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PRIMER NOTE

Characterization of microsatellite loci in red wood ants *Formica* (*s. str.*) spp. and the related genus *Polyergus*

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Abstract

Ants are interesting subjects for studies of evolution of altruism. We developed 13 microsatellite loci in a red wood ant *Formica* (*s. str.*) yessensis from random amplified polymorphic DNA fragments to study genetic structure within populations and colonies. Five loci bore two to five alleles in both *F.* (*s. str.*) yessensis and *F.* (*s. str.*) *truncorum* and two were also polymorphic in a related species, *Polyergus samurai*. Results suggest that the loci will be useful in evolutionary studies on *Formica* and *Polyergus* species.

Keywords: ants, *Formica truncorum*, *Formica yessensis*, microsatellite loci, *Polyergus*

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The evolution of polygyny is a major issue in evolutionary biology because this phenomenon can be regarded as the '2nd evolution of eusociality' (Rosengren & Pamilo 1983). There are many polygynous species in the common ant genus *Formica* that consists of four subgenera (*Raptiformica*, *Coptoformica*, *Serviformica* and *s. str.*). A highly polygynous ant, *Formica* (*s. str.*) yessensis, makes huge colonies (so-called super colonies) that range over several square kilometres and colony members share all the nests (Higashi 1976). New queens mate with nest-mate males on the ground and return to their own nests (Ito & Imamura 1974). These habits have attracted researchers to this ant from the viewpoints of both behavioural ecology (e.g. kin selection and evolution of sociality) and population genetics (e.g. population viscosity). For both viewpoints, microsatellite loci are very useful to examine genetic structure within a colony and / or a population. Microsatellite primers for European *F.* (*s. str.*) *lugubris* (Chapuisat 1996) have been reported to be applicable to European *F.* (*s. str.*) *truncorum* (Sundström 1993, 1995). Although we used the primers having the provided sequences and followed the polymerase chain reaction (PCR) procedure in the published papers (Sundström 1995; Chapuisat 1996), our tests failed to amplify the target regions of Japanese *F.* (*s. str.*) *yessensis* and *F.* (*s. str.*) *truncorum* (S. Imai, personal observations). Thus, new microsatellite loci are useful in studies on Japanese red wood ants.

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We developed microsatellite loci from random amplified polymorphic DNA (RAPD) fragments following the procedure reported in Hasegawa & Takahashi (2002). The RAPD reactions were conducted under 45 cycles of 94 °C for 30 s, 35 °C for 45 s and 72 °C for 2 min. The temperature ramp speed was 1.0 °C/s other than 0.3 °C/s between 35 and 72 °C (for detailed conditions, see Hasegawa & Takahashi 2002). The products of 40 RAPD primers that proved positive for microsatellites (10 repeats of any of AG, CG, TG or AT) were cloned into pUC19 and recombinants were subject to a second screening. In total, 20 of 189 recombinants were positive. Inserts of the positive recombinants were amplified independently using the vector's primers (*Bca*BESTTM Sequencing Primer RV-M and M13-47; TaKaRa) and sequenced using automated sequencers (ABI 373S, Applied Biosystems; CEQ2000, Beckman and Coulter). There were 14 different loci with more than five microsatellite repeats in these positive clones.

We could design primer pairs for 13 of the 14 loci (Table 1) because the cloned sequence of a locus does not have a long enough flanking region for primer design. To test the effectiveness of the obtained microsatellite loci, we checked the amplification of the primer pairs using *F.* (*s. str.*) yessensis (20 individuals from 10 nests) and a related species, *F.* (*s. str.*) truncorum (20 individuals from 10 nests). For the loci that were successfully amplified in this test, we

Table 1 Primers, polymerase chain reaction conditions and core sequence of 13 microsatellites obtained from *Formica yessensis*

