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The influence of invasive mongoose on the genetic structure of the endangered Amami rabbit populations

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Abstract Invasive carnivores often cause heavy damage to native species on small islands. Endangered Amami rabbit (*Pentalagus furnessi*) populations have been fragmented into a north-isolated population (NI) and a south-large population (SL) caused by predation pressure from the invasive small Indian mongoose (*Herpestes auropunctatus*) on Amami Oshima Island in southern Japan. We investigated the genetic structure of these rabbit populations to determine the effects of fragmentation. We collected rabbit feces from most of the Amami Oshima Island habitat and sequenced the mitochondrial DNA (mtDNA) control region along with its 5'-flanking region (312 bp) and genotyped eight microsatellite DNA loci. Genetic diversity was lower in the NI than that in the SL population, and F_{ST} values between the two populations were significantly higher than zero in both the mtDNA and microsatellite DNA. Bayesian clustering analyses suggested four ancestral clusters of Amami rabbit, but clear genetic structure was not observed. A partial Mantel test supported isolation-by-distance but not habitat fragmentation. These results suggest that the low genetic diversity in the NI population was caused by the small population size after fragmentation; however, the difference in genetic structure between the two populations was caused by isolation-by-distance and the structure has been maintained.

Keywords Genetic diversity · Isolation-by-distance · Invasive carnivore · Small Indian mongoose · *Pentalagus furnessi*

Introduction

The Amami rabbit (*Pentalagus furnessi*) is endemic to Amami Oshima Island and Tokunoshima Island, which is 40 km southwest of Amami Oshima Island, and is categorized as endangered on the IUCN Red List (Yamada and Sugimura 2008). The Amami rabbit previously inhabited most of the Amami Oshima Island, except the north-easternmost area until the 1970s (Fig. 1; Yamada and Cervantes 2005). However, the rabbit population fragmented into north-isolated (NI) and south-large (SL) populations in the 1990s, and both populations became smaller over the next decade. The reason of the fragmentation and the decrease in population size of the rabbit would be the expansion of invasive mongoose (*Herpestes auropunctatus*) released in 1979 (Watari et al. 2007).

Invasive species are a menace to island ecosystems. As most island species have co-evolved in the island-unique ecosystem, they have reduced resistance to predators or diseases from outside the island (Futuyma 1998). Therefore, they easily succumb to invasive species, which also decreases genetic diversity followed by genetic drift. However, only a few studies have empirically investigated such genetic loss caused by invasive predators. For example, the brown anole lizard (*Anolis sagrei*) lost allelic richness and heterozygosity because of invasive rats (*Rattus* spp.) on the Great Exuma Island, Bahamas (Gasc et al. 2010). Such an effect of an invasive predator on the genetic structure of animals on the Amami Oshima Island has been reported (Iwai and Shoda-Kagaya 2012). Amami Oshima Island has many endemic and/or endangered vertebrate species: e.g., Amami jay (*Garrulus lidithi*), Otton frog (*Babina subaspera*), Amami tip-nosed frog (*Odorrauna amamiensis*), Amami Ishikawa's frog (*Odorrana splendida*), and the Ryukyu long-haired rat (*Diplothrix legata*). A venomous

