Association between host wing morphology polymorphism and *Wolbachia* infection in *Vollenhovia emeryi* (Hymenoptera: Myrmicinae)

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**Abstract**

Many eusocial insects, including ants, show complex colony structures, distributions, and reproductive strategies. In the ant *Vollenhovia emeryi* Wheeler (Hymenoptera: Myrmicinae), queens and males are produced clonally, while sterile workers arise sexually, unlike other ant species and Hymenopteran insects in general. Furthermore, there is a wing length polymorphism in the queen caste. Despite its evolutionary remarkable traits, little is known about the population structure of this ant species, which may provide insight into its unique reproductive mode and polymorphic traits.

We performed in-depth analyses of ant populations from Korea, Japan, and North America using three mitochondrial genes (COI, COII, and Cytb). The long-winged (L) morph is predominant in Korean populations, and the short-winged (S) morph is very rare. Interestingly, all L morphs were infected with *Wolbachia*, while all Korean S morphs lacked *Wolbachia*, demonstrating an association between a symbiont and a phenotypic trait. A phylogenetic analysis revealed that the S morph is derived from the L morph. We propose that the S morph is associated with potential resistance to *Wolbachia* infection and that *Wolbachia* infection does not influence clonal reproduction (as is the case in other ant species).

**KEYWORDS**

divergence, population structure, *Vollenhovia emeryi*, wing polymorphism, *Wolbachia* infection

1 | INTRODUCTION

Population structure analyses using genetic data provide extensive information about populations, including genetic distribution, genetic diversity, gene flow, and selection. Furthermore, these analyses can be used to evaluate relationships between secondary traits such as phenotype, reproductive strategy, and symbiotic bacterial communities. Among secondary traits, wing morph is the principal phenotype associated with direct dispersal, distribution, and reproductive strategies in insects (Ikeda, Nishikawa, & Sota, 2012; Lin, Yao, Wang, Emlen, & Lavine, 2016; McCulloch et al., 2019; Roff, 1986). In ants, wings play a salient role in nuptial flight, which determines dispersal and breeding success. However, the wing is not
Vollenhovia emeryi Wheeler (Hymenoptera: Myrmicinidae) is a common ant species endemic to East Asia; this species has invaded North America (Kjar & Suman, 2007; Wetterer, Guenard, & Booher, 2015; Wright & Kubik, 2011). It is polymorphic for normal long and aberrant short wing length in queens. The two morphs are not thought to coexist in nature, and colonies of the long-winged (L) morph are typically monogynous, while short-winged (S) morph colonies are polygynous (Kinomura & Yamauchi, 1994). Unlike other ant species and Hymenopteran insects in general, queens and males are produced clonally, while sterile workers arise sexually (Kobayashi, Hasegawa, & Ohkawara, 2008, 2011; Ohkawara, Nakayama, Satoh, Trindl, & Heinze, 2006). This unusual clonal reproduction system is very similar to the system first found in some populations of the little fire ant *Wasmannia auropunctata* (Foucaud, Estoup, Loiseau, Rey, & Orivel, 2010; Foucaud et al., 2006, 2007; Fournier et al., 2005) and in the highly invasive longhorn crazy ant *Paratrechina longicornis* (Pearcy, Goodisman, & Keller, 2011). Selfish clonal reproduction in both sexes might evolve without allowing genetic contamination by the opposite sex, thereby giving rise to genetically homogenized clonal lineages, despite the cost for abandoning genetic diversity and thus the ability to tolerate environmental changes (Fournier et al., 2005; Matsuura, 2010; Pigneur, Hedtke, Etoundi, & Van Doninck, 2012). In some other hymenopteran insects, reproductive manipulators such as *Wolbachia* cause host’s clonal reproduction (Jeong & Stouthamer, 2004; Pannebakker, Pijnacker, Zwaan, & Ito, 2001; Tinaut & Heinze, 1992; Villet, 1991).

