Title: Isolation and characterization of eleven nuclear microsatellite loci for the parthenogenetic ant *Pristomyrmex punctatus*

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Abstract

Highly polymorphic genetic markers are very useful tools for analyzing population genetic structures of social insects. We developed eleven polymorphic nuclear microsatellite loci for the obligatory parthenogenetic ant, *Pristomyrmex punctatus*. The number of alleles per locus ranged from two to 12 and the average observed heterozygosity was 0.628. In addition, results of cross-species amplification tests are reported in three other species of *Pristomyrmex*.

In the past decade, our knowledge on the genetic structures of social insects has been dramatically extended, and these findings are greatly indebted to highly polymorphic genetic markers, especially microsatellite loci (Keller 2007). Here we report the isolation and characterization of eleven polymorphic microsatellite markers for the queenless parthenogenetic ant, *Pristomyrmex punctatus* (formerly *P. pungens*).

P. punctatus has a very unique social structure, in which the morphological queen caste is absent and all females are wingless monomorphic workers. They are involved in thelytokous parthenogenesis in their youth and shift to cooperative tasks as they age (Tsuji 1990). Recently, two remarkable phenomena were discovered in this species. First, colonies often consist of more than one clonal lineage (Hasegawa *et al*. 2001), and in laboratory conditions, genetically polymorphic colonies are harder to assemble into a single nest than those that are genetically monomorphic (Nishide *et al*. 2007). Second, Dobata *et al*. (2009) reported a genetically distinct cheater lineage, which lays more eggs and hardly takes part in cooperative tasks, in the field colonies. These studies were facilitated by some polymorphic microsatellite markers, but the levels of polymorphism were insufficient for more detailed analyses. The newly developed eleven microsatellites reported here would be useful in analyzing the bizarre social structure of *P. punctatus*.

We chiefly followed the protocol of Hamaguchi *et al*. (2007), which combined the methods of Hale *et al*. (2001) and Fischer & Bachmann (1998) with some modifications. Briefly, genomic DNA was extracted with DNeasy Blood & Tissue Kit (Qiagen) from the whole body of 10 individuals collected from Kihoku, Mie Prefecture, central Japan, into 100 μL of elution buffer (ca.10ng/μL). Extracted DNA was digested with *Sau*3AI and ligated to a *Sau*3AI cassette (TaKaRa), followed by 20 cycles of polymerase chain reaction (PCR) amplification (72 °C for 5 min prior to the first denaturation step, annealing temperature at 55 °C, extension for 4 min), using the linker-specific primer C1

(TaKaRa) and LA Taq DNA polymerase (TaKaRa). The PCR product was hybridized with 3′-biotin-labeled $(CA)_{12}$ oligonucleotide and captured with streptavidin-coated magnetic beads (Dynabeads-280; Dynal), followed by re-amplification by 30 cycles of PCR (annealing temperature at 55 °C, extension for 80 s) using the C1 primer and rTaq DNA polymerase. The PCR product was ligated into plasmid pGEM-T Easy Vector (Promega) and transformed in Epicurian Coli XL1-Blue MRF´ supercompetent cells (Stratagene). The recombinant colonies were picked and inserts were amplified by direct PCR using T7 and SP6 primers, and sequenced using an automated sequencer CEQ 8000 (Beckman & Coulter). Of these, eleven primer pairs were designed using the software Primer 3 (Rozen & Skaletsky 2000). PCRs were performed using Multilplex PCR Kit (QIAGEN) in a 5 μL volume containing 1×QIAGEN Multiplex Master Mix, 0.2 μM of each primer and 0.5 μL of genomic DNA. The reaction conditions were 15 min at 95°C, 35 cycles of 30 s at 94°C, 90 s at 60°C and 60 s at 72°C, followed by an additional extension for 30 min at 60°C. Among these markers, three sets of primers (three or four primer pairs each; see Table 1) were coamplified. The forward primer of each marker was 5'-end-labeled with a fluorescent dye (Beckman dye D3 or D4; Sigma-Aldrich). The PCR products were visualized on the CEQ8000 and allele sizes were determined using the attached fragment analysis software.

To assess the polymorphism of the eleven microsatellite loci, we genotyped 102 individuals ("S-types" in Dobata *et al*. (2009)) collected in 11 colonies (9.27 ± 1.42 individuals per colony) from Kihoku population. GENEPOP version 4.0.7 (Raymond & Rousset 1995) was used to estimate observed and expected heterozygosities, deviations from Hardy-Weinberg equilibrium (HWE) and to test for linkage disequilibrium (LD). The number of alleles per locus, the expected and observed heterozygosities ranged from two to 12, from 0.094 to 0.773 and from 0.098 to 1.000, respectively (Table 1). All but two loci (ppmb105 and ppmb132) deviated significantly from HWE after Bonferroni

correction (adjusted $p < 0.0045$). Of the 55 pairwise comparisons, 43 pairs of loci exhibited significant LD after Bonferroni correction (adjusted $p < 0.00091$). Although it should be considered preliminary, these results would be mainly due to clonal redundancy.

