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ORIGINAL ARTICLE

Clonal reproduction by males of the ant *Vollenhovia emeryi* (Wheeler)

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Abstract

In colonies of the queen-polymorphic ant *Vollenhovia emeryi*, some colonies produce only long-winged (L) queens, while others produce only short-winged (S) queens. At four nuclear microsatellite loci, males in the S colony had alleles that were different from those of their queen. This suggests that the nuclear genome of males is not inherited from their colony queen, as has also been described for *Wasmannia auropunctata* (Roger). In *V. emeryi* the possibility of male transfer from other colonies has not been ruled out because previous studies of this species have obtained only nuclear gene information. We analyzed both mitochondrial and nuclear genes for S queens, S males and L queens to clarify the origins of males. Sequence analyses showed that although S queens and S males shared the same mtDNA haplotype, they had a different genotype at a nuclear gene (long-wavelength opsin) locus. Neighbor-joining analysis based on the four microsatellite loci also suggested gene pool separation between S queens and S males. These results are consistent with predictions of clonal reproduction by males. While L queens share opsin genotypes with S males, they have very different mtDNA sequences. Hybridization in the near past between S queens and L males or gene transmission from S males to L queen populations in the present would explain these differences.

Key words: asexual reproduction, genetic caste determination, male clonality.

INTRODUCTION

In ants, as in other Hymenoptera, females are usually produced sexually and are diploid, whereas males develop from unfertilized eggs and are haploid. The fertilized diploid eggs generally develop into new queens (gynes) or workers, a process that is regulated by environmental factors such as food or temperature (Hölldobler & Wilson 1990). Ohkawara *et al.* (2006) found an atypical inter-caste genomic composition in colonies of the ant *Vollenhovia emeryi* (Wheeler). At three loci, sterile workers were mostly heterozygous, while queens and female reproductives were homozygous, as occurs in the genetic caste determination of *Pogonomyrmex* harvester ants (Anderson *et al.* 2006). One explanation for the difference in allele frequency between castes is interbreeding between genetically differentiated lineages, as has been proposed for *Pogonomyrmex* (Julian *et al.* 2006). The mating of queens with males of their own and of other lineages produces two types of diploid offspring: offspring of the former matings develop into queens whereas offspring of the latter matings develop into workers. This genetic caste determination (GCD) system causes different frequencies of heterozygosity to exist in queens and workers. In the GCD system, the genotype of a gyne differs from that of its mother, since it has its father's gene.

Another explanation for the genetic differentiation between queens and workers is the conditional use of

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sexual and asexual reproduction for workers and queens. This phenomenon has been found in a few ant species (Pearcy *et al.* 2004; Fournier *et al.* 2005; Pearcy *et al.* 2006). Asexual reproduction reduces heterozygosity through a process similar to inbreeding, whereas sexual reproduction maintains or induces heterozygosity through outbreeding (Pearcy *et al.* 2006), and thus workers become genetically differentiated from queens. Given this conditional use of different reproductive systems for each caste, gynes are genetically identical to their mothers and males cannot pass any genes to the next generation, meaning that males have zero fitness. In *Wasmannia auropunctata*, as a male strategy to counter female asexual reproduction, males reproduce via clones, probably by maternal genome loss from eggs (Fournier *et al.* 2005; Foucaud *et al.* 2007). In this situation, males and queens have separate gene pools.

In *V. emeryi*, S males carry a microsatellite locus allele that differs from that of their colony queens, as has also been found for males of *W. auropunctata* (Ohkawara *et al.* 2006). However, the existence of separate gene pools for S males and S queens has not been proven because an insufficient number of loci have been studied. Furthermore, as the study of Ohkawara *et al.* used only nuclear DNA information, the authors could not exclude the possibility that S males had been born in other colonies, and had transferred to the colony in question. Furthermore, this previous study did not clarify whether GCD or clonal reproduction occurs in *V. emeryi* females, because genetic data for males were not obtained. In GCD, males and queens form a single gene pool, whereas in the clonal reproduction system, males and queens have two separate gene pools based on sex.

The aim of this study was to clarify the above ambiguities and to assess the relationships among *V. emeryi* S males, S queens and L queens by using multiple microsatellite loci data and sequence data for a nuclear gene (long-wavelength opsin). We also investigated the mitochondrial DNA sequence that is cytoplasmically inherited and remains in males after maternal genome loss. Using these DNA markers, we intended to assess the hypothesis that S males with a curious genotype had transferred from other colonies.

