# Development of Genetic Markers for Sex and Individual Identification of the Japanese Giant Flying Squirrel (Petaurista leucogenys) by an Efficient Method Using High-Throughput DNA Sequencing

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Source: Zoological Science, 40(1) : 24-31

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zs220045

ZOOLOGICAL SCIENCE **40**: 24–31 (2023)


# Development of Genetic Markers for Sex and Individual Identification of the Japanese Giant Flying Squirrel (*Petaurista leucogenys*) by an Efficient Method Using High-throughput DNA Sequencing

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**DNA markers that detect differences in the number of microsatellite repeats can be highly effective for genotyping individuals that lack differences in external morphology. However, isolation of sequences with different microsatellite repeat numbers between individuals has been a time-consuming process in the development of DNA markers. Individual identification of Japanese giant flying squirrels (*Petaurista leucogenys*) has been challenging because this species is arboreal and nocturnal and exhibits little to no morphological variation between individuals. In this study, we developed DNA markers for sex and individual identification of this species by an efficient method using high-throughput DNA sequence data. Paired-end 5 Gb (2 × 250 bp) and 15 Gb (2 × 150 bp) genome sequences were determined from a female and a male Japanese giant flying squirrel, respectively. We searched SRY and XIST genes located on Y and X chromosomes, respectively, from high-throughput sequence data and designed primers to amplify these genes. Using these primer sets, we succeeded to identify the sex of individuals. In addition, we selected 12 loci containing microsatellites with different numbers of repeats between two individuals from the same data set, and designed primers to amplify these sequences. Twenty individuals from nine different locations were discriminated using these primer sets. Furthermore, both sex and microsatellite markers were amplified from DNA extracted non-invasively from single fecal pellet samples. Based on our results for flying squirrels, we expect our efficient method for developing non-invasive high-resolution individual- and sex-specific genotyping to be applicable to a diversity of mammalian species.**

**Key words:** sex identification, individual identification, microsatellite marker, fecal DNA, non-invasive sampling, Japanese giant flying squirrel

## INTRODUCTION

Identification of individuals and sexes is essential to studies of behavior, ecology, and population structure of animals (Waits et al., 2001; Kusahara et al., 2006; Rutledge et al., 2009). Observational studies have identified individuals and sexes based on external characteristics, such as differences in fur pattern and body size (Berry and Shine, 1980; Miththapala et al., 1989; Badyaev, 2002; Higashide et al., 2012; Takada and Minami, 2021). However, there are many animal species that do not show such external characteristics. In such cases, artificial markers including collars, ear tags, hair staining, and removal of body parts have been used to identify individuals (Southern, 1948; Bradford, 1976; Koyama, 1988; Rémy et al., 2011; Larsen et al., 2014). However, collars and hair staining can only be used for a limited period because they degrade and sometimes are dropped by animals. Compared to these, DNA markers are an effective method for identifying individuals that lack differences of external characteristics among individuals (for example, wolf:

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doi:10.2108/zs220045

Rutledge et al., 2009; brown bear: Barba et al., 2010; Sciuridae: Gorrell et al., 2012). DNA markers do not drop off, do not deteriorate, and can be used stably over a long period.

DNA marker analysis is possible with DNA extracted from feces, feathers, and hairs which can be collected non-invasively from animals (Rudnick et al., 2007; Brinkman and Hundertmark, 2009; Barba et al., 2010; Brinkman et al., 2011). DNA extracted from non-invasively sampled feces or other objects left by individuals allows researchers to collect information on animals without directly observing the individuals of interest (Beja-Pereira et al., 2009). This is particularly useful for studies of animals that are difficult to capture or observe. Therefore, the combination of non-invasive sampling and analysis with DNA markers is a powerful tool for the study of wild animal populations.

The Japanese giant flying squirrel (*Petaurista leucogenys*) is endemic to the Honshu, Shikoku, and Kyushu Islands of Japan. This species exhibits unique life history within the family of Sciuridae, such as arboreal (Ando and Imaizumi, 1982; Ando and Shiraishi, 1991; Stafford et al., 2003), nocturnal (Ando and Imaizumi, 1982; Baba et al., 1982; Funakoshi and Shiraishi, 1985), folivore ecology (Ando and Imaizumi, 1982; Baba et al., 1982; Funakoshi and Shiraishi, 1985; Sone et al., 1996; Kawamichi, 1997a; Shigeta et al., 2010), and gliding behavior (Ando and Shiraishi, 1991, 1993). Japanese giant flying squirrels are hard to observe continuously in their forest habitat because of their nocturnal and arboreal ecology. Individual Japanese giant flying squirrels are also hard to discriminate because they exhibit only small to no morphological differences between individuals and sexes. Individuals can only be identified when an individual has scars on the ears or distinctive coat colors on the tail (Kawamichi, 2015). The sex of adult animals can be determined by observing their reproductive organs during the breeding season (Kawamichi, 1997b). However, adults out of breeding condition and juveniles are difficult to identify sex using this method (Kawamichi, 1998). Because of these difficulties and the need for continuous observation and identification of individuals and sexes, studies of individual distribution, movement patterns, social structure, and population ecology of this species have been limited (Baba et al., 1982; Kawamichi, 2015). In recent years, forest fragmentation and an overall reduction of their available habitat have been reported (Okazaki, 1999; Aoki et al., 2006). For conservation planning and understanding of this species, further research using methods for individual discrimination and sex identification is required.

