



## Genetics, cuticular hydrocarbon profiles, and bacterial endosymbionts are associated with the nestmate recognition in the invasive African big-headed ant, *Pheidole megacephala*, in Taiwan

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### Abstract

Genetic and environmental cues have been suggested to affect cuticular hydrocarbon (CHC) profiles, in which the profile signature mediates aggressive behaviors in ants. Paradoxically, conflicting evidence exists, and none of these hypotheses has received decisive support. These cues have been studied independently without much attention to their potential mutual relationship. This study investigated the mutual relationships of cuticular hydrocarbons, genetics, and bacterial endosymbionts in mediating aggressive behavior in 14 colonies of the invasive African big-headed ant, *Pheidole megacephala*, in urban and forest areas of Taichung, Taiwan. Behavioral assays revealed that workers displayed aggression toward those from other colonies, indicating the absence of a supercolony in Taichung. However, urban ants did not necessarily exhibit aggression toward forest ants, indicating that environmental cues between these habitats play a minor role in mediating agonistic interactions. The aggression level corresponded to differences in dimethyl alkane. The dimethyl-alkane distances were significantly associated with genetic distances. Co-inertia analysis demonstrated a strong relationship between dimethyl alkanes and the bacterial endosymbionts of ants. In particular, colonies infected with *Wolbachia* exhibited a higher abundance of peaks corresponding to long-chain dimethyl alkanes ranging from carbon numbers C33 to C39. Based on these findings, we suggest that ant CHCs, shaped by heritable bacterial endosymbionts such as *Wolbachia*, lead to colony-level differences in dimethyl-alkane profiles and, thus, the observed agonistic interactions in *P. megacephala* regardless of habitat type.

**Key words:** Hymenoptera, Formicidae, biological invasion, genetic differentiation, aggressive behavior, supercolony, microbiota.

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### Introduction

Insect body surfaces are coated with cuticular hydrocarbons (CHCs), which serve as a waterproofing agent and as communication signals (MENZEL & al. 2019). The diversity of CHCs in insects is determined by the length of their carbon chains (typically ranging from C20 to C45), arrangement of methyl branches, and presence and locations of double bonds (SPRENGER & MENZEL 2020). In social insects, CHCs play crucial roles in transmitting essential information required for colony functioning (SPRENGER & MENZEL 2020) and in distinguishing between nestmates and non-nestmates (LAHAV & al. 1999, SOROKER & HEFETZ 2000).

Differences in CHC profiles between ant colonies are associated with varying levels of aggression (NOWBAHARI & al. 1990, SUAREZ & al. 2002, FOITZIK & al. 2007, LALZAR & al. 2010, VANDER MEER & MOREL 2019). Studies have suggested that these CHC profiles are affected by the genetic makeup of a colony (TESEO & al. 2014, WALSH & al. 2020, BLUMENFELD & al. 2022). However, some studies have reported conflicting results. For example, a study on the Mediterranean acrobat ant *Crematogaster scutellaris* determined no relationship between CHC profiles and genetic distances, suggesting that CHC profiles in this species are affected more by external environmental factors

than by genetic relationships (FRIZZI & al. 2015). Similarly, in *Formica exsecta*, aggression among conspecific ants is correlated with CHC distance but not genetic or spatial distances (MARTIN & al. 2012). The major enigma of ants' recognition mechanism still remains unsolved.

Environmental factors have a greater effect on CHCs than genetic factors (BUCZKOWSKI & al. 2005, MENZEL & al. 2018, VILLALTA & al. 2020, BLUMENFELD & al. 2022). For instance, dietary changes can alter CHCs, leading ants to exhibit aggression toward their former nestmates (LIANG & SILVERMAN 2000, LIANG & al. 2001, BUCZKOWSKI & al. 2005). Dietary effects on CHC profiles may not only be associated with food itself but also with changes in the gut microbiota (SPRENGER & MENZEL 2020). Microbiomes, such as bacterial endosymbionts and parasites, affect CHC production and profiles, thus influencing pheromone-based communication in insects (GUO & al. 1991, SHARON & al. 2010, ENGL & KALTENPOTH 2018, ENGL & al. 2018).

The African big-headed ant, *Pheidole megacephala*, a member of the Myrmicinae subfamily, is a tramp ant native to Africa (WETTERER 2012). Currently, *P. megacephala* has spread to numerous tropical regions worldwide (KE & al. 2024). Similar to other tramp ants, *P. megacephala* establishes expansive, polydomous, and highly polygynous supercolonies in its introduced territories. Workers of *P. megacephala* exhibit non-aggressive behavior toward conspecifics from different nests to reduce the cost associated with intraspecific competition and combine the labor forces of multiple nests, thus expanding into new territories up to 3000 km away (FOURNIER & al. 2009). The observed lack of aggression in introduced regions may be due to decreased genetic diversity among populations resulting from a genetic bottleneck (FOURNIER & al. 2009).

*Pheidole megacephala* was introduced into Taiwan a century ago, possibly during the colonization period of the Qing dynasty, through at least two separate events (WETTERER 2012, LIU & al. 2022). Since its introduction, *P. megacephala* has experienced a substantial decrease in genetic diversity in Taiwan (LIU & al. 2022). Despite the reduction in genetic diversity in *P. megacephala*, a substantial but low level of genetic differentiation is still observed between populations in different regions. Moreover, the colonies of *P. megacephala* exhibit aggressive behavior toward other colonies of the same species located more than 100 m away (LIU & al. 2022). This finding suggests that such aggressive behavior is associated with spatial factors arising from genetic differentiation. Therefore, we hypothesize that colonies of *P. megacephala* in a distance with genetic differentiation will exhibit aggressively toward each other, which prevents the formation of a supercolony.

PENG & al. (2023) observed that the urban population of *Pheidole megacephala* in Taiwan had higher nitrogen stable isotope ( $\delta^{15}\text{N}$ ) values than did their forest-dwelling counterparts. This finding indicates that ants in urban areas may occupy a higher trophic position than ants in forest areas due to their consumption of animal-based resources. Gut symbiotic bacteria in ants are closely related

to their dietary intake (RUSSELL & al. 2009, HE & al. 2014, HU & al. 2014, XU & al. 2016). Thus, the bacterial endosymbionts of *P. megacephala* may differ between forest and urban habitats, potentially affecting the expression of CHCs on the ants and their agonistic interactions.

