Cheater genotypes in the parthenogenetic ant *Pristomyrmex punctatus*

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Cooperation is subject to cheating strategies that exploit the benefits of cooperation without paying the fair costs, and it has been a major goal of evolutionary biology to explain the origin and maintenance of cooperation against such cheaters. Here, we report that cheater genotypes indeed coexist in field colonies of a social insect, the parthenogenetic ant *Pristomyrmex punctatus*. The life history of this species is exceptional, in that there is no reproductive division of labour: all females fulfil both reproduction and cooperative tasks. Previous studies reported sporadic occurrence of larger individuals when compared with their nest-mates. These larger ants lay more eggs and hardly take part in cooperative tasks, resulting in lower fitness of the whole colony. Population genetic analysis showed that at least some of these large-bodied individuals form a genetically distinct lineage, isolated from cooperators by parthenogenesis. A phylogenetic study confirmed that this cheater lineage originated intraspecifically. Coexistence of cheaters and cooperators in this species provides a good model system to investigate the evolution of cooperation in nature.

Keywords: evolutionary cheating; *Pristomyrmex punctatus*; *Pristomyrmex pungens*; parthenogenesis; social cancer; Emery's rule

1. INTRODUCTION

Cooperation, as well as competition, is a nearly ubiquitous feature of biological systems. Cooperative systems are subject to cheating, which can be defined as an evolutionary strategy that achieves higher individual fitness than cooperation by exploiting the benefits of cooperation selfishly without paying the fair costs (on such 'social semantics', see West *et al.* 2007a). It has been a major goal of evolutionary biologists to explain the origin and maintenance of cooperation against cheating (Maynard Smith & Szathmáry 1995; Keller 1999).

Although numerous theoretical models have been proposed (for references of ongoing controversies, see Lehmann & Keller 2006; Taylor & Nowak 2007; West *et al.* 2007b; Wilson 2008) and fascinating experiments using micro-organisms have been conducted in the laboratory (for review, see West *et al.* 2006), there are still a limited number of field studies on cooperation and cheating. For a thorough understanding of the nature of cooperation, it is important to find tractable systems containing both cooperators and cheaters under natural conditions. Here, we report that distinct cheater

genotypes coexist with cooperators in natural colonies of a social insect, the parthenogenetic ant *Pristomyrmex punctatus* (formerly *Pristomyrmex pungens*; Wang 2003).

Colonies of social insects provide a typical example of biological cooperation, which is characterized by a well-developed reproductive division of labour between queens and workers (Wilson 1971). However, an extraordinary life history has evolved in *P. punctatus*, i.e. the morphological queen caste is absent and all females are wingless morphological workers. Furthermore, males are rare and no evidence of sexual reproduction has been found (Itow *et al.* 1984; Tsuji 1988; T. Sasaki 2001–2005, personal observation). All monomorphic females are involved in thelytokous reproduction (female-producing parthenogenesis) in their youth and later shift to cooperative behaviour, such as colony defence and foraging, as they age (Tsuji 1990).

The characteristically cooperative society summarized above seems to be vulnerable to cheating, and this is suggested by previous studies that found unusual individuals in some field populations. (figure 1; Itow *et al.* 1984; Tsuji 1995; Sasaki & Tsuji 2003). Such individuals (hereafter referred to as the L-type versus normal S-type) are easily distinguished from S-types by their larger body size, ovariole number (four instead of two) and presence of spermathecae (Itow *et al.* 1984). Behavioural analysis revealed that L-types hardly ever take part in

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Figure 1. (a) S-type and (b) L-type of the parthenogenetic ant *P. punctatus*. Scale bar, 1 mm.

colonial tasks, except reproduction (Sasaki & Tsuji 2003). Therefore, the L-type individuals potentially exploit the cooperative benefit produced by S-types in the production of the next generation. Indeed, Tsuji (1995) investigated field colonies of *P. punctatus* and showed that an increase in the proportion of L-types lowers the reproductive success of nest-mates, which is a symptom of cheating. This trend is confirmed in subsequent field studies (T. Sasaki, H. Mori, S. Dobata & K. Tsuji 2001–2005, unpublished data).