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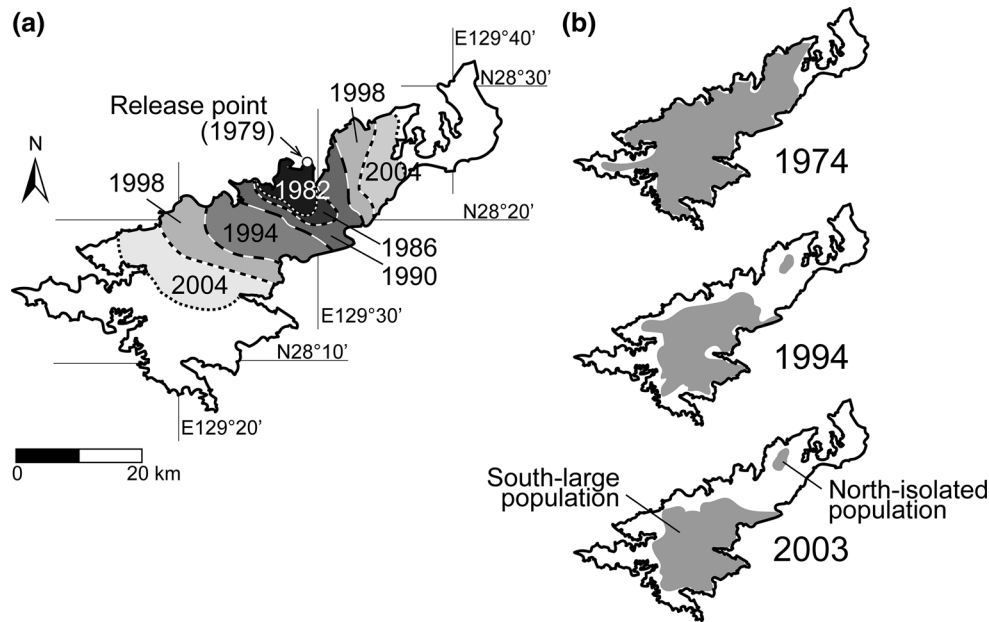


Fig. 1 **a** History of the small Indian mongoose distribution. White circle indicates release point of 30 mongooses in 1979. **b** History of the Amami rabbit distribution. *Shaded areas* represent the Amami rabbit distribution (modified from Yamada and Cervantes 2005)

snake, Habu (*Protobothrops flavoviridis*), is a top predator in the native food web on Amami Oshima Island and is toxic to humans. Thirty small Indian mongooses (*Herpestes auropunctatus*) were released in Naze city in 1979 and were expected to prey on the venomous snake. The mongoose population increased and expanded its range in a radial fashion (Fig. 1); however, mongooses rarely prey on the venomous snake but have decreased the populations of small native animals. The Otton frog population has also decreased in size since the mongoose was released, and their population has been fragmented into NI and SL populations. As the results suggest, the NI population is genetically fragmented and has a different genetic structure from the SL population (Iwai and Shoda-Kagaya 2012).

Although little information is available on the rabbit's status before 2000, we assumed that the population fragmented during the 1980s but low gene flow was maintained between them, as the impact of the mongoose was relatively mild. Gene flow between the two populations was obliterated during the 1990s because of the expansion and increase in the number of mongoose (Fig. 1).

We collected fecal samples during 2003–2005 and investigated the genetic structure of the two Amami rabbit populations to reveal the effect of population fragmentation and the decrease in population size due to the invasive mongoose.

Materials and methods

Fecal sample collection

We collected 164 Amami rabbit fecal piles on Amami Oshima Island during February and/or March from

2003 to 2005 (Fig. 2). We walked on roads and along rivers to find feces because the rabbits usually defecate in open areas. We determined the freshness of the fecal pile by smell, moistness, and stickiness of the surface and then picked up several fresh feces from each fecal pile. To avoid duplicate sampling, we ignored feces within 20 m from any other feces. The collected feces was stored at -20°C until DNA was extracted.

DNA investigations

We extracted DNA from the rabbit feces using the QIAamp DNA Stool Mini Kit (QIAGEN, Valencia, CA, USA). Before applying the kit, we placed three feces from each feces pile into a plastic bag and added 3 mL of ASL buffer (QIAGEN, Valencia, CA, USA). After 30 min incubation at 65°C , we broke up the feces using our fingers from outside the plastic bag. After an additional 3 h incubation at 65°C , 2 mL of the supernatant solution was applied to the extraction kit, and the manufacturer's instructions were followed.

