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短　報
Note

Molecular Sexing of the Ezo Mountain Hare (*Lepus timidus ainu*) based on *ZFX/ZFY* Genes and Application for Using Feces-Derived DNA

By

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Summary：The Ezo mountain hare (*Lepus timidus ainu*) is an endemic subspecies of Hokkaido, a northern island of Japan. Although the Ezo mountain hare is an important member of the ecosystem as a prey species for predators, its detailed ecological features are still unknown because of the nocturnal and solitary behavior that makes them difficult to find. In this study, we determined a partial sequence of 392-bp in exon 11 of the zinc finger protein X-linked gene (*ZFX*) and zinc finger protein Y-linked gene (*ZFY*) and detected the nucleotide substitution sites between the sexes. In addition, a nucleotide substitution was identified in exon 11 of *ZFY* between the Ezo mountain hare and *Lepus timidus*. In tissue-derived DNA, sex-specific band patterns were detected after digestion of polymerase chain reaction (PCR) products using the *Bgl*II restriction enzyme. Therefore, the *ZFX/ZFY* marker may be useful for molecular sexing in the Ezo mountain hare. Furthermore, sex could be distinguished in ～60% of the total tested feces-derived DNA. Although a relatively low success rate of sexing was shown in the analysis using feces-derived DNA, our study showed that the *ZFX/ZFY* marker may be useful for molecular sexing of the Ezo mountain hares using feces obtained non-invasively.

Key words：Ezo mountain hare, feces-derived DNA, molecular sexing, *ZFX/ZFY*

Introduction

Discrimination of sex (sexing) in individuals is essential in investigating the ecology of wild animal species. Molecular sexing, using DNA markers for the identification of sex, can provide precise and reproducible information and is widely applied. Zinc finger protein XY-linked (*ZFX/ZFY*), sex-determining region Y (*SRY*), and amelogenin X-linked (*AMELX*)-encoding genes are known to be useful sexing markers[1]. Among them, *ZFX/ZFY* can be amplified from DNA in both X and Y chromosomes and used to evaluate sequence differences between the sex chromosomes[2].

Molecular sexing may be useful for investigating the ecological features of the Ezo mountain hare (*Lepus timidus ainu*), an endemic subspecies of *Lepus timidus* in Hokkaido, a northern island in Japan. It is an herbivorous animal found in crops and trees, and an important prey species for predators in the ecosystem in Hokkaido. A few researchers have focused on the population decline of this species[3], but the precise population dynamics are unknown. To conserve these hares and ecosystems in Hokkaido, detailed ecological features, such as the sex ratio, should be known. Direct observation is difficult because of their nocturnal and solitary behavior; therefore, informative data from vestiges such as hairs and feces are required for research on this species. Recently, DNA extraction techniques from vestiges have been developed through advances in forensic medicine. Researchers can collect the DNA available for molecular sexing without disturbing the target population. Molecular sexing for a few genera in the order Lagomorpha has been successful: *Oryctolagus*, *Bunolagus*, *Sylvilagus*, *Pentalagus*, and *Lepus* were evaluated using the *ZFX/ZFY* marker[4,5]. Although sexing has not been attempted in the Ezo mountain hare, the *ZFX/ZFY* markers have proven effective in deter-

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mining the sex of the Japanese hare (*Lepus brachyurus*) and Amami rabbit (*Pentalagus furnessi*) in Japan[4,5].

In this study, to confirm the usefulness of the method established by FONTANESI *et al.* (2008) for the molecular sexing of the Ezo mountain hare, we determined the partial DNA sequences of exon 11 on *ZFX/ZFY* and detected the differences between the relevant region on X and Y chromosomes. In addition, we showed that the feces-derived DNA of this species collected from fields can be used for molecular sexing using the *ZFX/ZFY* marker.

Materials and Methods

Sample collection

Tissue samples of the Ezo mountain hares were provided by Dr. Gohta KINOSHITA at Kyoto University in 2017. We collected 48 fecal samples from forests around the Tokyo University of Agriculture (April 2017), Lake Abashiri (February 2018), and Mt. Mokoto (July 2017) in northeastern Hokkaido, Japan, and stored them at −20℃ until further use for DNA extraction (Table 1).

