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Biol. Lett. 2006 **2**, 359-363
doi: 10.1098/rsbl.2006.0491

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Clonal reproduction and genetic caste differences in a queen-polymorphic ant, *Vollenhovia emeryi*

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Most social Hymenoptera are characterized by simple haploid sex determination and environment-based caste differentiation. This appears to be strikingly different in the queen-polymorphic ant *Vollenhovia emeryi*. Almost all long- and short-winged queens from a population in Central Japan were homozygous at three microsatellite loci, whereas workers were mostly heterozygous, suggesting either a complex system of genetic caste determination or, more likely, the production of female sexuals from unfertilized eggs by thelytokous parthenogenesis and of workers from fertilized eggs. Furthermore, male genotypes were not compatible with those of the queens and had exclusively the paternal allele found in the sterile, heterozygous workers, probably because males are produced from fertilized eggs after the exclusion of maternal nuclear DNA as recently reported for *Wasmannia auropunctata*. The genus *Vollenhovia* might provide an interesting model system to trace the evolution of unusual caste and sex determination systems.

Keywords: ant; caste; genetic; parthenogenesis; *Vollenhovia*

1. INTRODUCTION

Eusocial insects by definition have a reproductive division of labour, which is often associated with a pronounced queen–worker polymorphism (Wilson 1971; Hölldobler & Wilson 1990). Though caste differentiation is mostly controlled by environmental and social factors (Wheeler 1986), recent studies have documented peculiar aberrations from this rule in a few ant genera. Queens in certain populations of *Pogonomyrmex* harvester ants are always homozygous, whereas workers are heterozygous hybrids (Helms Cahan *et al.* 2002; Julian *et al.* 2002; Volny & Gordon, 2002; Helms Cahan & Keller 2003). In *Cataglyphis cursor*, female sexuals are reared from unfertilized eggs through automictic parthenogenesis and are therefore more frequently homozygous than expected, whereas workers are produced from fertilized eggs (Pearcy *et al.* 2004). Finally, in the little fire ant *Wasmannia auropunctata*, female sexuals are produced by ameiotic parthenogenesis from unfertilized eggs, workers from fertilized eggs, and males from fertilized eggs after the elimination of the maternal nucleus (Fournier *et al.* 2005), resulting in a complete separation of male and female gene pools. Genetic data suggest a similar system of clonal reproduction by males and queens in the Japanese ant, *Vollenhovia emeryi*. This species is queen-polymorphic, i.e. some colonies produce only long-winged queens (L), while others produce only short-winged queens (S).

2. MATERIAL AND METHODS

Vollenhovia emeryi nests in dead trees and fallen branches in secondary forests in central and southern Japan. Colonies with long- and short-winged queens co-occur in sympatry and are polygynous (Ohkawara *et al.* 2002). Wing-size in female sexuals appears to be determined by a genetic factor. From 2001 to 2004, we collected *V. emeryi* colonies in mixed forest near the coast in Kanazawa City in Central Japan.

We amplified three DNA microsatellite loci using primers originally developed for *Temnothorax nylanderi* (L-5, L-18, Foitzik *et al.* 1997) and *Myrmica tahoensis* (Myrt-3, Evans 1993). First, we investigated the genotypes at all three loci in a sample consisting of 23 queens (1–6 per colony), 13 winged female sexuals (present only in five colonies, 1–4 per colony) and 103 workers (10–12 per colony) from 10 L-colonies and 85 queens (1–21 per colony), five female sexuals (present only in three colonies, 1–3 per colony) and 131 workers (10–19 per colony) from 11 S-colonies. Later, we increased the sample size for locus L-5 to 32 queens, 18 female sexuals, 128 workers, and 34 males from 16 L-colonies and 115 queens, 48 female sexuals, 252 workers, and 66 males from 23 S-colonies.

DNA was extracted from heads and thoraces of individual ants and microsatellites L-5 (five alleles: *a* 111 bp, *b* 113 bp, *c* 109 bp, *d* 107 bp, *e* 105 bp), L18 (19 alleles ranging from 163 to 230 bp, *a* 190 bp, *b* 214 bp, *c* 216 bp, *e* 204 bp) and Myrt-3 (14 alleles ranging from 1916 to 255 bp, *a* 223 bp, *b* 235 bp) were amplified as described by Foitzik *et al.* (1997) and Evans (1993). PCR products were analysed on a 310 ABI Genetic Analyser (Applied Biosystems). Microsatellite alleles were identified using GENESCAN v. 3.1 software (Applied Biosystems). Allele frequencies were calculated by weighting colonies according to the number of genotyped individuals. *F*-statistics were calculated using the program GDA (Weir & Cockerham 1984; Lewis & Zaykin 2001).

