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Microsatellite Loci and Genetic Polymorphism among Colony Members in the Parthenogenetic Ant *Pristomyrmex pungens*

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Abstract. Microsatellite loci were described for the parthenogenetic ant *Pristomyrmex pungens*. Two of four loci showed polymorphism, and the allelism of each locus was confirmed. Relatedness among colony members was extremely high (0.944), but one of the colonies contained two different genotypes. These results demonstrated that some colonies are comprised of multiple genetic lines, contrary to the existing assumption that parthenogenetic reproduction indicates monoclonality.

Key words: Microsatellites, parthenogenesis, Ant, reproductive conflict, relatedness.

Introduction

The polygynous colonies of social insects is a key to resolving evolutionary issues of conflict and cooperation among colony members. Kin selection theory predicts that altruistic behavior is more difficult to evolve in polygynous associations than in monogynous ones because genetic relatedness among members should be low in the former (Hamilton, 1964). However, recent researches showed that many species of social insect have multiple reproductives in a colony (see Keller, 1993). In several ant species, especially, each colony contains more than 1000 queens, producing a reproductive caste to some extent (Hölldobler & Wilson, 1990; Bourke & Franks, 1995). Thus, we can understand the maintenance of altruistic behavior in social association by elucidating genetic relationships between individuals and how they regulate conflict and cooperation over reproduction.

The queenless ant *Pristomyrmex pungens* is a special ant in that it has multiple reproductives that reproduce parthenogenetically (Itow *et al.*, 1984). All individuals in a colony engage in daily tasks and produce the next generation by thelytoky (Tsuji, 1990). If all individuals in a colony are of a single genetic clone having the same genetic composition, there should be no conflict over reproduction in a colony. But in some cases it is possible that colony members are not all genetic clones of each other, leading to conflict over reproduction. Tsuji (1995) found a size dimorphism among workers in several colonies (see also Itow *et al.*, 1984; Tsuji, 1988) and suggested that larger workers may be genetic cheaters because they engaged less in daily tasks and more in egg-laying. Thus, genetic relationships among colony members is very important information, but such data have yet to be presented.

Recently, microsatellite genetic markers have proven to be a powerful tool for analysing genetic structures at both colony and population levels (Hughes & Queller, 1993; Gertsch *et al.*, 1995; Chapuisat *et al.*, 1997). Microsatellites are more powerful than other allelic markers because they are usually highly polymorphic and can estimate genetic indices, such as relatedness or parent-offspring relationships more accurately.

In this paper we describe primers for four microsatellite loci for *P. pungens*. In addition, we examined the average relatedness within a colony and checked whether or not a colony consists of monoclonal individuals.

Materials and Methods

We developed microsatellite loci by using random amplified polymorphic DNA (RAPD) fragments. The experimental procedure will be described elsewhere (Hasegawa & Takahashi, in prep.). We designed primer pairs for the obtained microsatellite regions (Table 1) and amplified the target regions of each individual by polymerase chain reaction (PCR). In August 1998 we collected 15 colonies of *P. pungens*

from Fuchu, Tokyo, Japan. Five to ten individuals were selected randomly from each colony. Template DNA was extracted from each individual using the DNeasy Tissue Kit (QIAGEN) and was eluted to a final volume of 20 μl. Each 20 μl of PCR reaction contained 2.0 μl of template DNA (ca. 20 ng), 2.0 μl of 10 X reaction buffer (for TaKaRa Taq, TaKaRa), 2.0 μl of dNTP mix (1 mM of each dNTP), each 0.5 μl of primers (10 pmol/ μl), 0.2 U of taq polymerase (TaKaRa Taq, TaKaRa) and 16.4 μl of ddW. The reactions were incubated in the following temperature cycles by using a thermocycler (TaKaRa PCR Thermal Cycler MP, TaKaRa): 28 cycles of 1 min at 94℃, 1 min at 50–55℃ (see Table 1) and 3 min at 72℃. Five μl of each product was electrophorased on 8% acrylamide gel for 5 hrs at 200 V in 1X TBE buffer. DNA bands were visualized by a silver staining (Tegelström, 1986). Colonies of *P. pungens* rarely produce males. We also determined the male genotypes and compared them with the genotypes of workers in the same colony.