DNA sequencing of the partial *ZFX/ZFY* exon 11

Tissue-derived DNA was extracted using a Gentra DNA Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Feces-derived DNA was extracted using the QIAamp DNA Stool Kit (Qiagen) according to the method described by SONODA *et al.* (2014)[6] and the manufacturer's protocol. We designed the polymerase chain reaction (PCR) primers (F: 5'-GGT GCA GCA ACA TGC TCT TA-3', R: 5'-TTA AAG CCT GAG GCG TCT GT-3') for exon 11 of the *ZFX/ZFY* based on sequences reported by FONTANESI *et al.* (2008, acc. Nos. AM778417.1 and AM778418.1). PCR was performed using the PrimeTaq DNA polymerase (M&S TechnoSystems, Osaka, Japan) with tissue-derived DNA (40–100 ng) from the Ezo Mountain hare according to the manufacturer's protocol under the following conditions: 95℃ for 5 min, 40 cycles at 95℃ for 30 s, 56℃ for 30 s, 72℃ for 1 min, and a final extension at 72℃ for 5 min. PCR products were separated on a 1% agarose gel and purified using the MonoFas DNA purification kit (GL Sciences, Tokyo, Japan). Purified PCR products were ligated to the pT7Blue-T vector (Merck KGaA, Tokyo, Japan) and transformed into *Escherichia coli* (DH5α cells; NIPPON Gene, Tokyo, Japan). Colony-direct PCR was performed using PrimeTaq DNA polymerase (M&S TechnoSystems) with M13 primers[7]. PCR products were purified using ExoSAP IT (Thermo Fisher Scientific, Waltham, MA, USA) and sequenced using the Applied Biosystems 3730xl DNA analyzer (Thermo Fisher Scientific). DNA sequences were aligned using ClustalX ver. 2.1 software[8]. Previously submitted DNA sequence data of Lagomorpha included the following species were added to the analyses: *Lepus timidus* (AM778431.1 and AM778432.1), *Lepus europaeus* (AM778429.1 and AM778430.1), *Oryctolagus cuniculus* (AM778417.1 and AM778418.1), *Bunolagus monticularis* (AM778426.1 and AM778427.1), *Pentalagus furnessi* (AM778423.1 and AB497082.2) and *Sylvilagus floridanus* (AM778421.1 and AM778422.1).

Table 1 Information of collected feces in this study.

Location	Season	Year	Number of feces
Mt. Mokoto	July	2017	24
TUA*	April	2017	12
Lake Abashiri	Febrary	2018	12

*TUA: Department of Bioindustry, Tokyo University of Agriculture.

Molecular sexing

PCR-restriction fragment length polymorphism (RFLP) using *Bgl*II was performed to confirm the practicality of molecular sexing in the Ezo mountain hare. PCR was conducted using the PrimeTaq DNA polymerase (M&S TechnoSystems) with tissue-derived DNA according to the same conditions as mentioned above, and PCR products were digested using *Bgl*II (New England BioLabs, Ipswich, MA) and separated on a 4% or 2% agarose gel. We added 1 M betaine to the same reaction components as above with ～5–192 ng feces-derived genomic DNA. PCR was performed using the following conditions: 95℃ for 5 min, 45 cycles at 95℃ for 30 s, 54℃ for 30 s, 72℃ for 1 min, and a final extension at 72℃ for 5 min.

Results and Discussion

Partial DNA sequences of *ZFX/ZFY* exon 11 in the Ezo mountain hare

We sequenced 10 and 6 clones obtained from tissue-derived male and female DNA, respectively, and determined the partial sequences of exon 11 on *ZFX/ZFY* (392 bp) in Ezo mountain hares. The obtained sequences were submitted to GenBank under the accession numbers LC636310 and LC636311. The DNA sequences of *ZFX* and *ZFY* were aligned to those of Lagomorpha, and two substitution sites were found between them (Fig. 1), which was consistent with a previous study[4]. These two sites were able to distinguish sexes after treatment with restriction enzymes (*Bgl*II and *Alu*I) and were highly conserved in Lagomorpha, except for *B. monticularis* and *S. floridanus*. Although the partial sequence of exon 11 in *ZFX* for the Ezo mountain hare was identical to that of *L. timidus*, a nucleotide difference in *ZFY* was found between them (113A>G).