3. RESULTS

Genotype and allele frequencies differed strikingly between workers and queens and also between L- and S-queens. Almost all queens and female sexuals were homozygous at each of three analysed microsatellite loci. Most queens and female sexuals from L-colonies had the genotype *aa* at locus L-5 (85.7%); the genotypes *bb*, *cc*, *ee* at locus L-18 (78.8%); and the genotype *aa* at locus Myrt-3 (80.0%), while queens and female sexuals from S-colonies had *cc* and *dd* at L-5 (98.9%); *aa* at L-18 (98.8%); and *bb* at Myrt-3 (97.5%). In contrast, workers tended to be heterozygous. At locus L-5, 87% of workers from L-colonies and 98.4% of workers from S-colonies were heterozygous, at L-18 57.9% of L-workers and all S-workers, and at Myrt-3 98.4% of L-workers and 43.9% of S-workers (figure 1). A hierarchical analysis of molecular variance of the complete sample of worker genotypes clearly documented the considerable excess of heterozygotes at L-5 ($F = -0.385$, 95% CI -0.578 to -0.192) and in addition revealed a significant genetic differentiation both among individual colonies (averaged over all loci: $\theta_S = 0.480$, 95% CI 0.283–0.678) and between L- and S-colonies

The electronic supplementary material is available at http://dx.doi.org/10.1098/rsbl.2006.0491 or via http://www.journals.royalsoc.ac.uk.

Received 23 March 2006
Accepted 18 April 2006

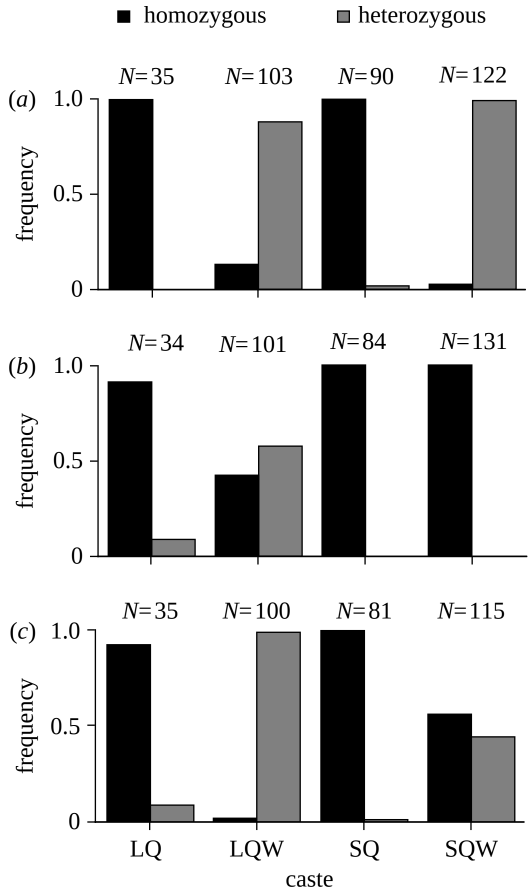

Figure 1. Frequency of homozygous and heterozygous queens and workers from *Vollenhovia emeryi* colonies with long-winged queens (LQ, LQW) and colonies with short-winged queens (SQ, SQW) at the microsatellite loci (*a*) L-5; (*b*) L-18; and (*c*) Myrt-3.

($\theta_S=0.306$, 95% CI 0.166–0.502). Analysing the workers from L- and S-colonies separately did not remove the heterozygote excess at L-5 (L: $F=-0.178$, 95% CI -0.407 to 0.102; S: $F=-0.947$, 95% CI -1.000 to -0.840). The significant differentiation between individual colonies (L: $\theta_S=0.299$, 95% CI 0.255–0.368; S: $=0.146$, 95% CI $=-0.015$ to 0.331) is reflected in the high relatedness among nest-mate workers (L: 0.56 ± s.e. 0.24; S: 0.71 ± 0.06).

