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Pedigree Analysis of the Sika Deer (*Cervus nippon*) using Microsatellite Markers

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ABSTRACT—The usefulness of microsatellite markers in pedigree analysis of the sika deer (*Cervus nippon*) was tested in a herd in which the maternal lineages were recorded. Eighteen sets of microsatellite primers originally designed for bovine, ovine, and cervine loci successfully amplified polymorphic DNA in the deer. The numbers of alleles per locus ranged from two to seven, and the observed heterozygosity ranged from 0.350 to 0.900. The resolution power of the markers in paternity testing was then determined by calculating exclusion probabilities and paternity indices. Parentages of the study population were efficiently discriminated by genotyping 17 microsatellite loci. The microsatellite data were also used to calculate the genetic relatedness between individuals, which significantly correlated with coancestry coefficients for the pairs. Our results demonstrate that the microsatellite markers are efficient tools in studying the social structure and behavior of the sika deer, as well as in monitoring the inbreeding status.

INTRODUCTION

Natural populations of the Japanese sika deer (*Cervus nippon*) have a promiscuous mating system at high population densities (Minami, personal communication). Because hinds at estrus usually mate with more than one stag, the paternity of calves cannot be easily determined by a behavioral study alone. Such ambiguous paternity of calves has been an obstacle for studying the reproductive success of males and the pedigree structure of the sika deer. This problem, however, can be solved if discrete genetic markers are available to examine the genetic profiles of dams, calves, and alleged sires. Recent studies have utilized microsatellite DNA analysis to obtain the genetic profiles of individuals in mainly species of wild animals. These microsatellite DNA are noncoding, short, and tandemly repeated simple nucleotide sequences. They are highly variable in the number of tandem repeats inherited in a simple Mendelian fashion (Tautz and Renz, 1984; Tautz *et al*., 1986; Tautz, 1989; Litt and Luty, 1989; Weber and May, 1989). Because of the high variability, microsatellite DNA markers have been successfully used in the pedigree analyses of primates (Takenaka *et al*., 1993, Deka *et al*., 1994), carnivores (Fredholm *et al*., 1995) and many avian species (Ellegren, 1992).

In recent studies, a number of microsatellite markers have been screened for red deer and sika deer, some of which have been found to be diagnostic for detecting variations within a population as well as between different populations (Abernethy, 1994; Slate *et al*., 1998; Nagata *et al*., 1998, Tamate *et al*., in press). These studies have demonstrated the usefulness of microsatellite markers in a population-based study of deer species. For individual-based study, on the other hand, the potential applicability of the microsatellite markers to pedigree analysis has only been inferred (Talbot *et al*., 1996; Mommens *et al*., 1998), and the reliability, efficiency, and appropriateness of this technique has not been evaluated in practice. In the present study, therefore, we carried out a pedigree analysis of sika deer in a captive population in which individual histories had been recorded, and tested the usefulness of the microsatellite markers in individual-based studies of the sika deer.

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MATERIALS AND METHODS

Animals

A study population of sika deer was kept in an enclosure from 1980 in Nogeyama Zoological Garden, Yokohama, Japan. This population was originally established with five founder animals, two stags, and three hinds. They are captured or introduced in Kanto area, Japan, and unrelated to each other except a stag and a hind introduced from a zoo. The coancestry coefficient (θ) between these individuals were unknown, so the θ between founders was regarded to be zero. The founders subsequently gave birth to 33 offspring during this period. For each newborn, the mother was identified but father was ambiguous. The maternal lineage of this population is shown in Fig. 1. Among 22 males in the study population, only two adult stags over the age of 3 were likely to have sired all the calves; nevertheless, we included all males above 1 year old as candidate males in

