Phylogenetic Relationships among Populations of *Vollenhovia* Ants, with Particular Focus on the Evolution of Wing Morphology

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Queen polymorphism in wing morphology and thoracic structure provides the opportunity to test hypotheses about mating strategies and colony founding modes. Polymorphic queens can be classified into two types: the winged morph that bears well-developed flight muscles and functional wings on the thorax, and the wingless morph that lacks functional wings because of reduction or absence of wings and flight muscles (Peeters 1991, Heinze and Tsuji 1995). Queen polymorphism with wing reduction is a widespread phenomenon in ant phylogeny (Heinze and Keller 2000, Peeters 2001). The pattern of winglessness varies among species and populations. In the Leptothorax (Leptothorax) sp. A. T. (L.) muscorum, and Harpagoxenus sublaevis (Heinze and Buschinger 1989, Heinze 1998), variation in queen morphology was confirmed in body size, thorax sclerites, and wing length. In these cases, because the variations in queens are continuous, the queen polymorphism is an intraspecific variation within a population, whereas in other cases wingless morphs completely replace winged morphs in reproductive roles in the colony and never cohabit in the same colony (Heinze 1998). Genetic differentiation between morphs may explain the absence of colonies producing both morphs. In the latter cases, genetic isolation between morphs may have occurred.

Some studies have indicated that mating behavior and queen polymorphism with wing reduction promotes genetic differentiation, leading to speciation (Bourke and Franks 1991, Seifert 2010). In general, wing reduction in queens is strongly associated with alternative dispersal strategies and colony-founding modes (Bolton 1986, Buschinger and Heinze 1992, Bourke and Heinze 1994, Heinze and Tsuji 1995). The winged morph founds new colonies independently after mating flights, whereas the wingless morph mates near or within the natal nest and founds new colonies by budding. This difference in mating behavior and colony-founding mode between winged and wingless queens may induce genetic isolation between the queen morphs. In particular, this difference in mating behavior may be a factor that promotes genetic isolation between the two groups because this difference may prevent mating between the morphs and lead to reproductive isolation between them. Thus, wingless and winged queens may be genetically isolated.

Vollenhovia emeryi is a polygynous ant species showing wing polymorphism in queens (Japanese Ant Image Database 2003), with long-winged (L-queen) and short-winged (S-queen) morphs (Fig. 1). The short-winged morph is unable to fly, and their wings do not extend beyond the length of the body, though their shape and length vary among populations (Kubota 1984). In the population of Kanazawa city in central Japan, the average wing length in L- and S-
queens was found to be $3.0 \pm SD 0.15 \text{ mm}$ ($N = 210$) and $2.0 \pm 0.24 \text{ mm}$ ($N = 68$), respectively (K. Ohkawara, unpublished data). In addition, the S-queens were slightly smaller than the L-queens. For example, the length of the thoracic flight muscles was shorter in S-queens ($0.59 \pm 0.02 \text{ mm}, N = 139$) than in L-queens ($0.68 \pm 0.04 \text{ mm}, N = 247$) and the head width of S- and L-queens was $0.54 \pm 0.02 \text{ mm}$ ($N = 160$) and $0.56 \pm 0.02 \text{ mm}$ ($N = 1602$), respectively. The two queen morphs have not been found coexisting in the same colony in the field (Ohkawara et al. 2002).

Because long wings are the typical morphological characteristic of ant queens and *Vollenhovia* species, the short-winged morph of *V. emeryi* may have evolved from the long-winged morph. However, the origin of an evolutionary process leading to the short-winged morph is unclear. The observation that the two queen morphs occur in separate colonies suggests that they may be genetically isolated. Kinomura and Yamauchi (1994) suggested that the two queen morphs belong to two different species based on the difference in the habitat of populations in Gifu city, central Japan: L-queen colonies mostly were distributed in mountainous forests, whereas S-queen colonies were distributed in flatland forests. Although differences in nuclear and mitochondrial DNA between L- and S-queens have been reported within a population of *V. emeryi* (Kobayashi et al. 2008, 2010), whether these differences exist across the distributional range of the species has not yet been investigated. In the current study, we examined the phylogenetic relationships among multiple populations of the two queen morphs of *V. emeryi* and other Japanese *Vollenhovia* species. Based on our findings, we discuss the relationship between wing morphology and genetic differentiation between the two queen morphs.