The frequencies of heterozygotes among queens and workers differed most at locus L-5 (figure 1; χ^2-test, L: $\chi^2=75.3$, $p<0.0001$; S: $\chi^2=193.0$, $p<0.0001$). Furthermore, the loci L-18 and Myrt-3 were characterized by high allelic diversity with several private alleles found only in S- or in L-colonies (see tables 2 and 3 in the electronic supplementary material). Subsequent analyses with a larger sample were therefore restricted to L-5. They corroborated the result that queens and female sexuals are typically homozygous, while workers are heterozygous with one maternal allele (usually *a*, *c* or *d*) and one allele not found in their respective mothers (table 1). To further investigate the transmission of alleles, we investigated the genotypes of young and old reproductives in the collected colonies. In L-colonies, 13 of the 18 female sexuals (72.2%) had the genotype *aa*, three (16.7%) were *ee*, and one each (5.6%) were *cc* and *dd*. In contrast, 33 of the 48 female sexuals (68.8%) from S-colonies were *cc*, 13 were *dd* (27.1%), and one each was an *aa*-homozygote (2.1%) and an *ad*-heterozygote (2.1%). The tendency was similar at the other two loci (see tables 2 and 3 in the electronic supplementary material). Males from L-colonies had the genotypes *a*, *d*, and *e*. Surprisingly, 57 out of 66 males (86.4%) from S-colonies had the allele *a*, though the queens in their maternal colonies had only the genotypes *cc* and *dd* (table 1). Workers in these colonies typically had the genotypes *ac* (31.3%) or *ad* (61.4%), and males had invariably the paternal allele of the workers' genotype and never the maternal allele.

4. DISCUSSION

Our study yielded three unexpected results. First, S- and L-colonies are genetically differentiated. Second, workers are mostly heterozygous, while queens and female sexuals are homozygous; and third, males at least in S-colonies appear to carry the allele of the queen's mate. The first result can easily be explained by assuming that gene flow between the two types of colony is restricted. It appears that our study population is effectively segregated into two genetic lineages linked with queen polymorphism. Though workers are morphologically indistinguishable, the two lineages might nevertheless constitute different species.

In both lineages, queens are typically homozygous, whereas workers are heterozygous. The data might be explained by a genetic system of caste differentiation as observed in hybridizing *Pogonomyrmex*. For example, the heterozygous L-5 genotypes of S-workers might result from mating between L-queens and S-males or vice versa. This explanation might appear particularly attractive because of the strongly split sex ratios of *V. emeryi* (Ohkawara *et al.* 2002): as many L-colonies produce only female sexuals and S-colonies produce only male sexuals, cross-breeding might be more frequent than mating within the respective lineage. Unfortunately, the different allele frequencies and the genotypes at L-18 and Myrt-3 do not support this hypothesis. Though some worker's genotypes can be explained by inter-lineage mating, others require male alleles that are neither found in L- nor S-queens.

It is more likely that the genotype pattern reflects a situation as in *C. cursor* where female sexuals are reared from unfertilized eggs through automictic thelytokous parthenogenesis, whereas workers are produced by sexual reproduction (Pearcy *et al.* 2004). Several mechanisms of thelytoky are associated with a gradual increase in homozygosity (Suomalainen *et al.* 1987; Simon *et al.* 2003), and the complete or almost complete homozygosity of *V. emeryi* queens might therefore be a consequence of prolonged parthenogenesis. Thelytoky sporadically occurs throughout the Hymenoptera, including social species such as the Cape honeybee (*Apis mellifera capensis*) and a few ants (Hartmann *et al.* 2003; Baudry *et al.* 2004). The combination of thelytokous reproduction to produce female sexual progeny and normal sexual reproduction to produce workers might be advantageous, in

Table 1. Genotype frequencies at microsatellite locus L-5 in 16 L-queen and 23 S-colonies. (‘*a–e*’ and ‘*u*’ mean each allele type and unknown, respectively. Almost queens and female sexuals were homozygous, while most workers were heterozygous for the maternal allele. Furthermore, in most males of S-colonies, the genotypes were not compatible with those of the queens.)

