Evaluation of the Genetic Diversity of the Ryukyu Long-furred Rat (*Diplothrix legata*) on Northern Okinawa-jima Island, Japan

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ABSTRACT

We examined the partial mitochondrial DNA sequences of the Ryukyu long-furred rats (*Diplothrix legata*) on northern Okinawa-jima Island to evaluate the genetic diversity of this species. Complete mitochondrial D-loop (40–933) and partial tRNA-Pro (1–39) and Phe (934–961) sequences, totaling 961 bp in length, were determined for 60 individuals. Three D-loop haplotypes were present in these animals, with mutations at six sites (GenBank accession numbers: AB699876–AB699878). The haplotype diversity (H) and the nucleotide diversity (π) of the D-loop sequences were 0.606 and 0.00238, respectively. The “twin-peak” phenomenon was observed for the mismatch distribution of pairwise differences, indicating that the population of these animals had experienced a population bottleneck.

Key words: D-loop, genetic diversity, Ryukyu long-furred rat

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The Ryukyu long-furred rat (*Diplothrix legata*) is endemic to Japan and is distributed on Amami-ohshima Island, Tokunoshima Island, and Okinawa-jima Island of the Nansei Islands (Fig. 1A, B) [1]. This species is one of the largest rodents in Japan; its population is in decline because of its limited habitat range, decreasing habitat quality, and predation by introduced species including the small Indian mongoose (*Herpestes auropunctatus*) and feral cats and dogs [2, 3]. It is listed as Endangered (EN) in the IUCN Red List of Threatened Species [2] and the Red list of Threatened mammals of Japan [4], and its conservation is urgently required.

In the area of the present study, northern Okinawa-jima Island, evergreen broad-leaved forest dominated by *Castanopsis sieboldii* is spreading. Many endemic and rare species also inhabit this region, including the Okinawa rail (*Gallirallus okinawae*), Okinawa woodpecker (*Sapheopipo noguchii*), and Okinawa spiny rat (*Tokudaia muenninki*).

Understanding genetic diversity is important to the

conservation of endangered species. Inbreeding depression, decreased immunity, and decreased adaptive evolution ability are caused by low genetic diversity, which can increase the possibility of extinction [5]. In this study, we examined the mitochondrial DNA D-loop region and partial t-RNA-Pro and Phe sequences of Ryukyu long-furred rats from northern Okinawa-jima Island, and we evaluated the genetic diversity of this species.

Sixty free-ranging Ryukyu long-furred rats that were thought to have been killed by vehicles or accidentally captured in mongoose traps were collected by the Ministry of the Environment, Government of Japan in northern Okinawa-jima Island (26°40′–26°50′N, 128°05′–128°20′E) from January 2007 to February 2012 (Fig. 1C and Appendix 1). The liver, muscle, and bone marrow samples were collected from these specimens and used for DNA extraction. These samples were cryopreserved in the Environmental Specimen Time Capsule Building, National Institute for Environmental Studies, Japan. DNA extraction was performed using an EZ1 DNA Tissue Kit (Qiagen) following the manufacturer's instructions. Mitochondrial DNA D-loop region and partial t-RNA-Pro and

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Fig. 1 Geographic distribution of the Ryukyu long-furred rat (*Diplothrix legata*) (A, B) and the sampling site for this study in northern Okinawa-jima Island (C). We determined the sequence of the mitochondrial DNA D-loop region. Three haplotypes were observed.

Phe sequences were amplified using the primer pair RLFRD-loopF/RLFRD-loopR, which was designed for this study (Table 1). AmpliTaq Gold® PCR Master Mix (Applied Biosystems) was used to conduct a polymerase chain reaction (PCR) for gene amplification in a final reaction volume of 50 µl, following the manufacturer's instructions. Amplification was performed on a T3000 Thermocycler (Biometra) with the following protocol: an initial denaturation step at 95°C for 10 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1.5 min; and a final extension at 72°C for 5 min. The PCR products were run on 2.0% agarose gel and purified using the MiniElute PCR Purification Kit (Qiagen).

