] Their color ranges from tan to buffy brown but may take on a slight greenish tinge in the wild due to green algae living on their hair. The digits

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of all four feet are syndactylous, and each bears a strongly curved claw.[6]

Figure 1. The basic morphology of the two-toed sloths. Reference: https://farukyalcinzoo.com/

Although members of *Choloepus didactylus* exhibit peculiar characteristics in morphology among other mammals, there is no distinct sexual dimorphism in this species. This situation, which makes it almost impossible to distinguish between males and females, leads to significant problems in raising individuals in zoos and wild parks. On the other hand, young animals need to be housed in pairs appropriately to increase the number of protected species. The absence of gender-specific morphologies also causes negativities for the species' future. The studies about molecular techniques reveal a better understanding of molecular mechanisms regarding many animal science applications, including determining the quantitative performance traits and genomic selection, taxonomic properties of different species, genotyping characteristics of various breeds, the genetic background of many diseases and defects, and evolution. From a clinical sense, genetic analyses may provide essential clues in evaluating the molecular basis of disease development, diagnosis, prognosis, and treatment. Molecular techniques also offer solutions for gender determination quite reliably in species that do not show sexual dimorphism. Like many other mammals, the sex-determining region of the chromosome Y (*SRY*) gene effectively contributes to gender identification.

SRY encodes a DNA-binding protein (gene-regulatory protein/transcription factor) responsible for initiating male sex determination in placental mammals and marsupials.[7] It is required for the differentiation of Sertoli cells to induce masculinization of the embryonic gonad. This gene encodes the master protein essential for initiating the cascade, leading to mammal testicular differentiation.[8] Therefore, the choice between male or female development is controlled by the sex chromosomes considering the presence of a Y chromosome results in male development, regardless of the number of X chromosomes in mammals. The Y chromosome must encode a dominant inducer of testis formation, and the Y-linked gene(s) controlling this process was named the testis determining factor (TDF).[9] During embryogenesis, TDF is activated to commit the undifferentiated genital ridge to the testicular pathway. Thus, hormonal production induces male sexual differentiation regarding testis formation.[8,9]

The mammalian Y chromosome is genetically degenerated and small (less than one-third the size of the X) compared to the X chromosome.[10,11] These chromosomes evolved ~180 Mya from an autosome pair. There are some distinctive differences between X and Y chromosomes regarding their evolution history. X chromosome roughly maintained its original structure. But the Y chromosome stopped combining and has undergone significant structural and genetic loss (~97% of its gene content).[11] Only the *SRY* gene and a few other genes, mostly spermatogenesis genes, remain on the Y chromosome.[10] Although the effect of the *SRY* gene is significant, some exceptions cannot be genetically overlooked,[12] such as people who develop female genitalia but have XY chromosomes with an intact *SRY* gene (Swyer syndrome).[13] Furthermore, XY females have also been described in various species without explanation.[14] Surprisingly, there exist so-called feminizing X chromosomes in which common X-linked mutations (in unknown genes) lead XY individuals to develop as females in some rodent species.[14,15] Here, it should be noted that recent evidence has shown some contradictions in the genetic regulation of mammalian sex determination. For instance, the first X-linked gene necessary for male sex determination has been introduced in mammals, *SDX* (also known as *PWWPB3* and *MUM1L1*).[16] Knockout of the gene leads to remarkably complete male-to-female sex reversal in some XY individuals, and reduced spermatogenesis in non-reversed individuals. Nevertheless, *SRY* is a very well-known gene for male development, and the mutations or absence of this gene can result in the development of female characteristics, underscoring its crucial role in sex determination. Female sloths have an irregular pattern of receptivity to mating. This irregularity is thought to be influenced by various factors, including hormonal fluctuations and environmental cues. Moreover, the absence of sexual dimorphism makes it almost impossible to determine sex in these animals based on morphological features. Behavioral features also do not provide reliable clues. This situation necessitates the use of genetic methods. The *SRY* gene is a significant marker in animals that do not show sexual dimorphism. Rapid PCR assays are crucially needed for breeding captive/protected species concerning animal health and welfare. In the present paper, we describe a PCR-based assay that allows gender determination of two-toed sloths

without requiring invasive applications using an *SRY* gene marker.

Materials and Methods

Hair samples were obtained from two-toed sloths in Faruk Yalcin Zoo, Kocaeli, Turkiye, and sent to Bursa Uludag University, Faculty of Veterinary Medicine, Department of Genetics. Hairs were washed with distilled water and cut at approximately 5-8 mm from the root using a sterile scalpel. Samples from male and female sloths were used as control samples (Figure 2A-D). Samples from the suspect individual were used in molecular analyses. In the present work, only molted hair samples were used for the DNA source, and no invasive procedure was applied to any of the animals.

Figure 2. The sloths that were sampled in the present study. A) female sloth 1; B) female sloth 2; C) male sloth 1; D) male sloth 2.

Genomic DNA was extracted from the hair follicles using the High Pure PCR Template Preparation Kit (Roche Diagnostics Corp., Indianapolis, IN) based on the manufacturer's indications. We measured the quantity and quality of the isolated DNA samples with a spectrophotometer (NanoDrop 2000c, Thermo Scientific, Wilmington, DE, USA). Thus, we determined the concentration (ng/µL) and purity (260/280 adsorbance value) of the obtained DNA samples. The samples within the 1.80-2.00 A260/A280 absorbance range were used for further analyses.