Feces of the Japanese giant flying squirrel are relatively easy to collect on the ground because Japanese giant flying squirrels excrete a large amount of pelleted feces under the canopies of large trees where they frequently stop during their gliding locomotion (Shigeta et al., 2010; Kawamichi, 2015). Accordingly, non-invasive DNA extraction and analysis using DNA markers is a suitable method to identify Japanese giant flying squirrels. However, no study has used Japanese giant flying squirrel DNA extracted from feces and markers for sex identification (Kusahara et al., 2006; Gorrell et al., 2012) or individual identification (Todd, 2000; Hale et al., 2001; Shibata et al., 2003; Gunn et al., 2005; Fike and Rhodes, 2009; Fike et al., 2013) that have been developed for placental mammals and species closely related to the Japanese giant flying squirrel.

DNA markers for individual identification detect differences in indels between individuals, mostly in the number of microsatellite repeats at multiple genomic loci. However, isolation of polymorphic sequences including microsatellites from genomic DNA has been a time-consuming process in the development of DNA markers. Even if a microsatellite-containing sequence is isolated, the number of repeats may be the same among individuals, making it impossible to discriminate among individuals. Although it is easy to isolate repeat sequences from assembled genomes in general, the genomes of species of interest are often unassembled. Many DNA markers are designed to amplify PCR products of around 100–300 bp (Sefc et al., 2003; Buchan et al., 2005; Broquet et al., 2007), and therefore long-assembled contigs are not always necessary to design primers. Recent studies have used high-throughput whole genome and cDNA sequencing to isolate targeted genomic regions including repeat sequences (Abdelkrim et al., 2009; Ariede et al., 2018; Fraga et al., 2020).

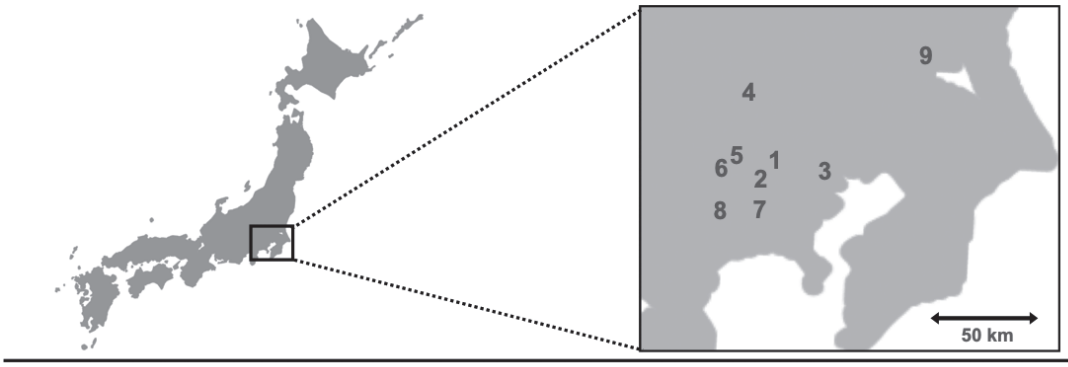
In most placentalia species, sex is determined by a combination of X and Y sex chromosomes. When the individual has a combination of XX, the sex is determined as female, and when the individual has XY, the sex is determined as male. The SRY and XIST genes are known to be located on the Y and X chromosomes, respectively (Gubbay et al., 1990; Sinclair et al., 1990; Borsani et al., 1991; Brockdorff et al., 1991; Herzing et al., 1997; Marahrens et al., 1998). Therefore, these genes can be used as DNA markers for sex identification (e.g., Dallas et al., 2000; Kusahara et al., 2006).

In this study, we developed DNA markers for sex and individual identification of the Japanese giant flying squirrel by an efficient method using high-throughput DNA sequence data. These DNA markers are expected to enable research that requires information for multiple individuals or continuous monitoring, such as distribution and dispersion, social structure, and population size estimation without the need to capture animals or observe them directly.