The *Wolbachia* bacterium is a maternally-inherited endosymbiont that infects a wide variety of invertebrates such as insects (including ants) and other arthropods (Bourtzis & Miller, 2008; Correa & Ballard, 2016; Hilgenboecker, Hammerstein, Schlattmann, Telschow, & Werren, 2010; Kautz, Rubin, Russell, & Moreau, 2013; Werren, 1997; Zientz, Feldhaar, Stoll, & Gross, 2005; Zug & Hammerstein, 2015). Infection induces various types of reproductive alterations in the host, including cytoplasmic incompatibility, feminization, male-killing, and parthenogenesis (Fujii, Kubo, Ishikawa, & Sasaki, 2004; Jeong & Suh, 2008; Stouthamer, Breeuwer, & Hurst, 1999). The ants are attractive taxa as the host of *Wolbachia* due to their eusocial haplodiploids with generally female-biased sex ratios (Russell, 2012). Approximately 30% of ant species have been estimated to be facultatively infected with *Wolbachia* due to their eusocial haplodiploids with generally female-biased sex ratios (Russell, 2012; Russell et al., 2012). Recent studies reported the evidence for *Wolbachia*-associated sex-, caste-ratio, and colony life cycle changing in ants (de Bekker, Will, Das, & Adams, 2018; Pontieri, Schmidt, Singh, Pedersen, & Links, 2017; Singh & Links, 2020; Wenseleers, Sundström, & Billen, 2002). However, the effect of *Wolbachia* infection is still poorly understood in their host ants.

The present study focused on the association between the ant species, *V. emeryi* with unusual clonal reproduction system, and *Wolbachia*. The specific aims of this study were to examine (a) the population genetic structure of the mitochondrial genes of *V. emeryi*; (b) the phylogeographic relationships among the two winged morphs from Korea and Japan; (c) the approximate divergence time of the two winged morphs; (d) the ubiquity of *Wolbachia* infection in this ant species; and (e) potential relationships between host phenotype and *Wolbachia* infection.
Kit (iNtRON Biotechnology, Seongnam, Korea) was used for each amplification along with 16 µl of distilled water, 1 µl of each primer (10 pmol), and 5 ng of template DNA. PCR amplification was conducted using either a PTC-100 Programmable Thermal Controller (MJ Research, Inc.) or a PeqSTAR Universal Gradient Thermocycler (Peqlab Gmbh). The PCR ampli-cons were visualized in a 1% agarose gel dyed with TopGreen Nucleic Acid Gel Stain (Genomic Base) and purified using a commercial kit (QIAquick PCR Purification Kit; Qiagen) prior to sequencing. In all cases, sequences were read in both directions for maximum clarity.

2.3 Data analysis

2.3.1 Population genetic structure and demographic analyses

The resultant sequences were aligned and analyzed using ClustalW embedded in MEGA (ver. 5.2; Kumar, Nei, Dudley, & Tamura, 2008; Kumar, Tamura, & Nei, 1994; Thompson, Higgins, & Gibson, 1994). The aligned sequences were submitted to GenBank along with the translated amino acid sequences. GenBank accession numbers are shown in Table S1. Haplotypes were determined using DnaSP (ver. 5.10; Librado & Rozas, 2009).

A good correlation has been reported between ground vegeta-tion and ant community diversity (Andersen, 1995, 1997; Lubertazzi & Tschinkel, 2003). Hence, sequence data were grouped according to regions on a vegetation map of the Korean peninsula overlaid with isothermal lines (Yi, 2011). Within the range of deciduous broad-leaved forests (temperate zone), the central area was designated region A, the southwestern area was designated region B, and the southeastern area was designated region C. Region D represented the evergreen broad-leaved forest (subtropical-warm temperate zone), and region E represented Yeosu-si, a central spot on the southern coast, based on the unique characteristics of the sample collected at this site. Jeju Island, a volcanic island far from the mainland of Korea, was labeled region F. Regions G and H were the USA and Japan, respectively.

Molecular diversity indices were calculated for all eight regions and each gene. Analysis of molecular variance (AMOVA) among regions, including the overall fixation index statistics ($F_{ST}$) and pairwise $F_{ST}$, was performed with 1,000 permutations. To test the model of evolution and demographic expansion for the COI gene, neutrality tests (Tajima’s $D$ and Fu’s $F_{S}$; Fu, 1997; Tajima, 1989) and mismatch distribution tests were performed with 1,000 replicates using Arlequin (ver. 3.5.1.2; Excoffier & Lischer, 2010). Based on the mismatch distribution, demo-graphic expansion patterns for seven regions (excluding region G, i.e., the USA, which lacks variation) were determined using DnaSP and ed-ited using Microsoft PowerPoint 2013.