A highly variable region of the mitochondrial DNA (mtDNA) control region along with its 5'-flanking region (312 bp) was sequenced. We used a polymerase chain reaction (PCR) primer pair and the following sequencing reactions: newly designed PF-1 (5'-TGGA-CAAGTAGCATCTATCC-3') and H14785 primers (Taberlet 1996). The target region was amplified using a DNA thermal cycler (GeneAmp PCR System 2700; Applied Biosystems, Foster City, CA, USA) with a 12 μL PCR mixture containing 50 mM KCl, 1.5 mM MgCl_2 , 10 mM Tris-HCl (pH 8.3), 1 $\mu\text{g}/\mu\text{L}$ bovine serum albumin, 0.5 μM of each primer, 1 unit Amplitaq Gold DNA polymerase (Applied Biosystems), and 0.5–1 μg total

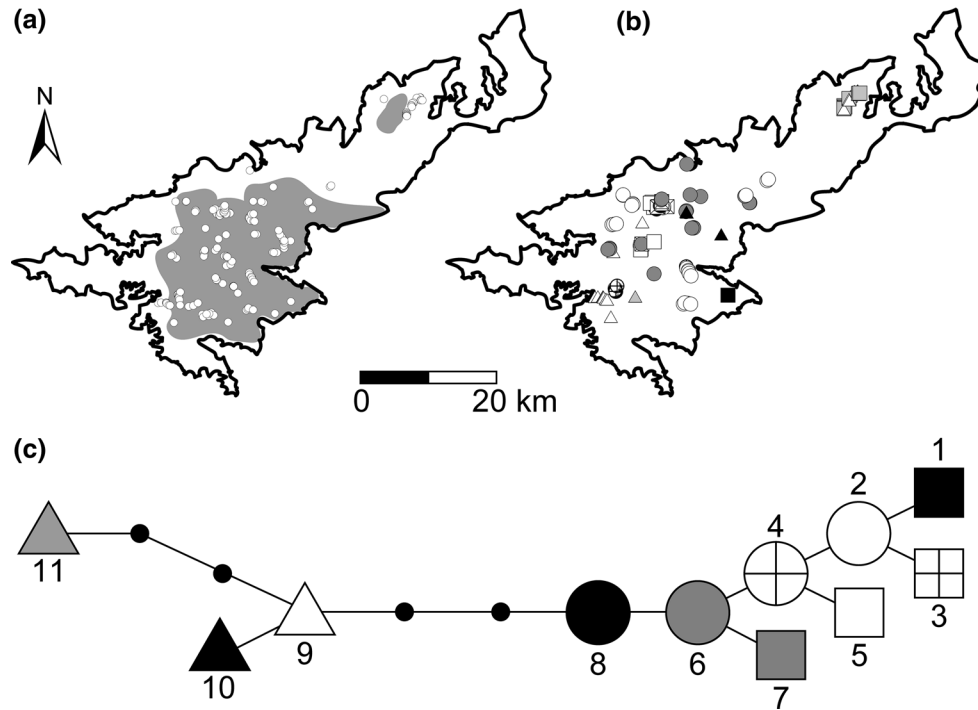


Fig. 2 a Fecal sampling for the microsatellite DNA and/or mitochondrial DNA (mtDNA) analyses. *Gray shaded area* indicates the distribution in 2003 (see Fig. 1). **b** Points showing mtDNA haplotypes where feces were sampled. **c** Statistical parsimony network for the mtDNA control region sequences.

Each line in the network represents a single mutational change. *Symbol* are the same as in **b**. *Numbers beside each symbol* are the haplotype designations (Hap 1–11). *Small black circles* indicate interior nodes that were absent from the sample or that are extinct haplotypes

genomic DNA as the template. After denaturation of the sample at 94 °C for 10 min, 50 cycles of PCR amplification were performed under the following conditions: 1 min at 94 °C, 2 min at 57 °C, and 2 min at 72 °C. The partial mtDNA sequences were determined using a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Pharmacia Biosciences, Piscataway, NJ, USA) and the 3100 Genetic Analyzer (Applied Biosystems).

The genotypes at eight microsatellite DNA loci (PF002, PF003, PF010, PF015, PF033, PF070, PF088, and PF098) were determined for all fecal samples using PCR following Nagata et al. (2009). To avoid mistyping, we amplified each sample a few times. When the same genotype was not detected in one sample, the sample was omitted from further analysis.