MATERIALS AND METHODS

Sampling

Queens and males of *V. emeryi* were collected in September 2003 and 2006 from mixed forest near the coast at Kanazawa City in central Japan. In the study population, L and S colonies exist sympatrically (Ohkawara *et al.* 2002). We used eight males and nine queens from six S colonies, and eight queens from eight L colonies. We could not collect a large number of S colonies in these years because colonies producing a short-winged queen were rare. We could not obtain males from L colonies because in this population L colonies produce almost no males (Ohkawara *et al.* 2002).

DNA extraction

DNA was extracted using a modified Chelex method (Walsh *et al.* 1991). The thoraxes of dried samples were crushed in a 1.5 mL tube and incubated at 55°C with 5 μL of proteinase K (20 mg/mL) and 200 μL of 5% Chelex solution (in 10 mmol L^{-1} Tris-HCl buffer, pH 8.0) for 3 h. After incubation, the solution was boiled at 100°C for 10 min to inactivate the proteinase K. We placed a water layer on top of the Chelex layer just after vortexing and centrifuging (1 min at 15 300*g*).

Microsatellite analysis

We used four microsatellite loci originally developed for *Temnothorax nylanderi* Förster (L-5, L-18; Foitzik *et al.* 1997), *Myrmica kotokui* Forel (MS26; Azuma *et al.* 2005) and *Myrmica tahoensis* Wheeler (Myrt-3; Evans 1993). Polymerase chain reaction (PCR) was carried out for 5 min at 94°C, followed by 30–44 cycles of denaturation for 75 s at 94°C, 75 s of annealing at 46–54°C (see below), and 75 s of extension at 72°C, using a programmable thermal controller (PTC-100TM; MJ Research, Watertown, MA, USA). The last extension step at 72°C was prolonged for 5 min to complete the extension. The annealing temperature and number of cycles for each primer were 54°C, 42 cycles for L5; 52°C, 42 cycles for L18; 46°C, 44 cycles for M3n; and 50°C, 30 cycles for MS26. The composition of the reaction mixtures was as follows: each 10 μL of reaction mixture contained 25 pmol L^{-1} of dye-labeled forward and unlabeled reverse primers, 1 μL of dNTP mixture (2.5 mmol L^{-1} each), 1 μL of 10× Ex Taq buffer, 0.25 U of Ex Taq DNA polymerase (TaKaRa, Osaka, Japan), 7 μL of distilled water and 0.5 μL template DNA. For analysis of fragment length, PCR products were run with the S400 size standard (Beckmann Coulter, Fullerton, CA, USA) using the CEQ2000 XL DNA Analysis System (Beckman-Coulter). After fragment analysis, pairwise individual allele shared distances were calculated as described in Fournier *et al.* (2005). A tree was constructed from the data matrix using the neighbor-joining procedure in MEGA ver. 3.1 (Kumar *et al.* 2004). We

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could not perform the bootstrap test because there was no automated tool to calculate it from the data set (Fournier *et al.* 2005).

Sequence analysis

The target regions were amplified by PCR using universal primers for mtDNA (COI-ATP8: 5′-TTA TAT CAA CAT CTA TTT TGA TTT T-3′ and 5′-ATT ACG TCW ACA AAA TGT CA-3′) and a nuclear gene (opsin: 5′-GCC CCA TTG TTY GGA TGG AA-3′ and 5′-AGA GCA SCT CGR TAC TTA GG-3′). The cycling protocol for the mtDNA primer set was 1 min at 94°C, followed by 40 cycles of denaturation for 1 min at 94°C, 60 s of annealing at 45°C, and 7 min of extension at 60°C. The last extension step was extended to 14 min. The cycling protocol for the opsin primer set was 1 min at 94°C, followed by 35 cycles of denaturation for 1 min at 94°C, 30 s of annealing at 42°C, and 3 min of extension at 72°C. The last extension step at 72°C was extended for 5 min. The composition of the PCR reaction mixtures was the same for microsatellite analysis. The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA), and were eluted with 10 μL sterilized water. The products were sequenced using the DTCS-Quick Start kit (Beckman Coulter) and a CEQ2000 XL automatic DNA sequencer (Beckman Coulter).