## MATERIALS AND METHODS

### Sample collection

We collected samples (tissues or feces) and extracted genomic DNA from 20 Japanese giant flying squirrels obtained from four breeding facilities and five field sites (Fig. 1). Tissue samples of four individuals were collected at Tama Forest Science Garden Hachioji and of one individual at Saitama children's Zoo Park (Fig. 1). Among them, three individuals collected at Tama Forest Science Garden were found dead at this site. The cause of death was presumed to be either predation or accidental collision. The entire specimens of two individuals and partial body (only skin) of one individual were stored at –80°C. Since one of these individuals was a partial body (K1), sex of this individual was not identifiable based on genital morphology. Sex of an adult male (K4) and an infant female (K3) were identified based on genital morphology. We note that the female sex of K3 should be interpreted with caution due to difficulty with morphological sex identification of infants. A fourth individual found at this location (K2) was also an infant and had fallen from a tree canopy but was kept alive. A tissue sample was obtained from this individual by swabbing the inside of its cheek with a cotton swab. The cotton swab was immediately soaked in Lysis buffer (0.1 M Tris-HCL, 0.1 M EDTA, 0.01 M NaCl, 0.5% SDS) and was stored at –30°C. Sex of this infant individual (K2) was not identifiable based on genital morphology. One female individual (S1) was collected at Saitama children's Zoo




| Location | Tissue type | Captive or wild | Sex | Sample ID |
|---|---|---|---|---|
| 1: Chuo University Junior and Senior High School | Feces | Captive | M | G1 |
| 2: Tama Zoological Park | Feces | Captive | F* | T1 |
| 2 | Feces | Captive | M* | T2 |
| 3: Ueno Zoological Gardens | Feces | Captive | F | U1 |
| 4: Saitama children's Zoo Park | Tissue | Captive | F | S1 |
| 4 | Feces | Captive | F | S1F |
| 5: Tama Forest Science Garden | Tissue | Wild | M* | K1 |
| 5 | Tissue | Wild | F* | K2 |
| 5 | Tissue | Wild | F* | K3 |
| 5 | Tissue | Wild | M | K4 |
| 5 | Feces | Wild | M* | K5 |
| 5 | Feces | Wild | F* | K6 |
| 6: Mt. Takao | Feces | Wild | F* | TK2 |
| 6 | Feces | Wild | M* | TK1 |
| 7: Private back yard forest | Feces | Wild | F* | MC1 |
| 7 | Feces | Wild | M* | MC2 |
| 8: Lake Tsukui Shiroyama Park | Feces | Wild | F* | SY1 |
| 8 | Feces | Wild | M* | SY2 |
| 9: Mt. Tsukuba | Feces | Wild | F* | TU2 |
| 9 | Feces | Wild | M* | TU1 |
| 9 | Feces | Wild | M* | TU3 |

*sex was identified by sex identification marker

**Fig. 1.** Sample information. Samples were collected from four breeding facilities (1–4) and five field sites (5–9). Arabic numbers on the map correspond to the location numbers in the table.

Park. A tissue sample was obtained from this individual by swabbing the inside of its cheek with a cotton swab.

Fecal samples of 16 individuals were collected from four breeding facilities and five field sites (Fig. 1). Fecal samples of five individuals (two males and three females) were collected from four breeding facilities (Chuo University Junior and Senior High School, Tama Zoological Park, Ueno Zoological Gardens, and Saitama Children's Zoo Park) (Fig. 1). All individuals were protected in a forest close to each facility when they were young. Fecal pellets defecated before night were collected from the floor of the breeding facility with disposable toothpicks or chop sticks. Samples were stored independently in 1.5 mL tubes with Lysis buffer (described above) and stored at −30°C. Because two individuals (one male and one female, T1 and T2 in Fig. 1) at Tama Zoological Park were kept in the same cage, it was unclear which feces belonged to which individual. Therefore, we identified defecating individuals by identifying the sex of the DNA samples extracted from each fecal pellet using sex identification markers. Feces collected from Tama Zoological Park were only used for testing of individual identification markers.

Fecal samples of 11 individuals were collected at five field sites (Tama Forest Science Garden, Mt. Takao, Mt. Tsukuba, Lake Tsukui Shiroyama Park and private forest site) from several different trees (Fig. 1). Each fecal sample was stored in separate 1.5 mL tubes containing Lysis buffer and stored at −30°C. To separate unique individuals from unidentified individuals, we performed sex identification for all samples after development of sex identification markers. Then, we selected two fecal samples with different sex from each site and treated these selected samples as unique individuals. In addition, two to three feces collected from a sufficient distance (1.2 km) apart, which could be safely assumed to have been defecated by different individuals, were also treated as unique individuals. These feces collected from presumably separate individuals were only used for testing individual identification markers. The guidelines for experimental animal management of SOKENDAI were followed throughout the study. The Institutional Animal Care and Use Committee of SOKENDAI approved the animal protocols and procedures (permission #2017004ar and #2018005ar).