Statistical analyses

The statistical parsimony network was constructed using TCS 1.18 m software (Clement et al. 2000). Departure of observed heterozygosity from the Hardy–Weinberg equilibrium was tested by the Markov chain method with 10,000 permutations (Guo and Thompson 1992). Genetic distance, F_{ST} , between populations was calculated according to Weir and Cockerham (1984), and a permutation test (10,000 replicates) was used to deter-

mine whether the genetic distances differed from zero. The Hardy–Weinberg equilibrium test, gene diversity, nucleotide diversity, and genetic distance were examined using Arlequin 3.5 (Excoffier et al. 2005). Allelic richness was calculated using Microsatellite Analyzer 4.05 (Dieringer and Schlotterer 2003). To test whether the low genetic diversity of the NI population was due to a small population size or sampling bias because of small sample size, we compared the observed genetic diversity indices with those of hypothetical populations. One thousand hypothetical populations of 10 individuals each were created by picking randomly from the SL population, and the genetic indices were calculated using Microsatellite Analyzer 4.05 (Dieringer and Schlotterer 2003). The low diversity was assumed to be caused by a small population size when most of the genetic diversity indices were <95% confidence intervals (CI: 26th–975th) of each value. We divided the 92 samples from the SL population into three hypothetical subpopulations ($n = 31, 31, \text{ and } 30$) by order of the value automatically obtained from the latitude and longitude products to determine if the Wahlund effect caused the heterozygous deficit. Then, we tested departure of the observed heterozygosity from Hardy–Weinberg equilibrium using the method described above. Bayesian clustering was investigated using STRUCTURE 2.3.4 (Pritchard et al. 2000) software to describe the genetic structure of the rabbit population. First, we calculated

P_{ID} 10 times for each K at 1–10 with 3,000,000 Markov chain Monte Carlo (MCMC) iterations, a burn-in of 300,000, independent allele frequencies (lambda 1.0), using an admixture model (alpha inferred, with initial alpha set to 1.0) with the LOCPRIOR option via the STRUCTURE. The log-likelihood value $\ln P(X|K)$ was highest and had one peak when $K = 4$. Moreover, the delta K values (Evanno et al. 2005) were highest when $K = 4$ based on the rate of change of $\ln P(X|K)$ between successive K values. Therefore, we set K to 4, and assumed the population inferred proportions with 10,000,000 MCMC iterations, a burn-in of 1,000,000, independent allele frequencies (lambda set to 1.0), and an admixture model (alpha inferred, with initial alpha set to 1.0).

A Mantel test (Manly 1997) and a partial Mantel test with 10,000 permutations were performed using ECO-DIST package in R ver. 2.14 (Goslee and Urban 2007). Bray–Curtis percent dissimilarity was calculated using ECODIST and was used as the genetic distance between individuals for both Mantel tests. The Bray–Curtis dissimilarity value was also used for a spatial autocorrelation analysis following Peakall et al. (2003) using GENALEX 6.2 software (Peakall and Smouse 2006).

Results

We successfully sequenced the 312 bp mtDNA control region and its 5'-flanking region in 106 of 164 fecal samples (NI: $n = 20$. SL: $n = 86$), and 11 haplotypes (Hap1–11) were observed (EMBL/GenBank/DBJ: LC107934–LC107944). Hap 7 and 9 were observed in the NI population, whereas all haplotypes except Hap 7 were observed in the SL population. Gene diversity was lower in the NI than the SL population (NI: 0.521, SL:

0.836); however, nucleotide diversity had little difference between the NI than the SL population (NI: 0.00835, SL: 0.00829). The haplotype network demonstrated two clusters of 11 haplotypes (Fig. 2). Five Haps (Haps 2, 4, 6, 8, and 9) on the stem of the network tree were broadly rather than regionally distributed.

We successfully identified 102 rabbits (10 for the NI and 92 for the SL) by genotyping on the microsatellite loci from the total of 164 fecal samples. All means of the genetic diversity indices, observed and expected heterozygosity values, and observed allele number and allelic richness were lower in the NI than the SL population (Table 1). All PF002 diversity indices were lower than the 95% CI of each value after comparing each genetic diversity index to 1000 hypothetical populations picked randomly from the SL population. The observed and expected heterozygosity values of PF033 and PF070 were also lower than the 95% CI of each value. The mean allele number was <95% CI of each value, and the other three indices were <99% CI (6th–995th) of each value. These results suggested that the low genetic diversity in the NI population was caused by the small population size not by sampling bias. The Hardy–Weinberg equilibrium was not observed in all loci in the NI population, whereas it was observed on three loci (PF002, PF015, and PF070) in the SL population ($P < 0.05$). However, these three loci did not show a sufficient heterozygosity deficit in all three hypothetical subpopulations to detect the Wahlund effect. PF003 and PF088 had significantly lower observed heterozygosity in one hypothetical subpopulation. These results suggested that these heterozygosity deficits were caused by the Wahlund effect.