Of particular interest is the genetic background of the L-types. To determine whether the L- and S-types share genetic interests, we investigated genetic differentiation between these two phenotypes. Our findings indicate that some L-type individuals are genetically distinct from the S-type nest-mates, i.e. they represent a lineage that has specialized in cheating.

2. MATERIAL AND METHODS

(a) Study species

Pristomyrmex punctatus is one of the most common ants in Japan (Japanese Ant Database Group 2003). Colonies can contain up to hundreds of thousands of individuals in their annual life cycles (adults live only 1 year, whereas each colony can last far longer and is potentially immortal; Tsuji 1995). We investigated a population in Kihoku, Mie Prefecture, where previous researchers found a colony containing many L-type individuals (Itow *et al.* 1984). Twenty-two colonies from one site and 54 colonies from six sites were sampled in July 2001 and July 2005, respectively. Each site was approximately 1–5 km apart. From each colony sampled, some hundreds of adult females were collected and were placed in pure ethanol. Some colonies contained several males in 2005, and these were also collected.

(b) Morphological analysis

Based on the number of ovarioles, each of the collected females was dissected and classified as the L- or S-type. Some colonies in 2005 contained callow adults, which will overwinter and reproduce the next year, and are characterized by lipid granules in their abdomens (Tsuji 1995); these callow adults are the offspring of the older adults found in their colony. The L-type individuals were present in 68.2 per cent (15/22) and 75.9 per cent (41/54) of the colonies collected in

2001 and 2005, respectively. The mean intracolony proportion of large workers was 3.5 ± 6.6 and $3.9 \pm 6.5\%$, respectively. Then we counted the number of ocelli for each individual sampled, because males and some L-types are known to have ocelli (whereas the S-type has no ocellus; Itow *et al.* 1984; Tsuji 1988). Furthermore, we measured the head width (across compound eyes) of the females collected in 2005.

(c) Genotypic analysis

From the preserved samples, 147 individuals from 17 colonies and 464 individuals from 24 colonies in 2001 and 2005, respectively (570 females and 41 males; 5 S-types and 3–10 L-types per colony in 2001; 10 S-types and 2–10 L-types per colony in the parent generation of 2005; 5 S-types, 1–6 L-types and 1 or 10 males per colony in the offspring generation of 2005), were used for the subsequent genotypic analyses. Total DNA was extracted from the thorax of each individual using the Qiagen DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA) and dissolved in 100 μ l of elution buffer.

(i) Mitochondrial markers

As the first screening, a downstream intron region of mitochondrial 12S ribosomal RNA gene was amplified for all samples. A preliminary survey (E. Hasegawa 2005–2006, unpublished data) had found 4 bp indels in this region among individuals. Polymerase chain reactions (PCRs) were conducted using Takara Ex Taq DNA polymerase (Takara Bio, Shiga, Japan) and its supplemented buffer system with the primer pair pmf102 (5'-CTACATTACTCTATATATAA-3') and pmr101 (5'-AAGATAATAATGAGTTACAGTT-3'). The reaction conditions were 35 cycles of 94°C for 30 s, 45°C for 30 s and 60°C for 1 min, followed by one cycle of 72°C for 5 min. The amplified fluorescent PCR products were analysed using an automated sequencer (CEQ 8000, Beckman & Coulter, Fullerton, CA, USA).

(ii) Nuclear microsatellite markers

In a preliminary survey, we amplified seven microsatellite markers, Pp1–Pp4 (originally developed for *P. punctatus*; Hasegawa *et al.* 2001), L-8, L-15 (Foitzik *et al.* 1997) and MYRT3 (known to be polymorphic in some ant species; Bourke *et al.* 1997), from 25 S-types and 15 L-types collected from eight colonies, following the protocols described in each reference. Polymorphism detection methods were the same as above. All markers were successfully amplified, but only three of them (Pp1, Pp2 and MYRT3) showed polymorphism among the samples. These three were used for subsequent amplification and analyses of all sampled individuals. As there were a large number of genotypes even within a colony (i.e. nest-mates differed in zygosity at the same locus), we carefully considered the results and performed re-genotyping when necessary. Using these polymorphic microsatellites, the average nest-mate relatedness within each colony was estimated using the software package RELATEDNESS v. 5.0.8 (Goodnight & Queller 2001).

