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RESEARCH ARTICLE

No gene flow between wing forms and clonal reproduction by males in the long-winged form of the ant *Vollenhovia emeryi*

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Abstract In *Vollenhovia emeryi*, males produced by the short-winged queens (S males) have the same genotype of genomic opsin gene as the long-winged queens (L queens) rather than the cohabiting S queens. This fact suggests that either one of the following two events might have occurred, (1) a recent gene flow between the S males and the L queens or (2) a past hybridization event between them. In order to test these hypotheses, we analyzed the nuclear genome and the mitochondrial DNA of L males, L queens, and sperm from the L queens' spermathecae. Results showed that genotype frequencies differed significantly between L males and L queens and between the sperm and L queens. Mitochondrial haplotypes of the sperm were consistent with their queen. These indicate that (1) queens of the L form mated only with the males that have been produced by L queens, and thus there is no gene flow from S males to L queens; (2) males of the L-form clonally produce sons as males of the S-form do; and (3) genotype similarity between S males and L queens indicates a past hybridization event.

Keywords Gene flow · Clonal reproduction · Microsatellites · Past hybridization event · *Vollenhovia emeryi*

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Introduction

In ants, as in other Hymenoptera, unfertilized haploid eggs develop into males, while females are raised from fertilized diploid eggs (Hölldobler and Wilson, 1990). There are, however, several exceptions to this rule. For example, some ants produce diploid eggs without insemination (Fournier and Aron, 2009; Rabeling et al., 2009). More conspicuous exceptions are male clonalities in *Wasmannia auropunctata* (Fournier et al., 2005) and *Vollenhovia emeryi* (Kobayashi et al., 2008). Although the cellular mechanisms that result in clonal reproduction are unknown, males clonally produce their sons in these species.

Two wing forms (long, L and short, S) are found in *V. emeryi* (Imai et al., 2003). In a population of the S form, Kobayashi et al. (2008) discovered a curious reproductive system in which queens and males clonally produce new queens and sons, respectively. Only workers are produced sexually (Ohkawara et al., 2006). This means that males and females are genetically independent from each other. In fact, each sex was clustered into separate clades on a phylogenetic tree constructed from a distance matrix based on four microsatellite loci (Kobayashi et al., 2008). The reproductive system of the L form is, however, still unknown because the L form colonies have produced few males

during the examined seasons (only 10 males were found in more than 300 sampled colonies during the past 5 years). Thus, we could not assess the genetic differentiation between L males and L queens in the previous study.

Another issue is the genetic similarity between S males and L queens. The S males have the same sequence of the nuclear opsin gene as in L queens, and this sequence differs from that found in the S queens (Kobayashi et al., 2008). It is difficult to explain the concordance between the S males and L queens by chance, because there are several nucleotide substitutions within the examined region of the opsin gene (3 substitutions in 320 nt). Transport of S males from L colonies can be ruled out because S males bear the same mtDNA haplotype as in S queens. The L and S colonies nested sympatrically in the examined population; therefore, the above fact can be explained by one of the following two hypotheses, (1) recent gene flow from S males to L queens, or (2) a past hybridization event. The first hypothesis is consistent with the fact that L colonies produce few males, while many males were found in S colonies during the study seasons (split sex ratio). If L forms have typical haplodiploid sexual reproduction, while S forms have clonal reproduction and reproduce many males, L queens need not produce males as the S forms produce enough. In order to get clarified on this, we require information on the mating partners of the L queens.

In this study, to determine the reproductive system of the L form we examined the nuclear genotypes and mtDNA haplotypes of somatic tissue and the sperm isolated from L queens. Mitochondrial sequence is highly differentiated between the S and L forms (Kobayashi et al., 2008). Individual spermatozoa have a few mitochondria at basal portion of the tail. Thus, we can examine both genomic and mitochondrial genotypes of sperm from a spermatheca. If mitochondrial haplotypes of sperm are different from L queens and similar to S forms, we can conclude that L queens mate with S males. If this is the case, then the genetic similarity between S males and L queens would be explained by the gene flow from S males to L queens. Even in the case of no gene flow (i.e., the same mtDNA haplotype between sperms and L queens), we can examine male clonality in the L form using microsatellite genotype comparisons between the queen's somatic tissue and the sperm isolated from her spermatheca. When there is a considerable differentiation between the sperm isolated from spermatheca and the somatic tissue of the L queen, we can conclude that the L males reproduce clonally. In that case, the similarity in the opsin gene between S males and L queens might have resulted from a past hybridization event between them. This paper provides results from genetic analyses that lead us to deeper insights into evolution of male clonality in *V. emeryi*.