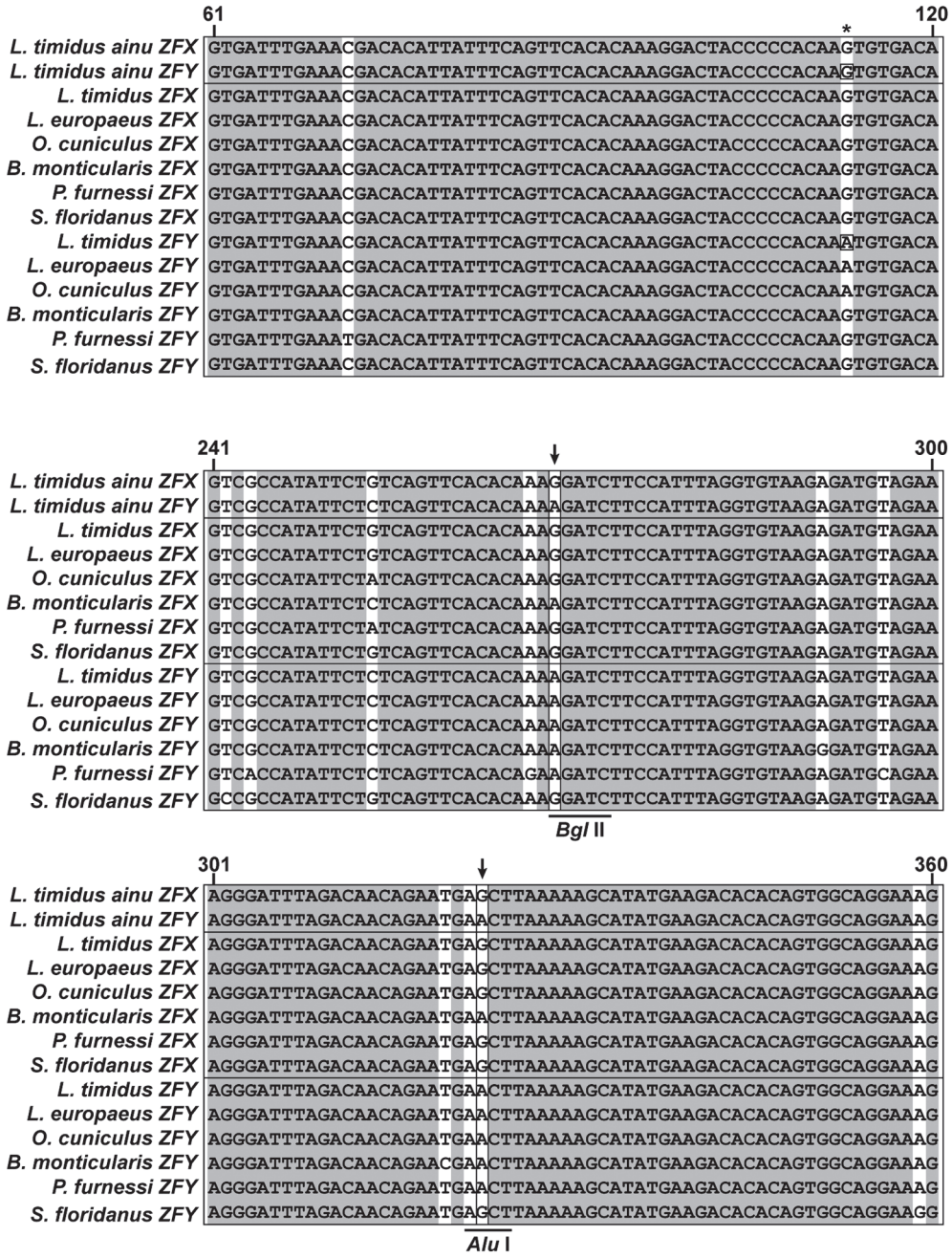

Fig. 1 Alignment of partial DNA sequences of the zinc finger protein X-linked (*ZFX*) and zinc finger protein Y-linked (*ZFY*) exon 11 in Lagomorpha. Gray-colored portions indicate the identical nucleotides in all of the tested Lagomorpha samples. Arrows indicate the substitution sites between *ZFX* and *ZFY*. Digits represent the nucleotide positions on the partial sequence of *ZFX/ZFY* exon 11. Underlines indicate the restriction sites of *Bgl*II and *Alu*I. The asterisk shows the nucleotide substitution site between *ZFY* of the Ezo mountain hare and *L. timidus* (113A>G).

Molecular sexing

We selected *Bgl*II as an optimal restriction enzyme to determine the sex using agarose gel electrophoresis because DNA fragments digested by *Alu*I were predicted to produce a small fragment of ~50-bp which is difficult to detect by electrophoresis. Predictably, *ZFX/X* derived from a female indicated an undigested intact band at ~450 bp (Fig. 2). Additionally, in males three bands were expected. *ZFY* was digested by *Bgl*II and fragmented bands were observed at ~300 and 150 bp in addition to an intact DNA fragment derived from *ZFX*. The results confirmed the utility of molecular sexing based on the *ZFX/ZFY* marker in the Ezo mountain hare, and in *L. timidus*, which is native to Europe.