Locus name	Core sequence in cloned alleles	Primer sequence (5′–3′)	$MgCl_2$ (mM)	Annealing temp. (°C)	Cycles	Allele size (bp)	GenBank Accession no.
Fy2	$(CA)_{19}$	F: 5′-CGATTAGGGGGATTGGTGTA-3′ R: 5′-GGCGTTCGTCAACAGATATG-3′	1.5	43	28	368	AB103291
Fy3	$(CT)_{16}$	F: 5′-ATTCATATGAGTTACATCGA-3′ R: 5′-AGGTAATCAATATATTTAAG-3′	1.5	45	26	199	AB103292
Fy4	$(CT)_{17}$	F: 5′-TTCCACTGGAGAACCATCGG-3′ R: 5′-TGCGTTTGAAATTTCCACAG-3′	1.5	45	27	286	AB103293
Fy5	$(CAT)_7$	F: 5′-ATTTTCACCAAACAATAAGA-3′ R: 5′-TATCATACTTGGTCTTACCC-3′	1.5	48	28	200	AB103294
Fy6	$(ATT)_7(CTT)_2$ $(CGT)_{13}$	F: 5′-TGTAAAGGTGGGTGTTTTTG-3′ R: 5′-CGTAACGTATATAAATTAAA-3′	1.5	NA	NA	205	AB103295
Fy7	$(CA)_{16}(TA)_3$	F: 5′-CACACTTATTTACTCTGGCC-3′ R: 5′-CAGGCAGAGATAATATTTGC-3′	1.5	53	28	248	AB103296
Fy8	$(GT)_{13}AT(GT)_5$	F: 5′-TGGCGGCCGTTCAAATTTCG-3′ R: 5′-AAAGTTGTTTCTCTCCGACC-3′	1.5	NA	NA	200	AB103297
Fy9	$(CG)_4(CA)_7$ $TCAT(GC)_4$	F: 5′-CAAAACACACCTCGTCCACC-3′ R: 5′-GTAACCCGAGCGAGCCCGAA-3′	1.5	48	28	228	AB103298
Fy10	$(CT)_9$	F: 5′-ATAAAGTTTTAAATAATCAA-3′ R: 5′-ACTTAGAATCAAATTCGTCC-3′	1.5	NA	NA	150	AB103299
Fy12	$(AG)_{12}$	F: 5′-ATGACGATTAGGGGGATTGG-3′ R: 5′-TTCTCTTACAACTATTCAAC-3′	1.5	48	28	188	AB103301
Fy13	$(CT)_{16}TTT(CT)_5$	F: 5′-TCGTCGCGGTAAATCATTCC-3′ R: 5′-CCCTTTTTCGCCCAATTTCC-3′	1.5	48	28	204	AB103302
Fy14	$(AT)_8T(AC)_{11}$ $(AT)_{13}$	F: 5′-GAAAGTTTTCATAACACATC-3′ R: 5′-AGGATTTATTCCGCGTAATC-3′	1.5	48	28	201	AB103303
Fy15	$(GA)_2G(GA)_7$ $A(GA)_5$	F: 5′-GATCGATAAATTCCAGTGCG-3′ R: 5′-AACCGTTCGAGCCTATCACC-3′	1.5	48	28	233	AB103304

NA, not amplified.

checked the degree of polymorphism using four populations of each species (see Table 2). For the cross-genus amplification test, we used a closely related species from a different genus, *Polyergus samurai*, and two males per nest were genotyped in nine nests for this species. The total DNA of each individual was extracted by boiling the ground tissue of a leg in 300 μL of 5% Chelex resin (Bio-Rad) for 2 h at 55 °C (Walsh *et al*. 1991). The PCR amplifications were carried out in a total volume of 10 μL, which contained 1.5 μL (*c*. 10 ng) of template DNA, 2.5 pmol of each microsatellite primer, 400 mM of dNTP, 1 μL of 10× reaction buffer, 1.5 mM $MgCl_2$ and 0.05 U of *Taq* (TaKaRa). Temperature cycles were as follows; 3 min at 94 °C followed by locus-specific numbers of cycles (see Table 1), i.e. 30 s at 94 °C, 30 s at 45–53 °C (for the annealing temperature of each locus, see Table 1) and 30 s at 72 °C. The PCR products were run on 8% polyacrylamide (mono : bis, 37.5 : 1) gels and visualized by silver staining (Tegelström 1986). A 2-bp difference in length could be discriminated by this method.

The sequence information of each locus is available from databases (GenBank Accession nos AB103291–AB103304). We checked the polymorphism at each locus using four populations of *F. yessensis* (four populations, total $n = 479$) and *F. truncorum* (four populations, total $n = 436$). Five of the 13 loci were polymorphic with two to five alleles (see Table 2). The allele detection method (acrylamide gel electrophoresis and silver staining) did not allow us to specify the length of alleles but the observed allele lengths were distributed around the length of the cloned allele. By sequencing some alleles, we confirmed that the observed minimum difference of allele length corresponded to a 2-bp difference.

We tested for deviations of genotype frequencies from Hardy–Weinberg equilibrium for each locus in each population using χ^2 tests (Table 2; tests were done by hand calculations). A significant deviation was observed at a single locus (Fy7) in the Kawayu population in Hokkaido prefecture (χ^2 test, $\chi^2 = 20.22$, $P < 0.001$ with Bonferroni correction for multiple comparisons). The cause of this deviation could not be attributed to the existence of null alleles because there was an excess of heterozygotes in this population. We also tested for linkage disequilibrium among the polymorphic loci using GENEPOP version 3.3 (Raymond & Rousset 1995). Linkage disequilibrium was detected for one pair of loci (Fy7 and Fy13, $P = 0.017$, Fisher's exact probability test with Bonferroni correction for multiple comparisons, cf. Bonferroni corrections were made by hand calculations) in one population (Gotenba in Shizuoka

Table 2 Number of alleles (N_a), expected heterozygosity (H_E) and observed heterozygosity (H_O) at all loci in three species of two different genera