(iii) Statistics

First of all, we tested whether the two phenotypes (L- or S-type) differed in the frequency of the multilocus genotypes detected (mitochondrial and nuclear markers combined) using Fisher's exact probability test. Then we conducted binomial tests to determine whether each multilocus

genotype significantly deviated in its ratio of L- to S-type from that of the population, i.e. the entire sample. The samples collected in 2001 and 2005 were treated independently, and the offspring generation of 2005 was excluded from the analyses due to small sample size. As this procedure took the form of multiple tests, we controlled for the false discovery rate by calculating q -values (Storey & Tibshirani 2003). All statistical analyses were implemented in R 2.7.1 (Ihaka & Gentleman 1996).

(d) Phylogenetic analysis

To confirm the phylogenetic position of the genotypes in this population, a portion of the mitochondrial cytochrome oxidase I gene (*COI*) was sequenced. Samples were chosen from the total so as to cover all of the multilocus genotypes and sampling years (one sample per genotype per year). Thirty-six samples were examined, together with two conspecific samples collected on Okinawa island, southern Japan, and one belonging to *Pristomyrmex rigidus*, the species most closely related to *P. punctatus* (Wang 2003), from Ulu Gombak, Malaysia. PCRs were conducted as described above (except with an extension time of 3 min) with a *COI* primer pair: ppcf (5'-GCAATTAATTTTATTTCAAC-3') and CI24 (5'-ACCTAAAAATGTTGAGGGAA-3'). After purification, sequencing was performed using a CEQ 8000 automated sequencer. The ant species *Myrmica rubra* and *Manica rubida* (GenBank accession nos. DQ074387 and AY280592, respectively) were chosen as out-groups, and their sequences were aligned by CLUSTALX (Thompson et al. 1997). A total sequence length of 595 bp was used for phylogenetic analysis. A neighbour-joining tree (Saitou & Nei 1987) was constructed, and a maximum-parsimony tree was estimated using the program package PAUP 3.1.1 (Swofford 1993). Bootstrap tests were conducted with 1000 resamplings. All the sequences analysed in this study have been deposited in GenBank under the accession nos. EU342353–EU342356.

3. RESULTS

The mitochondrial marker showed only two haplotypes in the study samples (product lengths: 338 and 342 bp). For the three polymorphic microsatellite markers, relatively few alleles were found (two, three and four alleles for Pp1, Pp2 and MYRT3, respectively). By combining these four markers, a total of 30 distinct multilocus genotypes were identified in females (table 1). According to Nishide et al. (2007) and due to the parthenogenetic nature of this species, we treated these genotypes as independent lineages in the subsequent analyses.

Next, we matched these marker genotypes with phenotypes (table 1). The two phenotypes (L- and S-type) differed significantly in the representation of the 30 multilocus genotypes both in 2001 and 2005 samples (Fisher's exact probability test, both $p < 10^{-15}$). This difference was due to some genotypes biasing their representation towards the L- or S-types. Nine genotypes (nos. 1, 4, 5, 6, 10, 13, 27, 29 and 30) were found only in the L-type individuals. The other 21 genotypes were present in the S-type individuals, and 11 of these genotypes (nos. 7, 12, 14, 15, 17, 18, 19, 20, 22, 23 and 25) existed in both the L- and S-types. The L-type individuals with these 11 genotypes were found only in 2005. Among them, the bias towards L-types was

Figure 2. Head width of *P. punctatus* females collected from Kihoku, Japan, in 2005. Only the parental generation was used in the analysis, and the rare genotypes (less than 1% of the sample) were omitted. L- and S-types are shown in grey and white bars, respectively, and the histograms are drawn in stacked columns. (a) S-type-specific genotypes (group 1; genotype nos. 2, 8 and 9); (b) L- and S-type-producing genotypes (groups 2 and 3; genotype nos. 7, 12, 14, 15, 17, 22 and 25); (c) L-type-specific genotypes (group 4; genotype nos. 4 and 5).