**DNA extraction**

From the stored body specimens, a piece of muscle tissue (~5 mm square) was cut out with a scalpel and used for DNA extraction. Genomic DNA was extracted from tissue samples using a DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany). Fecal DNA was extracted from a single fecal pellet sample using a Cica Geneus DNA Prep Kit for Stool (KANTO KAGAKU) following the manufacturer's instructions.

**Development of DNA markers to identify individuals**

The concentration of the genomic DNA extracted from tissue samples of the male (K4) and presumed female (K3) giant flying squirrels found at the Tama Forest Science Garden (Fig. 1) was measured using a Qubit Fluorometer 2.0 (Thermo Fisher Scientific). Sex of K3 was ambiguous because this individual was an infant. After development of a sex identification marker, this individual was confirmed to be female. The NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Bio Labs, Ipswich, MA, USA) was used to construct libraries from genomic DNA. Paired-end ($2 \times 250$ bp for a female [K3] and $2 \times 150$ bp for a male [K4]) sequencing was performed on the Illumina HiSeq X or Hiseq 2500 platform. In total, 5 Gb ($2 \times 250$ bp) and 15 Gb ($2 \times 150$ bp) sequences were determined from each individual. For sequences from a female (K3), the average length of DNA fragments was prepared to be less than 500 bp for Paired-end $2 \times 250$ bp sequences by Covaris M220 (Covaris. Inc., Woburn, MA, USA). When the paired-reads were overlapped, the two reads were merged as one sequence by the merge reads algorithm implemented in CLC Genomic Workbench 9.0 (https://www.qiagenbioinformatics.com/). The merged sequences were used as a database for BLASTN searches (Altschul et al., 1990). We searched for the sequences containing trinucleotide repeats using nine types of trinucleotide repeat (AATn, AGCn, AGTn, ATCn, ATTn, CATn, GATn, TAAn, TATn) sequence motifs as query sequences (see Supplementary Table S2). The sequence reads from a male (K4) were mapped to merged sequences (K3) using CLC Genomic Workbench. Reads showing high similarity (> 90% in > 90% of read length) were mapped to merged sequences to avoid mapping the low similarity reads. When the number of repeats was different between merged sequences and the mapped reads, the sequence was selected as a candidate for development of a marker region. We selected 12 candidate sequences (accession numbers: LC726459–LC726470) and designed two pairs of primers on the flanking regions of the repeats in each candidate sequence. The sequences of primers are listed in Supplementary Table S2.

**Development of DNA markers to identify sex**

SRY and XIST regions were searched from the sequences of the male (paired end 150 bp) and the female (paired end 250 bp) Japanese giant flying squirrels, using homologs of SRY (Gene ID 21674) and XIST (Gene ID 213742) from the house mouse (*Mus musculus*) as queries. To perform nested PCR, two sets of primers were designed on SRY and XIST sequences (accession numbers: LC726471, LC726472), respectively. The sequences of primers are listed in Supplementary Table S1.

**Test of DNA markers**

Since the amounts of Japanese giant flying squirrel DNA extracted from fecal samples were expected to be low, we used nested PCR to obtain a sufficient amount of PCR products. Furthermore, for efficient amplification, nested PCR was performed independently for each locus. Nested PCR reactions were performed with final volume of 6 μl with concentration of KOD Fx Neo (TOYOBO, Osaka, Japan) (0.12 U), 1× KOD Fx Neo buffer, dNTPs (0.4 mM), and 0.2 μM forward and reverse primer, distilled water and template DNA to amplify SRY, XIST, and microsatellite regions. For template DNA, we used 1 μl of DNA (0.05~376 ng) extracted from tissues or feces for the first round of nested PCR. For the second round of nested PCR, PCR products after the first round were diluted 100 times with water and 1 μl was used as a template. For the first and second round of nested PCR, different sets of primers were used (see Supplementary Table S1 and Supplementary Table S2: we used primers "F1" and "R1" for the first round of nested PCR and "F2" and "R2" for the second round of PCR. Primers without numbers (SRY_R and m6_R) were used for the first and the second rounds of PCR). The conditions of PCR were as follows: 94°C for 2 min of denaturation step, followed by 35 cycles of 98°C for 10 seconds of denaturation step, 58 – 62°C for 30 seconds of annealing step (listed in Table S1 and S2 for each locus), 68°C for 30 seconds of extension step, and final extension at 68°C for 1 minute. After the nested PCR, 3 μl of PCR product were electrophoresed on 2% agarose gels, with a size marker (φX174/HaeⅢ, Takara, Shiga, Japan). Samples were stained with DNA Fluorescent Staining Dye (SMOBIO, Hsinchu City, Taiwan).