Because *P. pungens* is parthenogenetic and queenless, we could not check allelism of the visualized bands by a normal crossing test or parent-offspring analysis. Thus, after each product was cloned we sequenced each band of polymorphic loci and then compared these sequences. Amplified DNA fragments in PCR products were blunted, kinated and ligated into SmaI cut pUC19 DNA by using the TaKaRa BKL Kit (TaKaRa). The treated pUC19 DNAs were transformed into comeitent *Escherichia coli* cells (Competent High -JM109-, TOYOBO). *E. coli* colonies that contained recombinant pUC19 were selected using the blue-white color selection method with agar plates containing X-Gal, IPTG and ampicillin. Inserted DNA was amplified by PCR using a primer pair for the multicloning site of pUC19 (*Bca*BEST™ Sequencing Primer RV-M and M13-47, TaKaRa). The length of each insert was checked by an acrylamide gel electrophoresis and silver staining under the same conditions mentioned above. Inserts having different lengths were sequenced by an automated sequencer (ABI373s, Applied Biosystems).

Relatedness among colony members was assessed by the method of Queller & Goodnight (1989) and calculated with the computer program Relatedness 4.2 b (Goodnight, 1994).

Results

We obtained four microsatellite regions using 40 primers for RAPD reaction (OPA01–20 and OPB01–20, Operon Technology). Table 1 shows the core sequences for each locus and the primer sequences. Two of the four loci were monomorphic, and the other two loci each had two bands of different length. The nucleotide sequences of these bands are shown in Fig. 1. For each polymorphic locus, the two different bands have almost the same sequence except for the microsatellite region, although two other regions (or sites) are different in the locus Pp1. This result strongly suggests that the 2 primer pairs successfully amplified two alleles for each locus.

Genotypic analysis of males and workers also sup-

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a. Pp1

Pp1-short   1:ggatacgcaagtaagaggaaAAGAGAGAAGATATATATA----GGGTCAGAGGATGGACC 56
Pp1-long    1:ggatacgcaagtaagaggaaAAGAGAGAAGATATATATATATAGGGTCAGAGGATGGACC 60
                                                  ****
Pp1-short  57:GGACGGAATATTGATCCGCGTACCTCACGATAGCTGCCTATGCTGCGCTATGCTTTGCCG 116
Pp1-long   61:GGACGGAATATTGATCCGCGTACCTCACGATAGCTGCCTATGCTGCGCTATGCTTTGCCG 120

Pp1-short 117:TATAGCCTATTCCTCTCCCTCTCTCCTATTTTCTCTCTCTCTCTCTCCCTCTCTTCCT 176
Pp1-long  121:TATAGCCTATTCCTCTCCCTCTCTCCTATTTTCTCTCTCTCTCTCTCCCTCTCTACCT 180
                                                                    *
Pp1-short 177:CCTTCTCCCTCCCT----CCCCCCCCgggccgagagtaagatagag          218
Pp1-long  181:CCTTCTCCCTCCCTTCCCCCCCCCCCCgggccgagagtaagatagag          226
                          ****

b. Pp2

Pp2-short   1:ttcaacgaaatgtacagtggGAGACAAGCAGCCCTCCTCTCTCTCTCTCTCTCT---- 56
Pp2-long    1:ttcaacgaaatgtacagtggGAGACAAGCAGCCCTCCTCTCTCTCTCTCTCTCTCT 60
                                                              ****
Pp2-short  57:---------CGCCCATTGCGAGTGGCGAAACGTAGCATGCGTGCGACTGTTCTCTTTCGTA 108
Pp2-long   61:CTCTCTCTCGCCCATTGCGAGTGGCGAAACGTAGCATGCGTGCGACTGTTCTCTTTCGTA 120
              ********
Pp2-short 109:ATTTTCACCAGCTTTTGTCGGCGCCGTATTTTCACTCATCGCTCGTTTATTAATTTTAAA 168
Pp2-long  121:ATTTTCACCAGCTTTTGTCGGCGCCGTATTTTCACTCATCGCTCGTTTATTAATTTTAAA 180

Pp2-short 169:AGTTATGGCGGCCGATATGACCAATTGCATCTCGGaggtgctgctcttcctccgg      223
Pp2-long  181:AGTTATGGCGGCCGATATGACCAATTGCATCTCGGaggtgctgctcttcctccgg      235
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Fig. 1. Nucleotide sequence of each allele. Lower characters are the primer region. – is a gap. Asterisks under sequences show that the two alleles are different at that position.