### Materials and Methods

**Collection of Samples.** L- and S-queen colonies were collected from 17 locations in central Japan from May to August 2005 and 2006. The collection locations and additional sample information are presented in Fig. 2 and Table 1. A distance of $>100 \text{ m}$ was maintained between collection locations. Branches in which *V. emeryi* nested were collected and transported to the laboratory. Because the resident egg-laying queen already had shed her wings in established colonies, we identified the queen morph by checking the wing morphology of the daughter queens in the collected colonies and excluded colonies without winged queens from later analysis. Only one queen from each colony was used for phylogenetic analyses because each nest mate would bear the same mtDNA. For phylogenetic analyses, queens of related species *V. nipponica* also were sampled. This ant is a workerless ant species that parasitizes *V. emeryi* colonies (Kinomura and Yamauchi 1992). Consequently, they were collected from *V. emeryi* colonies. In addition, the workers or queens of three congeneric species—*V. benzai*, *V. okinawana*, and *V. yambaru*—were used as outgroups. Two other congeneric species were known from isolated islands of Japan, but we could not collect samples of these species. After species identification, the specimens were preserved in 99% ethanol until DNA extraction.

**DNA Preparation and Sequencing.** Total DNA was extracted from each sample by using a DNA extraction...
kit (QIAquick DNA Tissue Kit, Qiagen, Valencia, CA). The DNA was eluted in 200 µl of the buffer provided with the kit. We amplified an approximately 2,600 nt region of the mtDNA genome encoding cytochrome oxidase subunit I (COI), tRNA-Leu, cytochrome oxidase subunit II (COII), tRNA-Lys, tRNA-Asp, ATP synthase subunit six (ATP6) and part of cytochrome oxidase subunit III (COIII), by using a long polymerase chain reaction (PCR) method with the following primer pair: (21v2; forward) 5' - ATATTCACAATTGGGTTAGATGGAG-3', (R8v2; reverse) 5' - AGCTGCGGCTTCAAATCACA-3'. The PCR cycle comprised 1 min at 94°C followed by 40 cycles of denaturation for 10 s at 96°C, extension for 3 min at 60°C, and a final extension step of 5 min at 72°C. Each 100-µl reaction mixtures contained 25 µM of each primer, 10 µl of dNTP mixture (2.5 mM each), 10 µl of 10× Ex Taq buffer, 2.5 U of Ex TaqDNA polymerase (Takara, Osaka, Japan), 65 µl of sterilized water, and 5 µl of template DNA. The PCR products were purified using a QIAquick PCR Purification Kit (Qiagen) and were eluted in 10 µl of sterilized water. The products were sequenced using the DTCS-Quick Start Kit (Beckman Coulter), and each primer for sequencing (Ve10: TCAATTCACGTAGTCCCTA, Ve04: GTATGGGTTATTTGAGGA, Ve14: ATATTTATATGGGTGAA, Ve25: CATTGTGAAAATATAGGGGA, Ve51: ATCGCCGGCCACCTACTATT, Ve41: CGGGTATTCCCATCACTAAT, Ve30: AAAATATGACCCCTCGTA, Ve38: TTTCATCTTCCTCTCCT). We confirmed that the sequences were mitochondrial and not pseudogenes in the nucleus by searching for open reading frames (Stothard 2000) and t-RNA (Schattner et al. 2005).

**Phylogenetic Analysis.** Whole sequences were aligned using ClustalW (ver. 1.8.3; Thompson et al. 1994) for all taxa. Because phylogenetic signals decay at sites where many replacements occur and mutations saturate (Misof et al. 2001, Hasegawa and Kasuya 2006), saturated sites often are excluded from analyses (Engstrom et al. 2004). In mtDNA, four partitions, namely, tRNA and three codon positions, are known as bearing different number of mutations (Li 1997). To evaluate the degree of saturation for these partitions, we plotted each substitution type against the Kimura 2-parameter distance, K2D (Kimura 1980), between each pair of sequences; saturation is indicated when a decrease is observed in the substitution rate over time.

![Map of Japan and locations of the sampling sites listed in Table 1. The species name and morph type (L, S, and P indicate L- and S-queens of *V. emeryi* and the parasite, *V. nipponica*, respectively) are given in parentheses.](456.jpg)
We excluded a partition from phylogenetic analysis when the saturation was observed in that partition.