The Bayesian clustering software STRUCTURE inferred four genetic clusters from the 102 genotyped samples (Fig. 3). Nine of ten rabbits in the NI popula-

Table 1 Observed and expected heterozygosities, the number of alleles and allelic richness in two populations

Population	Locus	Observed heterozygosity	Expected heterozygosity	No. alleles	Allelic richness
North-isolated ($n = 10$)	PF002	0.111 ^b	0.307 ^b	1 ^b	1 ^b
	PF003	0.5	0.409	3	2.6
	PF010	0.444	0.523	3	2.6
	PF015	0.2	0.637	3	2.6
	PF033	0.3 ^b	0.353 ^b	3	2.6
	PF070	0 ^b	0 ^b	2	2
	PF088	0.4	0.468	2	2
	PF098	0.5	0.511	3	3.0
	Mean	0.307 ^c	0.401 ^c	2.5 ^b	2.28 ^c
	South-large ($n = 92$)	PF002	0.422 ^a	0.68	4
PF003		0.379	0.54	5	3.7
PF010		0.405	0.565	4	2.6
PF015		0.369 ^a	0.488	3	2.9
PF033		0.494	0.647	5	3.4
PF070		0.205 ^a	0.378	4	2.5
PF088		0.326	0.412	5	2.8
PF098		0.626	0.625	4	2.9
Mean		0.403	0.542	4.25	2.89

^a Significantly lower observed heterozygosity than expected heterozygosity ($P < 0.05$)

^{b,c} Lower than the 25th and 5th lowest of each value in the 1000 hypothetical populations picked up randomly from south-large population, respectively

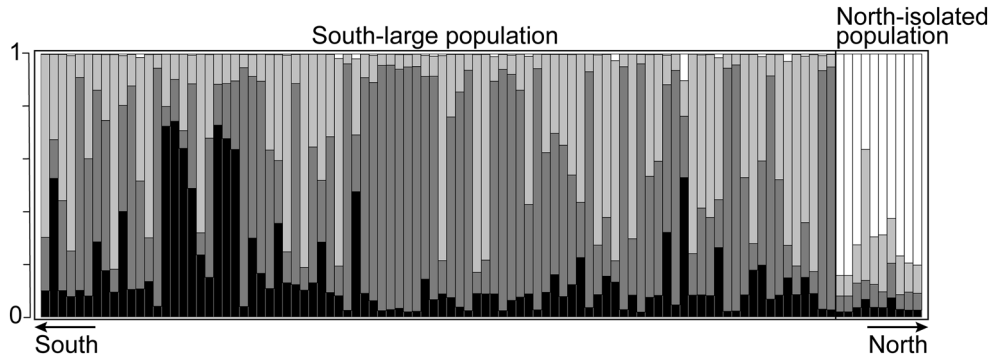


Fig. 3 Barplot of inferred proportions to the four genetic clusters of each individual analyzed by STRUCTURE. Individuals are sorted by the latitude of each sampling point. *White, light gray, dark gray and black bars* indicate Cluster I, II, III and IV, respectively