Next, we verified the performance of molecular sexing using the *ZFX/ZFY* marker in feces-derived DNA sam-

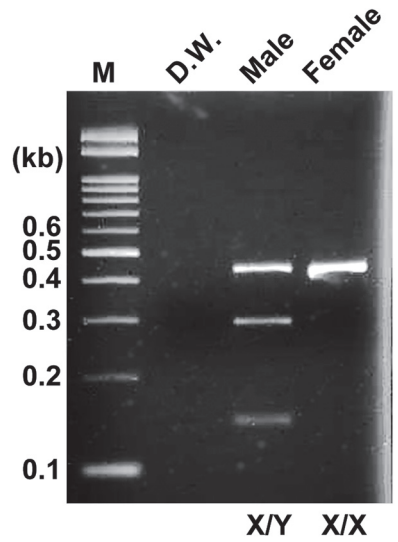

Fig. 2 Molecular sexing of Ezo mountain hare using tissue-derived DNA. M: Size standard (100 bp DNA ladder). Males (X/Y) showed DNA fragments with both intact and digested bands, whereas the intact band alone was detected in the females (X/X).

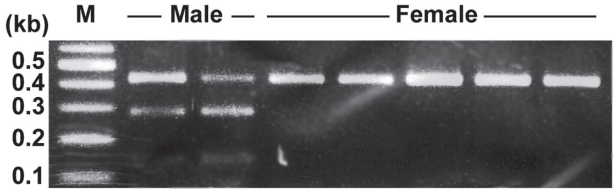

Fig. 3 Representative result of molecular sexing using feces-derived DNA from the Ezo mountain hare. M: Size standard (100 bp DNA ladder).

ples collected from fields. A total of 48 feces-derived DNA samples were tested in triplicate. Approximately 74% of the samples (35.3/48) were amplified from the target DNA fragment and 56% (27/48) were able to distinguish between the sexes. Representative results are shown in Fig. 3. Generally, degradation and fragmentation of DNA were observed in the fecal samples collected from the fields[9]. Thus, we could not determine the sex of nearly half of these samples. A reduction in the success rate of DNA analysis using feces has been observed in increased humidity[10] and temperature conditions[11]. A previous study indicated that the quality of the DNA collected in winter was better than of DNA collected in summer in the Japanese hare, *L. brachyurus*, living on the main island of Japan[4]. To confirm the influence of seasonality on the quality of DNA derived from collected feces, the success rate of determining sex was compared among the collected seasons. The success rates of determining sex were 45.8%–62.5%, 8.3%–25%, and 91.7%–100%

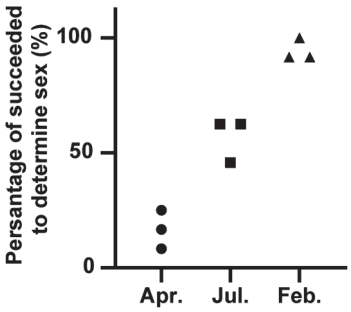

Fig. 4 Difference in the success rate of sexing among seasons in collected feces. Values indicated the percentage of successfully determining sex in the triplicate experiment.

in feces-derived DNA collected in July, April, and February, respectively (Fig. 4). This result indicated that a higher success rate of molecular sexing in winter may be obtained, consistent with the results of a previous study[4]. In the present study, the samples collected in April showed a lower success rate of sexing compared to the other seasons. In Hokkaido, the snows remain on the ground in April. This lower success rate in April may have been caused by the use of wet feces collected during the period of melting snow. However, it is necessary to improve the success rate of molecular sexing to determine seasonal changes in the population sex ratio in Ezo mountain hares. Thus, to improve the efficiency of molecular sexing and molecular ecological studies using the fece-derived DNA of the Ezo mountain hares, it is necessary to investigate the optimal conditions for sample collection, DNA extraction, and PCR amplification in future studies.