Materials and methods

In September 2007, we sampled ten colonies of the L form from a mixed forest near the coast in Kanazawa Prefecture (Fusho-ji population). Two queens were sampled randomly from each of the ten L form colonies. In addition, we collected ten males from three colonies in September 2009. The thorax of queens and males and the spermatheca of the living queens were removed under a binocular microscope. DNA was extracted using a DNeasy Blood & Tissue kit (Qiagen). Individual thoraxes were crushed in 1.5-ml microcentrifuge tubes with 180 µl of tissue lysis buffer (ATL) attached to the kit. Individual spermathecae were punctured by pulling the surface using two forceps under a binocular microscope, and the spermathecal contents were released into 180 µl of ATL in 1.5-ml tubes. Total DNA was extracted by following the protocol provided with the kit. The DNA was eluted into 50 µl of the elution buffer. Hereafter, the DNA isolated from the thorax of a male, the thorax of a queen, and the content of a spermatheca are termed as male DNA, female DNA, and sperm DNA, respectively.

For nuclear genotyping, we used two microsatellite loci that were originally developed for *Temnothorax nylanderi* Forster (Foitzik et al., 1997; L-5, L-18) and nine newly developed microsatellite loci that were identified by the method of Hamaguchi et al. (2007; Vems 14, Vems 21, Vems 25, Vems 40, Vems 66, Vems 70, Vems 77, Vems 78, Vems 86). The composition of each reaction mixture used was as follows, each 10-µl reaction mixture contained 25 pM of dye-labeled forward and unlabeled reverse primers, 1 µl of dNTP mixture (2.5 mM each), 1 µl of 10× Ex Taq buffer, 0.25 U of Ex Taq DNA polymerase (TaKaRa), 7 µl of distilled water, and 0.5 µl of template DNA. A programmable thermal cycler (PTC-100^{TM}: MJ Research, Inc.) was used for polymerase chain reaction (PCR) that was carried out using the following temperature cycle: 5 min at 94°C followed by 42 cycles of 75 s at 94°C, 75 s at 48, 52, or 54°C depending on the primer pair (details are shown in Table 1), and 75 s at 72°C. The final cycle was carried out at 72°C for 5 min to complete extension. For the analysis of fragment length, PCR products were run on the CEQ2000 XL DNA Analysis System (Beckman–Coulter) with the S400 size standard (Beckmann–Coulter).

After fragment analysis, the pair-wise individual allele sharing distances (ASD) among female DNA and male DNA were calculated (see Fournier et al., 2005). The data matrix was analyzed by the neighbor joining (NJ) procedure in MEGA Ver3.1 (Kumar et al., 2004). We could not estimate the bootstrap value because there was no program that estimates bootstrap values from the type of data set we generated.

