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Genome Size Estimation of the Two Wing Morphs of *Vollenhovia emeryi* (Hymenoptera: Myrmicinae)

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에메리개미 (*Vollenhovia emeryi* Wheeler)의 날개이형체의 유전체 크기 추정

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ABSTRACT: In *Vollenhovia emeryi* (Hymenoptera: Myrmicinae), the queen and the male are known to be clonally reproduced. Its colonies can be classified into the two morphs with the wing length of the queen caste. The morph with normal wings is called the long-winged and the other the short-winged that is brachypterous. Even though the two morphs are considered a species, investigation on the species status of the two morphs was suggested with natural separation in nature and the distinctive wing morphology. It has yet to be determined whether the clonally reproduced queen caste is haploid or diploid. Our data clearly show that the two morphs are the same species and the queen caste is diploid on the basis of the genome size data comparison.

Key words: *Vollenhovia emeryi*, Wing polymorphism, Genome size, Ploidy level

초 록: 에메리개미는 여왕개미와 수개미가 유전적으로 복제되어 번식한다고 알려져 있으며, 여왕개미의 날개형태가 장시형과 단시형으로 나타난다. 장시형은 정상적인 날개형태이고, 이보다 짧은 날개형태는 단시형이라고 한다. 장시형과 단시형 모두 한 종으로 취급되지만, 두 가지 점에서 종 지위에 대한 조사가 필요하다. 첫째, 자연 상태에서는 두 날개형이 함께 발견되지 않고, 둘째, 날개형이 육안으로 뚜렷하게 구분된다. 또한 복제되어 번식한 여왕개미가 단수체인지 배수체인지 조사가 필요하다. 따라서 우리는 본 연구에서 에메리개미 유전체 크기를 추정하여 두 날개형은 동종이며, 여왕개미는 배수체임을 확인하였다.

검색어: 에메리개미, 날개다형성, 유전체 크기, 배수성

Defining the boundary of species is of necessity because a species, a group having equivalent properties, is the basic units for science of living organisms. Morphological characteristics are principal for elucidating either the phylogeny or the taxonomic classification. In addition, morphological classifications, in spite of its limited resolution, afford fundamental information for diagnostic monophyletic estimation (Scotland et al., 2003). In insect taxa, the evolution of wings enabled the insects to become the most thrived group on Earth through provisioning new niches and mobility (Whiting et al., 2003). Judging by the tremendous diversity of their shape, size and color patterns, the wings fulfill the important role to classify the insects (Parchem et al., 2007).

Vollenhovia emeryi (Hymenoptera: Myrmicinae) is endemic in East Asia. It is further classified into the two morphs with the wing length of the queen caste. The morph with normal

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wings is called the long-winged (L) queen and the other the short-winged (S) queen with aberrantly small wings (Fig. 1) (Kubota, 1984). In contrast to the queen caste, the morphology of the worker and male caste of the two wing morphs is identical. Previous researchers suggested that it should be clarified whether the two morphs may be considered the same species with the reason of natural separation in nature and the distinctive wing morphology (Kinomura and Yamauchi, 1994; Kubota, 1984). Furthermore the mitochondrial genotyping of Japanese populations of the two morphs revealed substantial genetic differentiation between the two morphs (Kobayashi et al., 2012; Ohkawara et al., 2006). Likewise, *V. emeryi* samples collected in South Korea could easily be distinguished with the mt-CO1 genotype and the *Wolbachia* infection status (Noh, 2014). In addition, the gyne, i.e. newly emerged queen with wing, and the male are clonally reproduced in the ant (Kobayashi et al., 2008, 2011; Ohkawara et al., 2006), i.e. the queen caste holds only the maternal half of the genome and the male the paternal half of the genome. For this reason, we intend to measure the ploidy level and the genome size of the two morphs to determine the ploidy level of the queen caste and the species status of the two morphs.