statistically significant in genotype 5 in both 2001 and 2005 samples and genotype 4 in 2005 samples (binomial test, all $p < 0.01$), and the bias towards S-types was significant in genotype 8 in 2005, genotype 9 in 2005, genotype 11 in 2001 and genotype 17 in 2001 (binomial test, all $p < 0.01$). These results were neither due to very rare genotypes nor to false discoveries resulting from multiple tests (controlled by q -values). The L-type individuals belonging to the two genotype groups, one L-type specific and the other concomitant with S-types, also differed in other morphological features: the former had significantly larger head width (mean head width: 0.98 ± 0.018 s.d., $n = 72$) and all had three conspicuous ocelli, whereas the latter were smaller bodied (mean head width: 0.88 ± 0.020 s.d., $n = 76$; but still larger than the S-type; for details see below and figure 2) and

Table 1. Multilocus genotypes of females identified in 41 colonies of *P. punctatus* from Kihoku, Japan. (Multilocus genotypes were determined by combining one mitochondrial and three nuclear microsatellite (Pp1, Pp2 and MYRT3) markers. The *p*-values were calculated from binomial tests for each multilocus genotype found in each year, and the *q*-values estimate the probability of a result being a false discovery due to the multiple tests. Results are shown in italics when the phenotypes of individual multilocus genotypes were significantly biased towards the L- or S-type from that of the population, i.e. the entire sample. See text for details. MLG, multilocus genotype.)

MLG no.	genotype	2001			2005 parent				2005 offspring						
		mtDNA	Pp1	Pp2	MYRT3	L	S	p	q	L	S	p	q	L	S
1	338		220/220	238/238	179/179	1		0.422	0.759						
2	338		220/225	224/238	177/181						4	0.155	0.393		
3	338		220/225	228/238	181/185						1	1.000	1.000		
4	338		220/225	238/238	179/179							0.004	0.028	1	
5	338		220/225	238/238	179/181	60		<0.001	<0.001	66		<0.001	<0.001	11	
6	338		220/225	238/238	181/181					2		0.160	0.393		
7	338		225/225	224/238	177/179					3		0.384	0.568		
8	338		225/225	228/238	181/181						9	0.001	0.013		
9	338		225/225	228/238	181/185						14	<0.001	<0.001		
10	338		225/225	238/238	179/181	1		0.422	0.759		29				
11	342		220/225	228/238	177/177		12	0.002	0.006		3	0.280	0.556		
12	342		220/225	228/238	177/179		1	1.000	1.000	1	10	0.060	0.216		
13	342		220/225	228/238	177/181					3		0.064	0.216		
14	342		220/225	228/238	179/179					30	66	0.095	0.285	1	5
15	342		220/225	228/238	179/185					3	1	0.309	0.556		
16	342		220/225	228/238	181/181		2	0.512	0.768						
17	342		220/225	228/238	181/185		68	<0.001	<0.001	25	61	0.047	0.211		4
18	342		220/225	228/238	185/185					2	1	0.568	0.730		
19	342		220/225	238/238	177/179					1	1	1.000	1.000		
20	342		220/225	238/238	177/181						1	1.000	1.000		
21	342		220/225	238/238	179/179						1	1.000	1.000		
22	342		220/225	238/238	179/181					8	7	0.304	0.556		
23	342		220/225	238/238	181/181					1	2	1.000	1.000		
24	342		225/225	224/238	177/177						1	1.000	1.000		
25	342		225/225	224/238	177/181					6	23	0.037	0.199		
26	342		225/225	224/238	181/181						2	0.520	0.702		1
27	342		225/225	228/238	177/177		1	1.000	1.000	1		0.400	0.568		
28	342		225/225	228/238	177/181						3	0.280	0.556		
29	342		225/225	228/238	181/185		1	1.000	1.000	1		0.400	0.568		
30	342		225/225	238/238	179/181	62	85			1	240	0.400	0.568	13	10
total										160					