Table 1 Details of 11 primer pairs for *Vollenhovia emeryi*

Locus name	Primer sequence	Ta (°C)	A_{queens}	A_{sperms}	Ho	He	HWE test
L-5	F:CCCACGGTAACCCTCGAGAAC	54	5	6	0	0.595	$P < 0.001$
	R:AGCGGAGAAACAGGCGAGAAA						
L-18	F:TGAATTTGGATGGCGGTAGAC	52	12	16	0.15	0.826	$P < 0.001$
	R:ACCTAATGCACGCTTTAGAAT						
Vems 14	F:AAAAACACACGCTCCGTA	52	2	16	0.1	0.095	$P = 1$
	R:CACAGTTTCGCGCTTGAA						
Vems 21	F:AGAAACTAGAGCAACATGAAGTCATCGA	52	6	13	0.3	0.699	$P = 0.012$
	R:CCTCTTGCAAAATTTTCTCCGAAGCT						
Vems 25	F:GAACGCACGGGGGACAGGATTACAA	48	6	10	0.5	0.635	$P = 0.53$
	R:GTTCCAGCGACGAGCGAGGCTAGCCA						
Vems 40	F:AACTCGCGACGAGAACGCCGCTCGT	52	3	6	0.05	0.096	$P = 1$
	R:CAGACTTTCTGCGGTCAGTTGTGCT						
Vems 66	F:ACCATCGACTAGAAAACGGTATGACC	52	9	15	0.1	0.851	$P < 0.001$
	R:GTCGACCTGCAGGCGGCCGCGAAT						
Vems 70	F:ATTATATTTCCATGGGGACCGCCCT	48	2	9	0.1	0.095	$P = 1$
	R:AGGCCGAAGCAAATCCGGTCTTCGA						
Vems 77	F:ACTACCATGTTTCTACCACTCTACT	52	2	7	0.2	0.18	$P = 1$
	R:GATCCGTGGAGACACA						
Vems 78	F:ATTAAGTCAATTTTCAATGCCGCAA	52	9	14	0.15	0.844	$P < 0.001$
	R:CTGATGATGCATCTGTGGGATCT						
Vems 86	F:GTGTCCATTGGGCGAGGGGGAA	48	6	17	0.1	0.558	$P < 0.01$
	R:CTTTCGTTCTCTTCTTTCTAGCCCAT						

In six loci, heterozygosity is significantly less than Hardy–Weinberg proportions (L-5, L-18, Vems 21, Vems 66, Vems 78, Vems 86), which may be caused by automictic parthenogenesis (Pearcy et al., 2006)

Ta annealing temperature, A_{queens} and A_{sperm} number of observed alleles in queens and sperms, respectively, *Ho* observed heterozygosity in queens, *He* heterozygosity expected from the allele frequencies in queens, *HWE test* probability of deviation for Hardy–Weinberg equilibrium (Fisher's exact test with Holm's method)

A target region of the mitochondrial genome was amplified by PCR using a primer pair specific for mtDNA (COI; Mt-21:5′-TTA TAT CAA CAT CTA TTT TGA TTT T-3′, Mt-25:5′-GAT GTG AAA ATA TAG GGG AA-3′), and similarly a nuclear gene was amplified (Opsin; OP11: 5′-GCC CCA TTG TTY GGA TGG AA-3′ and OP16: 5′-AGA GCA SCT CGR TAC TTA GG-3′). The temperature cycle for amplification of COI was as follows: 1 min at 94°C followed by 40 cycles of 1 min at 94°C, 60 s at 45°C, and 7 min at 60°C, and for the opsin the profile was: 1 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 42°C, and 2 min at 72°C. The composition of the PCR reaction mixtures was similar to those used for the microsatellite analysis except that the volume of each reaction component was scaled up so that the total volume was 50 µl rather than 10 µl. The PCR products were purified using the QIAquick PCR purification kit (QIAGEN) and were eluted with 10 µl of sterilized water. The products were sequenced using the DTCS-Quick Start kit (Beckman–Coulter, Fullerton, CA, USA) and a CEQ2000 XL automatic DNA sequencer (Beckman–Coulter, Fullerton, CA, USA) according to the manufacturers' instructions.

