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Genetic Variations Revealed by Microsatellite Markers in a Small Population of the Sika Deer (*Cervus nippon*) on Kinkazan Island, Northern Japan

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ABSTRACT—Genetic variations within a population of the Japanese sika deer, *Cervus nippon*, on Kinkazan Island were studied by microsatellite analysis. Seventeen pairs of polymerase chain reaction primers designed for several species of ungulates successfully amplified polymorphic microsatellite DNA in sika deer. About 20% of the Kinkazan population was sampled and genotyped for nine diagnostic microsatellite loci. Alleles at those loci in the Kinkazan population were found to be under the Hardy-Weinberg equilibrium. To determine whether the Kinkazan deer have a reduced level of genetic variability, an average heterozygosity in the population was calculated and compared with the values determined for other populations from Hyogo, Yamaguchi, Shimane, Tsushima, and Nagasaki. Neither the observed nor the expected heterozygosity in the Kinkazan deer significantly differed from that in the other populations. Our result indicated that, despite its small population size, the Kinkazan deer preserve extensive microsatellite variations.

INTRODUCTION

The sika deer (*Cervus nippon*) is a common form of wildlife that was once distributed over the Japanese Islands. Its habitat has been fragmented during this century mainly due to the destruction of forests caused by human activities. As a consequence, local populations have been isolated and confined to relatively small areas. Most of the local populations are supposed to have experienced bottlenecking as a result of overexploitation and/or mass mortality occurring during severe winters.

Since gene flows between local populations rarely occur at present, the loss of genetic variation that may lead to local extinction has been anticipated in the Japanese sika deer. However, the genetic status of populations has not been studied well because of a lack of appropriate genetic markers. Molecular markers such as randomly amplified polymorphic DNAs (RAPD) and restriction fragment length polymorphism (RFLP) are not always diagnostic for detecting genetic variations within a population of sika deer because the genetic variability of those markers is reduced to an undetectable level in some cases (Tamate *et al*., 1995a, 1995b). Mitochondrial DNA sequences are good markers to track down the population history, although they are only informative for maternal lineages (Nagata *et al*., 1995; Tamate *et al*., 1998).

To overcome such difficulties, microsatellite markers have been sought for the genus *Cervus*. Microsatellite DNA consists of reiterated short sequences tandemly arrayed, and it usually provides genetic data with a greater information content than do mitochondrial DNA haplotypes or isoenzyme markers (Bruford *et al*., 1996). Previous studies demonstrated that artiodactyl microsatellite loci are conserved across closely related species (DeWoody *et al*., 1995; Pemberton *et al*., 1995; Engel *et al*., 1996; Kühn *et al*., 1996; Talbot *et al*., 1996; Slate *et al*., 1998). Some of those loci, from bovine and ovine microsatellites, were successfully used in population-genetic analysis for the sika deer (Nagata *et al*., 1998) and in the study of hybridization between the red deer and sika deer (Abernethy, 1994).

More intricate genetic analyses such as individual-based

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studies would require a number of diagnostic markers that detect genetic polymorphism within a single population. Screening of microsatellite markers has been continued for this purpose and, to date, 47 microsatellite loci have been found to be polymorphic in the sika deer in Scotland (Slate *et al*., 1998). However, it is not known whether those markers are diagnostic for detecting variations in a population of the Japanese sika deer.

In the present study, we took further effort to increase the number of informative markers for the Japanese sika deer. For this purpose, we screened microsatellite loci that were reported previously for other artiodactyls. To test the usefulness of the markers, we made an attempt to detect genetic variations in a small island population of the Japanese sika deer.

MATERIALS AND METHODS

Sampling

Sampling localities are shown in Fig.1. We collected samples from 109 deer from Kinkazan Island, a small island (960 ha) located off the northeastern coast of Japan (38°17' N, 141°39' E). During the collection, we took special care not to injure the animals, as described previously (Minami *et al*., 1992). Blood was taken from live-captured animals and subjected to DNA extraction by the standard method (Sambrook *et al*., 1984). We also collected DNA samples from the muscles of hunter-killed deer at the following localities in Japan (Fig. 1): Hyogo (n=26), Yamaguchi (n=24), Shimane (n=20), Tsushima (n=23), and Nagasaki (n=21). Those samples were used as representatives of "reference populations" to the Kinkazan population.