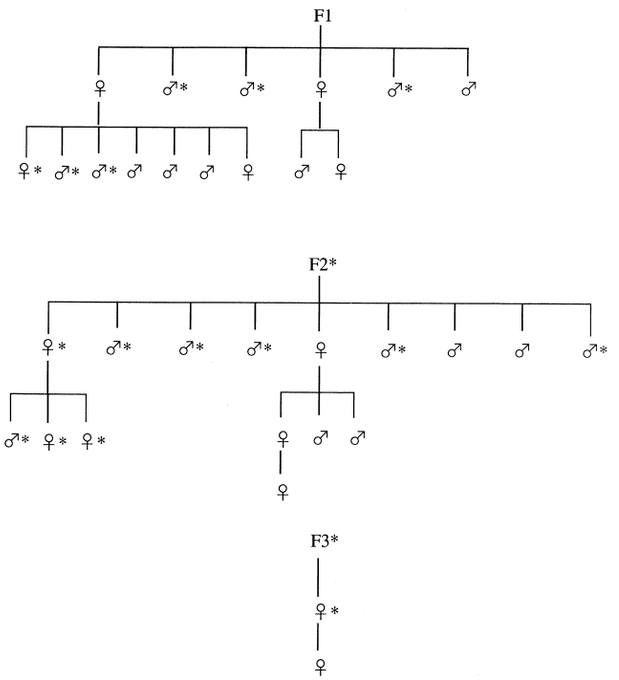

Fig. 1. Recorded maternal lineages in Nogeyama sika deer. Founders are shown by letters; F1, F2 and F3, respectively. Individuals of which DNA were not sampled are indicated by an asterisk.

the paternity analysis. The number of candidate males varied from two to nine for each year. Incestuous breeding was likely to have occurred because mating was not controlled in the study population.

Blood was collected from 20 sika deer of 17 pedigrees, and whole blood or white cell pellets were preserved in ethanol. DNA was extracted by the Phenol-Chloroform method as described in Sambrook *et al*. (1989).

Microsatellite analysis

Microsatellite primers were tested to amplify 50 ng of template DNA with the internal labeling method (Iwahana *et al*., 1996). PCR was performed in a total volume of 10 μl reaction mixture with 0.5 μM of each primer, 200 μM of each dNTP, 0.4 μM of [F]-dCTP, and 0.025 unit 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 $MgCl_2$. For some primers, 2.5 mM of $MgCl_2$ was added with DMSO for denaturation (Slate *et al*., 1998). The [F]-dCTPs were obtained from PE Applied Biosystems (PE-ABI). Amplification of microsatellite DNA was carried out with 37 cycles of a series of reactions for 30 sec at 96°C for denaturation, for 1 min for annealing, and for 30 sec at 72°C for elongation. For some primers, PCR was performed by a two-step PCR method, first with an amplification step of seven cycles, followed by a second step of 30 cycles, as described by Slate *et al*. (1998). Primer-specific annealing temperatures and modifications for the reaction mixture are shown in Table 1. A 0.5 μl sample of each PCR product was mixed with formamide and an internal size marker, ROX350 (PE-ABI), and then electrophoresed in an ABI-PRISM™ 310 Genetic Analyzer for 30 min as described previously (Tamate *et al*., in press). The fragment length was analyzed with GeneScan™ (PE-ABI).

Paternity testings was performed using the Cervus computation program (Marshall *et al*. 1998) with an error rate of 1%. The effectiveness of microsatellite markers for the parentage analyses was tested by calculating the exclusion probability (PE) according to formulae by Chakraborty *et al*. (1988). The individual's paternity was then determined by calculating the paternity index (PI) according to Evett and Weir (1998) and Essen-Möller (1938), with the prior probability of paternity being set to 0.5. The PI of each candidate male was determined separately for three different cases. Case 1 included six pedigrees in which the mothers were known, and all candidate fathers were included in the analysis. Case 2 included seven pedigrees in which the mothers were known, but not all of the possible fathers were sampled. Case 3 included four offspring, for whom the mothers were not sampled and the fathers were unknown. The paternity was judged to be resolved if the probability of the first candidate was above 0.900, while the probability of the second candidate was below 0.100. By using the Cervus program, we also calculated the polymorphic information content (PIC) according to a formula by Bostein *et al*. (1980).

