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Research Article

Establishing of Genetic Analyses Methods of Feces from the Water Shrew, *Chimarrogale platycephalus* (Erinaceidae, Eulipotyphla)

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- Analysis method development

Abstract

The Japanese endemic water shrew, *Chimarrogale platycephalus* is a small mammal adapted to mountain streams, and is the apex predator within its hierarchy preying on fish and aquatic benthos. Therefore, it is considered to be an extremely important species in the conservation of mountain stream ecosystems. Currently, a reduction in the number of habitats available and/or a decrease in populations, indicates that it is being threatened in various areas of Japan. They have been listed as being in critical status on the red list. With respect to the conservation of such endangered species, the accumulation of basic knowledge such as population structure and an understanding of genetic structure for each population are a very important. However, the accumulated ecological knowledge and knowledge of population genetics gathered to date is still at a poor level because this water shrew is relatively difficult to capture alive. It has been reported that severe damage occurs due to the stress of the capture method. We have tried to understand their population and genetic structures without capture by using their feces. We established genetic analyses methods using their feces.

INTRODUCTION

The Japanese water shrew, *Chimarrogale platycephalus* (Soricidae, Soricomorpha), is a small mammal adapted to underwater life in mountain streams (Figure 1A). This species is endemic to Japan, inhabiting only Honshu and Kyushu. This water shrew is the apex predator within its hierarchy, preying on fish and aquatic benthos (Figure 1B) [1]. They are basically nocturnal, doing their hunting at night. Recently, it has been reported that this water shrew also preys on salamanders [2]. As such, this water shrew is considered to be an extremely important species in the conservation of mountain stream ecosystems. Because they depend on the stability of their habitats for their staple diet prey in their sole environment of mountain streams, they are extremely vulnerable to any modifications to their habitat or environment [3]. In recent years, artificial modifications to the mountain stream environment (including construction of erosion control dams, bank protection construction work, and deterioration of water quality) are considered to have resulted in deterioration in their habitats.

Currently, with a reduction in the number of habitats available and/or a decrease in populations, this water shrew is being threatened in various areas of Japan. Although this water shrew has been recorded in 41 prefectures of Japan [3], in 37 of those prefectures, they have been listed as being in critical status on the local “Red List”. With respect to the conservation of such endangered species, the accumulation of basic knowledge such as population structures and an understanding of genetic structures for each population is a significantly important. However, for this water shrew, which is relatively difficult to capture alive (as will be described later), the accumulated ecological knowledge and knowledge of population genetics gathered to date are still at a poor level. Therefore, it is desirable to undertake further such research in the future [4].

In conventional biological research, some trap capture methods have been utilized. However, it has been reported that severe damage occurs due to stress of the capture method. This shrew, when captured in traps, often dies within about two hours [5]. When carrying out a trapping survey, it is essential to

Figure 1 A typical mountain stream habitat and places for predation and excretion, which the water shrew *Chimarrogale platycephalus* inhabits. 1A: Typical habitat landscape (Upper basin of the Kuma-gawa River, Hitoyoshi, Kumamoto Prefecture), 1B: A water shrew catching a Japanese white spotted char *Salvelinus leucomaenis*, while swimming around a mountain stream, 1C: Typical rock on which to find feces of the water shrew, 1D: Feces found on the rock, 1E-F: An accumulation of water shrew feces excreted under a large rock in the center of a mountain stream (E, F), and 1G: Feces found on the rock, and the color of the rock around shrew feces has a different appearance.

patrol any traps set frequently throughout the night. Although some important measures have been undertaken to improve this situation, such as the development of a trap which minimizes stress [6], this has only reduced researchers' difficulty slightly, and it is still hard to efficiently obtain a large amount of biological data on this water shrew.

As a result, we have tried to understand the population and genetic structures of this water shrew without capture by using their droppings. Because animal feces usually contain at least some remnants of epithelial cells from the gastrointestinal tract, it is possible to analyze the genetic information of the corresponding individuals. In addition, for this water shrew, *Chimarrogale platycephalus*, the feces can be relatively easily found on boulders in mountain streams. The water shrew has a strong tendency to defecate on top of relatively large boulders with a good surrounding view within mountain streams. This feature allow us to conduct genetic analyses on such water shrew feces and is a breakthrough which will enable us to develop a deeper understanding of their populations and genetic structures in their natural habitats via a non-lethal and non-invasive method.