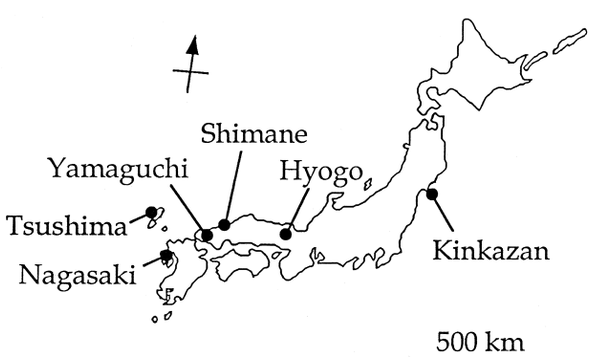

Fig. 1. Map showing locations of the sampled populations of the sika deer.

Polymerase Chain Reaction

To screen diagnostic markers, we selected 25 sets of primer pairs for loci that have been reported to be polymorphic in Cervidae (DeWoody *et al*., 1995; Pemberton *et al*., 1995; Engel *et al*., 1996; Kuhn *et al*., 1996; Talbot *et al*., 1996). Among them, twelve sets of primers were designed for bovine loci: BL42, BM203, BM888, BM4107, BM4208, BMC1009, BOVIRBP, ETH225, Haut14, Hel6, INRA040, and IOBT965. We also tested three ovine primer sets (OarFCB193, MAF65, and VH110), five caprine primer sets (SRCRSP2, SRCRSP3, SRCRSP6, SRCRSP9, and SRCRSP10), and five primer sets designed for the white-tailed deer (*Odocoileus virginianus*) (Cervid 1, Cervid 2, Cervid 3, Cervid 4, and Cervid 14). Except those primers, we used eleven sets of bovine primers (TGLA48, TGLA53, TGLA57, TGLA75, TGLA122, TGLA126, TGLA227, TGLA263, AGLA293, MGTG4B, and MGTG7) that were provided with the StockMarks™ for Cattle from the Applied Biosystem Division, Perkin-Elmer-Cetus, USA (ABI/PEC).

The polymerase chain reaction (PCR) was performed in a total volume of 20 μl, using the primer pair and 100 ng of genomic DNA. The reaction mixture contained 0.8 μM of each primer, 200 μM each of dATP, dCTP, dGTP, and dTTP, 0.4 μM of [F]-dCTP and 0.025 units of TaKaRa Taq DNA polymerase (Takara, Japan) in buffer consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$. The [F]-dCTPs R6G-dCTP (green, 100 μM), R110-dCTP (blue, 100 μM), and TAMRA-dCTP (yellow, 100 μM) were obtained from ABI/PEC (product number 402793). The primer sequences are listed in Table 1. Thirty cycles of a series of reactions at 96°C (denaturation) for 30 sec, 56°C (annealing) for 1 min, and 72°C (elongation) for 30 sec were carried out in a PE9600 thermal cycler (ABI/PEC). The annealing temperature was reduced to 46°C when BM888 marker was amplified. The first denaturation step at 96°C was extended to 2.5 min, and the final elongation step at 72°C was extended to 10 min.

Genotyping

After PCR, an aliquot of 0.5 μl for each locus, was taken from the products and mixed with 12 μl of deionized formamide and 0.5 μl of ROX350 marker. Gel electrophoresis was run in an ABI-PRISM 310 Genetic Analyzer (ABI/PEC) for 30 min by using a module STR55A with a sequencing capillary (product number 401821) cut to a length of 41 cm, sequencing polymer (product number 401673), and a genetic analyzer buffer (product number 401884). A matrix file for the GeneScan analysis was created by using an FdCTP matrix standard (product number 402792). The electrophoresis system used in our study was originally designed for DNA sequencing. This system is also applicable to separate DNA fragments with sizes of fewer than 300 base pairs.

In an initial screening of the markers, we used a small test panel of 20 individual DNA samples. Subsequently, a large panel of DNA samples from 109 deer was tested for polymorphisms within the population.

Data analyses

Genetic polymorphism was estimated as a number of alleles per locus, observed heterozygosity (*Ho*), and expected heterozygosity from Hardy-Weinberg assumptions (*He*), using the GENEPOP package, version 3.1b (Raymond and Rousset, 1995). This program was also used to test for deviations from Hardy-Weinberg equilibrium within a population at each locus and over all loci. To obtain unbiased estimates of the exact Fisher test through 1000 iterations, we used the Markov chain method for each locus that has more than four alleles. For each locus that has less than three alleles, we used the complete enumeration method for the test. The polymorphic information content (PIC) value, which indicates the potential usefulness of the markers in pedigree analysis (Bostein *et al*., 1980), was calculated as

$$1-(\sum_{i=1}^{n} p_i^{\,2})-\sum_{i=1}^{n-1}\sum_{j=i+1}^{n} 2p_i^{\,2}p_j^{\,2}$$

where the corresponding locus has *n* alleles and the frequency of the *i*th allele is p_i.