Table 2. Individual multilocus genotypes of colony 2005E03. (All individuals were adults and both generations (parent and offspring) were collected at the same time. Note that the offspring individuals are not necessarily the direct progeny of the parent individuals shown above them. MLG, multilocus genotype.)

phenotype	generation	no.	ocellus	mtDNA	Pp1	Pp2	MYRT3	MLG no.
L-type	parent	1	3	338	220/225	238/238	179/181	5
		2	3	338	220/225	238/238	179/181	5
		3	3	338	220/225	238/238	179/179	4
		4	3	338	220/225	238/238	179/181	5
		5	3	338	220/225	238/238	179/181	5
		6	3	338	220/225	238/238	179/181	5
		7	3	338	220/225	238/238	179/181	5
		8	3	338	220/225	238/238	179/181	5
		9	3	338	220/225	238/238	179/181	5
		10	3	338	220/225	238/238	179/181	5
	offspring	1	0	338	220/225	238/238	179/181	5
		2	3	338	220/225	238/238	179/179	4
		3	3	338	220/225	238/238	179/181	5
		4	3	338	220/225	238/238	179/181	5
		5	3	338	220/225	238/238	179/181	5
		6	3	338	220/225	238/238	179/181	5
		7	3	338	220/225	238/238	179/181	5
		8	3	338	220/225	238/238	179/181	5
		9	3	338	220/225	238/238	179/181	5
S-type	parent	1	0	342	220/225	228/238	179/179	14
		2	0	342	220/225	228/238	179/179	14
		3	0	342	220/225	228/238	179/179	14
		4	0	342	220/225	228/238	179/179	14
		5	0	342	220/225	228/238	179/179	14
		6	0	342	220/225	228/238	179/179	14
		7	0	338	225/225	228/238	181/181	8
		8	0	342	220/225	228/238	179/179	14
		9	0	342	220/225	228/238	179/179	14
		10	0	342	220/225	228/238	179/179	14
	offspring	1	0	342	220/225	228/238	181/185	17
		2	0	342	220/225	228/238	179/179	14
		3	0	342	220/225	228/238	179/179	14
		4	0	342	220/225	228/238	179/179	14
		5	0	342	220/225	228/238	179/179	14

rarely (13.2%) had three ocelli (mean number of ocelli: 1.34 ± 1.07 s.d., range 0–3, $n=76$). We performed pairwise multiple comparisons (Tukey's HSD test) among the four groups of individuals: (i) S-types of the S-type-specific genotypes (genotype nos. 2, 8 and 9), (ii) S-types of genotypes producing both the L- and S-types (genotype nos. 7, 12, 14, 15, 17, 22 and 25), (iii) L-types of genotypes producing both the L- and S-types, and (iv) L-types of the L-type-specific genotypes (genotype nos. 4 and 5). All differences were statistically highly significant ($p < 1 \times 10^{-7}$), except for the group 1 versus group 2 comparison (head width: $p=0.638$, ocellus: $p=1.000$). All males had three conspicuous ocelli.

A colony-level analysis revealed that most colonies contained more than one genotype, and the estimated average nest-mate relatedness within each colony varied from -0.0309 ± 0.6872 (s.e.) to 1.000 (see table S1 in the electronic supplementary material). In addition, the genotypes of males, which all belonged to the offspring generation, indicated that they were produced by females of the colonies in which they were found (see table S1 in the electronic supplementary material).

Table 2 lists the individual multilocus genotypes of one colony (2005E03), which contained two successive generations of both the L-type-specific genotypes (4 and 5) and S-type-producing genotypes (8, 14 and 17). In this colony, the mitochondrial haplotypes were mostly separated between these two genotype groups across

generations. These genotypic data show that the two genotype groups form separate lineages in this colony. Combined with the data from other colonies (see table S1 in the electronic supplementary material), we could rule out the possibility that the S-types are mainly produced by the L-type-specific genotypes through cross-breeding or facultative sexual reproduction, as reported in some ant species with genetic determination of reproductive castes (Helms Cahan & Keller 2003; Helms Cahan & Vinson 2003; Pearcy *et al.* 2004; Fournier *et al.* 2005; Ohkawara *et al.* 2006).