Results

A total of 20 queens and 10 males were examined. Two haplotypes were found in opsin DNA sequences (AB334117 or AB334119). These haplotypes are shared among L queens, L males and S males (Kobayashi et al., 2008; this study), and the allele frequency did not differ between males (sum of sperm and males) and queens ($P = 0.15$; data not shown). The NJ tree, which was generated based on the ASD calculated from 11 microsatellite loci, shows two clusters that correspond to each sex (Fig. 1). Although the sample size is small, allele frequencies were significantly different between the sexes (Fisher's Exact Test with Holm's method, $n = 50$, $P < 0.001$, for all the 11 loci). Heterozygosity was significantly lower at six loci in female DNA than the expectation under Hardy–Weinberg

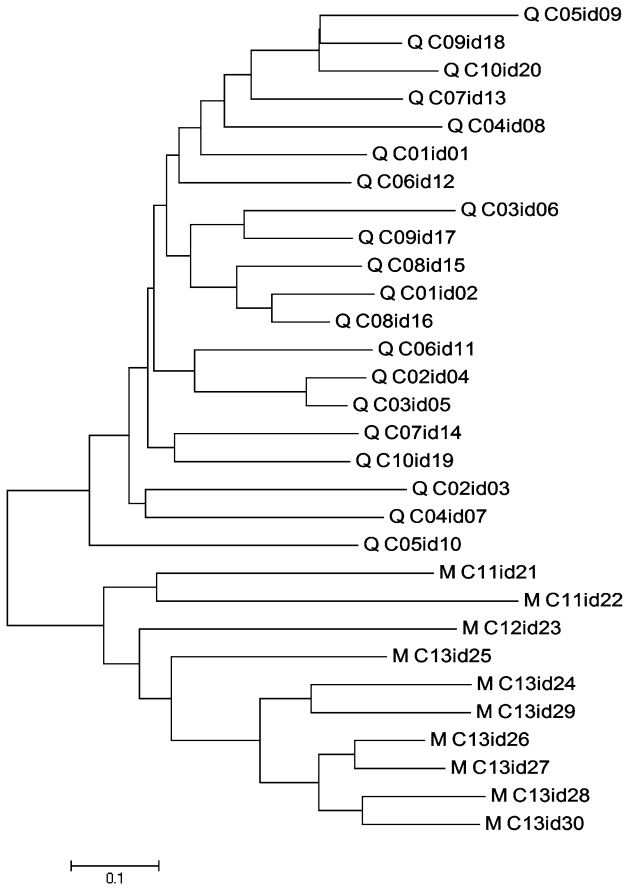

Fig. 1 Neighbor joining tree based on the genetic (microsatellite allele shared) distances. Each individual is identified by sex (*Q* queen, *M* male), colony number, and individual code; these unique identifiers are the same as those in Table 2. The scale bar corresponds to a 10% difference in the genetic distance for a given horizontal branch length

Equilibrium (Table 1). There are two possible causes for this observation: (1) existence of null alleles or (2) automictic parthenogenesis. Automictic parthenogenesis is the fusion of an oocyte and a secondary polar body (terminal fusion), and it has been observed in other ant species (see Pearcy et al., 2006). Even under the assumption that all the queens with one allele carried null alleles, the allele frequencies of the six loci, excluding the assumed null alleles, differed significantly between sexes (Fisher's Exact Test with Holm's method, $n = 50$, $P < 0.001$, for all the six loci).

The alleles of each sample of sperm DNA had at least one locus that had no overlap with the alleles of the queen from which the sperm was isolated (Supplementary online material Table S1). This result demonstrated that the sperm DNAs were not contaminated by queens' tissue. Some of the queens probably mated with multiple males because the sperm samples from these individual queens bore more than one allele and in some cases as many as four alleles (see Table S1).

All pairs of sperm and queen DNA from the L queens had the same mitochondrial haplotype (AB334120), which was different from that of the S type (AB334122; Table 2). This result showed that all the L queens have only mated with L males and not with S males; thus there was no gene flow between S and L forms. Therefore, we suggest that the similarity in the opsin genotypes between L queens and S males (Kobayashi et al., 2008) results from a past hybridization event.