We used the MEGA computer program (version 4.0; Kumar et al. 2008) to infer phylogenies by using the neighbor joining (NJ) and maximum parsimony (MP) methods. In the NJ method, evolutionary distances were computed using the maximum composite likelihood method (Tamura et al. 2004). In the MP method, MP trees were searched using the close-neighbor-interchange algorithm (Nei and Kumar 2000). PAUP* (version 4.0b10; Swofford 2003) was used for the maximum likelihood (ML) method and MrBayes (version 3.1.2; Ronquist and Huelsenbeck 2003) for the Bayesian inference analysis (Bayes). For the ML and Bayes methods, using the program KAKUSAN3 (version 3.0; Tanabe 2007), the most appropriate nucleotide substitution model for whole sequences was selected using the Akaike information criterion and Schwarz’s Bayesian information criterion, respectively. MrBayes was run for 4,000,000 generations with four chains and a tree sampling frequency of 1,000 generations. The Bayes consensus tree was generated from the last 1,000 sampled trees. To test whether the S-queens are a monophyletic group, we inferred an ML tree with negative constraint on the monophyly of S-queens by using the same method to infer the nonconstraint ML tree and compared the tree with the nonconstraint ML tree by using CONSEL (version 0.20; Shimodaira and Hasegawa 2001). The final data set included 14 S-queens, 14 liters-queens, 10 queens of V. nipponica, three queens of V. benzai, and one queen each of V. okinawana and V. yambaru.

## Results

### Sequence Characteristics and Alignment

In our sampling, L- and S-queens did not cohabit a single colony, even though both morphs were collected from the same locations, namely, Fusyohji, Utugimati, and Hirakamati, (~30 m apart in each location, Fig. 2). Ten queens of V. nipponica (social parasite) were collected only from S-queen colonies at seven locations. The sample included 14 S-queens, 14 liters-queens, 10 queens of V. nipponica, three queens of V. benzai, and one queen each of V. okinawana and V. yambaru.
open reading frames and tRNA search showed that all the sequences contained genes encoding part of COI (282 nt), tRNA-Leu (68 nt), COII (714 nt), tRNA-Lys (72 nt), tRNA-Asp (70 nt), ATP8 (156 nt), ATP6 (669 nt), and part of COIII (225 nt). The order of the protein-coding region in the mitochondria of these ants was the same as that of the honey bee (Crozier and Crozier 1993). However, in the sequence of V. emeryi and V. okinawana we found a region (~400 nt) in which no open reading frame or tRNA was found. Such a noncoding region has not been reported previously in other hymenopteran mtDNA sequences (Hasegawa et al. 2011). The alignment of the noncoding region was problematic because the alignment score for this region was low (6–63) between species. Considering the potential dangers associated with poor alignments (Yoshizawa 2010), we excluded this noncoding region from the phylogenetic analysis. Thus, the final data set included 2,210 positions, deposited in TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:S12129), and in which 559 variable sites, 484 parsimony-informative sites, and 1,651 conserved sites were found.

Phylogenetic Relationships Among Taxa. Saturation tests showed no evidence of saturation between sequences (data not shown) and showed that substitution rates linearly increased with time, even in the fastest-evolving region (transitions in the third codon position). These findings suggest that the dataset includes very little noise for phylogenetic inference, and that most commonly used analytical methods would yield accurate phylogenetic estimates. KAKUSAN3 showed that the most adequate model for whole sequences without the noncoding region is the HKY85 + gamma model for both the ML and Bayes methods. Figure 3 shows the Bayesian inference topology based on the HKY85 + gamma model. At the end of the run in the Bayes method, deviation of split frequencies was
low (<0.005). For major taxa, all methods produced phylogenetic trees with the same topology; (out-groups [V. nipponica; L, S-queen morphs]). The S-queen morphs were monophyletic in a clade of V. emeryi (95 and 90 bootstrap supports in the NJ and MP methods, respectively, and posterior probability 1.00), and the approximately unbiased test (Shimodaira 2002) showed a significant difference between the nonconstraint ML tree and the ML tree with negative constraint on the monophyly of S-queens (P = 0.021). In addition, queens of V. nipponica were monophyletic and inferred as sister to the V. emeryi clade.