Genetic distances among haplotypes were calculated after se-lecting the best-fit substitution model in MEGA. The median-joining algorithm was employed to infer phylogenetic relationships among the haplotypes using Network (ver. 4.6.10), with a fixed connection limit at 1,000 steps between haplotypes (Bandelt, Forster, & Röhl, 1999). The haplotype network was edited manually and reconstructed with the regional distribution data using Adobe Illustrator CS6 (Adobe Inc.).

**TABLE 1** Primers used in this study

| Locus   | Primer name | Primer sequences (5′−3′) Purpose | Annealing temperature (°C) | Reference                  |
|---------|-------------|----------------------------------|-----------------------------|----------------------------|
| COI     | F: LCO-a    | CCYCGWATAAAAYATAAGTTTGA TAAACTTGDGTRGWC CAAAAAATACA | 45–50 | Designed to be specific for V. emeryi |
|         | R: HCO-a    | GAGGAGGACCCCATTTTAT TCAATGCATAATCTGCCCATTATA | 45–50 | Designed to be specific for V. emeryi |
|         | F: Engel    | ATATTCAAAATGTTGATGAGTAGA AGCTCGGGCTTCAAATCCA | PCR and Sequencing | Simon et al. (1994) |
|         | R: Pat      | ATGTACATTTTGGAAGGCACTACG GAAGCTTGAGGTATAGGGCCAATTC | PCR and Sequencing | Kobayashi, Tamura, Okamoto, Hasegawa, and Ohkawara, (2012) |
| COII    | F: Ve13-sF1 | ATATGGGAGGAGCCTATCCAG AAGCTTGAGGGGCGAGATCC | Sequencing | — |
|         | R: Ve13-sR1 | AAGTCAAAATATTTGATGGGATAGA AGTACCTGGAAGGATAGC | — | Designed to be specific for V. emeryi |
|         | F: Ve13-sF2 | ATTAACCGGCCCTGAATATTT TTGTTAGAGATGGGACACAA | — | — |
|         | R: Ve13-sR2 | TTGTTAGAGATGGGACACAA | — | — |
| Cytb    | F: VeCB-F1  | TGCCTGAATCTCAATAGGGCCCTT | PCR and Sequencing | Designed to be specific for V. emeryi |
|         | R: VeCB-R1  | TGTATGGGGATCTAAATCTTGTG | 52–60 | — |
| 16s rRNA| F: WspecF   | CATACCTATCGAAGGGGATAG AGCTGCGGCTTCAAATCCA | Wolbachia-specific diagnostic PCR | Designed to be specific for V. emeryi |
|         | R: WspecR   | — | — | — |
| ftsZ    | F: ftsZ_F1  | ATYATGGARCATATAAARGATAG TCRAGYATGGGATGATAT | Wolbachia-specific diagnostic PCR | Designed to be specific for V. emeryi |
|         | R: ftsZ_R1  | ATATGGGAGGAGCCTATCCAG AAGCTTGAGGGGCGAGATCC | — | — |

Abbreviations: F, forward primer; R, reverse primer.

The relaxed clock method was used to estimate the approximate divergence date of the S gyne from the L queen. Three sets of
monophyletic lineages, thought to have diverged approximately 1 MYA (million years ago), 2 MYA, and 3 MYA, were used, that is, *Myrmica excelsa* and *M. taediosa*, *M. sulcinodis* and *M. xavieri*, and *M. tobiasi* and *M. georgica*, respectively (GenBank Accession No: FJ824432, GQ255131, GQ255141, GQ255197, GQ255192, and GQ255145; Jansen & Savolainen, 2010). Jansen and Savolainen (2010) estimated the divergence time of holarctic *Myrmica* ants using mitochondrial and nuclear genes; the COI data and their estimates were extracted.

The HKY + G + I model (gamma distribution shape value: 1.26247; proportion of invariant sites: 0.61287) was selected as the best fit evolutionary substitution model based on the Bayesian information criterion, as determined using MEGA (Kumar et al., 1994, 2008). For the clock method, Bayesian Markov chain Monte Carlo was run for 100 million generations. Trees were sampled every 1,000 generations using BEAST (ver. 1.8.0; Drummond & Rambaut, 2007). Posterior distributions for parameter estimates and likelihood scores were visualized using Tracer (ver. 1.5) to examine tree appropriateness. The trees were consolidated to a maximum clade credibility tree with median heights after discarding the first 15,000 trees as burn-in. The resultant tree was visualized, with 95% HPD (highest posterior density), using FigTree (ver. 1.40). It was further edited with additional data using Adobe Illustrator CS6 (Adobe Inc.).