MATERIALS AND METHODS

Collection, fixation and preservation of the feces of *Chimarrogale platycephalus*

To establish a genetic analysis technique of *Chimarrogale platycephalus* utilizing its feces, samples were taken under various conditions for use as source material. Basically, we used feces collected in the field from mountain streams (Figure 1). In addition, the feces of individuals bred in an aquarium were also added to the analysis. Furthermore, as a control, tissue specimens of *C. platycephalus*, which were fixed and stored in a freezer, were also used. With regard to the fixation and storage methods of the feces, we tried three post collection methods as follows: (1) Frozen storage; (2) Fixation and preservation utilizing 100% ethanol in 20 ml glass vials; (3) Preservation with "InhibitEX Buffer" contained in the QIAamp Fast DNA Stool Mini Kit (QIAGEN, Hilden). One piece of feces was preserved in 2.5 ml of this buffer. The specimens preserved using these buffers were mainly used for analysis of nuclear DNA.

DNA extraction

With respect to the extraction of total genomic DNA from the feces of *Chimarrogale platycephalus*, a QIAamp Fast DNA Stool Mini Kit (QIAGEN, Hilden) was used. For the control specimens, the total genomic DNA extraction from the frozen *C. platycephalus* tail tissue was obtained using a DNeasy Blood & Tissue Kit (QIAGEN, Hilden). The total genomic DNA was extracted and purified, predominantly according to the manufacturer's recommended instructions; however, we modified the process a little in order to improve the results.

For the DNA extraction process from frozen fecal specimens,

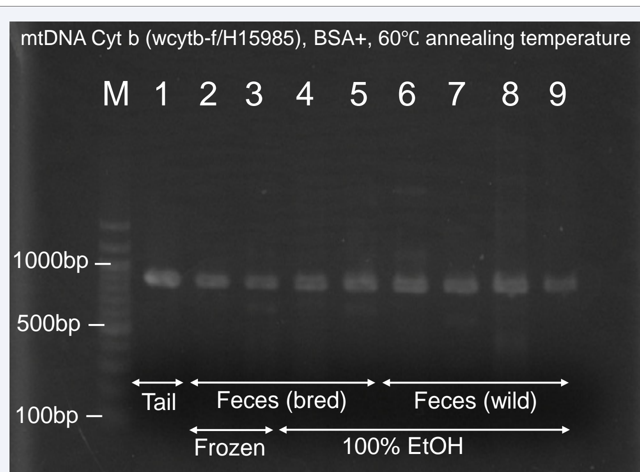

Figure 2 A PCR result of a DNA fragment of the mitochondrial DNA Cyt b region using the primer set, "wcytb-f/H15985", under conditions in which the BSA solution was added. PCR was carried out using an annealing temperature of 60°C. M: The molecular size marker of the 100-bp ladder. Results in column 1-9 respectively correspond to specimen No. 30 (column 1), No. 27-1 (col. 2), No. 28-1 (col. 3), No. 29-2 (col. 4), No. 29-3 (col. 5), No. 5 (col. 6), No. 10 (col. 7), 1 No. 5 (col. 8) and No. 20 (col. 9) in Table 4. Tail: DNA sample extracted from tail tissue used as a control experiment, Feces (bred): DNA samples extracted from fecal specimens in aquarium conditions, Feces (wild): DNA samples extracted from fecal specimens collected fin the field, Frozen: DNA samples extracted from cryopreserved fecal specimens, 100% EtOH: DNA samples extracted from 100% EtOH-preserved fecal specimens.

approximately 200 mg fragments of feces were used as samples. For the DNA extraction process from feces preserved in ethanol, because the feces had dissolved in the ethanol, we used about 200 ml of the feces in solution containing fragments of the feces as samples. The samples containing the feces were put in 1.5 ml collection tubes, and a solution that comes with the kit was added to the samples which were then suspended in that solution. For those specimens preserved in the InhibitEX Buffer, we proceeded directly to the next experimental step. Subsequently, the suspension was centrifuged to remove impurities. Thereafter a proteinase K decomposing protein enzyme treatment step was prepared and performed. Ethanol was then added in order to wash the DNA in a solution containing the *Chimarrogale platycephalus* DNA material and then collected by passing the solution through a filter kit, the DNA thereby accumulating on the filter. In addition, by adding a washing reagent, impurities were removed during the centrifuging process. Finally, DNA on the filter was eluted using an ethanol-free buffer solution, and purified DNA material was obtained. The total genomic DNA materials were then refrigerated. Prior to the storage of the total genomic DNA material, the degree of DNA concentration was measured using a spectrophotometer. In the case of DNA extraction from ethanol-preserved feces, it was conducted using only half the recommended amount of the buffer for the final DNA elution process. This was to compensate for the lower estimated concentration of DNA material expected to be contained within the ethanol-preserved feces samples. Meanwhile, as the frozen fecal samples were considered to contain a significant amount of impurities, the sampled material used for the extraction of DNA was decreased, taking the range of samples from the standard 200 mg down to about 50 mg. In addition, the time for the centrifugal separation of impurities from a suspension containing feces was extended as appropriate.