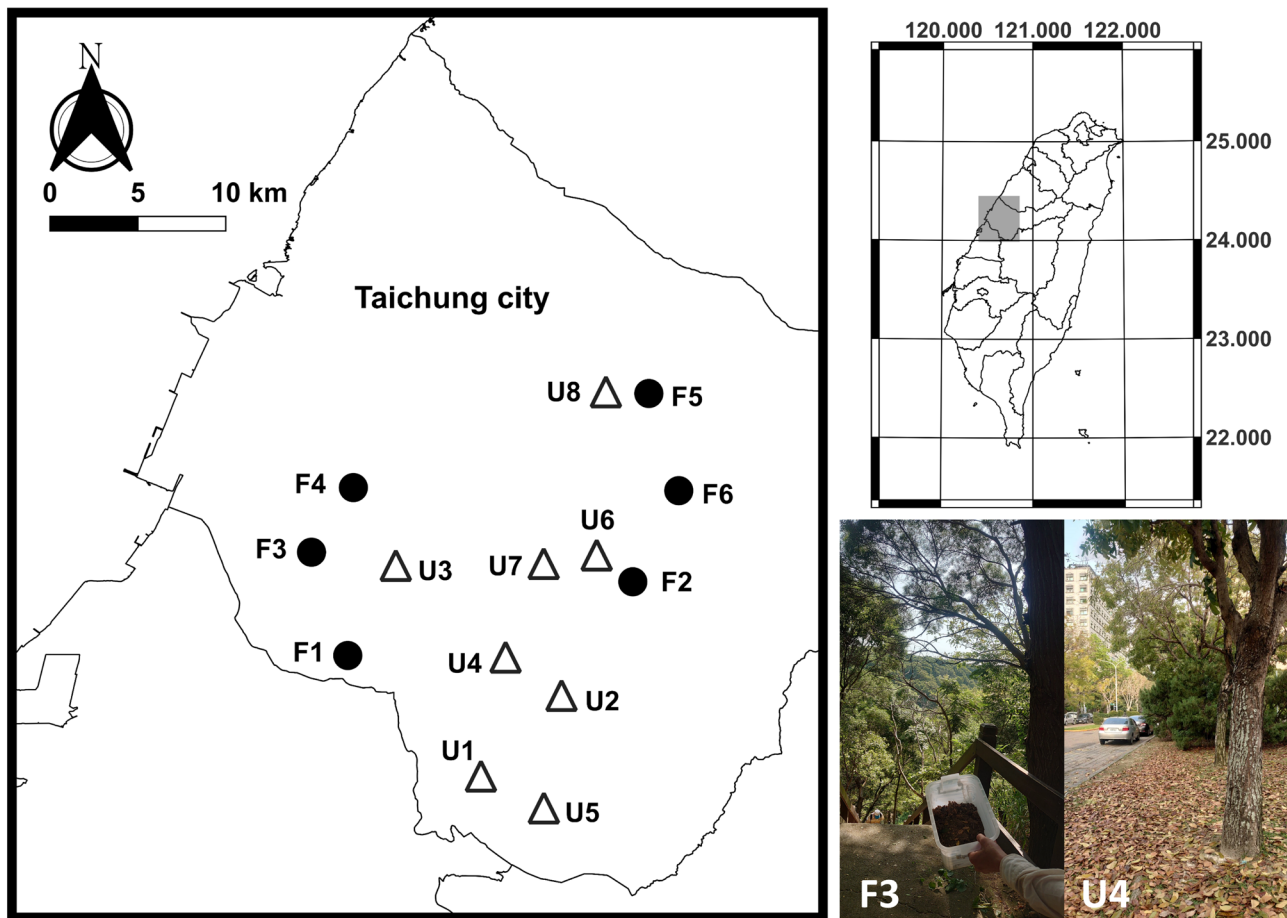
Several studies have investigated mechanisms underlying changes in the CHC profiles and recognition systems of ants (HOWARD & BLOMQUIST 2005, HEFETZ 2007, MARTIN & DRIJFHOUT 2009). However, most of these studies have examined the effects of either the genotype (using field specimens) or the gut microbiota (using laboratory experiments involving antibiotic treatments), without sufficiently exploring the potential association between genetic factors and the bacterial endosymbionts. This association can affect CHC profiles and mediate agonistic interactions among ants. To address this research gap, we collected *Pheidole megacephala* colonies from both forest and urban habitats in Taichung, Taiwan, and conducted pairwise agonistic interaction tests. We analyzed and quantified variations in CHC profiles through gas chromatography-mass spectrometry (GC-MS). The most suitable CHC profile model was selected on the basis of model-selection criteria for further analyses. In addition, we examined the genetic structure of colonies by using microsatellite DNA. Bacterial endosymbiont communities in *P. megacephala* colonies were identified by operational taxonomic units (OTUs) through next-generation sequencing.

## Material and methods

### Study site and ant sampling

Sampling was conducted in Taichung, Taiwan (24°04' - 24°21'N, 120°35' - 120°41'E), in August 2022. Taichung has a warm, humid, subtropical climate with monthly mean air temperatures ranging from 18.8 °C to 21.9 °C from December to March and from 25.1 °C to 28.1 °C from June to October in 2022. Rainfall is the highest during May and October and averages 2244.6 mm annually (TAIWAN CENTRAL WEATHER BUREAU 2023). Ants were identified based on the study by SARNAT & al. (2015).

*Pheidole megacephala* colonies were collected from 14 sampling sites, of which six were located in forests and eight in urban areas (Fig. 1 and Tab. S1, as digital supplementary material to this article, at the journal's web pages). To prevent pseudo-replication, sampling sites were spaced at least 1 km apart. Queens, workers, and broods were collected from each colony and transported to the laboratory in plastic containers (43 × 27 × 18 cm) coated with Fluon (Sigma-Aldrich, St. Louis, MO, USA) to prevent the ants from escaping. In addition, workers from each colony were preserved in 95% ethanol and stored at -20 °C for DNA extraction. All behavioral assays were conducted using minor workers because minor workers are typically the most abundant ant type (FOURNIER & al. 2009, LILLICO-OUACHOUR & ABOUHEIF 2017). Major workers and minor workers of *Pheidole* ants displayed similar aggression levels when confronted with non-nestmates (FOURNIER & al. 2016).



**Fig. 1:** The map shows the sampling sites, which include six colonies from forest areas (F1 - F6) and eight from urban areas (U1 - U8). The forest areas are located within forested environments, such as forest trails (F3 colony). In contrast, the urban areas are surrounded by artificial structures and include green spaces such as parks and campuses (U4 colony).

### Behavioral assays

A behavioral assay was conducted to examine interactions among 14 *Pheidole megacephala* colonies, and 91 intercolonial combinations were tested. For each combination, one worker from each colony was placed in a Petri dish with a diameter of 5 cm and a height of 1.5 cm. The walls of the dish were coated with Fluon to prevent the worker ants from escaping. Each dyadic encounter lasted 5 min. The behaviors observed were scored on a 4-level aggression scale following the methodology reported by SUAREZ & al. (1999) with minor modifications. These levels included (1) non-aggression, characterized by no physical contact or brief antennal contact; (2) antennation, involving reciprocal and prolonged antennal touching; (3) aggression, comprising biting, lunging, or spread-eagling; and (4) fighting, involving severe tension and prolonged biting and grappling between individuals. The highest level of aggression observed in each interaction was recorded. Each pairing was tested five times, and workers were discarded after each trial. The final score of each colony used in further analysis was calculated from the average score of the five replicates.

### Chemical analyses

For CHC lipid extraction, 50 workers were randomly selected from each colony. The ants were first immobilized at  $-20^{\circ}\text{C}$  for 5 min, then immersed in 0.1 mL of chromatography-grade n-hexane ( $\geq 99\%$  purity; Sigma Aldrich) for 10 min with gentle mixing to extract CHC lipids. The extracted solutions were transferred to a 100- $\mu\text{L}$  insert in a 1.5-mL auto-injection vial. Each sample was concentrated to dryness under a gentle nitrogen stream and stored at  $-20^{\circ}\text{C}$  until further analysis. For gas chromatography, the samples were reconstituted in 10  $\mu\text{L}$  of n-hexane. An auto-injector (AOC-20 i; Shimadzu Corporation, Kyoto, Japan) was used to inject 3  $\mu\text{L}$  of each sample in splitless mode for the initial 3 min. Nitrogen was used as the carrier gas at a constant flow rate of 35.6 mL / min. The injection port temperature was set to  $250^{\circ}\text{C}$ . A DB-1HT column (film thickness of 30 m  $\times$  0.25 mm  $\times$  0.1  $\mu\text{m}$ ; J&W Scientific, Agilent Tech, Santa Clara, CA, USA) was used for the analysis. The oven temperature program was started at  $160^{\circ}\text{C}$ , increased to  $300^{\circ}\text{C}$  at a rate of  $3^{\circ}\text{C} / \text{min}$ , and then held at  $300^{\circ}\text{C}$  for 5 min. CHCs were analyzed using gas chromatography (GC-2014; Shimadzu Corporation).

Data acquisition was performed using GC Solution v 2.4 (Shimadzu Corporation).