### 2.3.3 Association between wing morphology and *Wolbachia* infection status

The chi-square independence test in SPSS (Release 17.0) was used to examine whether there is a relationship between wing morph and *Wolbachia* infection. For statistical analysis, the three USA individuals were excluded owing to uncertainty with respect to their wing morphology.

### 3 RESULTS

#### 3.1 Molecular diversity

We analyzed the mitochondrial COI (1,224 bp), COII (663 bp), and Cytb (839 bp) genes for a specimen from each of the 145 ant colonies. We identified 37 (COI), 25 (COII), and 26 (Cytb) unique haplotypes (Table S2). Overall molecular diversity indices for eight regions and for each gene are shown in Table 2 (COI) and Table S3 (COII and Cytb). Both nucleotide diversity (\( \pi \)) and haplotype diversity (\( h \)) decreased in the order COI (\( \bar{\pi} = 0.086 \pm 0.078; h = 0.557 \pm 0.289 \)), Cytb (\( \bar{\pi} = 0.078 \pm 0.090; h = 0.455 \pm 0.233 \)), and COII (\( \bar{\pi} = 0.062 \pm 0.062; h = 0.430 \pm 0.278 \)) and were highest in region \( F \) (Jeju island; \( \bar{\pi} = 0.233, h = 0.867 \) for COI; \( \bar{\pi} = 0.261, h = 0.733 \) for Cytb; \( \bar{\pi} = 0.202, h = 0.733 \) for COII).

#### 3.2 Population genetic structure and demographic analyses

The observed \( F_{ST} \) values for COI, COII, and Cytb were 0.781, 0.687, and 0.803, respectively, indicating that the regional populations are genetically isolated. For COI, the estimated migration rate (\( N_{m} \), where \( N_{e} \) is the effective population size and \( m \) is the proportion of the population that migrates in each generation) was 0.07 migrants per generation (Slatkin, 1987; Slatkin & Barton, 1989). All three genes showed greater variation among regions (73.25%-75.90%) than within regions (0%-4.37%; Table 3; Table S4). We detected high \( F_{ST} \) in pairwise combinations between regions \( E, F, G, \) and \( H \) (Table 4; Tables S5 and S6). For the COI gene, 23 out of 28 pairwise combinations showed significant differentiation, and the highest pairwise \( F_{ST} \) was 0.91731 for the comparison between region \( C \) and region \( G \) (Table 4).

Neutrality and population expansion parameters for each gene are summarized in Table 5, Tables S7 and S8. For COI, we detected negative Tajima’s \( D \) values for regions \( A (-2.1452), \) \( C (-2.6215), \) and \( D (-2.0640) \) with 99% statistical significance, indicating that the current haplotype diversity resulted from selection on certain genotypes. Tajima’s \( D \) for regions \( B, E, F, \) and \( H \) was not statistically significant, indicating neutral evolution. The \( \tau \) values that represent the estimated time of expansion were very low in regions \( A, B, C, \) and \( D \) (min = 0.0 in region \( B \) and max = 1.6 in region \( D \)), indicating sudden and recent population growth (Table 5). The \( \tau \) values in regions \( E, F, \) and \( H \) were comparatively high (min = 9.2 in region \( E \) and max = 46.2 in region \( F \)), indicating that population growth was slower than that in regions \( A-D \). The observed mismatch distribution was used to evaluate the demographic expansion history. The raggedness indexes for all regions except region \( E \) were not significant.

### TABLE 2 Molecular diversity indices for eight regions of mitochondrial COI

| Region (N) | A (36) | B (14) | C (24) | D (17) | E (23) | F (6) | G (3) | H (22) | Total (145) |
|------------|--------|--------|--------|--------|--------|-------|-------|--------|-------------|
| \( N_{h} \) | 12     | 3      | 5      | 9      | 4      | 4     | 1     | 7      | 37          |
| \( nTi/nTv \) | 12.2   | 24.5   | 47     | 13.5   | —      | 10.5  | —     | 17     | 20.783 ± 13.769 |
| \( \bar{\pi} \) | 0.063  | 0.176  | 0.040  | 0.069  | 0.043  | 0.233 | 0     | 0.061  | 0.086 ± 0.078  |
| \( h \) | 0.560  | 0.473  | 0.377  | 0.853  | 0.549  | 0.867 | 0     | 0.779  | 0.557 ± 0.289  |

Abbreviations: \( h \), haplotype diversity; \( N_{h} \), number of haplotypes; \( N_{s} \), number of samples examined; \( nTi/nTv \), the ratio of transitions to transversions; \( \bar{\pi} \), nucleotide diversity.
suggesting that the expansion model could not be rejected, except in region E (Table 5). The analysis of region G, that is, the USA population, was not informative because the samples showed no haplotype variation.