Amplification of DNA fragments using the PCR method

The amplification of DNA fragments of the mitochondrial DNA cytochrome b (Cyt b) region, and the nuclear DNA apolipoprotein B (Apo B) and breast cancer susceptibility gene I (BRCA1) regions using the PCR method was attempted using the total genomic DNA of *C. platycephalus* as a template. As the primer set for amplification of the mtDNACyt b region, L14734/H15985 [7], and wcytb-f/wcytb-r [8] were used (Table 1). Also, as the primer set for amplification of the nuDNA BRCA1 and Apo B regions, BRCA1f/BRCA1r [9], and our designed ApoBf2/ApoBr2 were used, respectively. The Takara Ex Taq Kit was used to provide the polymerases for the PCR reagent and was prepared according to the manufacturer's protocol. In addition, since the DNA extracted from feces contains substances that inhibit the effectiveness of the PCR, BSA (bovine serum albumin) was added as a stabilizer for the PCR. The temperature conditions for the PCR which led to optimum results are shown in Table (2). General electrophoresis confirmed the successful amplification of DNA fragments using PCR.

Purification of the PCR product

The amplified PCR products, for which mtDNA fragments of the Cyt b region, and nuDNA fragments of the Apo B and BRCA1 regions were confirmed by electrophoresis, were then purified using illustra ExoProStar (GE Healthcare, Buckinghamshire). The composition of the reaction reagents and the reaction temperature conditions applied in this purification method are shown in Table (2,3).

DNA sequencing

The genetic material obtained from the purified PCR product was used as the sample on which the reaction was carried out in order to provide its DNA sequence. A Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Bisystems, California) was used for this reaction. The composition of the reaction reagents and the reaction temperature conditions for this sequencing reaction are shown in Table (4). After the reaction, the concentration of the DNA sample was increased by ethanol precipitation, and HiDi Formamide (Applied Biosystems, California) was added. Thereafter, by performing heat shock and a subsequent rapid cooling process, single-strand DNA was obtained whereby the nucleotide sequence was determined utilizing an auto DNA sequence (Applied Biosystems, California 3130/3130xl Genetic Analyzers).

Based on the nucleotide sequences which were analyzed from both the 5' and 3' sides using this method, a consensus sequence was determined using the software CLC DNA Workbench (Filgen, Nagoya). The sequence determined was compared with the sequences registered in GenBank using the "Blast Search" system, and it was thereby confirmed that the DNA sequence belonged to *C. platycephalus*.

With respect to judgment as to the success or failure of the

Table 1: Primers used in this study.

DNA	region	Primer name	Primer sequence (5' - 3')	TM (°C)	References
mtDNA	cyt b	wcytb-f	GAGGACAGATGTCCTTTTGAGGGGC	72.3	Yuan et al. (2013)
		wcytb-r	TCTGGGTCTCCGAGTAGGTCTGG	69.6	Yuan et al. (2013)
		L14734	AAAAACCATCGTTGTTATTCAACT	61.3	Ohdachi et al. (2001)
		H15985	TAGAATGTCAGCTTTGGGTGCT	64.7	Ohdachi et al. (2001)
nDNA	Apo B	ApoBf2	CACTTCCTTTGGATATGTTTTATGTGG	65.8	This study
		ApoB[illegible]2	AAGTCCAGCATTGGCATTCAGTGTAATG	71.7	This study
	BRCA	BRCA1f	TGAGAACAGCACTTTATTACTCAC	59.0	Dubey et al. (2006)
		BRCA1r	ATTCTAGTTCCATATTGCTTATACTG	67.7	Dubey et al. (2006)

Table 2: Preparation pf reaction regants in each experimental process.

Process	Product	Mixture Components	Volume (µl)
PCR	EX Taq (Takara)	SQ	5.55
		10x Ex Taq Buffer (Mg^{2+} free)	1
		dNTP Mixture (2.5 mM each)	0.8
		$MgCl_2$ (25 mM)	0.8
		Primer F	0.25
		Primer R	0.25
		BSA (3.0 µg/µl) +/-	0.30/0
		TaKaRa Ex Taq	0.05
		Template	1
Enzymatic PCR pulification	Illustra ExoProStar (GE Healthcare)	SQ	2.2
		Exonuclease	0.4
		Alkaline Phosphatase	0.4
		Template	2
Cycle Sequencing	Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems)	SQ	6.6
		5x Sequencing Buffer	1.8
		Primer (F or R)	0.2
		BigDye Reaction Mix	0.4
		Template	1

genetic analyses, the conclusion was evaluated based on the respective DNA sequencing results and not only on the PCR results (Table 4).