The purified PCR products were sequenced using the BigDye

Table 1 Ryukyu long-furred rat (*Diplothrix legata*) D-loop amplification primer sequences

Primer code	Nucleotide sequence
RLFRD-loopF	5' -TCA ACA CCC AAA GCT GAT ATT CT-3'
RLFRD-loopR	5' -TGC TTT GCT TTA TTA TAA GCT ACA TT-3'
RLFRD-loopF2	5' -GAT GCC TTC AGT CAA CAT AGC-3'
RLFRD-loopR2	5' -ACC AAT TGA TGG CCC TGA AGT-3'

Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and Applied Biosystems 3130 genetic analyzer (Applied Biosystems). The same primers were employed as those used for the PCR reactions, as well as the two internal primers

RLFRD-loopF2/RLFRD-loopR2, which were designed for this study (Table 1). The sequences were aligned using the BioEdit sequence alignment editor ver. 7.0.9.0 (Tom Hall). After alignment, 961 base pairs (bp) of the complete D-loop and partial tRNA-Pro and Phe regions were obtained.

The statistical analyses for the D-loop were performed using DnaSP v5 [6]. A phylogenetic tree for the D-loop haplotypes, with the Norway rat (*Rattus norvegicus*) as an outgroup, was constructed utilizing the neighbor-joining (NJ) method [7] with MEGA6 [8]. Genetic distances were computed using the Tajima-Nei method [9]. The bootstrap consensus tree was inferred from 1,000 replicates [10].

Complete mitochondrial D-loop (40–933) and partial tRNA-Pro (1–39) and Phe (934–961) sequences, totaling 961 bp in length, were determined for 60 individuals. Three D-loop haplotypes were present in the Ryukyu long-furred rats of northern Okinawa-jima Island, with mutations at six sites (GenBank accession numbers: AB699876–AB699878; Table 2). The haplotypes 1, 2, and 3 were present in 7 (11.7%), 26 (43.3%), and 27 (45.0%) individuals, respectively. Haplotype 2 seems to be more closely related to haplotype 3 than haplotype 1, but their relationship is unclear, due to low bootstrap value (78%). Haplotype diversity (H) and nucleotide diversity (π) of the D-loop were 0.606 and 0.00238, respectively; these values were higher than those of the endangered Okinawa rail of northern Okinawa-jima Island (0.499 and 0.00146, respectively) [11]. On the other hand, H and π in the Ryukyu long-furred rat were lower than those of the large Japanese field mouse (*Apodemus speciosus*) (0.70–0.80 and 0.013–0.029, respectively) collected from six habitats linked by forests or green tract [12]. The large Japanese field mouse is a species of the same murine subfamily and is distributed in mainland Japan.

The “twin-peak” phenomenon was observed for the mismatch distribution of pairwise differences among haplotypes (Fig. 2),

which is affected by past population events [13, 14]. The peak in the mismatch distribution was comparable to the population expansion period, and the trough between the peaks is comparable to a period of decline [15]. This result indicates that the population of Ryukyu long-furred rats has experienced a population bottleneck. The same phenomenon was observed in the Japanese rock ptarmigan (*Lagopus mutus japonicas*), an endangered alpine bird that experienced a bottleneck [15, 16]. Although twin peaks can be observed when contact has occurred with other populations that have different haplotypes, such contact is unlikely for the Ryukyu long-furred rat because of the isolation of the island. Moreover, relatively high H and low π values in the examined population also support the occurrence of a genetic bottleneck [17].

Many extinct plant and animal species have inhabited islands [18]. Island populations generally have lower genetic variation than do mainland populations [19, 20]. Island populations are thought to suffer increased inbreeding relative to mainland populations because of bottlenecks in the founding group and

Fig. 2 Mismatch distribution of 60 mtDNA D-loop sequences of Ryukyu long-furred rats (*Diplothrix legata*) in northern Okinawa-jima Island.

Table 2 Position of the mutation in D-loop haplotypes of the Ryukyu long-furred rat (*Diplothrix legata*) distributed in northern Okinawa-jima Island.