The microsatellite allele frequencies are significantly different between the males (sum of sperms and males) and the queens (Fisher's Exact Test with Holm's method, L-5 $n = 87$, $P < 0.001$, Fig. 2a; L-18 $n = 84$, $P < 0.001$, Fig. 2b; Vems 14 $n = 92$, $P < 0.001$, Fig. 2c; Vems 21 $n = 97$, $P < 0.001$, Fig. 2d; Vems 25 $n = 84$, $P = 0.006$, Fig. 2e; Vems 40 $n = 87$, $P < 0.001$, Fig. 2f; Vems 66 $n = 93$, $P = 0.005$, Fig. 2g; Vems 70 $n = 88$, $P < 0.001$, Fig. 2h; Vems 77 $n = 88$, $P < 0.001$, Fig. 2i; Vems 78 $n = 88$, $P = 0.003$, Fig. 2j; Vems 86 $n = 84$, $P < 0.001$, Fig. 2k). Some alleles are sex specific (Fig. 2). These results indicate there is no gene flow between queens and males in the L form as is the case in the S form.

Discussion

In *V. emeryi*, mtDNA sequences were considerably different between wing forms (Kobayashi et al., 2008). In each form, we concluded that queens mate only with males of the same form because mtDNA haplotypes are the same within the form and different between forms (Table 2). Thus, there is reproductive isolation between two wing forms. In spite of this evidence for reproductive isolation, the genomic opsin genotypes of S males are more similar to those of L queens than to those of cohabiting S queens (Kobayashi et al., 2008). The results of this study suggest that the concordance between the opsin genotypes of S males and L queens results from a past hybridization event because there is no evidence for current gene flow from S males to L queens.

Table 2 Summary of nuclear opsin gene and mitochondrial DNA sequences in *V. emeryi* complex

	Nuclear opsin			Mitochondrial DNA								
	30	153	319	14	15	34	108	176	282	283	304	330
L queens	T	G	R	–	–	–	C	A	G	G	A	C
L males	T	G	R	–	–	–	C	A	G	G	A	C
S queens	C	A	A	A	T	C	A	–	A	A	G	T
S males	T	G	R	A	T	C	A	–	A	A	G	T

Data of S males and S queens were used from Kobayashi et al., 2008. Accession numbers of each haplotype are AB334117–AB334122. R indicates G or A