For chemical identification, a Thermo MS DSQ II mass spectrometer coupled with a Thermo Trace GC Ultra gas chromatograph (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a DB-1HT capillary column was used. The temperature program was identical to that used for GC-2014. Helium was used as the carrier gas. The injection port and transfer line temperatures were maintained at 250 °C and 300 °C, respectively.

To determine equivalent chain length values (NELSON & SUKKESTAD 1975, HOWARD & al. 1978, CARLSON & al. 1998), an ASTM D5442 quantitative linearity standard diluted in n-hexane (C16-C44; Supelco, Bellefonte, PA, USA) was periodically injected between samples. Cuticular lipids were identified by analyzing mass spectra, which were generated using both electron impact and chemical ionization with methane in Qual Browser in Xcalibur software v 2.0.3 (Thermo Fisher Scientific). The relative peak areas of cuticular lipids were used as a quantitative measure for each profile. The peak areas of cuticular compounds were integrated using GC Solution v 2.4. Only compounds with a relative peak area greater than 0.5% were used for further analysis (BLIGHT & al. 2012).

#### **DNA extraction and microsatellite genotyping**

The ants used for CHC extraction were stored in 95% ethanol. Subsequently, genomic DNA was extracted individually from eight *Pheidole megacephala* workers per colony by using the Exgene Tissue SV kit following the manufacturer's protocol (GeneAll Biotechnology, Seoul, Korea). The extracted DNA was stored at -20 °C to maintain its stability.

To determine the genotypes of *Pheidole megacephala* workers, 8 polymorphic microsatellite loci were used: Pmeg-06, Pmeg-07, Pmeg-09, Pmeg-10, Pmeg-11, Pmeg-12, Pmeg-14, and Pmeg-15. Polymerase chain reaction (PCR) experiments were conducted in 25- $\mu$ L reaction tubes. Each tube contained 2  $\mu$ L of template DNA (25 - 50 ng), 12.5  $\mu$ L of TaKaRa EmeraldAmp Max PCR Master Mix (Takara Bio Inc., Shiga, Japan), 0.2  $\mu$ M of forward and reverse primers, and ddH<sub>2</sub>O. The PCR multiplex reactions were divided into 2 groups: Group 1 (Pmeg-06, Pmeg-09, Pmeg-12, and Pmeg-14) and Group 2 (Pmeg-07, Pmeg-10, Pmeg-11, and Pmeg-15) (FOURNIER & al. 2008). Group 1 was subjected to the following thermocycling conditions: initial denaturation at 95 °C for 15 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 90 s, and 72 °C for 60 s, with a final extension at 60 °C for 30 min. Group 2 was subjected to the following thermocycling conditions: initial denaturation at 95 °C for 15 min, followed by 35 cycles of 94 °C for 30 s, 56 °C for 90 s, and 72 °C for 60 s, with a final extension at 60 °C for 30 min. All PCR products were analyzed using the ABI 3730XL DNA Analyzer (Applied Biosystems, Genomics BioSci and Tech, Taipei, Taiwan). GeneMarker v 2.6.0 (SoftGenetics LLC, State College, PA, USA) was used for visualization and allele scoring.

#### **Bacterial endosymbiont DNA extraction and sequencing**

Ten ants were selected from each colony and rinsed with 75% ethanol to remove surface contaminants. These ants were then homogenized using a grinding pestle, combining all 10 into one sample. The DNA from the bacterial endosymbionts was extracted using the Exgene Soil DNA mini kit (GeneAll) and stored at -20 °C until further use.

Bacterial endosymbiont DNA was amplified and sequenced to target the variable V3 and V4 regions of the 16S rRNA gene. The amplification of the V3 + V4 regions was performed using the primers 341F (5'-CCTACGGG-NGGCWGCAG-3') and 806R (5'-GGACTACHVGGG-TATCTAAT-3') (XIAO & al. 2023). PCR was performed in 25- $\mu$ L reaction tubes containing 2  $\mu$ L of template DNA (25 - 50 ng), 12.5  $\mu$ L of TaKaRa EmeraldAmp Max PCR Master Mix (TaKaRa), 1  $\mu$ L each of forward and reverse primers, and 9.5  $\mu$ L of ddH<sub>2</sub>O. The PCR cycling conditions for amplification were as follows: initial denaturation at 95 °C for 10 min, followed by 30 cycles of 95 °C for 30 s, 52 °C for 30 s, and 70 °C for 1.5 min, with a final extension at 72 °C for 10 min. Subsequently, DNA library construction and next-generation sequencing (NGS) were performed using the Illumina MiSeq platform (Illumina, San Diego, CA, USA) by Genomics BioSci and Tech (Taipei, Taiwan), generating 2  $\times$  300 base pair paired-end reads. In the quality-trimming step, PCR-primer sequences were removed, and the reads were filtered using the DADA2 module, which involved length trimming, denoising, and chimera removal (CALLAHAN & al. 2016). The reference database for the taxonomic classification of the V3 and V4 region was generated from the SILVA database v 132 (QUAST & al. 2012) by using the "classify-sklearn" algorithm through the feature-classifier plugin (BOKULICH & al. 2018). The sequences were clustered into OTUs at an identity threshold of 97% to minimize sequencing errors (EDGAR 2013, PATIN & al. 2013). All sequencing data were analyzed using QIIME2 v 2020.11 (CAPORASO & al. 2010, BOLYEN & al. 2019). Bacterial composition was analyzed after excluding taxa with a relative abundance of less than 10%, thereby focusing on dominant members that are likely responsible for the majority of functional contributions.

#### **Statistical analyses**

**Behavioral assays and genetic distances:** The behavior scores of workers from colonies within the forest, urban, and between the two habitats were compared using non-parametric Kruskal-Wallis tests. Subsequently, a posteriori non-parametric pairwise comparisons were performed using Dunn's procedure (SIDNEY 1957). In addition, the effect of genetic differentiation on the level of behavioral aggression was examined. The colonies were categorized into two groups based on the fixation index (FST), derived from microsatellite loci: those with FST  $\leq$  0.25 and FST  $>$  0.25. FST values below 0.25 indicate low to substantial divergence, whereas values above 0.25 indicate significant divergence (WRIGHT 1984). The level

of aggression between the groups was compared using Wilcoxon rank sum tests.

**CHC analyses and chemical distances:** In this study, principal component analysis (PCA) was conducted based on the relative abundances of CHC peaks using the *ggfortify* and *cluster* packages in R in order to reduce dimensionality and visualize variation among colonies (BLIGHT & al. 2012, FOURNIER & al. 2016, BLUMENFELD & al. 2022). Peaks with loadings exceeding  $\pm 0.2$  were selected to determine the biological significance of these vectors.

To assess the similarity of selected CHCs among workers from different colonies, pairwise Euclidean chemical distances were calculated using the *philentropy* package in R. These distances were calculated based on the relative quantities of selected compounds. Chemical distances range from 0 to 1, with 0 indicating identical chemical profiles and 1 indicating no shared compounds between the two profiles (FOURNIER & al. 2009). Kruskal-Wallis tests were used to compare chemical distances between workers from forest, urban, and mixed habitat colonies. Subsequently, a posteriori non-parametric pairwise comparisons were performed using Dunn's procedure. In addition, the Wilcoxon rank sum tests were used to compare chemical distances between the aforementioned FST groups.