### 3.3 Haplotype network

In the haplotype network for COI, haplotype 1 was predominant in the Korean L morph samples, accounting for 40.0% of samples (58 individuals), including 41.4% of samples in region A, 32.8% in region C, 17.2% in region B, and 8.6% in region D (Figure 1). The USA samples belonged to haplotype 36 (Figure 1). Six haplotypes (haplotypes 1, 2, 4, 7, 17, and 34) were distributed in two or more regions and the other haplotypes were restricted to unique regions. Seventeen haplotypes were derived from haplotype 1, and 16 of these differed by a singleton mutation (Figure 1). Haplotype networks for COII and Cytb showed similar haplotype distribution patterns to that for COI (Figure S1). For all three genes, the Korean S morph haplotypes were more closely related to Japanese haplotypes than to the dominant L morph haplotypes in Korea (Figure 1; Figure S1).

### 3.4 Phylogenetic relationships and divergence time estimates

In the phylogenetic tree, we observed that the haplotypes were clearly divided into two clades, that is, clade 1 and clade 2, and the S morph was derived from the ancestral L morph (Figure 2). Clade 1 included only Korean L morph haplotypes, while clade 2 included haplotypes from Korea, Japan, and the USA, as well as both L and S morph haplotypes. The Korean and Japanese S morph haplotypes were monophyletic, implying that the wing transformation event took place only once in the history of the species. The USA haplotype (Hap 36) diverged earlier and was not monophyletic with the S morph haplotypes. Based on molecular dating, the two clades diverged approximately 2.7078 MYA (95% HPD: 0.0053–9.278 MYA), and the divergence of the S morph from the L morph occurred around 0.2 MYA (95% HPD: 0.0003–0.7164 MYA; Figure 2).

### TABLE 3 AMOVA for mitochondrial COI of V. emeryi

| Source of variation          | df | Percentage of variation |
|------------------------------|----|-------------------------|
| Among regions                | 7  | 74.81                   |
| Among populations within     | 53 | 3.27                    |
| regions                      |    |                         |
| Within populations           | 84 | 21.92                   |
| Total                        | 144| 100.00                  |

Abbreviation: df, degrees of freedom.

### TABLE 4 Population pairwise $F_{ST}$ values between regions for COI

| Region | A     | B           | C           | D           | E           | F           | G           | H       |
|--------|-------|-------------|-------------|-------------|-------------|-------------|-------------|---------|
| Region A | —     |             |             |             |             |             |             |         |
| Region B | 0.09915* | —           |             |             |             |             |             |         |
| Region C | —0.01541 | 0.09881*    | —           |             |             |             |             |         |
| Region D | —0.00838 | 0.04035     | —0.01882    | —           |             |             |             |         |
| Region E | 0.88699** | 0.75978**   | 0.91318**   | 0.88401**   | —           |             |             |         |
| Region F | 0.76830** | 0.50484**   | 0.80042**   | 0.72167**   | 0.69558**   | —           |             |         |
| Region G | 0.87031** | 0.64864**   | 0.91731**   | 0.86393**   | 0.83311**   | 0.54368     | —           |         |
| Region H | 0.87579** | 0.74100**   | 0.89815**   | 0.86605**   | 0.29224**   | 0.66380**   | 0.77577**   | —       |

* $p < .05$; ** $p < .01$.

### TABLE 5 Neutrality test for COI

| Region | A (36) | B (14) | C (24) | D (17) | E (23) | F (6) | G (3) | H (22) | Mean ± SD |
|--------|--------|--------|--------|--------|--------|------|-------|-------|-----------|
| Tajima's $D$ | −2.1452** | 0.6124 | −2.6215** | −2.4064** | 1.6972 | 1.4576 | — | 1.0687 | −0.2922 ± 1.8168 |
| Tau (τ) | 0.7 | 0.0 | 3.0 | 1.6 | 9.2 | 46.2 | — | 11.9 | 9.0728 ± 15.6562 |
| SSD    | 0.0161 | 0.3451** | 0.0124 | 0.0178** | 0.1672 | 0.1568** | — | 0.0854** | 0.1001 ± 0.1192 |
| Raggedness index | 0.1052 | 0.3843 | 0.1952 | 0.1103 | 0.3297** | 0.1867 | — | 0.1293 | 0.1801 ± 0.1253 |

Abbreviations: $N_s$, number of samples; SSD, sum of squared deviation.

