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Isolation of nine polymorphic microsatellite loci from the burying beetle, *Nicrophorus quadripunctatus* (Coleoptera: Silphidae)

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Abstract

The burying beetle, *Nicrophorus quadripunctatus* Kraatz, is a common species in Japan, and its biparental care has been well studied. It exploits small vertebrate carrion as food for its young. Therefore, its reproductive success is restricted to large habitats in which carrion is abundant and available. Hence, the abundance of *Nicrophorus* species can be used as an indicator of forest fragmentation. Forests have recently become fragmented due to anthropogenic activities and thus the population sizes of *Nicrophorus* species have decreased. To investigate the population genetic structure of *N. quadripunctatus*, we developed polymorphic microsatellite markers using magnetic particles. Nine microsatellites were polymorphic, with two to 12 alleles were observed in the samples collected from our study sites, Matsunoyama and Nagaoka A and B. Deviation from the Hardy-Weinberg equilibrium was detected at five to seven loci in each population examined, which is indicative of significant heterozygous deficiencies. Higher genetic diversity was found in the Matsunoyama and Nagaoka B populations, the collection plots of which were covered by continuous woodlands, compared to Nagaoka A. The isolated microsatellite markers will be used to determine the genetic structures of the fragmented populations of *N. quadripunctatus*.

Keywords: carcass, habitat size, forest fragmentation, magnetic particles, genetic diversity

Introduction

The burying beetle, *Nicrophorus quadripunctatus* Kraatz, is a common species in Japan, and its biparental care has been well studied (e.g., Suzuki and Nagano 2009). Typically a male–female pair prepares a carcass by burying it, removing its hair, and rounding it into a ball. Eggs are laid in the soil adjacent to the carrion ball. After the larvae hatch, they crawl to the carrion ball. Both parents feed on the carrion ball, and later regurgitate predigested carrion to the larvae. Forests have recently become fragmented due to anthropogenic activities, resulting in the emergence of small habitats. In small habitats, the available fresh carcasses are dominated by vertebrate scavengers. In addition, the abundance of *Nicrophorus* species is reduced at the edge of the woods because of the difficulty of controlling carcass decomposition at these sites (Trumbo and Bloch 2000). Such forest fragmentation causes decreases in the population size and reductions in the reproductive success of burying beetles relative to large habitats (Trumbo and Bloch 2000). Hence, the abundance and species diversity of *Nicrophorus* can be used as an indicator of the extent of forest fragmentation. However, in each of the fragmented populations, it is unclear to what extent inbreeding has occurred and whether genetic diversity has been reduced. To examine the population genetic structure and the degree of inbreeding of *N. quadripunctatus*, we developed nine polymorphic microsatellite markers.

Materials and methods

Nicrophorus quadripunctatus individuals were collected, using hanging bait traps, from the three sites; 32 individuals were collected at Matsunoyama (Tokamachi City) on 22

September 2012, 20 individuals at Nagaoka A (Takatomachi) on 29 September 2013, and 19 individuals at Nagaoka B (Miyamotomachi) on 29 September and 5 October 2013 (5 individuals in September and 14 individuals in October), Niigata prefecture, Japan. While natural forest is present in Matsunoyama, Nagaoka A and B contain secondary forest. The collection plots at Matsunoyama and Nagaoka B were surrounded by woodlands, but those at Nagaoka A were surrounded by urban areas. The collection plots covered an area of 100 square meters for each of the three sites.

We constructed an enriched genome library using magnetic particles based on the modified method of Fischer and Bachmann (1998), and we extracted genomic DNA from six legs of *N. quadripunctatus* using a modified Chelex-based method (Walsh et al. 1991). Four *N. quadripunctatus* collected in Matsunoyama were used for the DNA extraction, and their DNA were pooled. Five µg of genomic DNA were digested with 50 U of *Sau*3A I, and fragments were ligated to *Sau*3A I cassettes (Table S1) (TaKaRa-Bio, Shiga, Japan). Polymerase chain reaction (PCR) amplification was carried out with the Cassette Primer C1 (Table S1) (TaKaRa-Bio) in a T100™ thermal cycler (Bio-Rad, Tokyo). The PCR products were resolved in binding buffer (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 7.5) and hybridized to 5' biotin-labeled oligonucleotide probes $(CA)_{10}$ after denaturation. The hybrids were subsequently isolated by binding to Streptavidin Magnetic Particles (Roche Diagnostics, Tokyo). After the particles were rinsed in washing buffer (10 mM Tris-HCl, 1 mM EDTA, 1 M NaCl, pH 7.5), we recovered target DNAs by resuspending the particles in elution buffer (6 M Guanidine-HCl). The obtained fragments were then amplified by PCR. We ligated the enriched fragments into pGEM®-T Easy Vector (Promega, Tokyo) and transformed them into competent *Escherichia coli* cells, DH5α, (Promega).