Statistical significance ($P<0.01$) of the difference between heterozygosities was tested by a nonparametric multiple comparison using the computer program NP multi (distributed by Nagata O, 1995).

Computer Simulation

We used Monte Carlo computer simulations to test whether loss of alleles can occur in the the Kinkazan population if the herds are maintained at their current population size. We used a fixed effective population size $Ne = 220$ for each generation of the Kinkazan deer in the simulation. Ne of the Kinkazan population was estimated as follows:

1. The total number of the Kinkazan deer (*N*) was estimated to be

around 550 before the 1997 crush (Takatsuki *et al*., personal communication) by population census.
2. The number of deer in our study group (*N*') is 120.
3. Based on observations of mating behavior (Minami *et al*., personal communication), the numbers of reproductive stags (*Nm*') and hinds (*Nf*') in the study group were estimated to be 20 and 40, respectively.
4. Only the biased sex ratio was considered as a factor that affects *Ne*.

Based on the above assumption, the effective population size of the Kinkazan deer was calculated using the following equation:

$$Ne = (4Nm'Nf'/(Nm'+Nf'))N/N'$$

We began our simulation by creating an initial allele frequency distribution based on the observed data for each loci. We simulated a change in allele frequencies by sampling alleles randomly from the distribution and then generated a new distribution for the next generation. We repeated the entire simulation 1,000 times for each generation, and counted the number of events that ended in loss of alleles from the population. Migration, mutation, and difference in fitness among alleles were not considered in the simulation.

RESULTS

Table 1 shows a list of microsatellite loci tested in the present study and the result of the initial screening. Among the 36 loci used, twelve bovine microsatellites, BL42, BM203, BM888, BM4107, BM4208, BMC1009, BOVIRBP, ETH225, Haut14, IOBT965, TGLA53, and TGLA126, were found to be polymorphic, hence diagnostic, in the Kinkazan sika deer. Two caprine (SPCRSP3 and SPCRSP9), one ovine (OarFCB193), and two white-tailed deer loci (Cervid 2 and Cervid 14) were also found to be diagnostic in the sika deer. The rest of the loci tested in our study were monomorphic otherwise gave no PCR product. The number of alleles per locus and size-ranges of the PCR products for the diagnostic loci are shown in Table 2.

Mendelian traits of the diagnostic markers were confirmed by matching genotypes of mothers to that of offspring in 19 maternal pedigrees identified in the Kinkazan deer (Minami, personal communication). We found no discrepancies in matching the genotypes, except in the case of ETH225. When amplifying with ETH225 primers, we observed two different sets of alleles, with size ranges of 151–195 (designated as ETH225-1) and 284–293 (designated as ETH225-2). Statistical analysis showed that ETH225-1 and ETH225-2 were independent loci because there was no association of alleles between the two loci. We did not observe PCR products of ETH225-2 in 14% of 109 samples, while the same PCR gave products of ETH225-1 to all of the samples tested. Because of the possibility that the ETH225-2 locus has null alleles, we did not include this locus in the subsequent analysis of heterozygosities.

Genotypes of individuals were further tested for association of alleles among loci. We found no genotypic linkage disequilibrium between two loci, except for that between the BM203 and ETH225-1 loci ($P<0.01$).

Among the 17 diagnostic loci, we chose nine loci (BL42, BM203, BM888, BM4107, BMC1009, BOVIRBP, Cervid14, ETH225-1, and OarFCB193) and calculated observed heterozygosity (*Ho*), expected heterozygosity (*He*), and PIC values (Table 1). *Ho* in the Kinkazan population ranged from 0.301 to 0.846, it did not differ significantly from *He* calculated for each locus. An average PIC value was 0.483 for the nine loci, and three of the loci had a PIC value above 0.5.

Table 3 shows the size and frequency of microsatellite alleles at the nine diagnostic loci. We observed rare alleles in which the allele frequency is less than 0.05, at the BM888, BMC1009, Cervid14, ETH225, and OarFCB193 loci. Individuals with such rare alleles were genotyped again by a separate

Table 1. Microsatellite markers tested in the study.