Relatedness and coancestry coefficient

Based on the genotype data for the 17 loci, we calculated Queller and Goodnight's genetic relatedness (r) between individuals by using the Kinship computation program. The index is defined as:

$$\frac{\Sigma\Sigma\,(Py-P^*)}{\Sigma\Sigma\,(Px-P^*)}$$

where P^* is the population frequency of each allele excluding the compared individuals and Px and Py are the frequencies of each allele in the compared indivicuals, respectively (Queller and Goodnight, 1989).

The coancestry coefficient (θ) between a pair of individuals was calculated by the path analysis as described (Weir, 1996). This genetic measure is a consequence of sharing identical alleles in different individuals, and equivalent to an inbreeding coefficient of putative offspring of the pair.

RESULTS

Fifteen bovine, 1 ovine, 1 cervine primers successfully amplified polymorphic PCR products under the conditions shown in Table 1. Departure from Hardy-Weinberg equilibrium was not detected in any of those loci when more than 200 samples of the sika deer were genotyped in a separate experiment (data not shown), suggesting that the effect of null alleles is negligible in our analysis.

All individuals were genotyped at all 17 microsatellite loci except for an individual in which the alleles at the TGLA53 locus were not amplified. Each deer had a unique individual genotype. There was no conflict in matching the genotypes of mother and offspring. The number of alleles per locus, the observed heterozygosities, PIC, and the size-ranges of the alleles are summarized in Table 2. The most polymorphic locus, TGLA53, has seven alleles, and was found to be the most informative because of the highest PIC value 0.812. The average heterozygosity for the 17 loci was 0.656.

The exclusion probability for each locus (Pe) and the cumulative power of exclusion for the 17 loci (PE) are shown in Table 2. The PE reached 0.9997 for the two-parent test, and even for one-parent test, the PE exceeded 0.9889.

The genotype data of the 20 individuals were further subjected to paternity testing - the paternity index (PI) of each male was calculated to quantify individual's likelihood of being the true sire. In the two-parent test where the mothers were identified, paternity was resolved clearly with a high confidence level (>0.90) in all pedigrees. Table 3 shows the dis-

Table 1. Microsatellite information and amplification conditions.

Locus	Species	Annealling temperature (°C)	Modification	$MgCl_2$ (mM)
BL42	Bovine	56	no	1.5
BMC1009	Bovine	56	no	1.5
BM203	Bovine	56	no	1.5
BM3628	Bovine	56	no	1.5
BM4107	Bovine	56	no	1.5
BM6438	Bovine	56	no	1.5
BM6506	Bovine	58/60	5% DMSO	2.0
BM888	Bovine	48	5% DMSO	2.0
BOVIRBP	Bovine	56	no	1.5
Cervid14	Cervine	65	no	1.5
CSSM019	Bovine	56	no	1.5
CSSM043	Bovine	50/52	no	1.5
ETH225	Bovine	56	5% DMSO	2.0
IDVGA29	Bovine	50/52	no	1.5
OarFCB193	Ovine	56	no	1.5
RM188	Bovine	58/60	no	1.5
TGLA53	Bovine	56	5% DMSO	2.0

Table 2. Information of polymorphic markers for analysis of sika deer population.

Locus	Alleles (n)	Size-range (bp)	Heterozygosity	PIC	Exclusion probability for one parents	Exclusion probability for two parents
TGLA53	7	176–216	0.895	0.812	0.493	0.665
BM6506	6	196–212	0.850	0.734	0.380	0.561
ETH225	5	140–193	0.850	0.718	0.352	0.530
IDVGA29	4	137–157	0.900	0.694	0.319	0.494
Cervid14	4	214–231	0.800	0.685	0.311	0.485
BL42	4	246–258	0.650	0.623	0.253	0.419
BM888	4	197–203	0.700	0.618	0.251	0.413
CSSM019	3	138–156	0.700	0.585	0.217	0.364
CSSM043	3	256–277	0.700	0.580	0.215	0.361
BM203	3	212–221	0.650	0.548	0.196	0.335
BM4107	3	162–172	0.750	0.548	0.192	0.336
RM188	3	141–151	0.500	0.424	0.133	0.236
OarFCB193	3	107–125	0.450	0.413	0.113	0.236
BM3628	3	204–212	0.500	0.398	0.103	0.226
BMC1009	2	276–280	0.400	0.365	0.115	0.182
BOVIRBP	2	144–146	0.500	0.365	0.115	0.182
BM6438	2	262–274	0.350	0.247	0.042	0.124
Average	3.31		0.656	0.533	0.224	0.362
Cumulative power for exclusion					0.989	1.000