To determine gender, *SRY* primers were specifically determined using NCBI[17] and Ensembl[18] databases. We checked the specificity of the obtained primers with the BLAST program.[19]

The PCR amplification was performed with the following primer set (5'→3', Sequence ID: XM_037824592.1) using the FastStart High Fidelity PCR System, dNTPack (Roche):
Forward: AAGCGACCCATGAATGCCTTCATGGTGT-GGT
Reverse: GAGGTCGGTACTTATAGTCTGGGTATTTC-GCTCTGTG
PCR reaction mixtures (50 µL total volume) consisted of 5µL FastStart High Fidelity Reaction Buffer (10x with 18 mM $MgCl_2$), 1 µL PCR grade nucleotide mix (10 mM), forward and reverse primers (1 µL each, 10 µM), 0.5 µL FastStart High Fidelity Enzyme Blend (5 U/µl), DMSO (1 µL), 34.50 µL of molecular water (PCR grade water) and the DNA sample (6µL). The PCR condition was as follows: 94°C for 10 min, followed by 35 cycles of 94°C for 2 min, annealing temperature of 55°C for 30 sec, 72°C for 1 min, and final extension at 72 °C for 7 min. The quantification of PCR products was performed by 2% agarose gel electrophoresis. Afterward, the gel was visualized under UV transillumination.

Results

We successfully amplified the *SRY* fragment from the sloth genomic DNA obtained from the hair samples. We used the sloth samples of known gender as controls. The PCR amplification from samples of the young sloth with unknown gender indicated distinctive gel documentation. As shown in Figure 3, a specific band in the electrophoresis pattern is diagnostic for a male individual with a partial *SRY* region. The electrophoresis pattern of adult male sloth also had the 215 bp diagnostic band. As expected, the assay lacks the specific band regarding the female sloth. Hence, the PCR assay clearly demonstrated that the samples belonged to a male two-toed sloth.

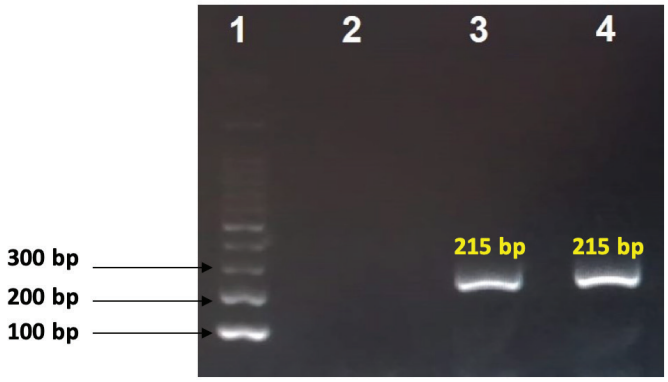

Figure 3. The electrophoresis pattern of the SRY-based PCR assay. 1: Marker (100 bp); 2: sample from the female sloth; 3: sample from the male sloth; 4: sample from the suspicious sloth. The PCR assay showed that the suspicious individual is a male two-toed sloth.

Discussion and Conclusion

Species of sloths have suffered from a low rate of reproductive success in captives and breeding under protection. It is

important to note that two-toed sloths, unlike most mammalian species, are not easy to sex from color variations, body size, and especially the anatomy of the external genitalia. Interestingly, the ovoid testicles are characterized by an intraabdominal position throughout life. What is more intriguing is that sloths (with the manatees) are the only mammalian species with an exceptional number of cervical vertebrae.[20] In fact, they are unique animals. However, one reason preventing their captive reproduction is the difficulty distinguishing their sex because of their peculiar characteristics. From a genetic perspective, they also have distinctive properties. To give an example, chromosomes in sloths are unusual and complex.[3] Several karyotypes have been reported consisting of highly polymorphic chromosomes, often unpaired.[3, 21] Among two-toed sloths, at least six different diploid numbers (2n=49, 50, 51, 52, 53, and 64) and seven distinct karyotypes have been reported.[3, 6, 21]

The mammalian Y chromosome acts as a dominant male determinant by the action of the *SRY* gene, whose role in sex determination is to initiate testis rather than ovary development from early bipotential gonads. This gene mainly triggers the differentiation of Sertoli cells from supporting cell precursors, which would otherwise give follicle cells.[22] It is located in the Y chromosome, typically as a single copy.[23] This gene exists exclusively in mammals (except prototherians, some mole vole species, and South American Akodon rodents). This clearly shows how important this gene is in the evolution of mammals.[24-27] *SRY* has been identified as a critical regulatory gene in mammalian sex determination and the detection of the male-specific signal by PCR amplification. The *SRY* gene initiates male sex determination in placental mammals and marsupials. This is an intronless gene on the Y chromosome.[27]

Using this gene as a marker for gender determination provides an efficient way in captive mammals such as *Choloepus didactylus*. On the other hand, sexing by PCR-based methods is safe because it can use a small amount of tissue sample, and the sample can be hair follicles.[1] Invasive procedures such as bloodletting can be pretty dangerous for animals not used to interacting with humans. Thus, molted hairs provide a convenient, accessible, and economical alternative to blood as a source of DNA for gender diagnostic analysis.

In the present work, we report a rapid, effective, and non-invasive *SRY*-based PCR assay for the gender determination of two-toed sloths. These genetic methods help raise captive mammals to keep the animals with the correct gender in the same area or location. Consequently, PCR assays from molted hair follicle samples allow DNA-based genetic analyzes of wild animals unaccustomed to humans. These techniques support good breeding programs for the animals under protection following animal welfare.

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