Marker	[a]Origin of marker	[b]Result	[c]Reference	Marker	[a]Origin of marker	[b]Result	[c]Reference
BL42	BO	p	1	TGLA227	BO	n	6
BM203	BO	p	1	TGLA263	BO	m	6
BM888	BO	p	1	AGLA293	BO	m	6
BM4107	BO	p	1	MGTG4B	BO	n	6
BM4208	BO	p	1	MGTG7	BO	m	6
BMC1009	BO	p	1	MAF65	OV	m	7
BOVIRBP	BO	p	2	VH110	OV	m	1
ETH225	BO	p	3	OarFCB193	OV	p	1
Haut14	BO	p	3	SRCRSP2	CA	m	7
Hel6	BO	m	4	SRCRSP3	CA	p	7
INRA040	BO	m	5	SRCRSP6	CA	m	7
IOBT965	BO	p	3	SRCRSP9	CA	p	7
TGLA48	BO	n	6	SRCRSP10	CA	m	7
TGLA53	BO	p	6	Cervid1	CE	m	8
TGLA57	BO	m	6	Cervid2	CE	p	8
TGLA73	BO	m	6	Cervid3	CE	m	8
TGLA122	BO	m	6	Cervid4	CE	m	8
TGLA126	BO	p	6	Cervid14	CE	p	8

[a] BO, bovine; OV, ovine; CA, caprine; CE, cervine.
[b] p, polymorphic; m, monomorphic; n, no PCR product.
[c] 1, Talbot *et al* (1996); 2, Bancroft *et al* (1995); 3, Kühn *et al* (1996); 4, Vaiman *et al* (1994); 5, Nagata *et al* (1998); 6, Bishop *et al* (1994); 7, Engel *et al* (1996); 8, DeWoody *et al* (1995).

Table 2. Number of alleles, expected heterozygosity (*He*), observed heterozygosity (*Ho*), and polymorphic information content (PIC) of microsatellite loci in the Kinkazan population.

Locus	Number of alleles	Product size (bps)	*He*	*Ho*	PIC
BL42	3	252–258	0.631	0.623	0.559
BM203	3	212–221	0.446	0.470	0.356
BM888	5	193–203	0.445	0.471	0.408
BM4107	2	162–170	0.286	0.311	0.239
BMC1009	3	276–290	0.325	0.319	0.277
BOVIRBP	2	144–146	0.502	0.556	0.374
Cervid14	3	217–231	0.462	0.451	0.373
ETH225-1	9	151–195	0.848	0.844	0.837
OarFCB193	6	105–127	0.703	0.670	0.605
BM4208	2	160–162	nd	nd	nd
Cervid2	2	155–157	nd	nd	nd
ETH225-2	2	284–293	nd	nd	nd
Haut14	2	115–119	nd	nd	nd
IOBT965	2	116–118	nd	nd	nd
SRCRSP3	2	96–101	nd	nd	nd
SRCRSP9	2	126–131	nd	nd	nd
TGLA53	4	176–196	nd	nd	nd

“nd, not determined.

Table 3. Allele frequencies at nine microsatellite loci in the Kinkazan population.

Locus	Allele*	Allele frequency	Locus	Allele*	Allele frequency
BL42	252	0.439	BOVIRBP	144	0.486
	254	0.195		146	0.514
	256	0.366			
			Cervid14	217	0.320
BM203	212	0.174		219	0.021
	217	0.679		231	0.660
	221	0.147			
			ETH225-1	151	0.130
BM888	193	0.010		153	0.159
	197	0.020		160	0.139
	199	0.721		163	0.005
	201	0.078		185	0.159
	203	0.172		187	0.024
	191	0.202		193	0.111
				195	0.072
BM4107	162	0.167			
	170	0.833			
			OarFCB193	105	0.096
BMC1009	276	0.799		107	0.394
	280	0.008		119	0.005
	290	0.193		121	0.328
				125	0.177

*Alleles are designated as their product sizes in base pairs (bp).

experiment to confirm that the alleles were correctly scored. None of the rare alleles was found to be an artifact in the repeated experiments.