RESULTS

Sampling of *Chimarrogale platycephalus* feces

C. platycephalus feces were often found on top of boulders or rocks among rapids zones within streams (Figure 1C-G). The shrew feces were not found on top of the rocks along stream edges. Boulders and rocks on which shrew feces were observed were almost always situated in locations surrounded entirely by flowing water (Figure 1C-D). The accumulation of feces was also often observed under large rocks around step flow (Figure 1E), and between stacked rocks beside the step flow of mountain streams (Figure 1F). It has been frequently observed that the color and surface of boulders or rocks around shrew feces has a different appearance (Figure 1G). This could be due to shrews frequently walking around those areas.

Eleven feces samples of *C. platycechala* were collected in the field from mountain streams, and were used in these analyses. Of these, two of the feces samples had a whitish surface, and were remarkably dry. One shrew feces sample was wet and had partially dissolved due to rain one or two days earlier. Of all 26 shrews' feces samples, total genomic DNA were successfully extracted from 25 of them, and successful subsequent genetic analysis of their respective mitochondrial DNA Cyt b regions was conducted. All of these obtained nucleotide sequences were confirmed to be the sequences of *C. platycephala*. Due to being

Table 3: Reaction conditions in each experimental process.

No.	Process	Temperature conditions
1	PCR	92°C min
		(92°C 30sec, 60°C 30sec,72°C 1min) × 35 cycles
		72°C 7min
2	PCR	92°C min
		(92°C 30sec, 55°C 30sec, 72°C 1min) × 35 cycles
		72°C 7min
3	Enzymatic PCR clean up	37°C 15min
		80°C 15min
4	Cycle Sequencing	96°C 30sec
		(96°C 10sec, 50°C 5sec, 60°C 4min) × 25 cycles

significantly dried one sample could not be used to conduct genetic analysis on it.

DNA analyses of *Chimarrogale platycephalus* feces

In this study, comparison of genetic analysis methods under various conditions listed below was conducted. An example of the results of electrophoresis performed after PCR is shown in Figure (2), results of the DNA sequence is shown in Figure (3), and a list of results up to the sequence is shown in Table (4).

The concentration of total genomic DNA and the PCR results

Each sample collected, its storage conditions, and the result of its DNA extraction (i.e., concentration) and DNA sequencing analysis, are shown in Table (4). The concentration of the extracted total genomic DNA was between 0.6-116.5 ng/µl. Excellent results were obtained using the PCR method for samples within the concentration range of 0.6-76.5 ng/µg (Table 1). However, higher concentration samples (i.e., over 76.5 ng/µl) were not successful in the amplification of their DNA fragments, despite attempts to use the PCR method after diluting the template.

The result of the genetic analysis using the feces storage method

With respect to genetic analyses performed on shrew feces either stored frozen or stored in ethanol, it was possible to analyze both without any problems (Table 1). In the results of the chi-square test comparing these preservation methods, no significant difference was observed ($p>0.05$). However, cases of failure in the amplification of DNA fragments did occur for the PCR experiments using feces samples of wild individuals that were frozen for a long period of time, i.e., about four months. For the chi-square test comparing the results of such long-term stored samples, a significant difference was observed ($P=0.002$).

The PCR results between the primer sets for amplifying the mitochondrial DNA Cyt b region

Two primer sets in this study were tested: wcytb-f/H15985 and L14734/wcytb-r. For both primer sets, it was possible to amplify the *C. platycephalus* DNA fragment. In particular, for the genetic analysis of feces from aquarium-bred individuals, there was a very high success rate.

Effect of BSA addition in genetic analyses of *Chimarrogale platycephalus* feces: Regardless of the presence or absence of BSA, genetic analysis for the feces of aquarium-bred shrews was successful. However, for the genetic analysis of wild shrew feces collected in the field, a positive effect of BSA addition was clearly observed in about a quarter of the samples (i.e., 9 of the total 27 feces samples). When limited to only using feces samples collected in the field, the chi-square test determined the effect of the BSA addition to be significant ($P=0.002$).