Sex was identified by electrophoresis of PCR products. When both SRY and XIST regions were amplified, sex was assigned as male. When only XIST region was amplified, sex was assigned as female. To verify the sex identification, amplification of XIST and SRY regions was performed two or more times.

For microsatellite regions, we performed an additional round of PCR using second round primers tagged with a fluorescent label. The labeled PCR products were purified, and the fragment length was analyzed using a 3500 Genetic Analyzer and the Gene Mapper software 6 (Applied Biosystems). For efficiency, when we analyzed the fragment length, one to four PCR products with different color labels or different length ranges were mixed. To verify the results of amplification, PCR for each microsatellite region was performed two to seven times. We only genotyped the sample if a clear peak was observed two or more times by fragment analysis (Taberlet et al., 1996; Sefc et al., 2003). When peak observation was less than two times, the sample-specific locus was treated as failed amplification. Also, when we observed more than two peaks at a locus, the sample-specific locus was treated as a failed allele. We therefore excluded loci with either failed amplifications or failed alleles from the data analyses. Contamination was checked by negative controls for all PCR reactions.

**Data analysis**

To measure the discrimination efficiency of each individual identification marker, $P_{(ID)}$ and $P_{(ID)}$sib values were calculated. $P_{(ID)}$ is the probability that two individuals chosen from a random mating population have the same alleles and $P_{(ID)}$sib is the probability that two siblings chosen from a random mating population have the same alleles.

$$P_{(ID)} = \Sigma p_i^4 + \Sigma\Sigma\left(2p_i p_j\right)^2 \quad (1)$$

$p_i$ and $p_j$ are ith and jth allele frequencies. i is not equal to j. (Paetkau and Strobeck, 1994)

$$P_{(ID)}\text{sib} = 0.25 + \left(0.5\Sigma p_i^2\right) + \left[0.5\left(\Sigma p_i^2\right)^2\right] - \left(0.25\Sigma p_i^4\right) \quad (2)$$

(Woods et al., 1999)

Cumulative $P_{(ID)}$ and total $P_{(ID)}$sib were calculated by multiplying $P_{(ID)}$ or $P_{(ID)}$sib values from the lowest $P_{(ID)}$ or $P_{(ID)}$sib loci to obtain the discrimination efficiency when multiple markers were analyzed. Deviation from Hardy–Weinberg equilibrium was tested for each locus using the software Genepop (ver 4.7.5.) (Raymond and Rousset, 1995).

**Availability of data**

The nucleotide sequences were deposited in the DDBJ Sequenced Read Archive under accession numbers DRX346687 and DRX346688.

## RESULTS

### Development of sex identification markers

We isolated the sequences of the SRY and XIST genes and designed two sets of primers based on the sequences of these genes. We used these primers as sex identification markers. To assess the sex identification markers, we used each of two individuals of males and females of the Japanese giant flying squirrel (G1, U1, S1, K4: Fig. 1). DNAs extracted from two samples each of tissue and feces were used to

amplify XIST and SRY regions. We predicted that the XIST region on the X chromosome is amplified from both males and females, whereas the SRY region on the Y chromosome is amplified from only males. Indeed, the XIST region (258 bp) was amplified from both males and females, while the SRY region (103 bp) was amplified only from males (Fig. 2). Therefore, sex of Japanese giant flying squirrel individuals could be identified using DNA markers on the X and Y chromosomes. Furthermore, both regions were amplified from DNA extracted from even a single fecal pellet, suggesting that DNA extracted from a fecal sample is sufficient to non-invasively identify sex.