We also measured allele frequency at the nine diagnostic loci in the reference populations. By using this data set, we calculated an average observed heterozygosity (*HO*) for each population to determine whether there is a significant difference in heterozygosity between the Kinkazan and the reference populations (Fig. 2). An average expected heterozygosity (*HE*) was also calculated for each population. *HO* and *HE* in the Kinkazan population were 0.525 and 0.516, respectively. A multiple comparison test revealed that *HO* in the Kinkazan population did not statistically differ from *HO* in the other populations except for that at Nagasaki; *HO* in the Nagasaki population was significantly ($p<0.01$) lower than *HO* in the Kinkazan, Hyogo, Shimane, Tsushima, and Yamaguchi populations. We also found no significant difference between *HO* and *HE* in any of the populations.

Figure 3 shows the result of the simulation of the genetic drift that leads to the loss of rare alleles at BMC1009 locus for 50 generations of the Kinkazan deer, under the condition that *Ne* is fixed at 220. The result shows that allele 280, which has the lowest frequency (0.008) among the alleles at the loci, is most likely to be lost within 50 generations.

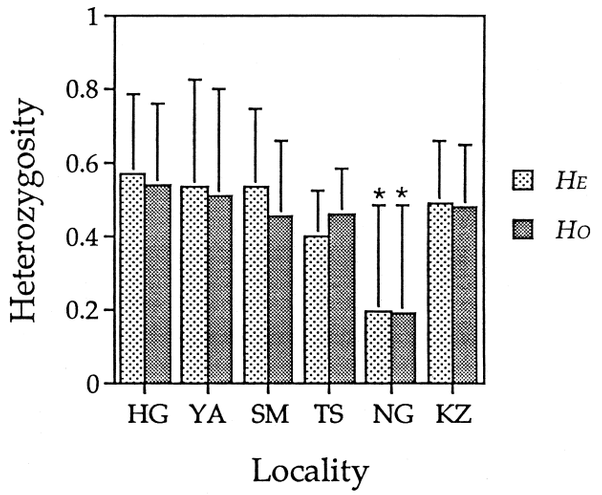

Fig. 2. Average observed (*HO*) and expected (*HE*) heterozygosities in local populations of the Japanese sika deer. Capital letters denote localities of the sampled populations shown in Fig.1: HG, Hyogo; YA, Yamaguchi; SM, Shimane; TS, Tsushima; NG, Nagasaki; KZ, Kinkazan. Bars indicate standard deviations. The heterozygosities in the Nagasaki population are significantly lower than the others, as indicated by the asterisks.

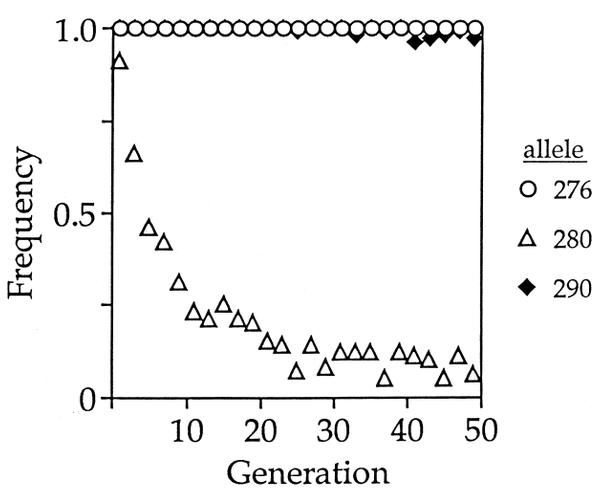

Fig. 3. Computer simulation of the effect of random genetic drift over the three alleles at the BMC1009 locus. Each generation alleles are sampled with replacements, as described in the text. The frequency of the event that a given allele is retained in the population at each generation is shown for each the allele.

DISCUSSION

Resolution power of microsatellite markers

The usefulness of microsatellite markers in deer population study has been demonstrated by previous studies (Abernethy, 1994; Pemberton *et al*., 1995; Nagata *et al*., 1998; Slate *et al*., 1998), although the number of diagnostic markers was limited. In the present study, we added more microsatellite loci to a list of the diagnostic markers for population genetic analysis of the sika deer. By using those markers, we detected considerable genetic variations in the Kinkazan population in which little polymorphism of mitochondrial DNA and RAPD has been observed (Tamate *et al*., 1995a, 1995b). The same magnitude of genetic variations that has been observed in the Kinkazan population was also demonstrated in the other populations by using the markers. The microsatellite markers tested in our study were, therefore, proved to be effective tools in dissecting the population genetic structure of the sika deer, not only for the Kinkazan population but for other local populations. Moreover, our result indicated high resolution power of the markers in individual-based analyses. Three of the markers had high PIC values and, therefore, were judged to be particularly useful in identifying individuals. The microsatellite markers tested in the present paper are now utilized successfully in both population-based and individual-based studies of the sika deer (Okada *et al*., in press).