We picked a total of 960 recombinant colonies and suspended them independently in 20 µL of distilled water. Inserts were amplified by PCR using a pair of M13 primers (Table S1) (Promega). After PCR, 1 µL of each amplified product was dropped separately on a positively charged MagnaGraph nylon membrane (OSMONICS) (Funakoshi, Tokyo). After drying at room temperature, the DNA samples on the membrane were denaturalized by normal alkaline transfer (0.5M NaOH, 1.5M NaCl), neutralized by buffer (0.5M Tris-HCl [pH 7.5], 1.5M NaCl), and crosslinked using a UV transilluminator. We detected PCR products containing microsatellite regions using 5' biotin-labeled oligonucleotide probes $(CA)_{10}$. For the positive clones (78 of the 960 clones), 1 µL of the remaining PCR product was checked for its length on 8% polyacrylamide gels in TBE. There were 42 unique inserts (length 400–1000 bp). We sequenced plasmids from the positive clones using an automated sequencer (CEQ 2000XL, Beckman Coulter, Tokyo). From the 42 sequences, we designed 13 primer pairs using the online primer design software PRIMER 3.0 (Rozen and Skaletsky 1998). Primer-designed microsatellite loci were tested in the three populations mentioned above. Of the 13 primer pairs, ten successfully amplified the target regions in *N. quadripunctatus*, and nine primers were polymorphic on the samples examined. Sequences of the ten plasmids were deposited in the DNA Data Bank of Japan (Table 1). To examine the degree of genetic differentiation among the three populations, genomic DNA was extracted from individual beetles of the three populations using the modified Chelex-based method mentioned above. PCR amplification was performed in 10-µL reaction volumes containing 2 µl of 5×KAPATaq EXtra Buffer (Mg2+ free) (Nippon Genetics, Tokyo), 1 µL of 25 mM MgCl2, 0.3 µL of dNTP mix (10 mM of each dNTP), 0.5 µL each of primers (10 pmol/µL), 0.25 U of KAPATaq Extra DNA polymerase, 1 µL

(50 ng) DNA and 4.65 µL of sterilized distilled water. The PCR cycles were as follows: 2 min at 94°C, followed by 30 cycles of 20 s at 94°C, 20 s at 55°C (see Table 1), and 1 min at 68°C. The PCR products were electrophoresed in 8% non-denaturing polyacrylamide gels. The allele sizes were assigned by comparison with a size standard, Marker 10 (pBR322/*Msp* I digest) (Nippon Gene, Tokyo). Linkage disequilibrium across the three populations and genotype frequency deviations from the Hardy-Weinberg Equilibrium (HWE) in each population were tested for each locus using GenePop 4.2 (web version, Raymond and Rousset 2010).

Results and Discussion

Linkage disequilibrium was found between Nq-04 and Nq-10. For each of the three populations, the numbers of alleles, observed and expected heterozygosities, and inbreeding coefficients, are presented in Table 2 and Table S2. Significant deviations from the HWE were observed in the heterozygosity frequencies at five to seven loci in the three populations; all of these five to seven loci showed heterozygous deficiency (F_{IS} scores in Table 2 indicate positive values). The heterozygous deficiency would be caused by inbreeding. Although significant genetic differentiation was found in all pairs drawn from the three populations, the degree of genetic differentiation was weak (Table 3), suggesting that *N. quadripunctatus* has a high capability of dispersion. Genetic diversity defined by averaged heterozygosity was higher in both the Matsunoyama and Nagaoka B populations compared to the Nagaoka A population (Table 2). This may be due to the difference in the vicinities of forest. While Nagaoka A is fragmented by urban areas, Matsunoyama and Nagaoka B are surrounded by forest, suggesting that continuous woodlands provide a suitable habitat for *N. quadripunctatus*, which mainly