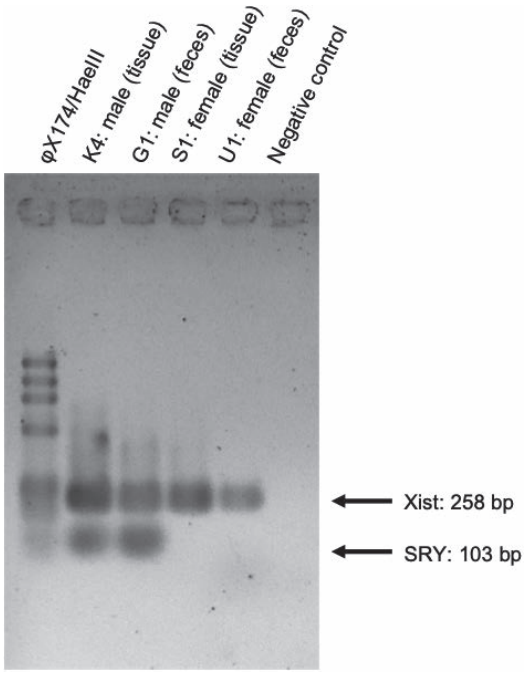



**Fig. 2.** Amplification of XIST and SRY genes from males and females of Japanese giant flying squirrels. Arrows indicate the expected length of XIST (258 bp) and SRY (103 bp) genes. Amplification from DNA extracted from tissue and feces are shown as (tissue) and (feces), respectively.

### Individual identification markers

We isolated 12 sequences containing microsatellites with different numbers of repeats between two individuals, male (K4) and female (K3), and designed primers to amplify these sequences. To assess the efficiency of individual

**Table 1.** Alleles, *P*(ID) and P(IS)sib values of 12 microsatellite loci.

| Microsatellite loci | allele size range (bp) | Number of alleles | *P*(ID) | *P*(ID)sib | cummulative *P*(ID) | cummulative *P*(ID)sib |
|---|---|---|---|---|---|---|
| 12 | 130–157 | 10 | 0.05 | 0.34 | | |
| 5 | 104–125 | 7 | 0.08 | 0.38 | 0.0038 | 0.1325 |
| 4 | 121–139 | 6 | 0.1 | 0.41 | 0.0004 | 0.0537 |
| 14 | 172–190 | 6 | 0.11 | 0.41 | 0 | 0.0219 |
| 2 | 121–130 | 4 | 0.12 | 0.41 | 0 | 0.009 |
| 9 | 87–105 | 7 | 0.11 | 0.42 | 0 | 0.0038 |
| 10 | 81–96 | 5 | 0.14 | 0.43 | 0 | 0.0016 |
| 1 | 83–99 | 5 | 0.15 | 0.45 | 0 | 0.0007 |
| 3 | 110–125 | 5 | 0.16 | 0.45 | 0 | 0.0003 |
| 7 | 117–138 | 5 | 0.21 | 0.49 | 0 | 0.0002 |
| 8 | 101–113 | 5 | 0.23 | 0.53 | 0 | 0.0001 |
| 6 | 174–177 | 2 | 0.46 | 0.68 | 0 | 0.0001 |

**Table 2.** Amplification patterns of 12 microsatellite loci for each sample.

| sample ID | Microsatellite 1 | Microsatellite 2 | Microsatellite 3 | Microsatellite 4 | Microsatellite 5 | Microsatellite 6 | Microsatellite 7 | Microsatellite 8 | Microsatellite 9 | Microsatellite 10 | Microsatellite 12 | Microsatellite 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| G1 | 93/93 | 121/124 | – | 127/133 | – | 174/177 | 120/123 | 101/107 | 96/99 | 84/84 | 133/136 | 184/187 |
| T1 | FA | 127/130 | 113/119 | 127/127 | 116/116 | 174/177 | 120/138 | FA | 90/105 | FA | 133/154 | 181/184 |
| T2 | FA | 124/130 | 110/125 | FA | 113/116 | 177/177 | 120/123 | FA | 96/105 | FA | 151/154 | FA |
| U1 | 93/99 | 124/130 | 113/113 | 130/133 | 116/125 | 174/174 | 120/120 | 104/107 | 99/102 | 87/93 | 130/157 | 178/184 |
| S1F | – | – | – | – | – | 177/177 | 138/138 | 104/107 | 96/99 | 84/93 | 130/154 | FA |
| K1 | 90/93 | 121/124 | 110/119 | 127/127 | 104/119 | 177/177 | 120/120 | 101/104 | 99/99 | 84/93 | 148/154 | 187/187 |
| K2 | 90/93 | 121/130 | 110/113 | 130/136 | 107/116 | 177/177 | 120/138 | 104/110 | 99/99 | 93/93 | 136/154 | 181/187 |
| K4 | 93/96 | 121/127 | 113/119 | 130/139 | 116/119 | 177/177 | 120/120 | 104/113 | 96/105 | 93/96 | 130/151 | 181/190 |
| K3 | 93/96 | 121/124 | 110/113 | 127/133 | 104/113 | 174/177 | 120/123 | 101/107 | 96/99 | 84/84 | 133/151 | 184/187 |
| K5 | 83/96 | 124/130 | 110/113 | 130/136 | 107/110 | 174/177 | FA | 104/104 | 99/99 | 81/84 | 136/154 | – |
| K6 | 93/96 | 124/130 | – | 127/136 | 116/116 | 177/177 | 120/120 | FA | FA | 81/84 | – | 184/187 |
| TK1 | 90/93 | 124/127 | 113/113 | 130/130 | 113/116 | 174/177 | 128/138 | 104/104 | 90/93 | 93/93 | 133/145 | 172/181 |
| TK2 | 96/96 | 121/124 | 110/110 | 130/136 | 110/119 | 177/177 | 138/138 | 104/110 | 99/99 | FA | 133/133 | 181/184 |
| MC1 | 96/96 | 130/130 | 110/113 | 139/139 | 104/116 | 174/177 | 138/138 | 104/113 | 99/99 | 84/93 | 139/142 | 172/187 |
| MC2 | 99/99 | 130/130 | 110/110 | 136/136 | 116/116 | 174/177 | 138/138 | 104/110 | 93/93 | 93/93 | 130/133 | 181/187 |
| SY1 | 93/96 | 121/124 | 110/110 | 130/130 | 113/113 | 177/177 | 120/120 | 104/104 | 90/99 | 96/96 | 133/145 | 181/181 |
| SY2 | 93/96 | 127/127 | 110/125 | 136/139 | 110/119 | 174/177 | 117/120 | 104/104 | 87/90 | 87/96 | FA | 181/181 |
| TU1 | 93/93 | 124/127 | – | 130/130 | 113/116 | 177/177 | 120/120 | 104/104 | 90/99 | FA | – | – |
| TU2 | 93/96 | 127/127 | 113/113 | 130/130 | 119/119 | 177/177 | 123/138 | 104/104 | 96/99 | 84/93 | 154/154 | 184/184 |
| TU3 | – | 124/124 | 122/122 | 130/130 | – | 177/177 | FA | 104/104 | FA | 93/96 | – | – |