In the phylogenetic analysis, only two haplotypes of the *COI* portion were found in the study population, which was completely congruent with the indel pattern of the 12S rRNA flanking region. The two haplotypes differed only by a single base-pair substitution, indicating that all genotypes in the study population were more closely related to one another than to the conspecific S-types from Okinawa island (figure 3).

4. DISCUSSION

Of the 30 genotypes, nine were found only in the L-type individuals. Among them, genotype 5 was the most abundant, statistically significantly biased towards L-types, and found in a total of 16 colonies over all the studied generations. In addition, we confirmed that the individuals with genotype 5 laid eggs parthenogenetically

Figure 3. Phylogenetic relationships among genotypes (nos. 1–30) found in the Kihoku population of *P. punctatus*, combined with conspecifics (S-type) from another population (Okinawa) and the congeneric species *P. rigidus* from Malaysia. Two ant species (*M. rubra* and *M. rubida*) were taken as out-groups. The neighbour-joining tree based on the 595 bp fragment of *COI* is shown. Maximum parsimony analysis gave essentially the same result. The bootstrap values are shown at the branches, numbers in parentheses are sampling years (2001 and 2005, shown in the last two digits), and numbers in square brackets are GenBank accession numbers. See text for the scheme of phylogenetic sampling from the study population.

(see table S2 in the electronic supplementary material). Together with the cross-generational genotypic analysis of L-types in the same colonies, we concluded that at least genotype 5 is a distinct lineage of cheaters. Unfortunately, the number of alleles of each locus used in this study was too small to analyse genealogical relationships of genotype 5 to the other L-type-specific genotypes, and thus we cannot determine at present whether these L-type-specific genotypes have a single origin. Additional polymorphic markers and more information about the mode of parthenogenesis in this species are needed to clarify this issue.

Our phylogenetic analysis demonstrated that this cheater lineage is more closely related to nest-mate cooperators than to conspecifics (S-type) of another population. These findings indicate that the cheater genotypes originated intraspecifically, thus excluding the possibility that they belong to a different congeneric species that is parasitizing *P. punctatus*.

Our study revealed two unexpected results. First, the identical multilocus genotypes were shared by the L- and S-types in 2005 while some of them (typically genotype 17) produced only S-types in 2001. This implies that lineages that usually become only S-types have the potential to develop into L-types under certain environmental conditions (e.g. with more food available), although having only the S-type is sufficient for *P. punctatus* colonies to flourish (Tsuji 1990, 1994). Developmental plasticity is a widespread feature of social insect caste systems (Wilson 1971), and the dual loss of morphological castes and lifetime reproductive division of labour is a rare derived event in ants (the only other example is *Cerapachys biroi*, also known to be parthenogenetic; Tsuji & Yamauchi 1995; Ravary & Jaisson 2004). Therefore *P. punctatus* might retain the potential for developmental plasticity, and this would result in the occasional production of L-types, which corresponds to the

intercastes in other ant species (Peeters 1991). In fact, occasional production of large-bodied ‘intercastes’ has been reported in *C. biroi* as well (Ravary & Jaisson 2004). Furthermore, our morphometrical analysis revealed that the L-types produced by the L-type-specific genotypes are larger than those produced by genotypes producing both the L- and S-types. This finding would shed light on the possible mechanism of the development of L-type-specific genotypes. There are two explanations for the micro-evolutionary shift of the ant caste determination system in relation to larval size (Yang *et al.* 2004; see also Nonacs & Tobin 1992). One is the evolutionary change in threshold size above which larvae develop into larger sized caste. The other is the evolutionary shift in larval size distribution beyond a fixed threshold size. The latter would be achieved by the evolved larvae obtaining more food from nursing S-types or requiring less food to reach a larger size, and this would result in individual larvae being more likely to switch to the L-type developmental trajectory in *P. punctatus*. If this is the case, it is predicted that individuals of the L-type-specific genotypes will be larger in body size than those of the other genotypes, which is exactly what is observed for L-types (figure 2). The developmental basis of L-types deserves further study, especially in the context of the evolutionary origin of cheaters in *P. punctatus*. It is also worth examining if the L-type-specific genotypes could develop into S-types in certain environmental conditions and if higher resolution genetic study would find other L-type-specific lineages within the multilocus genotypes found in both L- and S-types.