Generalized linear mixed models (GLMMs) were used to investigate the effects of chemical distances on aggression levels and to identify chemicals that exert the most significant effect on aggressive behavior. GLMMs were separately applied to analyze each of the seven main CHC groups (all peaks, *n*-alkanes, monomethyl alkanes, dimethyl alkanes, trimethyl alkanes, alkenes, and peaks with PCA loadings exceeding  $\pm 0.2$ ), using the full set of chemical distance measurements as predictors in each model. In the GLMM, habitat (forest and urban) was treated as a random effect, whereas chemical distance was treated as a fixed effect. Errors in the analysis of response variables were accounted for by using a Gaussian distribution. The GLMM analysis was performed using the *lme4* package in R. Model performance was evaluated using the Akaike information criterion (AIC). Model selection was based on the corrected AIC ( $\Delta\text{AICc}$ ) by using the *MuMIn* package in R. The CHC profile model that had the best goodness of fit, as determined by  $\Delta\text{AICc}$ , was used for subsequent analyses.

**Genetic analyses:** GenAlEx v 6.5 (PEAKALL & SMOUSE 2006) was used to analyze the frequency of alleles, number of alleles (NA), expected heterozygosity (HE), and observed heterozygosity (HO) for each microsatellite locus. Allelic richness (Ar) was calculated using the *hierfstat* package in R. To compare genetic diversity among the 14 colonies across these microsatellite loci, the Kruskal-Wallis test in R was used. Subsequently, a posteriori non-parametric pairwise comparisons were performed using Dunn's procedure.

To estimate genetic differences among the 14 colonies in Taichung, pairwise FST values, representing genetic distances between colony pairs, were calculated from

the microsatellite data using GenAlEx. These genetic differences among workers from colonies within forest, urban, and between the two habitats were compared using Kruskal-Wallis tests. Subsequently, posteriori pairwise comparisons were performed using Dunn's procedure. To examine the relationship between selected CHC and genetic distances, Mantel tests were conducted using the *vegan* package in R with 100,000 permutations.

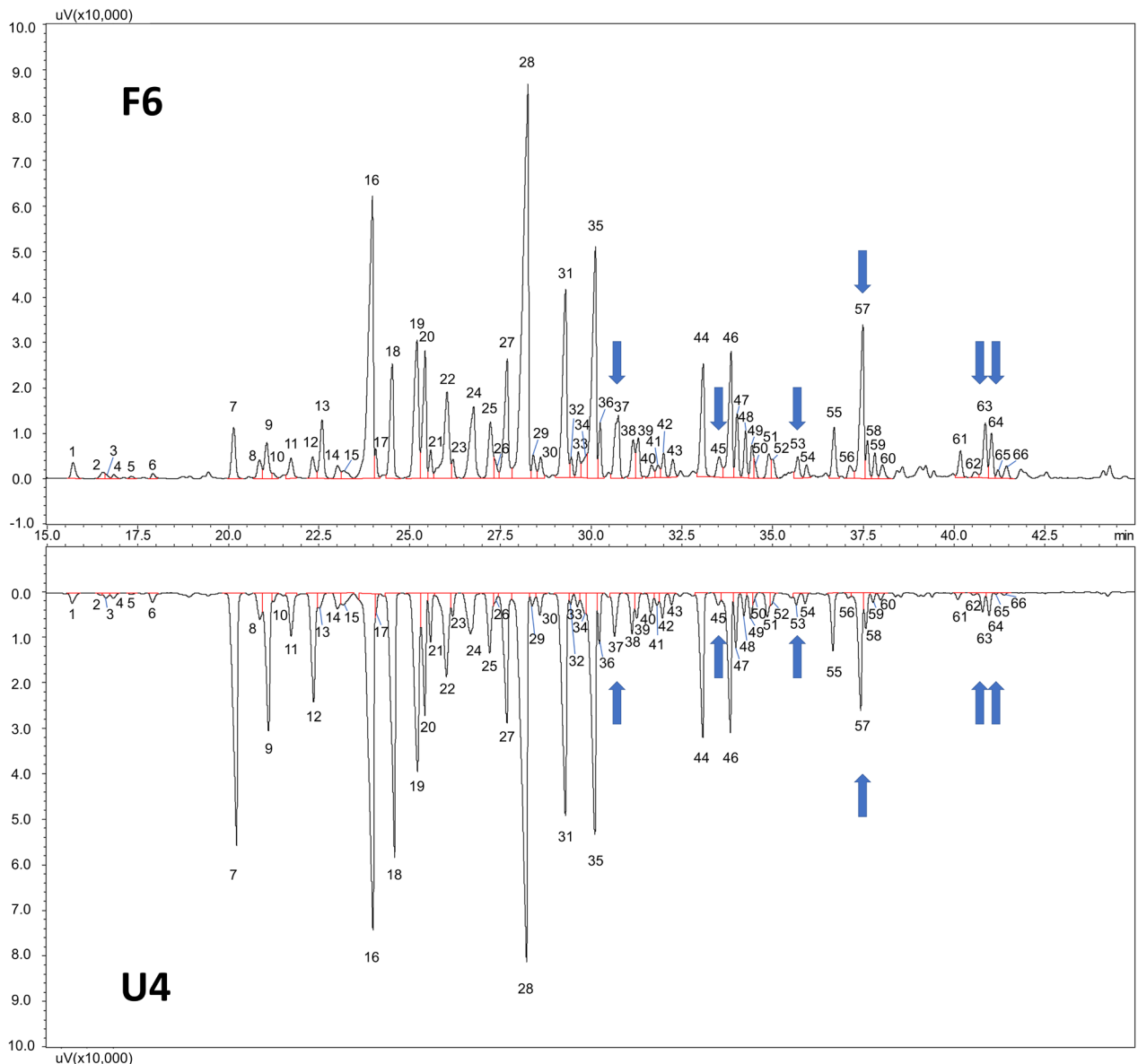
**Bacterial endosymbiont analyses:** An OTU script was generated using QIIME 2. Rarefaction curves were obtained using QIIME 2 for all samples to evaluate sequencing saturation and species diversity. Alpha diversity metrics, including observed species count and the Shannon index, were calculated on the basis of the OTU abundance of each ant colony. Significant differences in alpha diversity between the forest and urban habitats were determined using Student's *t* test. Beta diversity analyses were conducted using Bray-Curtis distance metrics to investigate structural variations in microbial communities among colonies (HU & al. 2014, TESEO & al. 2019). Non-metric multidimensional scaling (NMDS) was performed to ordinate and visualize the effects of environmental factors on the microbial communities of ants. These analyses were conducted and visualized in R by using the *vegan*, *ggrepel* and *ggplot2* packages. The significance of the differences in microbial community structures among the colonies was determined through permutational multivariate analysis of variance, conducted with 10,000 permutations by using the *adonis2* package.

To examine the correlations between selected CHC distances and bacterial OTUs similarity (measured using OTU Bray-Curtis distances; HU & al. 2014), a coinertia analysis (CoIA) (DOLÉDEC & CHESSEL 1994, TESEO & al. 2019) was conducted with Z-transformation and 10,000 Monte Carlo permutations by using the *ade4* package in R. The association between the two data sets was quantified using the RV coefficient. The RV coefficient is a multivariate generalization of the squared correlation coefficient, with values ranging from 0 to 1; a value closer to 1 indicates a stronger correlation between the data sets (CULHANE & al. 2003). Finally, in this study, all analyses were performed using R v 4.2.1 (R CORE TEAM 2022). A P-value of  $< 0.05$  indicated statistical significance.