Materials and Methods

Collection of *V. emeryi* samples

Colonies of the long-winged were collected from Yeongju-si, Gyeongsangbuk-do, Gimhae-si, Gyeongsangnam-do, and the short-winged from Yeosu-si, Jeollanam-do in 2013 (Table 1). Each colony was kept in an insect breeding jar (SPL life sciences, cat. No. 310102) with rotten wood chips for main-

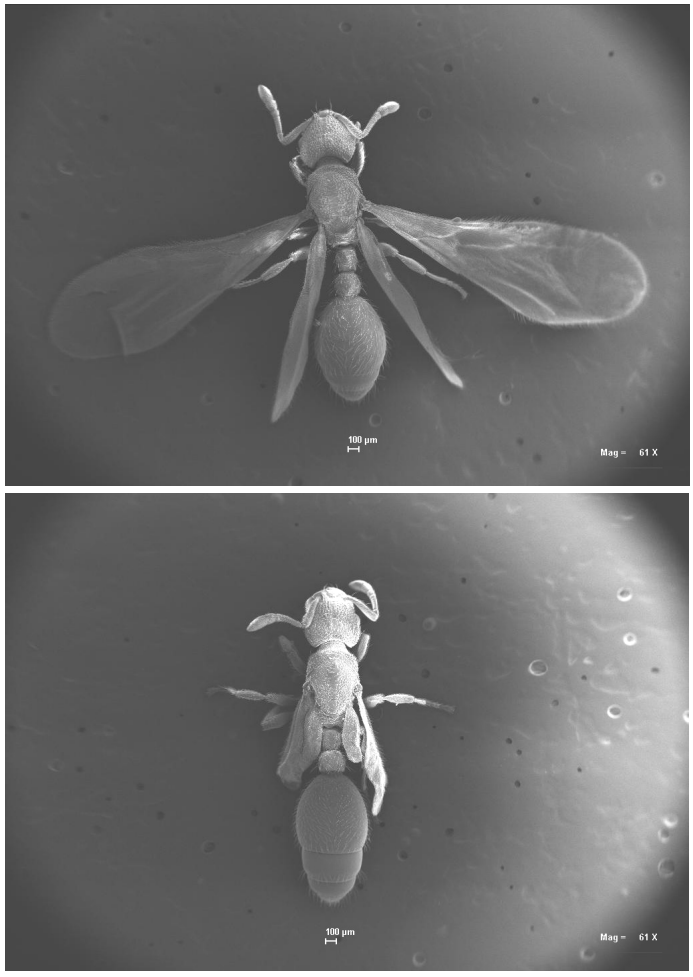

Fig. 1. Two morphological types of *V. emeryi* queen: (a) long-winged morph (L-queen) and (b) short-winged morph (S-queen).

taining moisture until the measurement was performed. They were provided with 100% non-sterile honey and commercial dried mill worm as nutrient source.

Flow cytometry analyses

Flow cytometry was performed to estimate the genome size of the two wing morphs. Whole castes of the two wing morphs were examined (i.e. queen, worker, male). Sample preparation was modified from the method for plants and a wasp species (Galbraith et al., 1983; Tulgetske, 2010). Individual heads of adult *V. emeryi* were cut and ground with a pestle in 0.4 ml of the ice-cold Galbraith buffer (21 mM $MgCl_2$ 2 g, 30 mM tris-sodium citrate dehydrate 8.823 g, 20 mM MOPS solution 20 ml, 0.2% Triton X-100 2 ml, 1 mg/L RNase A 0.001 g in 1 L of distilled water, and adjusted to pH 7.2) in order for extracting the brain cells. Brain cells of *Drosophila melanogaster* prepared simultaneously were mixed into queen and worker samples as the internal standard. In the case of males, to avoid being piled up with the *D. melanogaster* peak, queen brain cells were used as the internal standard for measurement. After the volume of the buffer was adjusted to 0.75 ml, the sample was filtered through 20 μm nylon mesh filter (Millipore, Ireland). Each sample was mixed with 55 μl of Propidium Iodide (1 mg/ml) (Sigma-Aldrich, USA) and incubated for 4 hours at 4°C. The mean fluorescence of stained nuclei in each sample was measured using a Becton Dickinson FACSCalibur with a laser source (488 nm). CELLquest Pro software was used for analyzing 10,000 nuclei. Additionally the mean genome size of each castes of the two wing morphs was calculated according to the following formula (Dolezel and Bartos, 2005):

Sample 2C DNA content (pg) =
(Mean G_0/G_1 peak of the sample × Standard 2C DNA content) × (Mean G_0/G_1 peak of the standard)$^{-1}$.