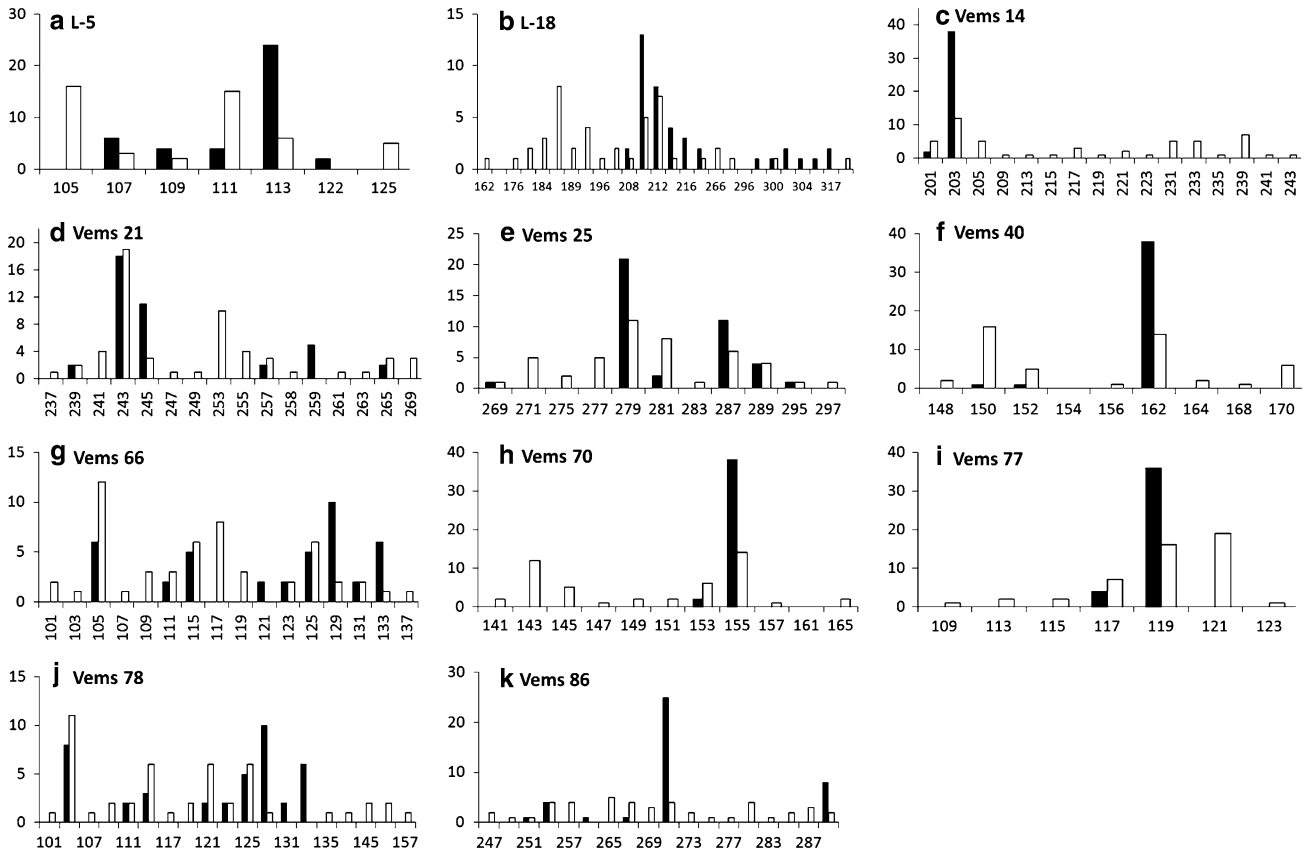

Fig. 2 Allele frequencies of queens (*filled bars*) and males (males and sperms; *open bars*). The *X* axis represents fragment length of alleles and the *Y* axis represents allele frequency. Each graph represents one microsatellite locus. Some alleles were observed only in males or queens, and allele frequencies were significantly different between the sexes. These results indicate that there is no gene flow between the sexes of long-wing form

Concordance by chance is unlikely because there are several nucleotide substitutions within the opsin region (Table 2; Kobayashi et al., 2008; this study). Usual sexual reproduction is needed for gene flows at least in the past. Thus, there are several evolutionary events in the *V. emeryi* complex, including the differentiation of wing forms, the appearance of clonal reproduction of females and/or males, hybridizations between forms, and loss of sexual reproduction. A robust phylogeny is needed to establish the order of these events in the evolution of the *V. emeryi* complex. In this study, we could not obtain a robust phylogeny with an out-group species because there is no candidate out-group species in which all 11 microsatellite primer pairs successfully amplified the target loci. Moreover, the opsin gene sequences do not include enough substitutions between L queens and males (Table 2) to construct a robust phylogeny. An analysis of more rapidly evolving genes of *V. emeryi* with an out-group will bring us important insights into the evolutionary history of *V. emeryi*; including the origin of clonal reproduction by both sexes, past hybridization events, and the loss of sexual reproduction.

In *W. auropunctata*, a typical sexual system coexists with male clonality within populations (Foucaud et al., 2006, 2007, 2009a), and some queens can produce daughter queens clonally or both sexes sexually (Foucaud et al., 2009b). The phylogenetic analyses by Foucaud et al. show polyphyletic occurrence of clonal reproduction and that clonal reproduction by males are only found under existence of female clonality. Based on these results, they argued that male clonality in *W. auropunctata* is likely to be a strategy of the queens rather than the males (Foucaud et al., 2007).

The hypothesis that male clonality is a strategy of the queens can be assessed by determining the cellular mechanism of male-egg production. If queens produce fertile eggs that do not contain a maternal genome, these eggs may develop into clonal males when fertilized by a spermatozoon. In this case, male clonality can be regarded as a queen's tool. Why queens would benefit from clonal males is an issue for future studies. In *Pogonomyrmex*, a mating experiment between two genetic lines has showed that only heterozygotic offspring develop into workers (Cahan et al.,

2004). Physiological constraints on worker production may be a cause of male clonality in *V. emeryi*. Phylogenetic and cytological knowledge will bring us many important insights into the evolution of this extraordinary reproductive system.

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