Table 3. PI for the first and the second candidate of parentage

	case 1	case 2	case 3
Mean PI	(n=6)	(n=7)	(n=4)
First candidate	0.985	0.999	0.867
(range)	(0.939–0.999)	(0.994–1.000)	(0.556–1.000)
Second candidate	1.27×10-2	5.63×10^{-7}	1.29×10^{-2}
(range)	$(3.08\times10^{-11}$–$7.12\times10^{-2})$	$(6.79\times10^{-11}$–$3.87\times10^{-6})$	$(1.38\times10^{-7}$–$5.17\times10^{-2})$

tribution of PIs of the first candidate (the individual having the highest PI among the possible sires in a test of a certain pedigree) and of the second candidate (an individual that has the second highest PI in the same test). In all pedigrees in cases 1 and 2, where the mothers were sampled, the PI values for the first candidates were above 0.90, while those of the second candidates were below 0.10. In the one-parent test, on the other hand, the PI values of the first candidates ranged from 0.5559 to 0.9998 (Table 3). According to the standard for the confident parentage above, three of four parentages were judged to be resolved.

Figure 2 shows the pedigrees of the Nogeyama deer

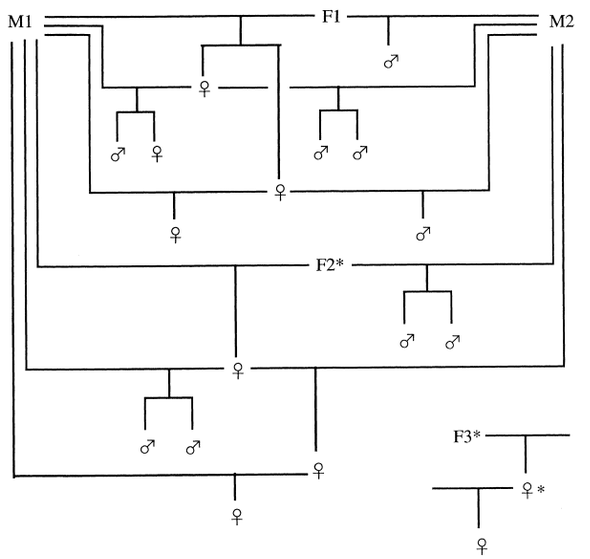

Fig. 2. Resolved pedigrees in Nogeyama sika deer. Founders are shown by letters; F1, F2 and F3, respecitively. Individuals of which DNA were not sampled are indicated by an asterisk. Offsprings without DNA samples are omitted if not necessary to construct pedigrees.

population reconstructed from the data of the parentage analysis. These results indicate that the two founder stags sired all the calves sampled. The results also suggest that there was incestuous breeding in the study population as was anticipated.

Based on the resolved pedigrees, pairwise coancestry coefficients (θ) from path analysis were calculated. θ ranged from 0 to 0.375, while r ranged from –0.741 to 0.712. Relatedness between the founders was lower than –0.349 in any combination, supporting our assumption that all the founders were unrelated. We then tested the correlation between θ and r. The two indices showed strong and significant corrrelation with the coefficient 0.844 (Fig. 3, Pearson's correlation coefficient, $p<0.001$).