	genotype frequency																									
	queen							worker										female sexuals					male sexuals			
colony no.	*aa*	*bb*	*cc*	*dd*	*ee*	*ae*	*u*	*ab*	*ac*	*ad*	*ae*	*bd*	*de*	*aa*	*ce*	*dd*	*u*	*aa*	*cc*	*dd*	*ee*	*ad*	*a*	*c*	*d*	*e*
L-colony group																										
2010717(1)	3						1	1		1	8							1								
2010722(8)	1										11						1									
2010504(7)	1							4		1	2			4												
2010521(16)	1								1		6			3												
2010718(3)	1										10							3								
2010717(3)	6										5			5				3								
2010717(5)	1									7	3							3	1							
2030701(37)	1								1	10																
2010722(7)	4		1				2	2			3			4	1			2								
2040913(4)	1		2	2														1		1					1	
2010711(2)		1										10														
2030701(39)				1									11													
2040915(1)				2																					6	4
2010711(4)				2									11													
2040915(2)					1																3					10
2040915(5)[a]											3												12	1		
total	20	1	3	7	1	0	3	7	2	19	51	10	22	16	1	0	1	13	1	1	3	0	12	1	7	14
%	57.1	2.9	8.6	20	2.9	0	8.6	5.4	1.6	14.7	39.5	7.8	17.1	12.4	0.8	0	0.8	72.2	5.6	5.6	16.7	0	35.3	2.9	20.6	41.2
S-colony group																										
2030610(33)			1						8	1																
2030923(74)			4						10										5				11			
2030923(76)			1						11										1				6			
2030708(47)			2						9						1											
2030911(68)			5						9						1				9				7			5
2030911(69)			2						10										2				7			
2030911(67)			5						2						8				9					1		3
2030911(70)			5						9						2				7				1			
2010511(4)				3						10																
2010515(14)				5						10																
2010515(20)				1						10																
2010515(1)				8						10																
2010920(2)				6						12																
2010920(3)				11						13							1									
2030923(87)				2						10													7			

(*Continued.*)

Table 1. (*Continued*.)

	genotype frequency																									
	queen							worker										female sexuals					male sexuals			
colony no.	*aa*	*bb*	*cc*	*dd*	*ee*	*ae*	*u*	*ab*	*ac*	*ad*	*ae*	*bd*	*de*	*aa*	*ce*	*dd*	*u*	*aa*	*cc*	*dd*	*ee*	*ad*	*a*	*c*	*d*	*e*
2030923(82)				1						10										9	1		2			
2030923(83)				2						11																
2010919(3)				20		1	7			16						2				3			12			
2010504(5)				7			1			9							1			1						
2010504(6)				2			2			10																
2010515(18)				5			3			9							1									
2010920(1)				16			1			10				1				1					1			
2030923(80)[a]									9														3			
total	0	0	25	89	0	1	4	0	77	151	0	0	0	1	12	2	3	1	33	13	0	1	57	1	0	8
%	0	0	19.4	79	0	0.8	10.9	0	31.3	61.4	0	0	0	0.4	4.9	0.8	1.2	2.1	68.8	27.1	0	2.1	86.3	1.5	0	12.1

[a] Data for the queen's genotype are missing as the queen was not sampled.

that mother queens save the twofold costs of sex, while at the same time benefiting from a genetically diverse worker force that protects them from environmental variation (Gadagkar 2004; Pearcy *et al.* 2004). In such a system, males father only workers. In queenless colonies of *C. cursor*, workers produce female offspring by parthenogenesis (Cagniant 1979), resulting in a considerable fitness return for males through their granddaughters. *V. emeryi* workers, however, appear to lack ovaries (K. Ohkawara 2003, unpublished work).

This leads to our third result: males, in particular those in S-colonies, have alleles that cannot be explained from the genotypes of the queens. Ant males are haploid and normally develop from unfertilized eggs laid by the queen or occasionally also workers. Mothers bequeath one of the two alleles at each locus to their sons. Furthermore, males carry only the paternal allele of the workers' heterozygous genotype. A similar incompatibility of queen and male genotypes was recently reported from *W. auropunctata*, and it was suggested that males arise from fertilized eggs after eliminating the maternal genome and are therefore genetically identical with their father (Fournier *et al.* 2005). This clonal male production might be the response of males to thelytokous production of female sexuals from unfertilized queen eggs in a species with sterile workers, which otherwise would leave males without reproductive success. A phylogenetic analysis that might clearly document the separation of male and female gene pools as in *Wasmannia* (Fournier *et al.* 2005) is not yet possible in *V. emeryi*.

The puzzling genetic composition of *V. emeryi* colonies adds further complexity to the sociogenetic diversity of insect societies. Research on *Cataglyphis*, *Wasmannia*, *Pogonomyrmex*, and now *Vollenhovia* documents that considerably more variation exists concerning the genetic basis of sex and caste determination. To look more into the details of such phenomena will be necessary to fully understand the details of social evolution.

We are grateful to E. Hasegawa and K. Yamauchi for their help on the genetic analysis and field research. Cordial thanks to Y. Fukushima, H. Ishii, and S. Iwanishi for their help in field collection and experiments.

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