The PCR results and annealing temperature conditions: For each primer set, PCR was carried out under the annealing temperature conditions shown in Table (2,3), and their corresponding results are also shown in Table (4). With respect to the PCR conditions using the wcytb-f/H15985 primer set, lowering the annealing temperature to 55°C resulted in an extra band being detected in about a quarter of the samples (i.e., 7 of the total 27 samples). Similarly, for the PCR conditions using the L14734/wcytb-r primer set, lowering the annealing temperature to 55°C, resulted in an extra band being detected in about 30% of the samples (i.e., 8 of the total of 27 samples). However, in PCR examination using the L14734/wcytb-r primer set, the nucleotide sequence of cattle (*Bosprimigenius*) was amplified in only a few samples: i.e., three specimens under 55°C annealing conditions, and two specimens under 60°C annealing conditions. These results constitute a technical error due to the addition of BSA as a PCR stabilizer.

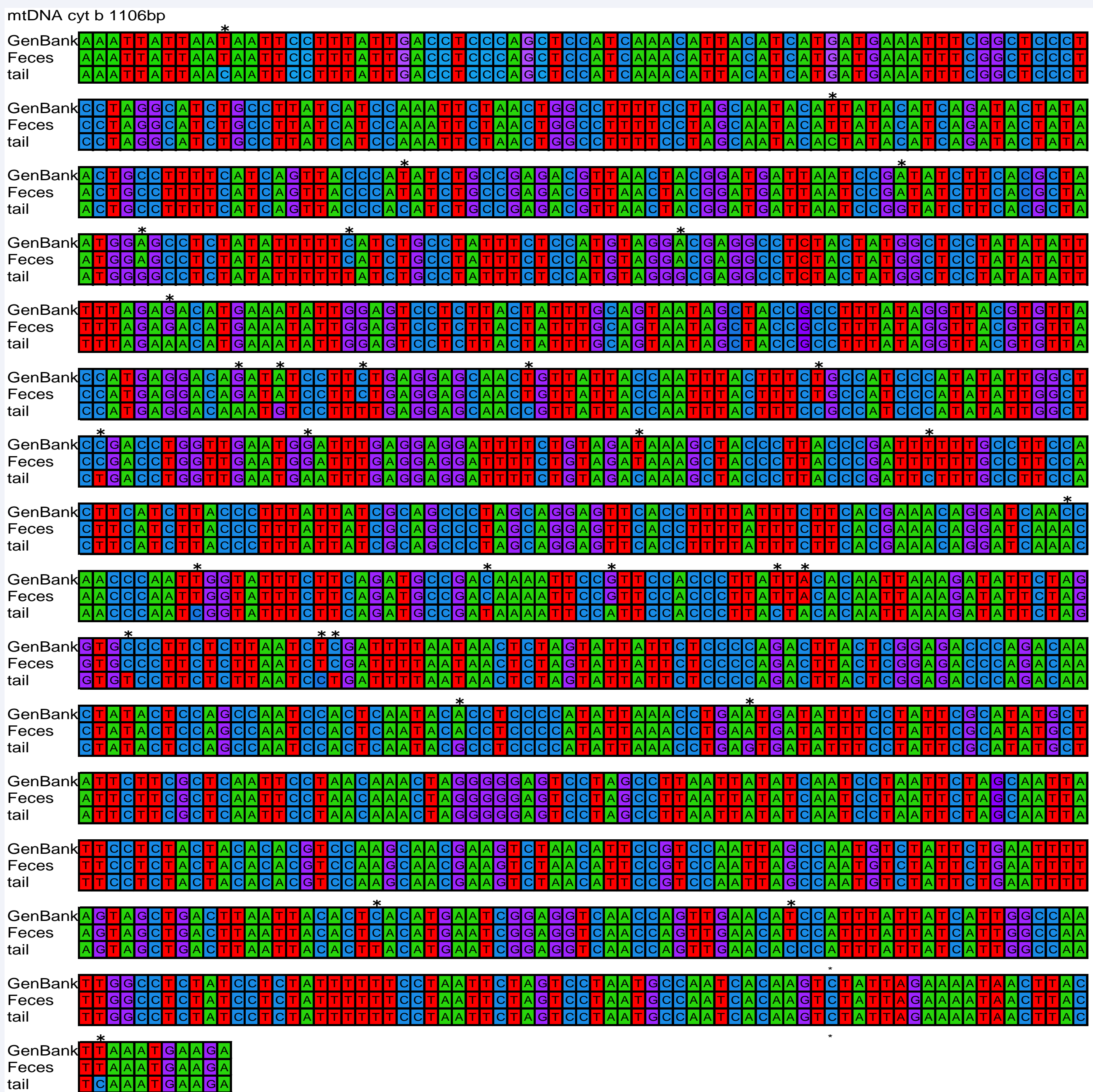

Figure 3 A result of DNA sequencing (mtDNA Cyt b region, 1,106-bp) from the water shrew *Chimarrogale platycephalus* feces (Specimen No. 1; Table 4). Comparison with DNA sequencing result from the fresh tail tissue (as a control, Specimen No. 30; Table 4), and with a GenBank registered DNA sequence of *C. platycephalus* (accession No. AB108707).