DISCUSSION

Resolution power of the microsatellite markers in paternity testing

Genetic analysis using microsatellite markers provides useful information in studying the social structure of animals, although its resolution power is fully dependent on the number of available markers. In a behavioral study of the sika deer, which is promiscuous at least at high population densities, a number of diagnostic microsatellite markers will be required to clarify even a simple aspects of their society such as the father-offspring relationship. In the present study, we demonstrated that genotyping 17 loci of the microsatellite markers provides sufficient data to determine the paternity at a high confidence level in relation to the exclusion probabilities and the paternity index. Theoretically, in a one parent test where the mothers are not sampled, as in case 3, the resolution power is not superior to that of the two-parent test. In the present study, however, we were able to determine some of the paternity relationship by means of one-parent testing through the use of a set of markers. The genotyping system described in the present paper has been successfully applied

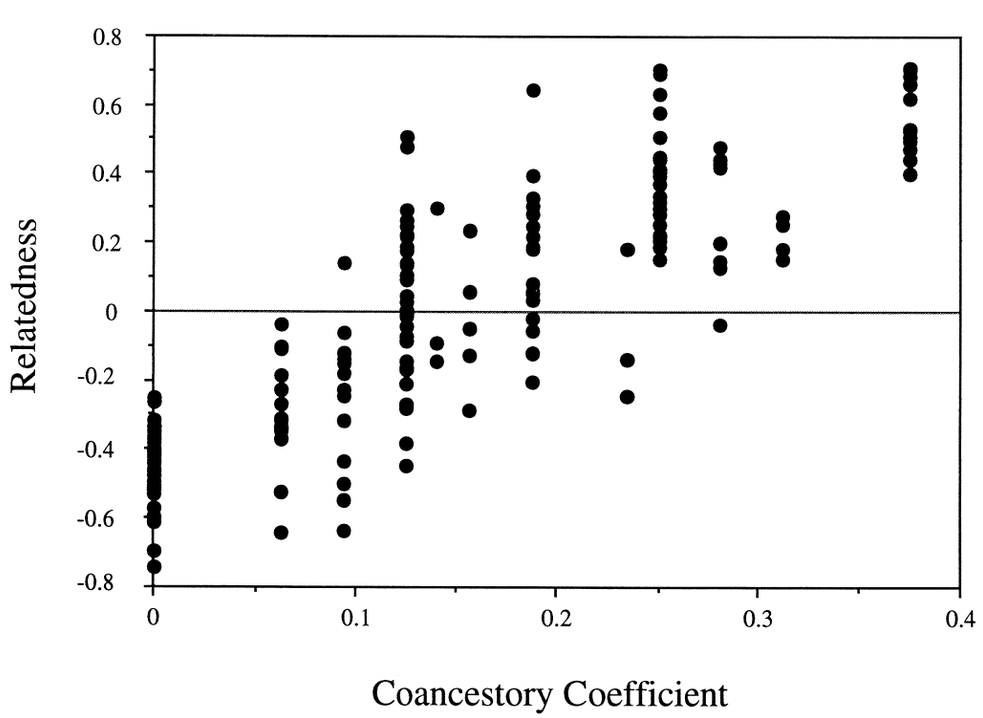

Fig. 3. Correlation between coancestry coefficient and genetic relatedness.

to the study of a wild population of the sika deer in the Kinkazan Island (Tamate *et al*., in press).

Use of microsatellite markers in estimating relatedness

Microsatellite data are also utilized in estimating genetic relatedness (r) between individuals in wild populations. Theoretically, r is a measure of the genetic similarity between a pair of individuals, which corresponds directly to the proportion of shared alleles in the genomes of the pair. The genetic relatedness has been a powerful tool in dissecting the social structures of many mammalian species (Girman *et al*., 1997), although, its relationship with classic genetic measures, such as the coancestry coefficient (θ), has not been studied well.