The calculated genome size represented in C-values (pico-grams) was converted in megabase pairs based on Dolezel et al. (2003) (1 pg = 978 Mbp). For haploid males, the sample 2C DNA content in the above formula was substituted to 1C DNA.

Colony examination and molecular identification

A long-winged gyne was found in the short-winged colony by the periodic observation and scan of whole colonies reared in the laboratory. To examine potential invasion of the gyne into the colony by accident and the heredity of the long-winged gyne from the short-winged colony, she was isolated with ten workers and a male. After a month, a larva and a pupa produced by her were collected from that colony and genomic DNA was extracted from them using a commercial kit (Qiagen DNeasy Blood and Tissue kit, Hilden, Germany). PCR targeting the mitochondrial cytochrome C oxidase I (COI) region was carried out to confirm the CO1 sequence identity. PCR was performed with two primer sets, LCO-a (5'-CCY CGW ATA AAT AAY ATA AGA TTT TGA-3')/HCO-a (5'- TAA ACT TDG GRT GWC CAA AAA ATC A-3') and Engel (5'-GAG GAG GAG ACC CCA TTT TAT-3')/Pat (5'- TCC AAT GCA CTA ATC TGC CAT ATT A-3'), to obtain two parts of partial COI sequence. Maxime™ PCR PreMix Kit (iNtRON biotechnology, Korea) was used, with 16 μl double distilled water, 1 μl 10 pmol of each primer and 2 μl DNA template. The temperature profile for the PCR was an initial denaturation step of 3 min at 95℃ followed by 35 cycles at 95℃ for 1 min, 48℃ for 30 sec, 72℃ for 1 min and the final extension step at 72℃ for 2 min. To test the infection status of *Wolbachia*, diagnostic PCR was performed using the *Wolbachia* specific primer set: Wol438-f (5'-CAT ACC TAT TCG AAG GGA TAG-3')/Wol438-r (5'-AGA TTC GAG TGA AAC CAA TTC-3'), with the same experimental materials mentioned above. The temperature profile for the PCR was an initial denaturation step of 2 min at 94℃, followed by 38 cycles of 30 s

Table 1. Sampling localities and geographical information of the *V. emeryi* colonies

Sample lab code.	Wing morph	Location	Latitude	Longitude	Collection date
YNJ	Long	Yeongju-si, Gyeongsangbuk-do	36°48'N	128°36'E	Aug 2013
GH	Long	Gimhae-si, Gyeongsangnam-do	35°15'N	128°52'E	May 2013
DS34	Short	Yeosu-si, Jeollanam-do	34°43'N	127°44'E	Apr 2013

at 94℃, 45 s at 55℃, and 90 s at 72℃, and a final extension step of 10 min at 72℃ (Jeong et al., 2009). PCR amplifications were conducted in PTC-100™ Programmable Thermal Controller (MJ Research INC. USA) or PeqStar Universal gradient (Peqlab Gmbh, Germany). The PCR amplicons were separated on a 1% agarose gel dyed with TopGreen Nucleic Acid Gel Stain (Biopure). The two mt-CO1 PCR amplicons were purified using a commercial kit (Qiagen Qiaquick PCR purification kit, Hilden, Germany) and sequenced. The resultant sequences were combined into 1239 bp and aligned on MEGA (ver. 5.2) (Kumar et al., 2008; Kumar et al., 1994).