## Results

### Chemical analyses

We identified 66 peaks ranging in size from C25 to C40 (Fig. 2, Tab. S2). The relative proportions of these peaks indicated the presence of CHC lipids in *Pheidole megacephala*: linear alkanes (13%), monomethyl alkanes (31%), dimethyl alkanes (28%), trimethyl alkanes (4%), alkenes (22%), and unknown compounds (1%). Additional analysis by using electronic ionization revealed a similar distribution of CHC lipids: linear alkanes (9%), monomethyl alkanes (33%), dimethyl alkanes (33%), trimethyl alkanes (12%), alkenes (4%), and unknown compounds (6%). In addition, a cholesterol-like compound was identified in



**Fig. 2:** Chromatograms of cuticular hydrocarbon profiles of *Pheidole megacephala* minor workers from forest (F6 colony) and urban areas (U4 colony). Blue arrows indicate the peaks corresponding to the relative abundances of *Wolbachia*; these peaks contribute to colony-level differences in dimethyl-alkane profiles, and the observed agonistic interactions. The X-axis denotes retention time (min), and the Y-axis denotes detector response ( $\mu\text{V}$ ). Numbers on the peaks correspond to those listed in the Table S2.

the CHC profiles of *P. megacephala* (peak no. 27). However, this compound was excluded from further analysis because it does not belong to the CHC family. Thus, a total of 65 peaks were selected for subsequent analyses. A PCA of all cuticular profiles reduced the dimensionality of the dataset from 65 variables to the first two principal components, which together accounted for 50.61% of the total variance – 32.32% for PC1 and 18.29% for PC2 (Fig. 3).

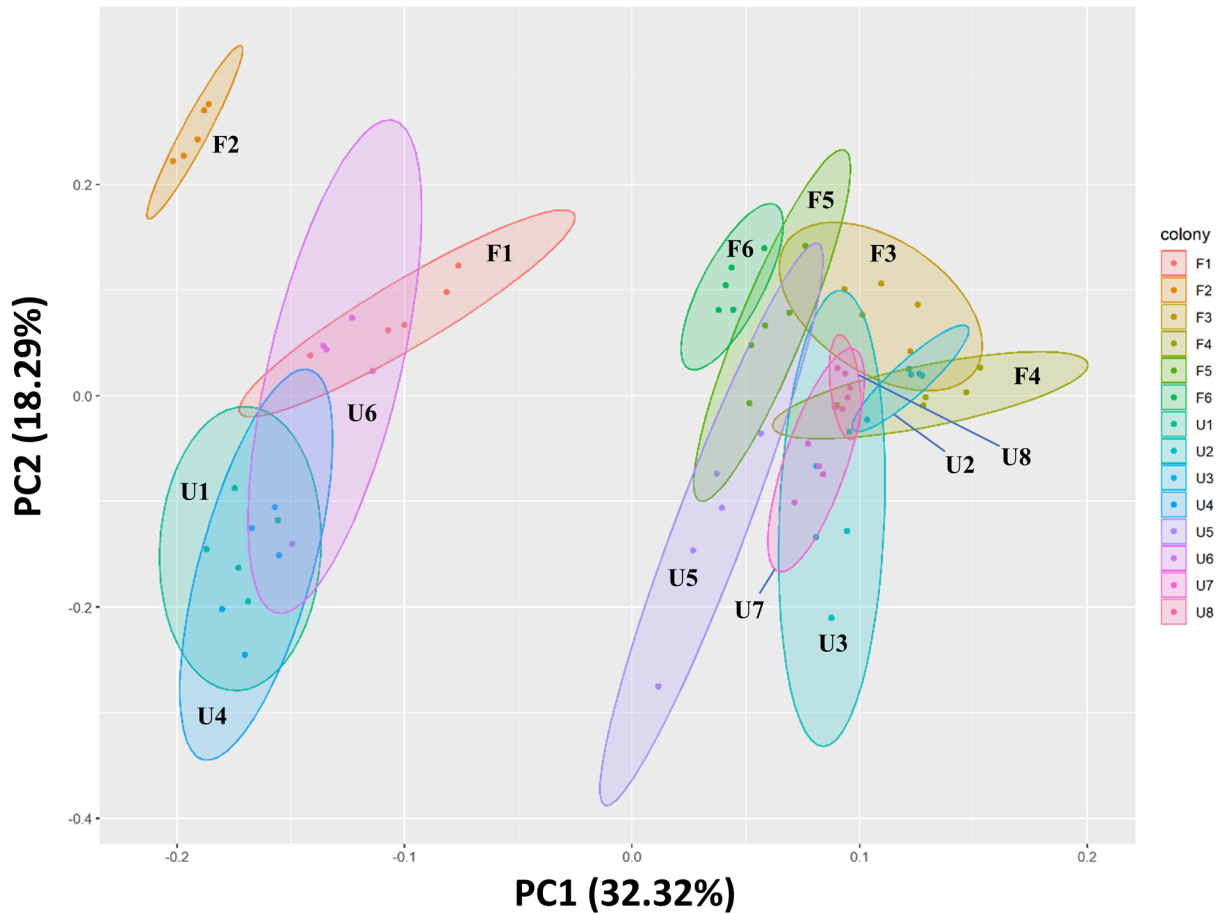
### Microsatellites

One to five alleles were identified across eight polymorphic microsatellite loci (Tab.S3). A total of nine private alleles were detected in six out of eight microsatellite loci. The average private allele frequencies were 0.063, 0.125,

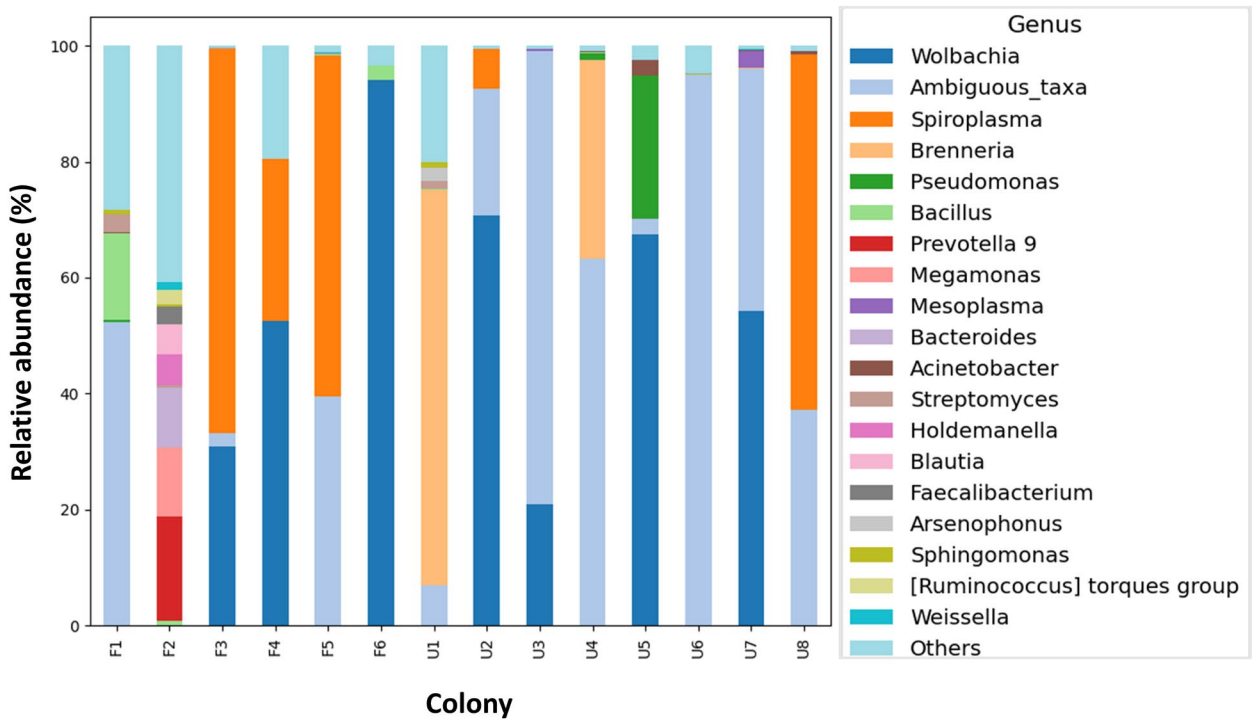
0.063, 0.063, 0.063, and 0.271 in colonies F1, F3, F4, F5, U7, and U8, respectively. The medians for NA, Ar, HE, and HO were 2.000 (interquartile range, IQR = 2.000), 1.786 (IQR = 0.993), 0.305 (IQR = 0.492), and 0.250 (IQR = 0.531), respectively. No significant differences were observed in NA ( $\chi^2 = 17.82$ ,  $df = 13$ ,  $P = 0.164$ ), Ar ( $\chi^2 = 14.48$ ,  $df = 13$ ,  $P = 0.341$ ), HE ( $\chi^2 = 13.52$ ,  $df = 13$ ,  $P = 0.408$ ), and HO ( $\chi^2 = 10.11$ ,  $df = 13$ ,  $P = 0.685$ ) among the 14 colonies. The pairwise  $F_{ST}$  values among populations ranged from 0.012 to 0.660.