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References

1) STRAH R, KUNEJ T (2019) Molecular sexing assays in 114 mammalian species: In silico sequence reanalysis and a unified graphical visualization of diagnostic tests. *Ecol. Evol.* **9** : 5018–5028.
2) AASEN E, MEDRANO J F (1990) Amplification of the *ZFY* and *ZFX* genes for sex identification in humans, cattle, sheep and goats. *Biotechnology* **8** : 1279–1281.
3) NOZAKI S (2020) Distribution of the mountain hare (*Lepus timidus ainu*) during the snow season in the central Tokachi district, Hokkaido. *Obihiro Otani Junior College Center for Regional Collaboration* **7** : 51–57.
4) MATSUKI R, YATAKE H, TAKEUCHI T, ABE S, ISHII T, NASHIMOTO

M (2004) Study on the ecosystem sustaining a pair of Golden Eagles-Population size estimation of Japanese hare by fecal DNA typing-. *Abiko Research Laboratory Rep.* No. U03066. In Japanese with English abstract.

5) Fontanesi L, Tazzoli m, Pecchioli E, Hauffe H C, Robinson T J, Russo V (2008) Sexing European rabbits (*Oryctolagus cuniculus*), European brown hares (*Lepus europaeus*) and mountain hares (*Lepus timidus*) with *ZFX* and *ZFY* loci. *Mol. Ecol. Resour.* **8** : 1294–1296.

6) Sonoda Y, Nakamura M, Matsue M, Kubo M, Ueno Y, Kurihara M (2014) Assessing the feasibility of identifying individual mammals by fecal DNA analysis to evaluate the environmental impacts of a road. *Journal of JSCE* **70** : 341–350. In Japanese with English abstract.

7) Koshiishi Y, Murata-Okubo M, Fujisawa S I, Shimoi G, Hirayama H, Kameyama Y, Souma K, Wada K (2020) Development and characterization of ten novel microsatellite loci for the emu (*Dromaius novaehollandiae*) and genetic diversity of Japanese farm populations. *Mol. Biol. Rep.* **47** : 2521–2527.

8) Thompson J D, Gibson T J, Plewniak F, Jeanmougin F, Higgins D G (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic. Acids Res.* **25** : 4876–4882.

9) Zhang B W, Li M, Ma L C, Wei F W (2006) A widely applicable protocol for DNA isolation from fecal samples. *Biochem. Genet.* **44** : 503–512.

10) Murphy M A, Waits L P, Kendall K C, Wasser S K, Higbee J A, Bogden R (2002) An evaluation of long-term preservation methods for brown bear (*Ursus arctos*) faecal DNA samples. *Conserv Genet* **3** : 435–440.

11) Nsubuga A M, Robbins M M, Roeder A D, Morin P A, Boesch C, Vigilant L (2004) Factors affecting the amount of genomic DNA extracted from ape feces and the identification of an improved sample storage method. *Mol. Ecol.* **13** : 2089–2094.

エゾユキウサギ糞由来 DNA を用いた *ZFX/ZFY* 遺伝子による雌雄の DNA 鑑定

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要約：エゾユキウサギ（*Lepus timidus ainu*）はユキウサギの北海道固有亜種であり，肉食動物の餌資源として重要な生態学的役割を担うと考えられる。その一方で，その詳細な生態学的特性は明らかにされていない。それはエゾユキウサギが夜行性の単独行動であり，周囲の環境に隠れて生活しているためと推測される。本研究は，エゾユキウサギの *ZFX* および *ZFY* 遺伝子におけるエキソン 11 の部分配列を決定し，*Bgl*II および *Alu*I によって雌雄を区別できる塩基置換部位を明らかにした。PCR-RFLP の結果，オスにおいて *Bgl*II によって消化された *ZFY* 由来の PCR 産物と未消化の *ZFX* 由来の断片が検出され，メスでは未消化のバンドのみが確認された。以上の結果はこれまで報告された他のウサギ目と一致した。したがって，*ZFX/ZFY* を用いた雌雄鑑定はエゾユキウサギにおいても有用であることが確認された。一方，エゾユキウサギの *ZFY* にはヨーロッパに生息するユキウサギ（*L. timidus*）との間に 1 塩基置換が検出された。加えて，本研究は非侵襲試料である糞由来 DNA を用いた雌雄判定を試みた。その結果，成功率は約 56% と低いものの，糞由来を用いた *ZFX/ZFY* システムによる雌雄鑑定は可能であった。加えて，冬季（2 月）に採集された糞を用いた雌雄判定は，ほぼ 100% の成功率であった。これらの結果は，フィールドに残された痕跡物，特に冬季に採集された糞を利用したエゾユキウサギの DNA 分析が可能であることを示唆した。

キーワード：エゾユキウサギ，糞由来 DNA，雌雄鑑定，*ZFX/ZFY* 遺伝子

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