The results of PCR amplification comparing the primer sets of the nuclear DNA Apo B region

Regarding amplification of the DNA fragment of the nuDNA Apo B region, amplification of the targeted DNA fragment was not achieved at all in either of the ethanol-preserved feces, the frozen-preserved feces specimens. However, amplification of the targeted DNA fragment (nuDNA Apo B region) was achieved for the fecal specimens preserved in the "InhibitEX Buffer". The amplification success rate under this PCR condition was 66.7%.

On the other hand, with regard to PCR amplification of the nuclear DNA BRCA1 region, amplification of the targeted DNA fragment could not be achieved, even for the fecal specimens preserved in the "InhibitEX Buffer". In the positive control experiment, using the total genomic DNA extracted from the tail tissue of water shrews, DNA fragments of the nuDNA "BRCA1" region were amplified without any of the above problems, which are considered to be qualitative problems of examining the specimens.

Table 4 Results for each attempt to conduct a set of genetic analyses of feces from *Chimarrogale platycephalus*

Specimen No.	Analysis No.	Specimen condition	Storage condition of specimen	Concentration of total genomic DNA (ng/µl)	PCR condition and the amplification of results							GenBank accession No.
					Primers used; wcytb-f/H15985			Primers used; L14734/wcytb-r		Primers used; ApoBf2/ApoBr2	Primers used; BRCA1F/BRCA1r	
					BSA added/non-added, and annealing temperature			BSA added/non-added, and annealing temperature		BSA added/non-added, and annealing temperature		
					BSA+, 60°C	BSA-, 60°C	BSA+, 55°C	BSA+, 60°C	BSA-, 60°C	BSA+, 60°C	BSA-, 60°C	mtDNA Cyt b
1	1-1	Feces collected in the wild	frozen	88.0	Failure	no attempt made	no attempt made	no attempt made	no attempt made	Failure	Failure	-
1	1-2	Feces collected in the wild	frozen	22.5	Success *2	no attempt made	no attempt made	no attempt made	no attempt made	Failure	Failure	LC269788
2	2-1	Feces collected in the wild	frozen	116.5	Failure	no attempt made	no attempt made	no attempt made	no attempt made	Failure	Failure	-
2	2-2	Feces collected in the wild	frozen	7.0	Success *2	no attempt made	no attempt made	no attempt made	no attempt made	Failure	Failure	LC269789
3	3	Feces collected in the wild	frozen	19.0	Success *2	no attempt made	no attempt made	no attempt made	no attempt made	Failure	Failure	LC269790
4	4	Feces collected in the wild	frozen	41.0	Success *2	no attempt made	no attempt made	no attempt made	no attempt made	Failure	Failure	LC269791
5	5	Feces collected in the wild	100% EtOH	4.6	Success	Failure	Success *1	Failure	Failure	Failure	Failure	LC269792
6	6	Feces collected in the wild	100% EtOH	2.2	Success	Failure	Success	Failure	Failure	Failure	Failure	LC269793
7	7	Feces collected in the wild	100% EtOH	4.8	Success	Failure	Success	Success	Success *1	Failure	Failure	LC269794
8	8	Feces collected in the wild	100% EtOH	3.0	Success	Success	Success *1	Success	Failure	Failure	Failure	LC269795
9	9	Feces collected in the wild	100% EtOH	2.0	Success	Success	Success	Success	Success *1	Failure	Failure	LC269796
10	10	Feces collected in the wild	100% EtOH	6.1	Success	Failure	Success	Success	Success	Failure	Failure	LC269797
11	11	Feces collected in the wild	100% EtOH	1.2	Success	Success	Success *1	Success	Success	Failure	Failure	LC269798
12	12	Feces collected in the wild	100% EtOH	2.0	Success	Success	Success	Success	Success *1	Failure	Failure	LC269799
13	13	Feces collected in the wild*3	100% EtOH	2.7	Failure	Failure	Failure	Success	Success *1	Failure	Failure	LC269800
14	14	Feces collected in the wild*4	100% EtOH	2.9	Success	Failure	Success	Success	Success	Failure	Failure	LC269801
15	15	Feces collected in the wild	100% EtOH	8.6	Success	Success	Success	Success *1	Success *1	Failure	Failure	LC269802