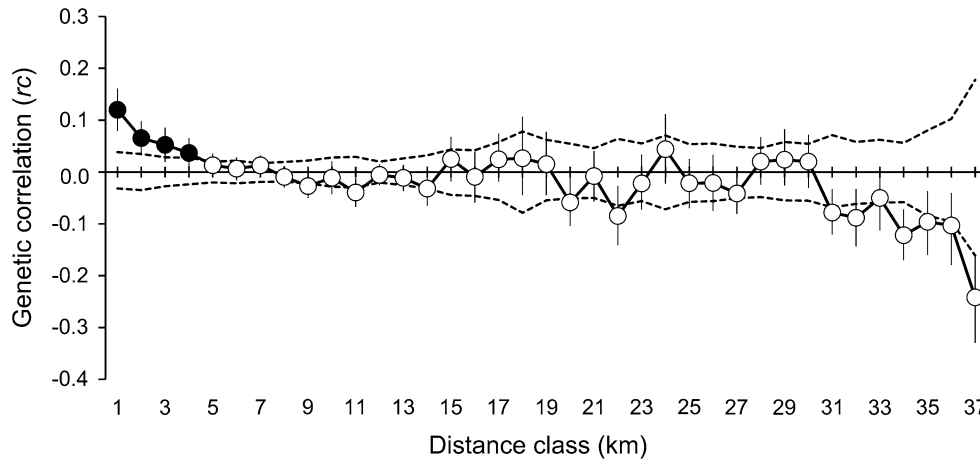


Fig. 4 Correlogram plots of the genetic correlation coefficients (r_c) among individuals as a function of distance. *Black circles* indicate a significantly positive r_c value ($P < 0.05$). *Dashed lines* are the 95%

confidence intervals about the null hypothesis for a random distribution of genotypes, and the bootstrapped 95% confidence error bars are also shown

tion were assigned to Cluster I, even though inferred proportions to the Cluster I of these rabbits were < 0.85 . The inferred proportions of the rest were 0.36 and 0.50 to the Cluster I and II, respectively. 92 rabbits in the SL population was mostly inferred to the Clusters II, III and IV. Some rabbits with high-inferred proportions to the cluster III observed in the southern area. However, spatial clustering structure in the SL populations was not clear, and 30 rabbits did not have high-inferred proportions to each cluster (< 0.60).

The genetic differences between the NI and SL populations were significantly high (mtDNA: $F_{ST} = 0.287$, $P < 0.0001$; microsatellite DNA: $F_{ST} = 0.171$, $P < 0.0001$). The Mantel test between the genetic and geographic distance supported isolation-by-distance (Mantel $r = 0.144$, $P < 0.01$). The partial Mantel test after partialling out the effect of population fragmentation also detected a significant correlation (partial Mantel $r = 0.133$, $P < 0.0001$). The correlation between effect of population fragmentation and genetic distance was not supported after partialling out geographic distance (partial Mantel $r = -0.067$, $P > 0.05$). Isolation-by-distance was detected within the SL population by the simple Mantel test (Mantel

$r = 0.085$, $P < 0.05$), but not within the NI population (Mantel $r = -0.070$, $P > 0.05$). The spatial autocorrelation analysis also revealed isolation-by-distance (Fig. 4). The r_c value was highest within the 1 km distance class and decreased with fluctuating and increasing distance. Significantly positive r_c values were observed within the 4 km distance class ($P < 0.005$), and no r_c value was significantly positive over the 4 km distance class ($P > 0.05$). Similar results were found in the autocorrelation analysis within each population: The r_c value decreased with distance classes, and was significantly positive ($P < 0.05$) within the 3 km distance class in the SL population and within the 1 km distance class in the NI population (data not shown).

Discussion

The main aim of the present study was to estimate the effect of population fragmentation and decrease in the number of Amami rabbits in the northeastern part of Amami Oshima Island. The NI population had lower genetic diversity than that of the SL population. The

significantly higher F_{ST} value and the Bayesian clustering analysis revealed the genetic differentiation between the NI and SL populations (Fig. 3). However, the results of the partial Mantel test rejected the effect of population fragmentation, which had a potential to increase genetic differentiation between these two populations. Why were such inconsistent results observed? The simple and partial Mantel tests and the autocorrelation analysis (Fig. 4) showed isolation-by-distance over the entire island. Additionally, not only Isolation-by-distance but also indistinct spatial structure by the Bayesian clustering were observed within the SL population. These results suggest that the genetic structure gradually changes based on geographic distance. That is to say, different genetic structures exist within the SL population (e.g. at both ends of the SL population), which would have brought the Wahlund effect there. As we apply this process to the entire island, it is concluded that differences between the NI and SL populations were caused by geographic distance not population fragmentation.