Discussion

The inferred phylogenetic relationship of L- and S-queens was different from that found for other queen polymorphic ants in previous studies. Rüppell et al. (2001) investigated the phylogenetic relationships between the subpopulations of dimorphic queens, microgyne, and macrogyne in Temnothorax (Leptothorax) cf. andrei. The two queen morphs had similar allele distributions for four microsatellite loci; they were clustered in each population, showing that the two queen morphs could be regarded as the same species. In the harvester ant Pogonomymex piina Wheeler with winged and wingless queens, Johnson et al. (2007) showed that these two queen phenotypes belonged to the same gene pool and the same mitochondrial clade. Some winged and wingless queens had identical mitochondrial haplotypes. In these cases, queen polymorphism represented inaspecific variation in a population. Our current study, however, revealed that L- and S-queen morphs of V. emeryi are genetically differentiated in their maternal genomes as predicted by Kinomura and Yamauchi (1994). S-queen morphs are clustered and form a monophyletic group apart from L-queen morphs. Furthermore, the approximately unbiased test supported the robustness of the monophyly of S-queens. Although both morphs were collected from the same sampling locations in many cases (Fig. 2), samples clustered phylogenetically based on winged morphs and not on collection locations. The current study is the first of which we are aware in which two queen morphs can be regarded as a polymorphism that is genetically differentiated. In some cases of queen-polymorphic ants, wingless queens fulfilled a reproductive role in a colony without cohabiting with winged queens like S-queens in V. emeryi. Our results suggest that wingless queens can be genetically differentiated from the winged morph in ant species in which the morphs never cohabit within a colony.

In V. emeryi, wing reduction is considered to have occurred once, as shown in the phylogenetic tree (Fig. 3). Factors promoting the evolution of the short-winged morph in V. emeryi remain unclear. As suggested in some studies on queen polymorphism, the wing reduction may be an adaptation to environments where resources and habitats are patchily distributed (Bourke and Heinze 1994). In general, dependent colony founding and short dispersal are favorable for new queens in such environments. The density and distribution of resources for V. emeryi may act as selective pressures that favor wing reduction in queens. Moreover, the mating behavior of S-queens is different from that of L-queens (Ohkawara et al. 2002). L-queens mate during the nuptial flight, whereas S-queens almost always mate with males in the natal nest. The evolution of intranest mating in S-queens probably is related to wing reduction. The difference in mating behavior may therefore promote the genetic isolation of the two queen morphs.

In Vollenhovia species, several remarkable characters have evolved—wing reduction, social parasitism, and male clonality. The phylogenetic analysis presented here provides insights into the evolution of wing reduction. Additional phylogenetic analyses will permit further exploration of the evolution of other characters. For example, in this study the social parasite V. nipponica formed a monophyletic sister clade to the host species V. emeryi. The cluster showed that this parasitic ant diverged before the divergence of L- and S-queen groups. Further phylogenetic analysis will be useful for clarifying the evolutionary origin of social parasitism in the genus Vollenhovia. In other social parasitic ants, phylogenies have indicated that social parasites evolved from the host (Parker and Rissing 2002, Savolainen and Vepsäläinen 2003, Sumner et al. 2004). Additional sequence data will allow us to test more rigorously whether the phylogenetic relationships found between host and parasite of other ants also occurs in the genus Vollenhovia. Furthermore, a unique reproductive system in which each sex was clonally produced by each sex was discovered in L- and S-queen populations of V. emeryi (Ohkawara et al. 2006; Kobayashi et al. 2008, 2010; Okamoto and Ohkawara 2009, 2010). Whereas workers are produced sexually, queens are produced by parthenogenesis, which gives rise to genetically homogeneous clonal lineages. More remarkably, haploid males are produced as a clone of their father by some mechanism. In another ant, Wasmannia auropunctata Roger, this unique reproductive system was observed in some populations but not in others (Fournier et al. 2005, Foucaud et al. 2006). In contrast, the male clonality in V. emeryi was observed in both morphs, which are genetically isolated in their maternal genomes. Thus, unlike male clonality in W. auropunctata, that in V. emeryi may occur across all populations. In addition to our phylogenetic analyses based on mitochondrial genes, other genomic analyses in V. emeryi and closely related species will provide important information about the evolution of this unique reproductive system. Thus, the results of our phylogenetic analysis should be useful in future studies of reproduction systems and social parasitism in the genus Vollenhovia.

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