We examined the conservation of the primer sequences and the levels of polymorphism of the eleven loci in three other *Pristomyrmex* species (*P. rigidus*, *P. brevispinosus* and *P. bicolor*). For each species, DNA extraction and PCR amplification were performed following the procedure described above, except that each locus was separately amplified. Six loci successfully cross-amplified in at least one species, but levels of polymorphism were relatively low, with only one or two alleles per locus (Table 2).

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107 **Table legends**

108 **Table 1**. Characteristics of eleven microsatellite loci for the ant *Pristomyrmex punctatus.*

109 **Footnote:** N_A, number of alleles; N_{ind}, number of genotyped individuals; (H_E) expected and (H_O)
110 observed heterozygosities; *, Significant deviation from Hardy-Weinberg equilibrium after Bonferroni
111 corrections; †, Loci with the same alphabets (A–C) were coamplified by multiplex PCR.

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113 **Table 2**. Cross-species PCR tests for 11 *P. punctatus* microsatellite loci in three congeneric ant species.

114 **Footnote:** The number of alleles and the allelic size range are based on *N* workers. Amplification
115 failure is indicated by a dash.

Table 1 Characteristics of eleven microsatellite loci for the ant *Pristomyrmex punctatus.*

Locus†	Primer (5'-3')	Allele size range (bp)	Repeat motif	N_A	N_{ind}	H_E	H_O	Accession no.
ppmb13[A]	f: GAAACGCCACGAGTGTAAAAA	272-308	$(AC)_{10}(TC)_7$.....$(TC)_2(CT)_3$	4	102	0.561	0.873*	AB479990
	r: GATAAAAAGTGTGGTCTTTATGATG		....$(CTTTTT)_2$					
ppmb27[A]	f: TAAAGATCGGAGGCGACATC	157-160	$(GTT)_9(GCTGTT)_3(GTT)_3$	2	102	0.450	0.657*	AB479991
	r: GGAGAATCTCAGCACGAAGC							
ppmb33	f: ATGGGACACCCTGTATATTTATG	310-324	$(CG)_5(CA)_4..(CA)_{18}$	3	102	0.547	0.971*	AB479992
	r: GATTCCAGGCAGTACCGAAA							
ppmb104[B]	f: GCCGAATTTACATCGCAATA	160-162	$(CA)_{11}$	2	102	0.417	0.118*	AB479993
	r: GACAGTTCTGCGGAATCCAT							
ppmb105[A]	f: GCAACATTCTTTCCCTGCTT	190-192	$(GT)_{16}(TG)_2…(TG)_3$	2	102	0.094	0.098	AB479994
	r: CCACGATGTTACATTGCACA							
ppmb106[B]	f: CCGACGCCTCAGATTTGTAT	312-344	$(GT)_{22}(CG)_3$	7	102	0.685	0.922*	AB479995
	r: GCTTGCCGGCAGTGGTGTAA							
ppmb109[B]	f: CTTTTCAAACTGACCGCTATTGT	238-390	$(GT)_{35}$	12	102	0.773	0.961*	AB479996
	r: AAAGTTGGGTCTCTTCTGCTGT							
ppmb114[C]	f: CGCCGTGTACGATACGATTA	308-312	$(TG)_{13}$	3	102	0.277	0.206*	AB479997
	r: CGTAGCCCATACACACATGC							
ppmb132[C]	f: CGGCCACTTAATAACGTCCA	268-276	$(CA)_{18}(TA)_3(CA)_2$	4	102	0.300	0.343	AB479998
	r: GACCCCACAGTTCGTCACTT							
ppmb139[B]	f: CACCCCTGAGCACACTAACA	142-147	$(CA)_{11}(CG)_3(CGCA)_3(CA)_3$	3	102	0.594	0.765*	AB479999
	r: CATCCCGTGTGCCTGTAACT							
ppmb204[C]	f: GCTATTAGGGTTAGCCGGTTG	191-231	$(AC)_{27}$	6	102	0.650	1.000*	AB480000
	r: GCTTGTGTCGACTTGTGTTGTT							

N_A, number of alleles; N_{ind}, number of genotyped individuals; (H_E) expected and (H_O) observed heterozygosities; * Significant deviation from Hardy-Weinberg equilibrium after Bonferroni corrections; †, Loci with the same alphabets (A–C) were coamplified by multiplex PCR.

Table 2 Cross-species PCR tests for 11 *P. punctatus* microsatellite loci in three congeneric ant species.

	ppmb13	ppmb27	ppmb33	ppmb104	ppmb105	ppmb106	ppmb109	ppmb114	ppmb132	ppmb139	ppmb204
P. rigidus	–	1	–	1	–	2	1	2	–	–	1
(*N*=1)		141		156		292-294	192	309-311			166
P. brevispinosus	–	2	–	2	–	–	–	–	–	–	–
(*N*=4)		129-142		150-152							
P. bicolor	–	1	–	1	–	–	–	–	–	–	–
(*N*=3)		129		150							

The number of alleles and the allelic size range are based on *N* workers. Amplification failure is indicated by a dash.