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16	16	Feces collected in the wild	100% EtOH	7.2	Success	Success	Success *1	Success *1	Success *1	Failure	Failure	LC269803
17	17	Feces collected in the wild	100% EtOH	16.5	Success	Success	Success	Success	Success	Failure	Failure	LC269804
18	18	Feces collected in the wild	100% EtOH	4.0	Success	Failure	Success	Success	Success *1	Failure	Failure	LC269805
19	19	Feces collected in the wild	100% EtOH	5.2	Success	Success	Success *1	Success *1	Success *1	Failure	Failure	LC269806
20	20	Feces collected in the wild	100% EtOH	2.2	Success	Failure	Success	Success	Success *1	Failure	Failure	LC269807
21	21	Feces collected in the wild*4	100% EtOH	3.1	Failure	Failure	Failure	Failure	Failure	Failure	Failure	-
22	22	Feces collected in the wild	100% EtOH	6.5	Success	Failure	Success	Success	Success	Failure	Failure	LC269808
23	23	Feces collected in the wild	100% EtOH	11.1	Success	Failure	Success	Failure	Failure	Failure	Failure	LC269809
24	24	Feces collected in the wild	InhibitEX buffer	8.2	Success	no attempt made	no attempt made	no attempt made	Success	Success	Failure	LC269810
25	25	Feces collected in the wild	InhibitEX buffer	5.2	Success	no attempt made	no attempt made	no attempt made	Success	Success	Failure	LC269811
26	26	Feces collected in the wild	InhibitEX buffer	41	Success	no attempt made	no attempt made	no attempt made	Success	Failure	Failure	LC269812
27	27-1	Feces collected under captive breeding conditions	frozen	22.0	Success	Success	Success	Success	Success	Failure	Failure	LC269813
27	27-2	Feces collected under captive breeding conditions	frozen	76.5	Success	Success	Success	Success	Success *1	Failure	Failure	
28	28-1	Feces collected under captive breeding conditions	frozen	65.0	Success	Success	Success *1	Success	Success	Failure	Failure	LC269814
28	28-2	Feces collected under captive breeding conditions	100% EtOH	12.5	Success	Success	Success *1	Success	Success *1	Failure	Failure	
28	28-3	Feces collected under captive breeding conditions	100% EtOH	1.2	Success	Success	Success	Success	Success	Failure	Failure	
29	29-1	Feces collected under captive breeding conditions	frozen	24.0	Success	Success	Success	Success *1	Success	Failure	Failure	LC269815
29	29-2	Feces collected under captive breeding conditions	100% EtOH	0.6	Success	Success	Success	Success	Success	Failure	Failure	
29	29-3	Feces collected under captive breeding conditions	100% EtOH	3.4	Success	Success	Success	Success	Success	Failure	Failure	
30	30	Actual tail tissue	100% EtOH	15.4	Success	Success	Success	Success	Success	Success	Success	LC269816

*1: Non-target extra-band was also amplified.

*2:Succesful amplification of DNA fragments under these conditions was possible without problems. However, when PCR was performed using total genomic DNA extracted from the same fecal specimens stored frozen for about 4 months, good results were not obtained.

*3: This feces specimen was in a slightly damp condition due to rainfall.

*4: This feces specimen was extremely dry.

DISCUSSION

Selective feces sampling of the water shrew, *Chimarrogale platycephalus*

In this study, we carried out genetic analysis of feces samples collected in the field from 26 individual animals. Of these, genetic analysis of 25 of the samples was successful, and achieving a success rate of around 95% is considered to be a very satisfactory outcome. In addition, it was also an important achievement to be able to ultimately verify that all of the sequences analyzed were in fact those of the water shrew, *Chimarrogale platycephalus*. There was only one sample from which a genetic sequence could not be obtained due to its having become extremely dry over the passage of time. Thus, genetic analysis could not be performed on it. It is considered that the DNA on the dried feces sample had likely already been damaged by the time of its collection. Notably, in previous studies attempting to conduct genetic analyses using the feces of bears (Ursidae, *Ursusthibetanus*) [10], the rate of successful analysis was reported to decrease in many of the dried feces samples. It is considered that collecting only the fresh feces of this water shrew is what made it possible to achieve such a high rate of successful genetic analysis.

However, with regard to PCR amplification of the partial nuDNA fragments of the nuDNA regions (i.e., the Apo B and the BRCA 1 regions), good results were not obtained in either the Apo B or BRCA 1 regions when the total genomic DNA was extracted and purified from either the frozen fecal specimens or the 100% EtOH-preserved specimens used as templates.