Sample IDs are listed in Figure 1

Allele names for each microsatellite are listed in Table 1

"–" indicates the locus failed to amplify two or more times for genotyping and "FA" indicates the samples showed three or more peaks at a locus (failed allele)

identification, DNA fragments were amplified from each of 12 microsatellite loci (see Supplementary Table S2) using DNA from 20 individuals (Fig. 1). Two to 10 DNA fragments with different lengths were amplified from each of 12 loci (Table 1). The frequencies of the DNA fragments with different lengths did not deviate from Hardy-Weinberg equilibrium ($P > 0.05$) for all loci, indicating that the DNA fragments were the alleles of each locus. Values of $P_{(ID)}$ and $P_{(ID)}$sib were lowest on the 12th microsatellite locus (0.05 and 0.34) and highest on the 6th microsatellite locus (0.46 and 0.68) (Table 1). Cumulative $P_{(ID)}$ and Cumulative $P_{(ID)}$sib values were calculated to obtain the probability of individual discrimination when multiple markers were used (Schwartz and Monfort, 2008). When three or more microsatellite markers were selected from the loci with lowest $P_{(ID)}$ values, Cumulative $P_{(ID)}$ became lower than 0.001 (Waits et al., 2001). Similarly, when four or more microsatellite markers selected from the loci with lowest $P_{(ID)}$ value, Cumulative $P_{(ID)}$sib became lower than 0.05 (Woods et al., 1999). As shown in Table 2, PCR results showed that six individuals at seven loci were unreliable due to low amplification rate (labeled "-") and eight individuals at eight loci were unreliable due to genotyping errors (labeled "FA"). We excluded these unreliable PCR results from our analysis.

Even after excluding the unreliable PCR results, none of the PCR products amplified from the 12 microsatellite loci of the 20 individuals showed the same amplification pattern as the others. Thus, all 20 individuals from five different field sites and four breeding facilities (Fig. 1) were discriminated by newly developed microsatellite markers (Table 2). These identifications were successfully analyzed from DNA extracted from a single fecal pellet.

## DISCUSSION

The Japanese giant flying squirrel exhibits a unique life history within the family Sciuridae, such as arboreal and folivore ecology, and gliding behavior (Ando and Imaizumi, 1982; Baba et al., 1982; Funakoshi and Shiraishi, 1985; Ando and Shiraishi, 1991, 1993; Sone et al., 1996; Kawamichi, 1997a; Stafford et al., 2003; Shigeta et al., 2010). However, it is still not well understood how Japanese giant flying squirrels behave and adapt to the forest environment due to the difficulty of continuously observing individuals. In addition, the Japanese giant flying squirrel is a solitary animal (Kawamichi, 2015), and relationships and sociality between individuals, such as male-female, parent-offspring, and mating relationships, have not been well studied. If the information on individual distribution, use of nests and foraging sites, and patterns of individual distribution during breeding and non-breeding seasons is collected using DNA markers, it can reveal the social relationships and ecological factors related to survival and reproduction of the Japanese giant flying squirrel. DNA markers for sex identification and individual identification would help to study this species, but these markers have not been reported until now. In addition, no high-throughput DNA sequences of Japanese giant flying squirrels that could be used to isolate microsatellite sequences for novel marker development were available in the database. In this study, we used high-throughput DNA sequencing to determine the genome sequence of Japanese giant flying squirrels and developed DNA markers for sex and individual identification.