Results

Genome size estimation and ploidy examination

The histogram shows that the number of nuclei peaks of the queen and worker exhibited two-fold values than that of *D.*

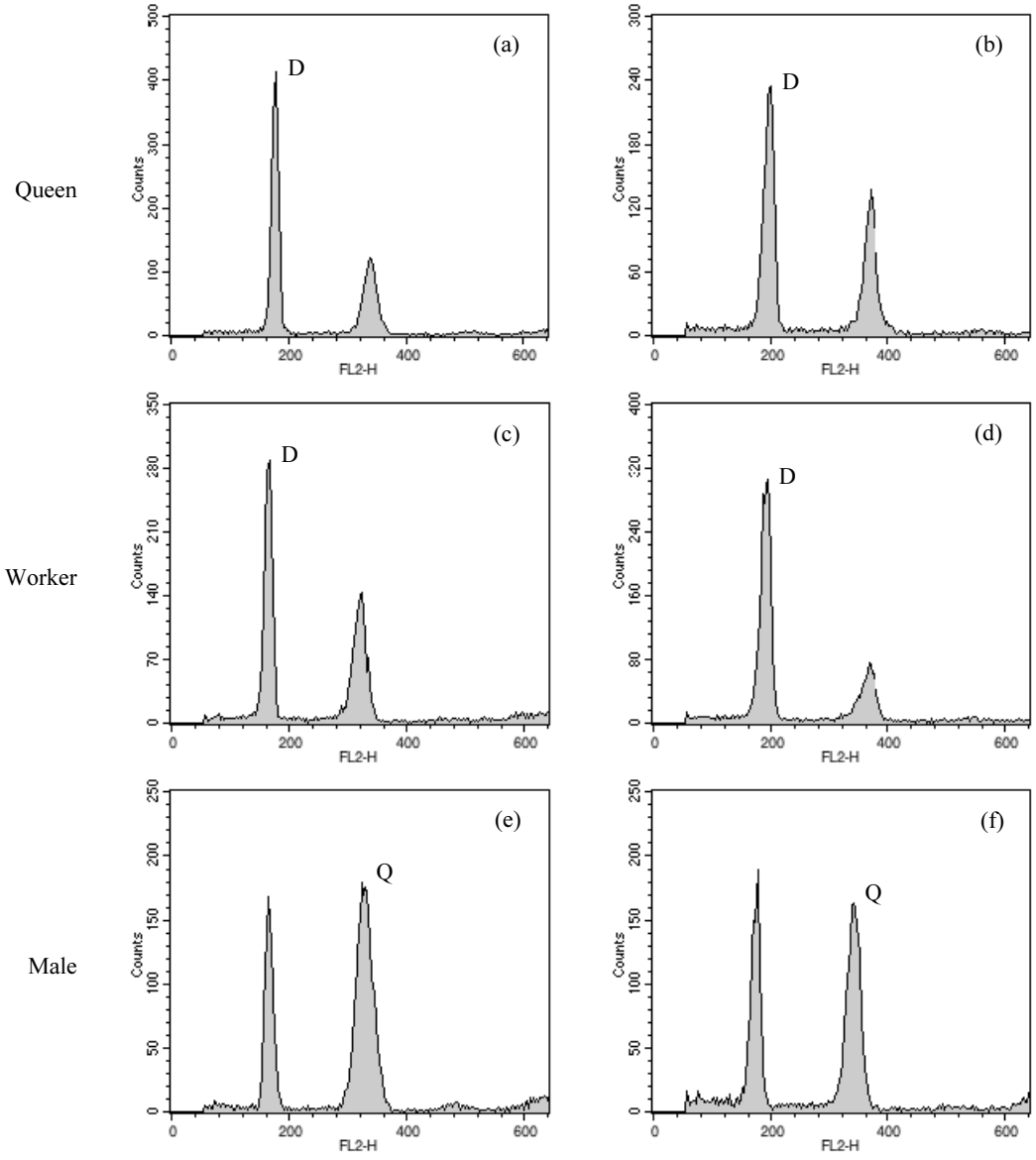

Fig. 2. Flow cytometric analysis of *V. emeryi* two wing morphs. As the internal standard, *D. melanogaster* (D) was added in queen and worker samples, and queen (Q) with the corresponding wing morph with sampled male was added in the male samples. (A) Long-winged queen (B) Short-winged queen (C) Long-winged worker (D) Short-winged worker (E) Long-winged male (F) Short-winged male. Vertical axis: number of nuclei and horizontal axis: fluorescence intensity.