In general, mtDNA regions retain a large number of copies per cell, whereas nuDNA retains only a single copy in each cell. Therefore, nuDNA analysis from feces has also been considered to be very difficult [11]. Actually, the poor analysis efficiency of nuDNA has also been reported in comparative analysis of mtDNA and nuDNA regions from fecal specimens of other animal species [12,13]. In particular, since the nuDNA regions treated in this study were comparatively long sequences of 517-bp. (Apo B) and 775-bp. (BRCA 1), respectively, they may have been susceptible to DNA degradation. Other study [14] also pointed out that short sequence analyses (e.g., less than 200-bp.) are preferable for specimens that are susceptible to fragmentation of their DNA, such as animal feces.

However, in the trial in this study, we succeeded in analyzing the nuDNA Apo B region (517-bp.) of the water shrew by using the "InhibitEX Buffer" for sample preservation (as detailed in the next section). It is considered a significant development that the successful analysis of the nuDNA region from fecal samples became possible. Establishing such a method for the analysis of nuDNA will expand the potential to conduct a greater range of analyses in the future. For example, by establishing this method, the possibility of successful microsatellite analyses of the water shrew has been increased very much. In response to this, we are currently working on developing markers for microsatellite analysis.

In conducting this study, one minor issue faced was that in the mountain streams of central Honshu, Japan, also observed on top of boulders and rocks in streams is the feces of an ouvriere bird, *Cincluspallasii* (Cinclidae), often inhabiting a sympatric habitat with the shrews. However, it was possible to relatively easily differentiate its droppings as they contained uric acid. In addition, in the cases of comparatively fresh feces, it was possible to identify the feces of *C. platycephala* by its peculiar musk-like scent. Since the water shrew also tends to deposit its feces in selected locations, this is another key factor in identifying it.

Storage method of *Chimarrogale platycephalus* feces

The results of this study show that there should be no problem even with frozen feces storage, or storage in ethanol. However, due to the somewhat poor results of long-term frozen storage, ethanol storage is preferable. We could not identify the issue with respect to the problem of long-term preservation by freezing because the concentration of total genomic DNA yielded higher values when obtained from DNA extracted from frozen feces samples. Perhaps some impurities were also preserved at a high concentration along with the shrew's DNA in the feces. Thus, it may be best to utilize cryogenic preservation methods or frozen storage after having dipped the feces samples in ethanol.

As mentioned above, in analysis of the nuDNA Apo B region, preservation of water shrew feces using the "InhibitEX Buffer" resulted in a large positive effect. Originally, the purpose of this InhibitEX Buffer was as a kit developed for medical examination of human feces. Although the details are unknown as to what kind of reaction actually occurs, it is considered that binding of a substance in the InhibitEX Buffer matrix may function effectively in the trapping of substances that inhibit genetic analysis. In addition, this method is extremely simple and easy, since the feces specimens can be immediately stored directly in the InhibitEX Buffer in the field. Furthermore, we can extract DNA directly from the sample eluted into this buffer. As a result, it is a great advantage to minimize the loss of DNA material originally contained only in trace amounts in feces.

Amplification of DNA fragments by PCR: Removal of the PCR inhibitors and use of the PCR stabilizer, BSA

Since only a very small amount of DNA is present in *Chimarrogale platycephalus* feces samples, and the feces also contains a lot of impurities, the efficient amplification of DNA fragments was extremely difficult when applying the standard PCR method. However, the addition of BSA as a PCR stabilizer was extremely effective. Since the BSA originated from cattle serum, situations in which the mitochondrial DNA Cyt b sequences of cattle were also amplified occurred in some samples. Despite this problem, it may be possible to successfully extract the water shrew's DNA fragments by a combination of PCR and DNA cloning techniques. Another workaround is by maintaining a high concentration of total genomic DNA, and this can overcome the problem. As for the temperature conditions and primer sets for PCR, it was possible to obtain good results by performing the analysis as in Table (4).

Establishment of non-lethal sampling for genetic analysis

As noted even in the "Introduction" section, the water shrew *Chimarrogale platycephalus* is a highly endangered mountain stream-dwelling mammal. Due to the extreme stress sensitivity of *C. platycephalus* if trapping, acquiring an adequate number of genetic research samples is a very difficult problem. Under

such circumstances, progress in genetic structure analysis of this shrew has stagnated. Therefore, it is an extremely valuable and significant development to establish a genetic structure analysis method using *C. platycephalus* feces.

In the future, by accumulating genetic analysis results from feces targeting more local populations, we would like to investigate the systematic evolutionary history and structure of the population genetics of *C. platycephalus* in detail. Beyond achieving that basic knowledge it is believed that it will also contribute greatly toward conservation efforts of the endemic Japanese water shrew, *C. platycephalus*.

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