### Bacterial endosymbiont communities

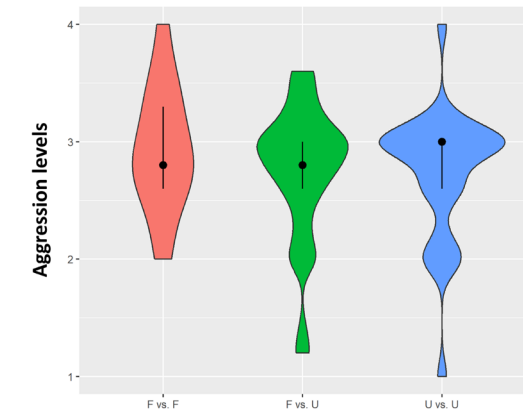
We generated a total of 1,231,403 paired-end reads of the V3 and V4 amplicon sequences of 16S rDNA. After



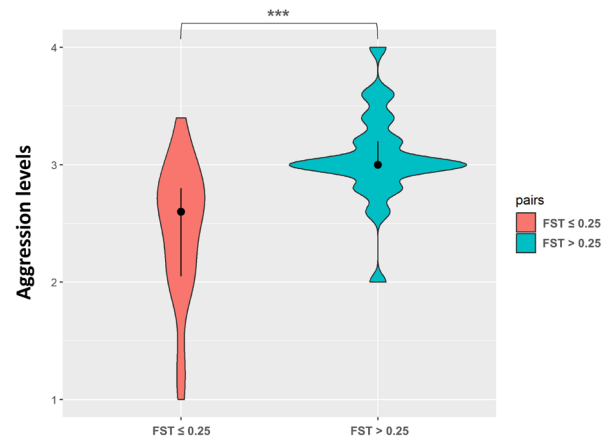
**Fig. 3:** Principal component analysis (PCA) of worker cuticular hydrocarbons from 14 *Pheidole megacephala* colonies in Taichung, Taiwan. The ellipses correspond to the 95% confidence limit of the data from each colony. Colonies F1 - F6 were collected from forest areas, while colonies U1 - U8 were collected from urban areas.



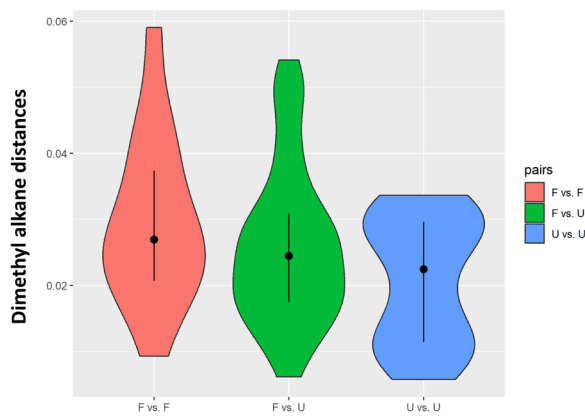
**Fig. 4:** Taxonomic composition of bacterial communities in *Pheidole megacephala* collected from forest (colonies F1 - F6) and urban (colonies U1 - U8) areas. Genera with less than 1% of the relative abundance are grouped as “others.”



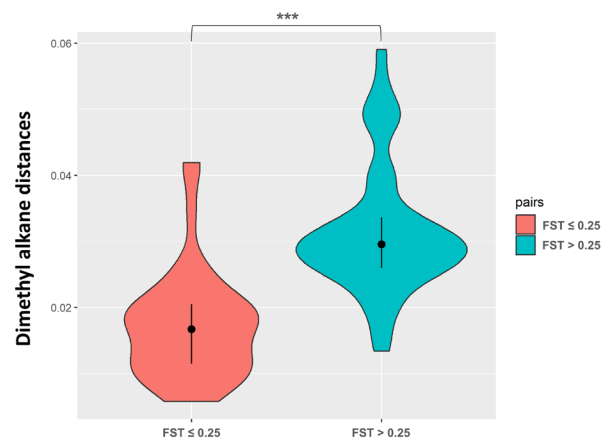
**A** Habitat pairwise comparison



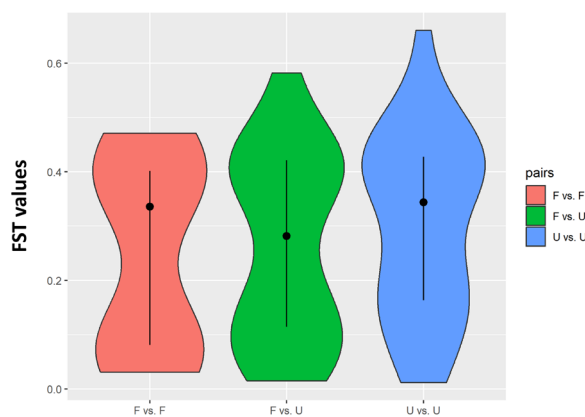
**B** FST groups



**C** Habitat pairwise comparison



**D** FST groups

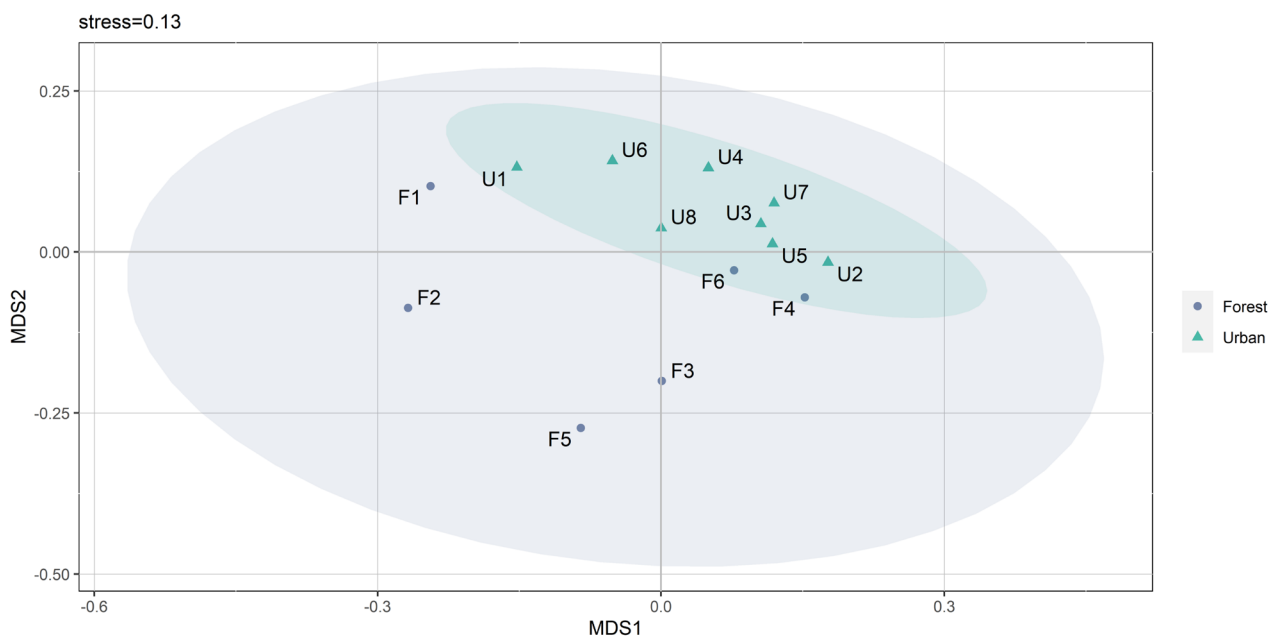


**E** Habitat pairwise comparison

**Fig. 5:** Violin plots showing aggression levels, dimethyl-alkane distances, and FST values across habitat pairwise comparison and FST groups. (A) Aggression levels between colonies, compared within and among habitat types. (B) Aggression levels between colonies, grouped by low and high FST values. (C) Dimethyl-alkane distances between colonies, compared within and among habitat types. (D) Dimethyl-alkane distances between colonies, grouped by low and high FST values. (E) FST values between colonies, compared within and among habitat types. (F: Forest; U: Urban; \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ).