Ideally, primer sets designed in conserved regions on the X and Y chromosomes should be used to isolate orthologs of these genes by PCR. However, due to the low conservation of sex chromosome sequences in squirrels (Peakall et al., 2006; Gorrell et al., 2012), it was expected to be difficult to design primers in the conserved regions of these genes. Therefore, we searched for the sequences of SRY and XIST genes from the genome sequences of both female and male Japanese giant flying squirrels and designed the primers based on these sequences. Indeed, these markers successfully identified the sexes. According to these results, the method of isolating SRY and XIST genes from the high-throughput sequences of one male and one female is expected to be applicable to other mammalian species.

To identify individuals using microsatellite markers, the number of repeats must be different between individuals (Abdelkrim et al., 2009; Ariede et al., 2018; Fraga et al., 2020). Therefore, we expected that markers for individual identification would be efficiently developed by screening for microsatellite regions with different numbers of repeats between individuals. Thus, loci with different numbers of microsatellite repeats were selected by mapping sequences from a male to sequences containing repeats from a female. Indeed, all 12 microsatellite markers showed individual differences in the number of repeats. This method allowed us to avoid microsatellites with the same number of repeats between individuals, indicating efficient development of individual identification markers. Since microsatellite sequences are present in many organisms, not only in animals, it is also expected that this method will be applicable to a broad range of diverse species. In this study, we used (AGCn, AGTn, ATTn, CATn, TATn, TAAn, TGAn) as the query repeat sequences. Many more novel markers could be developed by using other tandem repeat motifs (e.g., GTCTn) for the query sequence.

We applied the DNA markers to 20 samples that were from different locations and thought to be from different local populations and successfully discriminated between the individuals. Since the newly developed DNA markers were applicable to individuals from different local populations, the individual identification markers are likely to be applicable not only to Japanese giant flying squirrel individuals in a particular area, but also to individuals from other local populations.

Individuals and sex were able to be identified from DNA extracted from a single fecal pellet, indicating that non-invasive sampling is effective for this species. In the case of non-invasive sampling from feces, the amount of DNA of the target species in the extracted DNA was estimated to be low because of the high content of DNA from bacteria and food remains in the feces and the degradation of target DNA over time due to exposure in the external environment (Murphy et al., 2003; Broquet et al., 2007; Santini et al., 2007; Brinkman et al., 2010; Woodruff et al., 2015). Indeed, nested PCR was necessary for sex identification and individual identification using DNA extracted from feces. Despite the nested PCR, PCR using DNA extracted from feces sometimes yielded unreliable data. This was presumably due to the low amount of target DNA which was caused by the low amount of fiber contained in foods consumed under captivity, degradation of target DNA, and contamination by DNA of other Japanese

giant flying squirrels and other organisms in the feces. Therefore, collecting fresh and less-degraded feces for study should lead to a higher PCR success rate.

The method used in this study is suitable for the study of organisms that are difficult to observe but whose feces are easily sampled. Non-invasive collection of Japanese giant flying squirrel feces and the use of the markers developed in this study will lead to further research on the ecology and the social and population structure of Japanese giant flying squirrels.

## ACKNOWLEDGMENTS

We thank Tama Zoological Park, Ueno Zoological Gardens, and Saitama children's Zoo Park for providing tissue and fecal samples.

## COMPETING INTERESTS

The authors declare that they have no competing interests.

## AUTHOR CONTRIBUTIONS

DNA extraction and PCR: AS. Data analysis: AS. Conceptualization: AS, NK, YT. Next-generation sequencing: AS, YT. Sampling: AS, MS, NT, HO. Writing (original draft): AS, YT. Editing manuscript: NK, YT, MS, NT.

## SUPPLEMENTARY MATERIALS

Supplementary materials for this article are available online. (URL: https://doi.org/10.2108/zs220045)

**Supplementary Table S1.** Primer sequences of sex identification markers.

**Supplementary Table S2.** Primer sequences of individual identification markers.

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(Received May 5, 2022 / Accepted September 12, 2022 /
Published online October 28, 2022)