** $p < .01$.**
3.5 | Wing morphology and Wolbachia infection

All of the L morph individuals proved to be infected with Wolbachia. However, Wolbachia infection was polymorphic in the S morph individuals. None of the Korean S morph populations (Hap 7) harbored Wolbachia, but the Japanese S morph populations collected from the mid-northern part of Japan, that is, Ishikawa and Toyama (Hap 22), were completely infected whereas populations from Tokyo (Hap 25, 26) and Gifu (Hap 24) were free of Wolbachia (Figure 2). The wing development pattern correlated strongly with Wolbachia infection status in this ant species ($n = 142$, Pearson $\chi^2 = 100.339$, df = 1, $p < .001$). These results also suggest that Wolbachia is not involved in clonal reproduction in the ant species because clonal reproduction occurs in both wing morphs (Kobayashi et al., 2011).

3.6 | COI clade and haplotype frequencies in the eight regions

The COI haplotypes were divided into two clades in the Bayesian phylogenetic tree (Figure 2). The demarcated vegetation maps, with clade and haplotype composition data, are shown in Figure 3a. Regions A, C, and D showed similar ratios of clade 1 to clade 2. Clade 2 was slightly more highly represented in regions B and F than in regions A, C, and D. Even though region E belongs to the Korean peninsula, all haplotypes from the region formed a group with region H (Japan) and region G (USA) haplotypes in clade 2. Moreover, the ratio of the L morph to the S morph in region E was similar to that in region H (Japan).

Hap 1 was a dominant haplotype in regions A, B, and C. In region D, the frequency of hap 1 was lower than that in the other regions (Figure 3b). In Korean populations, region E (Yeosu-si), in which the S morph can be found, and region F (Jeju), which is isolated from the mainland of the Korean peninsula, had haplotype compositions distant from those of regions A to D. The haplotype compositions and frequencies in region H (Japan) were different from those in Korea (Figure 3b).

4 | DISCUSSION

The strong genetic isolation among regions (overall fixation index for COI: 0.781) indicates an extremely low dispersal rate after
NOH et al. regional colonization, similar to the situation in Japan (Miyakawa & Mikheyev, 2015). In the Korean populations, other than the island (region F), pairwise $F_{ST}$ values indicated more limited dispersal in the region E population than in the other populations; the S morph is found in this region, and its mating almost always occurs in the natal nest (Table 4; Ohkawara, Ishii, Fukushima, Yamauchi, & Heinze, 2002). The haplotype network and pairwise $F_{ST}$ results indicate that populations in region E (the Korean S morph population) are closely related to populations in region H (Japan; Figure 1 and Table 4). In the COI phylogenetic tree, we detected a migration event(s) between region E and region H (Japan) after the S morph divericated from the L morph (Figure 2). Our divergence estimation indicates that the emergence of S morph and loss of infection are evolutionarily very recent events (Figure 2). However,
when interpreting these divergence data, caution is necessary because Hymenopteran insects show lineage-specific variation with respect to mitochondrial evolution (Dowton, Cameron, Austin, & Whiting, 2009).

Populations in three regions (region A, C, and D) seem to be undergoing purifying selection, although mitochondrial DNA is a neutral marker (but see Morales, Pavlova, Joseph, & Sunnucks, 2015; Mossman, Jennifer, Navarro, & Rand, 2019; Table 5). Our results may be explained by the reproductive strategy and sib-mating behavior. Selfish clonal reproduction forms strong maternal nuclear-mitochondrial bonds in gynes, and sib-mating behavior enhances paternal nuclear-mitochondrial bondage in males, similar to linkage disequilibrium. Therefore, the signature of selection on a neutral marker may reflect selection for linked loci or nonrandomly associated genotypes.