quality filtering, we retained 843,957 high-quality sequences, averaging 60,283 reads per sample, and identified 1039 OTUs across 14 samples. The rarefaction curve reached a plateau (Fig. S1), indicating that the sequencing depth was sufficient to capture the majority of microorganisms present in the samples. Taxonomic analysis at the genus level (Fig. 4) revealed that the predominant bacterial

communities in *Pheidole megacephala* were *Wolbachia*, followed by ambiguous taxa from the family Rhizobiaceae, *Spiroplasma*, *Brenneria*, *Pseudomonas*, *Bacillus*, *Prevotella* 9, *Megamonas*, *Mesoplasma*, *Bacteroides*, and various uncultured and unidentified bacteria, including those from the phylum Rsa HF231 and the family Microbacteriaceae.



**Fig. 6:** Non-metric multidimensional scaling ordinations of Bray-Curtis distances of bacterial endosymbionts for *Pheidole megacephala* collected from forest (colonies F1 - F6) and urban areas (U1 - U8). Each ellipse represents a 95% confidence interval of the data from colonies collected from forest (green color) and urban areas (gray color).

### Aggressive behaviors in association with habitat types, genetic differentiation, and chemical distances

In the 91 pairwise encounter tests conducted, the aggression level varied from non-aggression (score = 1) to fighting (score = 4). The findings revealed no significant differences in the aggression level among the three groups: forest versus forest (median = 2.8, IQR = 0.7, N = 15 pairs), urban versus urban (median = 3, IQR = 0.4, N = 28 pairs), and forest versus urban (median = 2.8, IQR = 0.4, N = 48 pairs;  $\chi^2 = 0.54$ , df = 2, P = 0.76; Fig. 5A). However, the group with high  $F_{ST}$  values exhibited a significantly higher aggression level than did the group with low  $F_{ST}$  values ( $F_{ST} \leq 0.25$ : median = 2.6, IQR = 0.75, N = 42 pairs;  $F_{ST} > 0.25$ : median = 3, IQR = 0.2, N = 49 pairs; W = 337.5,

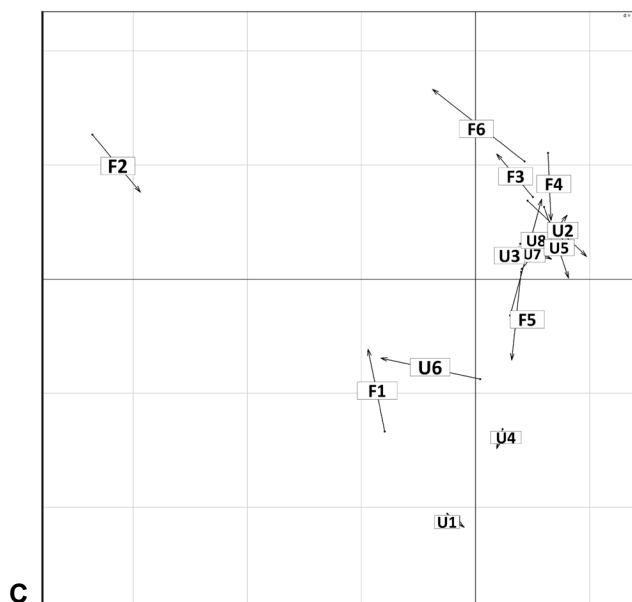
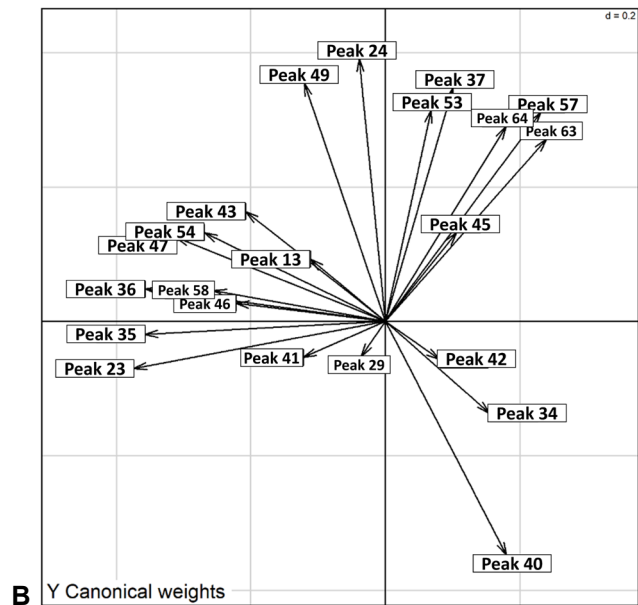
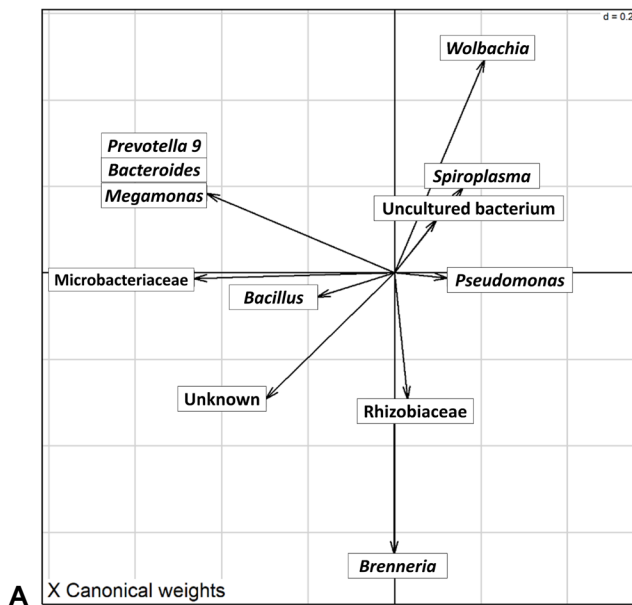
P < 0.001; Fig. 5B). A significant correlation was observed between aggression level and chemical distance, with the dimethyl-alkane group exhibiting the best goodness of fit ( $\Delta AICc = 0.00$ ,  $r^2 = 0.40$ , P < 0.001; Tab. 1). Therefore, distances of the dimethyl alkanes were selected and indicated as the chemical distances in the following analysis. Additionally, no correlation was observed between aggression level and isolation-by-distance (Mantel test,  $r = -0.08$ , P = 0.762).

### Associations among chemical distances, habitat types, and genetic differentiation

Dimethyl-alkane distances did not significantly differ among the habitat types (forest vs. forest: median = 0.027, IQR = 0.017, N = 15 pairs; urban vs. urban: median = 0.022,

**Tab. 1:** Results of generalized linear mixed models used for investigating the effect of chemical distances on aggression levels. Log-likelihood values indicate model fit, with higher values representing a better fit. Abbreviations: AICc, the second-order Akaike information Criterion;  $\Delta AICc$ , AIC differences; AICw, Akaike weights; PC1, Principal component 1.