Isolation-by-distance was observed all over the island and within the SL population, indicating that the rabbit population has reached genetic equilibrium. Isolation-by-distance has also been observed in the Otton frog on Amami Oshima Island, and their populations were fragmented into NI and SL populations by the invasive mongoose. The frog and rabbit cases differ in that all individuals in the NI and SL populations of the frog were strongly assigned to each population by a Bayesian clustering analysis. Moreover, the frog had one dominant haplotype occurred all over the island, which did not suggest genetic fragmentation in a historical timescale (Iwai and Shoda-Kagaya 2012). Therefore, the genetic characteristics of the NI frog population was likely caused by genetic drift after fragmentation by the mongoose (Iwai and Shoda-Kagaya 2012). In contrast to the Otton frog, the Amami rabbit had two clusters in the haplotype network (Fig. 2). The reason why a few clusters were observed would be conjectured by historical geology. Amami rabbits inhabit not only Amami Oshima Island but also Tokuno Island, which is 40 km southwest of Amami Oshima Island. As these two islands have been repeatedly connected at some times during the Quaternary period (Kizaki and Oshiro 1977), the rabbits in Tokuno Island, which have a different genetic structure, probably immigrated to Amami Oshima Island a few times. After that, it is possibly that their dispersal increased gene flow and haplotype distributions were mingled up.

Although we have revealed low genetic diversity caused by a population decline in the Amami rabbit, the population is currently increasing because of a decrease in the mongoose population (Watari et al. 2013). The Ministry of the Environment started a mongoose eradication project in 2000, and approximately 2500 mongoose were captured (culled or removed) per year until 2006. The number captured has decreased to < 1000 per year since 2006, and only 71 mongoose were captured in 2014. As a consequence, the number of mongoose in-

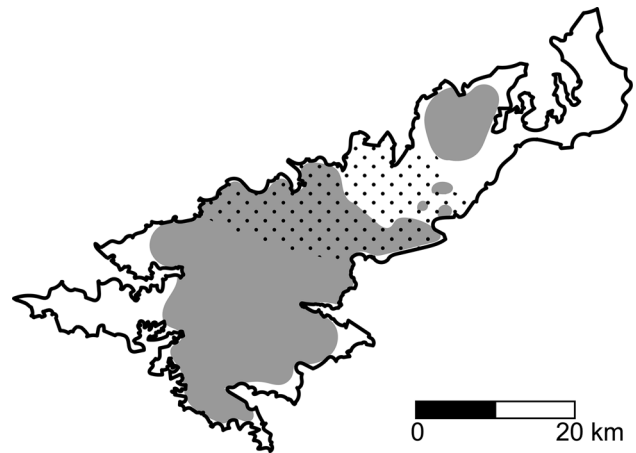


Fig. 5 Distributions of the small Indian mongoose (dotted area) and Amami rabbit (gray shaded area) in 2013 (Ministry of the Environment 2015)

creased and reached > 5000 by 2000 but decreased to < 100 in 2014 (Ministry of the Environment 2015). As the results, the number of Amami rabbits started to recover after 2006, and their distribution area has expanded (Watari et al. 2013). The NI population also expanded, and some observations were reported from an area between the NI and SL populations where the rabbit had been previously reported as extirpated (Fig. 5; Ministry of the Environment 2015). In the present study, we revealed a decrease in genetic diversity of the NI population due to small population size; however, no effect of habitat fragmentation was observed. We collected fecal samples in 2003, which was 24 years after the mongoose was released. Given that gene flow stopped in 1990, considering that generation time of the Amami rabbit is 3 years (Di Marco et al. 2013), this effect took about four generations. From the present results, it would not enough to visualize the effect of fragmentation on genetic differentiation between the two populations even though genetic diversity decreased more in the NI population than the SL population (Landguth et al. 2010). Moreover, both populations are now expanding, which will restart gene flow between them in the near future. And also, expansion of the NI population suggests that it will escape from crisis. Changes in genetic status should be monitored continuously to detect the bottleneck effect during the small-population period. The recent low catch per unit effort by the mongoose eradication program argues the need to continue based on cost-effectiveness. However, once the pressure to capture mongoose decreases, they will quickly recover their population size. It would be necessary to extinguish mongoose completely from Amami Oshima Island.

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