Second, males were found in the study population only in 2005. Previous studies have shown that these occasional males in *P. punctatus* colonies are probably reproductively functional (haploid with active sperm; Itow *et al.* 1984; Hasegawa *et al.* 2001). Normal S-types do not have spermathecae (Itow *et al.* 1984), as is the case with

workers in many other ant species, and they are not able to mate with males. However, L-types have spermathecae (Itow *et al.* 1984), suggesting that they have the potential to mate with males. Nevertheless, after dissecting more than 1000 L-types from the study population, no L-type individuals have been found with sperm in their spermathecae (T. Sasaki 2001–2005, personal observation). Due to the lack of sufficient genetic markers, however, at present we cannot exclude the possibility of occasional sexual reproduction.

A variety of selfish strategies resulting from evolutionary conflict within social insect colonies have been reported, from traditional examples of sex-ratio manipulation (Trivers & Hare 1976) and interspecific social parasitism (Wilson 1971) to more recent findings of intraspecific parasites (Oldroyd *et al.* 1994; Abbot *et al.* 2001; Lopez-Vaamonde *et al.* 2004; Nanork *et al.* 2005), green beard genes (Keller & Ross 1998), conditional use of sex (Pearcy *et al.* 2004), clonal males (Fournier *et al.* 2005; Ohkawara *et al.* 2006) and putative genetic royal cheats (Hughes & Boomsma 2008; see also Schwander & Keller 2008). The cheater lineage in *P. punctatus* gives a novel addition to these diverse systems.

The cheaters in *P. punctatus* resemble obligate inter-specific social parasites. Although the phylogeny indicates that we have observed an intraspecific phenomenon and the definition of 'species' is essentially semantic in parthenogenetic organisms such as *P. punctatus*, our finding has an important implication for the evolution of social parasite species. It has long been suggested that workerless obligate social parasite (i.e. inquiline) species arose from intraspecific parasites followed by sympatric speciation. This proposal is based on the fact that inquiline species are often the closest relatives of host species (i.e. Emery's rule; Emery 1909). The phylogeny shows that Emery's rule holds true in *P. punctatus*. The system most similar to that of *P. punctatus* is that of the Cape honeybee (*Apis mellifera capensis*). In this subspecies, clonal mutant workers invade colonies of the neighbouring subspecies, *Apis mellifera scutellata*, and behave as 'pseudoqueens' in those colonies, a process that has caused rapid mass extinction of the host colonies (i.e. within 10 years; Neumann & Moritz 2002; Dietemann *et al.* 2007). The Cape honeybee system is an example of speciation by secondary sympatry. Likewise, the fact that the identical cheater lineage (genotype 5) of *P. punctatus* was found in more than one colony where nest-mate S-types have different genotypes suggests that L-type individuals can move and infect new colonies. These thelytokous social parasites could be interpreted as the transmissible 'social cancer' in these superorganisms. This raises two questions. First, previous studies have shown that strict nest-mate discrimination inhibits the movement of individuals between *P. punctatus* colonies, analogous to immunity in organisms (Tsuji & Ito 1986; see also Sanada-Morimura *et al.* 2003). Thus, future studies should also clarify how cheaters can break through nest-mate discrimination systems. Second, it should be noted that cheaters and cooperators in *P. punctatus* have coexisted at the same locality for at least 25 years: the L-type individuals were first discovered in 1982 (Itow *et al.* 1984), and the locality name 'Nagashima' referred to by those authors is identical to Kihoku of this study. Future studies should investigate what causes the difference between the Cape honeybee

and *P. punctatus* systems, with one resulting in mass extinction and the other leading to long-term coexistence.

The cheater genotypes found in field colonies of *P. punctatus* are a promising model system to investigate selfish strategies in social insects, the evolution of social parasitism and the nature of cooperation.

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