							Deviation from Hardy–Weinberg equilibrium		
Locus	Species	Population	*n*	N_a	H_E	H_O	χ^2	d.f.	Significance
Fy3	*Formica* (*s. str.*) *truncorum*	Obihiro	99	4	0.286	0.263	9.309	6	NS
		Kawayu	99	4	0.721	0.556	18.935*	5	NS
		Moshiri	90	4	0.632	0.622	1.811	6	NS
		Furano	100	4	0.700	0.571	12.119	6	NS
	F. (*s. str.*) *yessensis*	Ishikari	100	4	0.574	0.500	8.970	6	NS
		Ohnuma	100	3	0.156	0.170	0.863	3	NS
		Gotenba	100	2	0.180	0.180	0.000	1	NS
		Norikura	100	1	—	—	—	—	—
	Polyergus samurai	Chiba	18	2	0.198	—	—	—	—
Fy4	*F.* (*s. str.*) *truncorum*	Obihiro	99	2	0.010	0.010	0.003	1	NS
		Kawayu	99	2	0.030	0.030	0.023	1	NS
		Moshiri	90	2	0.054	0.056	0.074	1	NS
		Furano	99	2	0.114	0.101	1.262	1	NS
	F. (*s. str.*) *yessensis*	Ishikari	100	2	0.458	0.570	5.987	1	NS
		Ohnuma	100	2	0.394	0.420	0.428	1	NS
		Gotenba	95	2	0.172	0.168	0.031	1	NS
		Norikura	100	2	0.188	0.210	1.376	1	NS
	P. samurai	Chiba	18	1	—	—	—	—	—
Fy7	*F.* (*s. str.*) *truncorum*	Obihiro	93	3	0.515	0.538	5.600*	2	NS
		Kawayu	96	3	0.520	0.750	21.140	3	$P = 0.003$
		Moshiri	85	4	0.500	0.541	6.226	6	NS
		Furano	95	3	0.631	0.516	9.124*	2	NS
	F. (*s. str.*) *yessensis*	Ishikari	98	3	0.533	0.622	4.682*	1	NS
		Ohnuma	100	2	0.446	0.490	0.995	1	NS
		Gotenba	100	2	0.494	0.530	0.534	1	
		Norikura	99	2	0.448	0.495	1.100	1	NS
	P. samurai	Chiba	18	NA	—	—	—	—	—
Fy13	*F.* (*s. str.*) *truncorum*	Obihiro	99	3	0.335	0.354	2.520	3	NS
		Kawayu	99	4	0.472	0.505	3.050	6	NS
		Moshiri	89	3	0.269	0.213	5.766	3	NS
		Furano	100	3	0.411	0.380	0.086*	1	NS
	F. (*s. str.*) *yessensis*	Ishikari	100	3	0.289	0.220	2.474*	1	NS
		Ohnuma	100	3	0.406	0.340	3.241	3	NS
		Gotenba	100	2	0.086	0.090	0.222	1	NS
		Norikura	100	1	—	—	—	—	—
	P. samurai	Chiba	18	1	—	—	—	—	—
Fy15	*F.* (*s. str.*) *truncorum*	Obihiro	—	—	—	—	—	—	—
		Kawayu	—	—	—	—	—	—	—
		Moshiri	10	3	0.461	0.500	–†	—	—
		Furano	—	—	—	—	–	—	—
	F. (*s. str.*) *yessensis*	Ishikari	8	4	0.690	0.500	–†	—	—
		Ohnuma	10	3	0.555	0.600	–†	—	—
		Gotenba	—	—	—	—	—	—	—
		Norikura	—	—	—	—	—	—	—
	P. samurai	Chiba	18	2	0.278	—	—	—	—

*χ^2 values were corrected for combining cells with expected values of < 5.
†χ^2 test was not done because sample size is too small ($n < 10$).
Deviation from Hardy–Weinberg equilibrium was tested statistically for each locus in each population. NA, not amplified; ND, no data; NS, not significant; —, calculation is impossible. For *P. samurai*, H_O cannot be calculated because all the examined samples were males, which are haploid in Hymenoptera.

prefecture). In the Gotenba population, one of the four alleles at Fy13 (the shortest allele, frequency 0.045) was detected only with a specific allele at Fy7 in this population but this linkage disequilibrium between these two alleles was not observed in the other populations.

In *P. samurai*, although the two loci were monomorphic, two of the five loci were polymorphic (two alleles) and a primer pair could not amplify the target region (Table 2). The observed alleles at the polymorphic loci corresponded in length with the alleles of *Formica*. A phylogenetic study showed that *P. samurai* is the closest sister genus of the genus *Formica*, in which four subgenera are known (Hasegawa *et al*. 2001). The reported primers are likely to work in these *Formica* subgenera. Thus, we concluded that these microsatellite markers will be useful in the genetic analysis of *Formica* ants.

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