Haplotype	n	Mutation site in D-loop region						Accession No.
		61	134	143	177	219	578	
Haplotype 1	7	C	C	T	A	C	G	AB699876
Haplotype 2	26	C	T	C	G	T	A	AB699877
Haplotype 3	27	T	T	C	A	C	A	AB699878

lower subsequent population size [20]. Moreover, introduced species are one of the most important factors for extinction of island population [5]. Native animals that inhabit islands have not experienced coevolution with introduced predators [21], and they are easy prey for those predators. In fact, it is thought that recent risk of extinction of the Ryukyu long-furred rat is a result of predation by feral cats and reduction of suitable habitat, *C. sieboldii* forest, in Okinawa-jima Island [22]. Habitat conservation and control of introduced predators are important for preventing extinction and loss of genetic diversity in Ryukyu long-furred rats.

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Appendix 1 Sample list of Ryukyu long-furred rats used in this study.

Year	Collected Date	Sample ID	Haplotype	Sex	Year	Collected Date	Sample ID	Haplotype	Sex
2007	Jan. 3	397M	1	Male	2011	Apr. 14	2053M	2	Male
	Mar. 16	420M	2	Female		Mar. 22	2054M	2	Male
	Nov. 23	636M	2	Female		May. 16	2065M	2	Male
	Dec. 9	645M	3	Female		May. 22	2075M	2	Male
	Dec. 12	646M	3	Female		May. 31	2079M	2	Male
2009	Apr. 9	989M	2	Female		Jun. 20	2096M	2	Male
	May. 9	1012M	3	Female		Jun. 27	2104M	2	Female
	Jun. 28	1078M	3	Male		Jun. 30	2105M	3	Female
	Jun. 30	1079M	1	Male		Jul. 4	2107M	1	Male
	Jul. 27	1100M	3	Male		Jul. 7	2110M	3	Female
	Oct. 20	1191M	3	Male		Jul. 13	2117M	2	Male
	Dec. 10	1204M	2	Male		Jul. 31	2146M	2	Female
2010	Feb. 19	1280M	3	Male		Sep. 2	2190M	2	Female
	Feb. 27	1284M	3	Male		Aug. 30	2202M	3	Male
	Mar. 2	1288M	2	Female		Sep. 11	2203M	3	Female
	Mar. 26	1294M	1	Female		Nov. 29	2259M	2	Male
	Apr. 12	1311M	3	Male		Dec. 9	2263M	1	Male
	Apr. 19	1318M	3	ND		Jul. 22	2295M	1	ND
	May. 15	1344M	3	Female		Aug. 1	2296M	3	ND
	Jun. 18	1377M	3	Female		Sep. 29	2300M	3	ND
	Jun. 26	1405M	3	Male		Oct. 3	2301M	2	ND
	Jun. 29	1406M	3	Male		Oct. 22	2302M	2	ND
	Sep. 10	1768M	2	Male		Nov. 22	2304M	3	ND
	Sep. 29	1827M	3	Female		Dec. 18	2306M	3	Female
	Oct. 15	1831M	2	Male	2012	Jan. 13	2343M	2	Male
	Oct. 20	1844M	2	Male		Jan. 14	2344M	3	ND
	Nov. 2	1994M	3	Male		Jan. 14	2345M	1	Female
	Nov. 10	1995M	3	Male		Jan. 24	2349M	2	Male
	Dec. 5	1998M	3	Male		Jan. 24	2398M	2	Female
	Oct. 10	2001M	2	Male		Jan. 30	2401M	2	Female

研究短報 動物遺伝学

沖縄島北部におけるケナガネズミ (*Diplothrix legata*) の遺伝的多様性の評価

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要 約

沖縄島北部のケナガネズミのミトコンドリア DNA の部分塩基配列を調べ, その遺伝的多様性を評価した。D-loop 領域の全塩基配列と, tRNA-Pro および Phe の部分塩基配列を 60 個体において決定した。その結果, D-loop で 6 か所の塩基置換部位を持つ 3 つのハプロタイプが存在した。ハプロタイプ多様度 (H) および塩基多様度 (π) は, それぞれ 0.606 および 0.00238 であった。ミスマッチ分布解析により, 沖縄島北部の集団がボトルネックを経験していることが示された。

キーワード: ケナガネズミ, 遺伝的多様性, D-loop

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