In a population starting from a few founders, microsatellite alleles of the same size are likely to have originated from a single ancestral allele. Under this condition, r can be an alternative genetic measure to θ, because θ is a measure that quantifies the proportion of alleles that are identical by descent in a pair of individuals. The assumption was supported by our data —we demonstrated a strong correlation between θ and r. These results suggested that even without pedigree structure, we can assess the level of inbreeding (or outbreeding) of the sika deer populations by the genetic relatedness calculated from microsatellite data. Moreover, in a captive population, the inbreeding status may be monitored by measuring the genetic relatedness between individuals.

Incestuous breeding, which may lead to an inbreeding depression, may occur in many captive populations where mating is not controlled. In our study population, among 16 calves for whom the paternity was resolved, six calves were found to be inbred to some extent because of the incestuous mating. The microsatellite analysis described in the present paper should be an efficient tool not only in behavioral studies of wild deer populations, but in creating precise breeding programs and monitoring the genetic status of the population.

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Appendix 1. Microsatellite primer sequences and repeat motif.

Locus	Primer sequence	Repeat motif	Reference
BL42	5' CAAGGTCAAGTCCAAATGCC	$(AC)_n$	1
	5' GCATTTTTGTGTTAATTTCATGC		
BMC1009	5' GCACCAGCAGAGAGGACATT	$(AC)_n$	1
	5' ACCGGCTATTGTCCATCTTG		
BM203	5' GGGTGTGACATTTTGTTCCC	$(AC)_n$	2
	5' CTGCTCGCCACTAGTCCTTC		
BM3628	5' CTGAGATGGACTCAGGGAGG	$(AC)_n$	1
	5' GTTGGATTGGAAAGGTTAGGC		
BM4107	5' AGCCCCTGCTATTGTGTGAG	$(TC)_n(TG)_n(AC)_n$	2
	5' ATAGGCTTTGCATTGTTCAGG		
BM6438	5' TTGAGCACAGACACAGACTGG	$(AC)_n$	1
	5' ACTGAATGCCTCCTTTGTGC		
BM6506	5' GCACGTGGTAAAGAGATGGC	$(AC)_n$	1
	5' AGCAACTTGAGCATGGCAC		
BM888	5' AGGCCATATAGGAGGCAAGCTT	$(AC)_n$	2
	5' CTCGGTGAGCTCAAAACGAG		
BOVIRBP	5' GTATGATCACCTTCTATGCTTCC	$(TC)_n(AC)_n(AT)_n$	3
	5' CCCTAAATACTACCATCTAGAAG		
Cervid14	5' TCTCTTGCGTCTCCTGCATTGAC	$(AC)_n$	4
	5' AATGGCACCCACTCCAGTATTCTTC		
CSSM019	5' TTGTCAGCAACTTCTTGTATCTTT	$(AC)_n$	5
	5' TGTTTTAAGCCACCCAATTATTTG		
CSSM043	5' AAAACTCTGGGAACTTGAAAACTA	$(AC)_n(TC)_n$	5
	5' GTTACAAATTTAAGAGACAGAGTT		
ETH225	5' GATCACCTTGCCACTATTTCCT	$(AC)_n$	6
	5' ACATGACAGCCAGCTGCTACT		
IDVGA29	5' CCCACAAGGTTATCTATCTCCAG	$(AC)_n$	7
	5' CCAAGAAGGTCCAAAGCATCCAC		
OarFCB193	5' TTCATCTCAGACTGGGATTCAGAAAGGC	$(AC)_n$	2
	5' GCTTGGAAATAACCCTCCTGCATCCC		
RM188	5' GGGTTCACAAAGAGCTGGAC	$(AC)_n$	8
	5' GCACTATTGGGCTGGTGATT		
TGLA53	5' GCTTTCAGAAATAGTTTGCATTCA	$(TG)_n(TA)_n$	1
	5' ATCTTCACATGATATTACAGCAGA		

1, Bishop *et al* . (1994); 2, Talbot *et al* . (1996); 3, Bancroft et al. (1995);
4, DeWoody et al. (1995); 5, Moore et al. (1994); 6, Kühn et al. (1996);
7, Mezzelani et al. (1995); 8, Barendse et al. (1994)