colonizes in forests (Ohkawara et al 1998) and such a habitat would promote the availability of fresh carcasses. Although similar scores with respect to genetic diversity were obtained in the populations at both Matsunoyama and Nagaoka B, the inbreeding efficient is higher in Nagaoka B population. Of the nine loci, three (Nq-03, Nq-05, and Nq-06) in the Nagaoka B population had a monomorphic allele, implying that a small population has recently colonized Nagaoka B and has rapidly increased in number. Therefore higher genetic diversity and higher inbreeding might both be observed if the population has only been present for a short period of time. Further investigation is needed to clarify the cause of the higher inbreeding coefficient in the Nagaoka B population. Burying beetle species show elaborate biparental care, which is rare in other animal species because paternity assurance is low compared to maternity assurance (Wade and Shuster 2002; Westneat and Sherman 1993). However, few studies have examined the relationship between paternity and biparental care in insects. In addition, most of the past parentage analyses for biparental insects were conducted using dominant genetic markers (Scott and Williams 1993; Simmons et al. 2004), and it has been difficult to analyze multiple paternity scenarios in field conditions. Hence, these nine microsatellite loci will be useful for estimating genetic diversity and for genetic analyses concerning parental care.

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Table 1. Characteristics of the ten microsatellites of *Nicrophorus quadripunctatus*.

Locus	Repeat motif	Primer sequences (5'-3')	Size range (bp)	Ta[a] (℃)	GenBank Accession no.
Nq-01	$(GT)_5$…$(GT)_7$	F: TGAGCGGGAGTTTCATTAC	218-239	55	AB827941
		R: TCACAGCCTCATCAACGTGT			
Nq-02	GTT$(GT)_2$… $(GTT)_2$	F: TGAAAGTTTTGCGGGAATTG	178	55	AB827942
		R: CCTTCCTCCCTCGGATATACA			
Nq-03	$(GT)_9$	F: GATCGGGATGATCCATTGTC	168-182	55	AB827943
		R: TCATTATCAGGGCGTCATTG			
Nq-04	$(CA)_8$… $(AT)_4(CA)_3$	F: TTTCCCGTTTAGTCGTCGTT	195-215	55	AB827944
		R: GCGACGTCGTCTTCTAAATCC			
Nq-05	$(ATGT)_2$…$(GT)_6$	F: CGACCCGTGTAGTTTTGCTT	210-218	55	AB827945
		R: GGAATGCAGAAAGGGAATCA			
Nq-06	$(CA)_8$	F: AGTGATGCTCTACCCCCGTA	217-219	55	AB827946
		R: GGTTGGACGGAAGAATTGTG			
Nq-07	$(GT)_7(AT)_4$	F: AATTGAGCTCGTGTGGGTTC	238-244	55	AB827947
		R: ATGCTCGAACGCCAATAAAT			
Nq-08	$(AC)_5(CT)_3$	F: ATCGGTCCATTATTCCGTTG	236-242	55	AB827948
		R: TGTGTTTTGCCAACGTTCTG			
Nq-09	$(TA)_9(CA)_{10}$	F: AAGGAGGAGTAGACGACTAT	240-246	55	AB827949
		R: AACGCGCTTTAAACGTGTGGTGT			
Nq-10	$(TA)_2(GT)_8$	F: TCCCAGTGAATGCGATGGAA	238-242	55	AB827950
		R: TCGAATGCCAATTCCTCTTT			

a. Locus-specific annealing temperature.

Table 2. Population genetic structure at the study sites.

	Matsunoyama						Nagaoka A						Nagaoka B					
Locus	*N* (32)[a]	*Na*[b]	*Ho*[c]	*He*[d]	HWE[e]	F_{IS}[f]	*N* (20)	*Na*	*Ho*	*He*	HWE	F_{IS}	*N* (19)	*Na*	*Ho*	*He*	HWE	F_{IS}
Nq-01	32	12	0.31	0.75	**	0.59	20	5	0.35	0.65	*	0.47	19	2	0.11	0.48	**	0.78
Nq-03	32	4	0.06	0.32	***	0.81	20	2	0.05	0.05	-	0	19	1	0.00	0.00	-	-
Nq-04	32	8	0.50	0.60	**	0.18	19	5	0.47	0.68	*	0.31	19	8	0.53	0.74	*	0.30
Nq-05	31	2	0.10	0.09	NS	-0.03	20	3	0.10	0.10	NS	-0.01	19	1	0.00	0.00	-	-
Nq-06	32	2	0.03	0.03	-	0.00	20	1	0.00	0.00	-	-	19	1	0.00	0.00	-	-
Nq-07	32	6	0.28	0.69	***	0.60	20	5	0.15	0.78	***	0.81	19	4	0.00	0.64	***	1
Nq-08	28	3	0.04	0.58	***	0.94	20	2	0.00	0.10	*	1	19	5	0.11	0.57	***	0.82
Nq-09	32	5	0.53	0.70	*	0.25	20	5	0.15	0.79	***	0.81	19	6	0.79	0.76	NS	-0.04
Nq-10	28	5	0.18	0.75	***	0.77	19	3	0.32	0.68	**	0.54	19	4	0.47	0.69	**	0.32
Average	31	5.2	0.23	0.50		0.45	19.8	3.4	0.18	0.43		0.49	19	3.6	0.22	0.43		0.53