Genetic variations in the Kinkazan population

It should be noted that the microsatellite alleles were under the Hardy-Weinberg equilibrium (HWE) in the Kinkazan deer, even though the population size is small. The population density is very high (50 deer/km^2), and the mating system is promiscuous (Minami, personal communication). These conditions should drive the intermixing of genes within a population, and, therefore, may have contributed to keeping the Kinkazan population at HWE.

HO in the Kinkazan population was greater than *HO* reported for microsatellite loci in other Cervus species (Engel *et al*., 1996), indicating that the genetic variability of the Kinkazan deer is not necessarily reduced, as had been anticipated (Tamate *et al*., 1995b). This was further supported by the data from other local populations: there was no significant difference in either *HO* or *HE*, between the Kinkazan and the other populations, except the Nagasaki population. We therefore concluded that heterozygosity of the Kinkazan population is not reduced in comparison with other local populations of the Japanese sika deer. The Nagasaki, Yamaguchi, and Tsushima populations are closely related in mitochondrial DNA phylogeny (Tamate *et al*., 1998); however, only the Nagasaki population displayed a remarkable reduction in heterozygosity. The lowered heterozygosities could be explained if the Nagasaki population is recovering from a recent bottlenecking. We also found differences in allele frequencies among the populations — the result and its implications for the population differentiation will be discussed in separate papers.

Nagata *et al*. (1998) reported that *HO* in the Hokkaido and Chiba populations of sika deer were 0.21 and 0.23, respectively. These values are much smaller than those observed in our study. The difference in the heterozygosity can be attributed to types of microsatellite markers used in their study, which were different from ours. Alternatively, such a difference in *HO* may indicate that our study populations have greater genetic variability than both the Hokkaido and Chiba populations.

Presence of rare alleles

The Kinkazan deer population has been confined to a small island and has been maintained at a small size (500 animals at the maximum) for at least two hundred years. It is not known whether the Kinkazan deer originated from a natural population or from an introduced population because historical records of the deer are scarce before the 18th century. The population has experienced mass mortalities repeatedly in recent years (Takatsuki *et al*., 1994). In theory, an isolated small population like the Kinkazan deer will lose rare alleles within a small number of generations due to the effect of genetic drift. In the present study, on the contrary, we observed such rare alleles in the Kinkazan population. If the Kinkazan population has been maintained at the current population size for many generations, and if no mutation or migration had occurred, the rare alleles should have been lost. Our simulation, for example, demonstrated that the rare allele at the BMC1009 locus will be lost within 50 generations in the Kinkazan population.

The presence of rare alleles in the Kinkazan population can be explained by either migration, mutation, or heterosis. Migration from the mainland may introduce rare alleles to the island. This possibility cannot be excluded because local people have occasionally witnessed deer swimming in a channel between Kinkazan Island and the Honshu mainland of which distance is only 500 m. We have no direct evidence of the migration of deer to or from the island, and we have not yet collected genetic data from a mainland population in this area.

Alternatively, mutation can also provide rare alleles in a population. If the mutation rate at microsatellite loci is relatively high, as suggested by Dallas (1992), we may have a chance to observe rare alleles as a result of recent mutation in the population, even if the rare alleles are of short duration. However, some of the rare alleles that were found in the Kinkazan population are not universally rare and exist at a higher frequency in other populations (data not shown). It is, therefore, difficult to adopt the mutation theory to account for the presence of such alleles in multiple populations.

Heterosis may also contribute to the existence of rare alleles in small island populations. Coulson *et al*. (1998) reported heterosis in the red deer population on Rum, Scotland, and they demonstrated that individuals heterozygous for microsatellite loci had higher fitness than did homozygotes. Heterosis at microsatellite loci was also reported for a harbor seal population in Nova Scotia (Coltman *et al*., 1998). If the Kinkazan population is under such selective conditions, rare alleles will persist longer than what we expected under nonselective conditions. To clarify this possibility, we are now investigating the correlation between individual fitness and genotypes in the Kinkazan population by recording lifetime histories of individual deer.

The maintenance of genetic polymorphism in a small island population has been a relevant issue in both genetics and conservation biology (Pemberton *et al*., 1996). Studying microsatellite variations in the Kinkazan deer population will provide further information to clarify the mechanism that controls the level of genetic variability in island animals.

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