melanogaster (Fig. 2A-2D). The mean haploid genome size values of the *V. emeryi* was estimated as 332.5 Mbp (C = 0.34 pg), and coefficient of variations ranged from 3.19 to 4.77%. Therefore, the queen caste should be diploid as the worker caste is, and the male caste is haploid. In addition the genome size of the whole castes of the two morphs is extremely similar with each other, if not identical (Fig. 2).

Production of a long-winged gyne from a short-winged colony

The partial CO1 sequences of the larva and the pupa from this long-winged gyne were identical to that of the original colony where the long-winged gyne emerged (Date not shown). All collected sequences were deposited in GenBank (accession number: KF815883, KF850415, KF850416). Furthermore, the larva and the pupa are free of *Wolbachia* as the short-winged colony (Sample lab code: DS34), the origin of their long-winged mother, is (Fig. 3) (Noh, 2014). Therefore, such facts confirm that the long-winged gyne is from the short-winged colony.

Discussion

As is the case in most other bees and ants species, the two female forms are diploid and the male is haploid. The interesting question arising in the genome size analysis is how the queen-destined egg, i.e. egg, restores the diploid status. The reproductive parasite, *Wolbachia*, induce diploid restoration in such Hymenopteran insects as *Trichogramma* complex and *Muscidifurax uniraptor* via gamete duplication and nuclear fusion and *Rickettsia*-induced ameiotic mechanism (Adachi-Hagimori et al., 2008; Giorgini et al., 2010; Jeong and Suh, 2008). Unlike the diploidization induced by infection, diploid restoration in the queen caste occurs regardless of infection status.

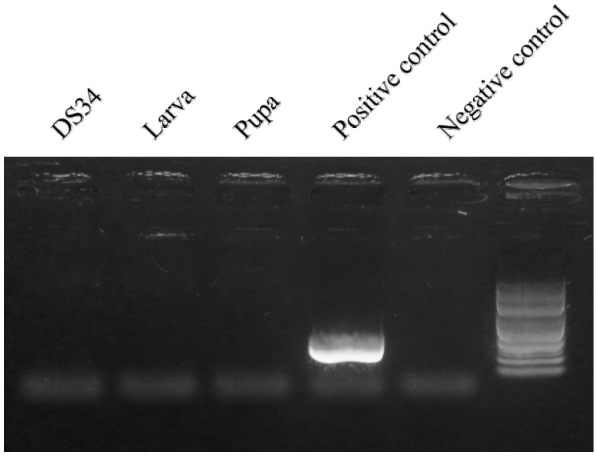

Fig. 3. Amplification results of the *Wolbachia* specific PCR. The size of the PCR amplicons was examined with the 100 bp ladder.

The anomalous emergence of the long-winged gyne from a short-winged colony may not be too rare. It provides the conclusive evidence about the species status of two wing morphs of the ant. Moreover, the result strongly suggests that the wing formation is under the control of epigenetic mechanisms in this species. Our results can be grounds for the hypothesis that the wing formation is governed by the silencing or re-expression of relevant genes, then it induces a feature being lost and later recovered in an evolutionary lineage. Because the development and formation of wing is regulated by the multiple developmental hierarchy: gene, transcription, protein and receptor, a simple malfunction in any of these stages may lead to winglessness (Kim et al., 1996; Whiting et al., 2003). Given this point, it is probable that the reason of the emergence of the long-winged gyne from the short-winged colony is a temporary malfunction in wing developmental hierarchy. In the ecological perspective, it may occur in response to the environmental stress that the artificial rearing condition may induce.

Collectively, the two morphs of the ant are the same species and the queen caste is diploid on the basis of the genome size estimation data, partial CO1 sequence and *Wolbachia* infection. Moreover, the species status of the two morphs is conclusively supported by the emergence of a long-winged queen from a short-winged colony.

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