a. *N* indicates the number of samples that successfully underwent PCR amplification. The number of individuals used in PCR amplification is in parentheses.
b. The number of alleles.
c, d. Observed and expected heterozygosities. The average of observed heterozygosities corresponds to the genetic diversity in each population.
e. A significant deviation from the Hardy-Weinberg Equilibrium when an alternative hypothesis is set to heterozygote deficiency.

Table 3. Population differentiation for all pairs of populations. Upper cells above the diagonal show genetic differentiation between populations (F_{ST}) . Lower cells below the diagonal show the geographical distance between populations (km). *** indicates $P <$

	Matsunoyama	Nagaoka A	Nagaoka B
Matsunoyama	-	0.0811***	0.0647***
Nagaoka A	38	-	0.0522***
Nagaoka B	42	9	-

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Table S1. Sequences of *Sau*3A I cassette, cassette primer C1, and M13 primers.

*Sau*3A I Cassette
5'-GTACATATTGTCGTTAGAACGCGTAATACGACTCACTATAGGGA-3'
3'-CATGTATAACAGCAATCTTGCGCATTATGCTGAGTGATATCCCTCTAG-5'

Cassette Primer C1
5'-GTACATATTGTCGTTAGAACGCGTAATACGACTCA-3'

M13 Primers	
Forward	5'-d(GTTTTCCCAGTCACGAC)-3'
Reverse	5'-d(CAGGAAACAGCTATGAC)-3'

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Table S2. Alleles (bp) and their frequency detected in each locus in each population.

Matsunoyama

Nq-01	frequency	Nq-03	frequency	Nq-04	frequency	Nq-05	frequency	Nq-06	frequency	Nq-07	frequency	Nq-08	frequency	Nq-09	frequency	Nq-10	frequency
218	0.0156	168	0.0156	197	0.0469	217	0.9516	217	0.0156	239	0.0781	238	0.5000	241	0.4531	238	0.3571
221	0.0469	178	0.8125	202	0.0156	218	0.0484	219	0.9844	240	0.5000	239	0.4107	242	0.1094	239	0.0714
222	0.0156	180	0.1563	203	0.1250					241	0.2188	240	0.0893	243	0.1875	240	0.1964
223	0.0469	182	0.0156	205	0.1094					242	0.0469			244	0.2344	241	0.2857
224	0.3281			209	0.0313					243	0.0781			245	0.0156	242	0.0893
227	0.3750			210	0.0469					244	0.0781						
228	0.0313			211	0.6094												
231	0.0625			212	0.0156												
232	0.0156																
236	0.0313																
238	0.0156																
239	0.0156																

Nagaoka A

Nq-01	frequency	Nq-03	frequency	Nq-04	frequency	Nq-05	frequency	Nq-06	frequency	Nq-07	frequency	Nq-08	frequency	Nq-09	frequency	Nq-10	frequency
224	0.2500	176	0.0250	195	0.0263	210	0.0250	219	1	238	0.0500	236	0.0500	240	0.2750	238	0.3158
227	0.5250	178	0.9750	200	0.0526	217	0.9500			239	0.2500	238	0.9500	241	0.2500	239	0.3947
229	0.0500			203	0.2895	218	0.0250			240	0.3250			242	0.2500	240	0.2895
230	0.0250			204	0.1579					241	0.2000			243	0.1500		
234	0.1500			211	0.4737					242	0.1750			244	0.0750		

Nagaoka B

Nq-01	frequency	Nq-03	frequency	Nq-04	frequency	Nq-05	frequency	Nq-06	frequency	Nq-07	frequency	Nq-08	frequency	Nq-09	frequency	Nq-10	frequency
224	0.3684	178	1	203	0.2368	217	1	219	1	238	0.3684	236	0.0526	241	0.3947	238	0.4474
227	0.6316			204	0.0263					239	0.4737	238	0.6053	242	0.1579	239	0.2105
				207	0.0263					240	0.0526	239	0.2632	243	0.1842	240	0.2895
				208	0.0263					241	0.1053	240	0.0526	244	0.2105	241	0.0526
				209	0.1053							242	0.0263	245	0.0263		
				211	0.4474									246	0.0263		
				212	0.0790												
				215	0.0526												