RESULTS

In the neighbor-joining analysis using four microsatellite loci, S queens formed a separate clade from S males, which formed two separate clades within a larger clade with L queens. As a result, within a colony S males formed a different clade from the S queens (Fig. 1). We could not perform a bootstrap test for the robustness of clades because there is no software available that automatically calculates bootstrap probabilities, owing to the unusual nature of the distance matrix used, which includes the genetic distance between diploids and haploids (see Fournier *et al.* 2005). Differences in allele frequencies between S queens and S males were significant at three loci (Fisher's exact test with Holms method: $P < 0.001$; L5, L18 and Myrt3) and marginally significant for the remaining locus ($P = 0.051$; MS26). When we used one male and/or queen from each colony in order to avoid sampling bias arising from use of multiple samples from one colony, similar trends were observed ($P < 0.005$; L5, L18 and Myrt3, $P = 0.673$; MS26). These results suggest that the gene pool is separated between S queens and S males.

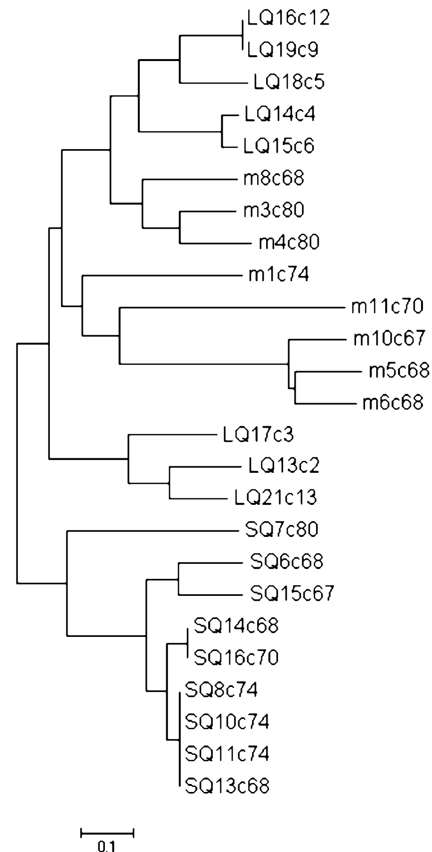

Figure 1 Neighbor-joining dendrogram of genetic (allele-shared) distances for four microsatellite loci (see text for explanations of how the distances were calculated). SQ, queens from S colonies; m, males from S colonies; LQ, queens from L colonies. Individual codes are the same as those used in Table 1.

Sequence analysis of a nuclear gene (long-wavelength opsin) showed that there were three sites with substitutions within the 320 bp examined (GenBank accession no.s AB334117–AB334119). Sequence comparisons among reproductives showed that the same two sequences existed in S males and L queens, whereas all S queens shared a different sequence from S males and L queens (Table 1). Statistically, the difference in gene frequency between S females and S males was highly significant under the assumption that they form a single gene pool ($P < 0.001$, Fisher's exact test). The result also suggested separation of the gene pool between S queens and S males, despite the small sample size.

Mitochondrial DNA analysis revealed a high level of differentiation (nine substitutions in 400 bp; GenBank accession no.s AB334120–AB334122) between S and L

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Table 1 Individual sequences at positions with substitutions for the opsin gene (320 bp) and mtDNA (400 bp)

	Opsin (nuclear gene)			Opsin (nuclear gene)								
Individual code	30	153	319	14	15	34	108	176	282	283	304	330
LQ13c2	T	G	A	A	T	–	C	A	G	G	A	C
LQ14c4	T	G	G	–	–	–	C	A	G	G	A	C
LQ15c6	T	G	G	–	–	–	C	A	G	G	A	C
LQ16c12	T	G	G	–	–	–	C	A	G	G	A	C
LQ17c3	T	G	A	–	–	–	C	A	G	G	A	C
LQ18c5	T	G	G	–	–	–	C	A	G	G	A	C
LQ19c9	T	G	G	–	–	–	C	A	G	G	A	C
LQ21c13	T	G	A	–	–	–	C	A	G	G	A	C
m1c74	T	G	A	A	T	C	A	–	A	A	G	T
m3c80	T	G	A	A	T	C	A	–	A	A	G	T
m4c80	T	G	A	A	T	C	A	–	A	A	G	T
m5c68	T	G	A	A	T	C	A	–	A	A	G	T
m6c68	T	G	G	A	T	C	A	–	A	A	G	T
m8c68	T	G	A	A	T	C	A	–	A	A	G	T
m10c67	T	G	G	A	T	C	A	–	A	A	G	T
m11c70	T	G	G	A	T	C	A	–	A	A	G	T
SQ6c68	C	A	A	A	T	C	A	–	A	A	G	T
SQ7c80	C	A	A	A	T	C	A	–	A	A	G	T
SQ8c74	C	A	A	A	T	C	A	–	A	A	G	T
SQ10c74	C	A	A	A	T	C	A	–	A	A	G	T
SQ11c74	C	A	A	A	T	C	A	–	A	A	G	T
SQ13c68	C	A	A	A	T	C	A	–	A	A	G	T
SQ14c68	C	A	A	A	T	C	A	–	A	A	G	T
SQ15c67	C	A	A	A	T	C	A	–	A	A	G	T
SQ16c70	C	A	A	A	T	C	A	–	A	A	G	T