Founder effect and relaxed selection may result in the loss of Wolbachia infection in some invasive ant species while exploring new habitats (Bouwma, Ahrens, DeHeer, & Shoemaker, 2006; Reuter, Pedersen, & Keller, 2005; Rey et al., 2013; Tsutsui, Kauppinen, Oyafuso, & Grosberg, 2003). In the endemic V. emeryi population in East Asia, the phylogenetic tree of the COI haplotypes shows that Wolbachia infection is evident in the ancestral L morph, but disappeared in the S morph (Figure 2). The speculation that the loss of Wolbachia of the S morph was caused by the colony founding process is less plausible because the S morph populations are endemic and the malformed wings of S morph queen restrict dispersal to a new habitats.

Łukasiewicz, Sanak, and Węgrzyn (2016) reported the correlation between malformation of wings and the absence of Wolbachia in apollo butterfly, Parnassius apollo. Their result is correspond to the phenomenon of the loss of Wolbachia in V. emeryi we investigated. In light of these results, it is reasonable to argue that the presence of Wolbachia be associated with wing development in these species. Although the mechanism of this connection remains to be elucidated, we suggest hypothesis from two perspectives, host or Wolbachia as a main driver of evolutionary outcome. In the former perspective, there might be a positive relationship between short wing formation and evolution of resistance to Wolbachia infection in this species. Epigenetic factors might be involved in wing formation in this ant based on intermittent L gyne production from the S morph colonies (Noh, 2014; Noh, Park, Choe, & Jeong, 2018; Okamoto, Kobayashi, Hasegawa, & Ohkawara, 2015). If that is the case, it is possible that the gene(s) responsible for the wing formation, and the gene(s) resistant to Wolbachia infection, exhibit epistatic interactions. Wing polymorphism is regulated by hormones mainly the juvenile hormone titer and certain genes (Zera, 2016). Such genes may encode antigens-converting enzyme for bacterial immunity (Dani, Richards, Isaac, & Edwards, 2003). It will be meaningful to investigate such gene(s) to elucidate the prevalence of Wolbachia in insects from a mechanistic evolutionary perspective. Another speculation, in the latter perspective, is that Wolbachia might play a contributive role in the ontogenic stage of host wing development in these species, as suggested by Łukasiewicz et al. (2016). A further
study that examines the effects of elimination of Wolbachia infection on these host species by antibiotic treatment will provide more insightful explanation for this uncommon association between host wing morphology and Wolbachia infection.

The Wolbachia bacterium is known for its manipulative effects on host reproduction (Fuji et al., 2004; Jeong & Suh, 2008; Stouthamer et al., 1999). The Wolbachia-induced parthenogenesis is similar to queen developmental procedure in the ant V. emeryi. In the ant species, however, the clonal reproduction takes place in both Wolbachia-infected L morph and Wolbachia-free S morph. Therefore, Wolbachia may not contribute to clonal production system of the queen caste, as is the case in W. auropunctata (Rey et al., 2013).

In conclusion, all L morphs, the predominant ancestral form, were infected with Wolbachia, while the rare derived S morphs were free of Wolbachia, at least in Korean populations, and were partially infected in Japanese populations in parallel with the potential evolution of Wolbachia infection resistance. This is the significant report of an uncommon association between Wolbachia infection and host morphological characteristics.

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CONFLICT OF INTEREST
The authors have no conflict of interest.

AUTHOR CONTRIBUTIONS
Pureum Noh: Data curation (lead); Formal analysis (lead); Investigation (lead); Methodology (lead); Writing-original draft (lead). Seung-Yoon Oh: Data curation (lead); Formal analysis (supporting); Investigation (supporting); Methodology (supporting); Resources (lead); Software (supporting); Writing-original draft (lead). Soyeon Park: Data curation (supporting); Investigation (supporting); Methodology (supporting); Resources (supporting); Software (supporting); Visualization (supporting); Writing-original draft (supporting). Taeung Kwon: Methodology (supporting); Resources (supporting). Yongsan Kim: Investigation (supporting); Methodology (supporting). Jae Chun Choe: Funding acquisition (supporting); Methodology (supporting); Project administration (supporting); Supervision (lead). Gilsang Jeong: Conceptualization (lead); Data curation (lead); Formal analysis (lead); Funding acquisition (lead); Investigation (lead); Methodology (lead); Project administration (lead); Resources (lead); Software (lead); Supervision (lead); Validation (lead); Writing-original draft (lead); Writing-review & editing (lead).

DATA AVAILABILITY STATEMENT
Data are available at the Dryad Digital Repository, https://doi.org/10.5061/dryad.j6q573n1b1. Sampling locations and GenBank accession numbers for the sequences of each sample are included in the Supporting information section (Table S1).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.