Table 1. Core sequence, primers, annealing temperatures of four microsatellite loci of *Pristomyrmex pungens*. Number of alleles, fragment size and heterozygosity are also shown. N and n means number of sampled individuals and colonies, respectively.

Locus	Size (bp)	Core sequence in cloned allele	primer sequence (5′–3′)	No. of alleles	Annealing temp. (℃)	Heterozygosity (H_O)	N (n)
Pp1	226	$(TA)_6$...	f: GGATACGCAAGTAAGAGGAA	2	55	0.74	127 (15)
		$(CT)_3$...$(CT)_8$	r: GAGATAGAATGAGAGCCGGG				
Pp2	235	$(CCT)_8(CT)_{15}$...	f: TTCAACGAAATGTACAGTGG	2	55	0.63	125 (15)
		$(GCC)_8$...$(AT)_8$	r: CCGGAGGAAGAGCAGCACCT				
Pp3	220	$(CAG)_9$...$(GT)_8$	f: TAAGTCCCTTTTTCGGATCC	1	50	–	98 (10)
			r: CCTCGGGTCTCGACAAGATC				
Pp4	210	$(GA)_8$	f: TCTGTGCTGGCCGGAGTGAC	1	50	–	94 (10)
			r: TCATCTGGTCCAAGCAAGGG				

Table 2. Genotype frequency at the loci Pp1 and Pp2. Number in parenthes means male genotype. Genotype 11 and 22 means homozygote, and 12 means heterozygote for each locus.

Colony No.	Pp1			Pp1		
	11	12	22	11	12	22
1		9		9		
2		10		9		
3		8		8		
4			10		9	
5		10		10		
6		10		10		
7			10		10	
8		10		10		
9		5		5		
10	(1)	10		(1)	10	
11		10	(1)		10	(1)
12		5			5	
13		5			5	
14		5			5	
15		2	8	2	8	

ported this result. We collected two males, each from a different colony. All of the workers examined in these two colonies were heterozygotes at least one of the two loci (see Table 2), and each of the males had one of the two bands found in workers in the respective colony. This genotypic analysis also showed that the two primer pairs correctly amplified both of the alleles of a locus.

Observed heterozygosities (H_o) were 0.74 at Pp1 and 0.63 at Pp2, respectively (Table 1). The average relatedness within each colony was 0.944 ± 0.056 (mean $\pm$ S.E.). Table 2 shows genotype distribution in the examined colonies. In 14 of the colonies, all individuals were of the same genotype at both loci, but colony 15 contained each two genotypes at each locus.

Discussion

At least two of the four microsatellite loci were proven to be useful for genetic analysis in *P. pungens*. Although the other two showed no polymorphism in this study, it does not necessarily follow that there are no polymorphic alleles in this or other population(s). We easily obtained these four loci from RAPD products, and the primers were designed within two weeks from the start of the experiment. RAPD reaction amplifies randomly regions of a genome depending on the sequence of the primer used. Thus, we can add more loci by using other RAPD primers.

The mechanisms of parthenogenesis in *P. pungens* are not clear, but the occurrence of meiosis has been confirmed (Itow *et al.*, 1984). If haploid oocytes rejoin randomly and become a diploid egg, all individuals are expected to become homozygotes finally at all loci, because any locus that becomes homozygote can never return to being a heterozygote without mutation. However, there were many heterozygotes in this population, and the heterozygosities in the population are high (Tables 1 and 2). Therefore, the microsatellite markers obtained here will be useful for investigating the mechanism of thelytoky in this species through more detailed genotypic analysis on mothers and their daughters.

The average relatedness between colony members is extremely high, compared with other ants or eusocial Hymenoptera (Crozier & Pamilo, 1996). The genotypic distribution within colonies suggested that many colonies are monoclonal (Table 2). If all colony members are of a single genetic clone, genetic conflict over reproduction is unlikely even when all individuals lay viable eggs. However, the data also showed that one of the 15 colonies contained different genotypes. This colony had at least two different matrilines and

the possibility of conflicts over reproductive interests between individuals. In some *P. pungens* colonies, larger workers achieved reproductive benefit by engaging less in work and more in egg-laying (Tsuji, 1988, 1995; Sasaki & Tsuji, in prep.). It will be interesting to investigate, using the microsatellites reported here, whether or not these two types of workers are of different genotypic lines. Using more microsatellite loci, *P. pungens* will be a model system for understanding the evolution of conflict and cooperation over reproduction in social associations.

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