The numbers in the column headings represent the position within each region. A gap (deletion) is shown by "–".

colony members (Table 1). All S males had the same sequence as cohabiting S queens.

These results indicate that S males have a different nuclear genome composition from cohabiting S queens, but share a mitochondrial sequence with them, and that the genome composition of S males is more similar to that of L queens than that of the cohabiting S queens.

DISCUSSION

Our results clarified several points that have remained ambiguous in previous studies (Ohkawara *et al.* 2002, 2006). First, S males are produced clonally from the father's genome, and are not drifters from other colonies. Second, sexual reproduction, but not GCD, is conditionally used in S females. Third, there exist curious genetic relationships among S queens, S males and L queens.

Clonal reproduction by S males is supported because separation of the gene pool for S males and cohabiting S queens was observed for both the opsin locus and the microsatellite loci. Although we could not perform a bootstrap test for the phylogenetic tree, statistical tests at each locus supported gene pool separation despite the small sample size used. There is no evidence for linkage among the loci used, except between L5 and opsin, suggesting that the loci were independent. Thus, it is unlikely that the small sample size would have caused a type I error because the four loci (three microsatellites + opsin) independently suggest gene pool separation ($P < 0.001$).

The opsin sequence of the S queens was different from that of the S males, although their mtDNA were similar. As S males have the same opsin sequence as L queens, but have quite different mtDNA, we can conclude that the S males did not migrate from L queen colonies. Only the male clonality hypothesis could explain all of the observed facts. A possible criticism of this conclusion may be that the opsin gene is closely linked to the sex determining region while the rest of the genome may be a genetic admixture. However, the microsatellite data are inconsistent with this hypothesis

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and a long mtDNA sequence data set (~3000 bp) has also clearly demonstrated the separation between L queens and S queens (K. Tamura *et al*., unpubl. data). Furthermore, our preliminary data for another nuclear gene (transferrine) demonstrate differentiation between S queens and S males (K. Kobayashi, unpubl. data). Thus, all of the available evidence supports male clonality in *V. emeryi*.

In *W. auropunctata*, all queens in a colony have a single genotype, and their gene pool is separate from that of males (Fournier *et al.* 2005), so they can be regarded as clones. In *V. emeryi*, whether the S queen produces new queens clonally or whether GCD operates remains obscure because in a previous study gene pool separation between males and females was not found (Ohkawara *et al.* 2006). Data from the present study clearly support the conditional use of sex by S queens, not GCD, because the gene pools of S queens and S males were shown to be separated, and the male drift hypothesis was rejected. Thus, both the S queens and the S males reproduce clonally in this population. We could not determine the reproductive mode of the L queens because no males were collected. Genotyping of the mates of L queens would be important for further investigating this matter.

The shared nuclear sequences of S males and L queens can be explained by two hypotheses. One involves gene flow from S males to L queens, while the other involves hybridization between S queens and L males in the past. If there were gene flow, the different allele frequencies in L queens and L workers would be caused by GCD and not by conditional use of sexual reproduction, because the existence of gene flow between S males and L queens contradicts the asexual reproduction of gynes. In this case, the GCD gene would be close to L5 and opsin, which have clearer inter-caste differences in heterozygosity than other loci. In a previous study (Ohkawara *et al.* 2002), a split sex ratio was found for *V. emeryi*: most L colonies were found to produce only female sexuals, whereas many S colonies produced mostly male sexuals. Thus, cross-morph mating might be more frequent than expected given random mating. Under the hybridization hypothesis, S and L colonies would have been previously isolated populations, and there would be no gene flow between them at present. GCD resulting from interspecific hybridization has been shown in a few ant species (Cahan & Vinson 2003; Anderson *et al.* 2006). If hybridization caused an atypical reproductive system in *V. emeryi*, it may occur only in populations in which the two morphs cohabit sympatrically. It will be interesting to test these hypotheses by analyzing DNA in the spermathecae of queens and the genetic compositions of allopatric populations.

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