Compound groups	AICc	$\Delta AICc$	AICw	Log-likelihood	R <sup>2</sup>	P-value
All peaks	131.4	19.57	0.000	-61.490	0.28	< 0.001
n-alkane	149.3	37.47	0.000	-70.441	0.16	< 0.01
Mono-methyl	138.1	26.19	0.000	-64.797	0.20	< 0.001
Di-methyl	111.9	0.00	0.966	-51.704	0.40	< 0.001
Tri-methyl	143.9	32.06	0.000	-67.736	0.12	< 0.001
Alkene	149.2	37.34	0.000	-70.375	0.11	< 0.01
Peaks with PC1 loadings exceeding $\pm 0.2$	118.6	6.68	0.034	-55.046	0.35	< 0.001



**Fig. 7:** (A) Ordinations of 12 bacterial OTU variables (only OTUs present in at least 10% of the samples were used in the analysis), (B) ordinations of 22 dimethyl alkanes, and (C) coinertia analysis showing the relationship between bacterial OTUs in the gut and 22 dimethyl alkanes found on the cuticle of 14 *Pheidole megacephala* colonies collected from forest (colonies F) and urban (colonies U) areas in Taichung, Taiwan. In Fig. 7C, the starting point of each arrow represents the OTU relative abundance dataset, while the endpoint corresponds to the di-methyl alkane relative abundance dataset. Shorter arrows indicate a higher degree of covariation between the two datasets (CULHANE & al. 2003). The first and second components of the CoIA explained 64.56% and 19.99% of the total variance, respectively.

IQR = 0.018, N = 28 pairs; forest vs. urban: median = 0.024, IQR = 0.013, N = 48 pairs;  $\chi^2 = 3.82$ , df = 2, P = 0.15; Fig. 5C). However, the group with high FST values exhibited significantly higher differences than did the group with low FST values (FST  $\leq$  0.25: median = 0.017, IQR = 0.009, N = 42 pairs; FST > 0.25: median = 0.030, IQR = 0.008, N = 49 pairs; W = 207, P < 0.001; Fig. 5D). A positive correlation was also observed between genetic and dimethyl-alkane distances (Mantel test, r = 0.59, P < 0.001). No significant differences in FST values were observed among the 3 combinations (forest vs. forest: median = 0.336, IQR = 0.321, N = 15 pairs; urban vs. urban: median = 0.344, IQR = 0.264, N = 28 pairs; forest vs. urban: median = 0.282, IQR = 0.307, N = 48 pairs;  $\chi^2 = 1.47$ , df = 2, P = 0.48; Fig. 5E). Additionally, no correlation was observed between isolation-by-dis-

tance and FST values (Mantel test, r = -0.23, P = 0.990), and dimethyl-alkane distances (Mantel test, r = -0.06, P = 0.655).

### Bacterial endosymbionts in associations with habitat types, genetic differentiation, and chemical distances

In terms of alpha diversity, bacterial species richness (observed OTUs: t = 1.456, df = 12, P = 0.17) and species diversity (Shannon index: t = 1.315, df = 12, P = 0.21) did not significantly differ between habitats (Tab.S4). Furthermore, NMDS analysis demonstrated no significant separation between the bacterial endosymbiont communities of ant colonies from forest and urban areas (F = 1.343, df = 12, P = 0.25; Fig. 6). In addition, we observed a significant correlation between the genetic distance (FST) of ants

from different colonies and OTU Bray-Curtis distances (Mantel test,  $r = 0.40$ ,  $P < 0.01$ ).

### Association between bacterial endosymbionts and dimethyl-alkane profiles of *Pheidole megacephala* from forest and urban areas

The first principal component of the bacterial endosymbionts explained 35.50% of the total variance, with a positive loading for *Pseudomonas* and negative loadings for unclassified bacteria of the Microbacteriaceae family, *Megamonas*, *Prevotella* 9, *Bacteroides*, *Bacillus*, and unknown bacteria. The second principal component explained 19.23% of the variance, with positive loadings for *Wolbachia*, *Spiroplasma*, and uncultured bacteria of the phylum Rsa HF231 and negative loadings for ambiguous taxa of the Rhizobiaceae family and *Brenneria* (Fig. 7A).

For dimethyl-alkane profiles, the first principal component explained 33.57% of the total variance, with positive loadings for peaks no. 34 and 42 and negative loadings for peaks no. 13, 23, 35, 36, 41, 43, 46, 47, 54, and 58. The second principal component explained 28.37% of the total variance, with positive loadings for peaks no. 24, 37, 45, 49, 53, 57, 63, and 64 and negative loadings for peaks no. 29 and 40 (Fig. 7B and Tab. S2).

The first and second components of the CoIA explained 64.56% and 19.99% of the total variance, respectively. The CoIA revealed a significant association between genus-level microbiota composition and dimethyl-alkane profiles (RV coefficient = 0.69; Monte Carlo  $P < 0.001$ ; Fig. 7C). In particular, colonies F3, F4, F6, U2, U3, U5, and U7, which were infected with *Wolbachia*, exhibited a higher abundance of peaks corresponding to long-chain dimethyl alkanes (peaks no. 37, 45, 53, 57, 63, and 64; Figs. 2, 7C and Tab. S2), ranging from carbon numbers C33 to C39.

## Discussion

As a rule, genetic and environmental factors both contribute to variance in aggression among ants (BEYE & al. 1998, STUART & HERBERS 2000, THURIN & ARON 2008, VAN ZWEDEEN & al. 2009, VILLALTA & al. 2020). Although the African big-headed ant, *Pheidole megacephala*, experienced a substantial reduction in genetic diversity in Taiwan due to a genetic bottleneck (LIU & al. 2021), workers still exhibited aggression toward workers from different colonies at the study sites. Moreover, the results revealed that habitat type did not significantly affect the level of aggression in *P. megacephala*. Variations in CHC profiles among conspecific colonies were associated with the level of genetic differentiation and composition of the bacterial endosymbionts in *P. megacephala*. These factors collectively mediated agonistic behaviors.

Unicoloniality is common among invasive ants (HELANTERÄ & al. 2009). FOURNIER & al. (2009) studied *Pheidole megacephala* populations in Darwin and Brisbane, Australia, which are approximately 3000 km apart, and observed considerable genetic differences in cuticular

profiles between the populations. However, they found that *P. megacephala* did not exhibit aggressive behavior toward conspecifics from different colonies, indicating the formation of a large unicolonial population across northeastern Australia (FOURNIER & al. 2009). The authors proposed that decreased genetic diversity is the underlying reason for the lack of intraspecific aggression in *P. megacephala* in Australia (FOURNIER & al. 2009). Similarly, *P. megacephala* in Taiwan has also experienced a substantial reduction in genetic diversity (LIU & al. 2022). However, in the present study, we observed significant genetic differences among *P. megacephala* colonies at the study sites, with  $F_{ST}$  values reaching up to 0.660. Workers exhibited aggressive behavior toward conspecific workers from different nests, suggesting the absence of unicolonial populations at the study locations.

In ants, the recognition of nestmates typically depends on differences in CHCs between colonies (LAHAV & al. 1999, STURGIS & GORDON 2012). However, a study on native *Pheidole megacephala* populations in Cameroon found no significant correlation between aggressiveness and differences in CHC profile and no dichotomy in CHC profiles between two genetically distinct supercolonies (FOURNIER & al. 2012). This result may be due to including a wide range of chemical profiles in the analysis. In the present study, the cuticular profiles of *P. megacephala* workers were primarily composed of methyl-branched alkanes (mono-, di-, and tri-methyl) and some straight-chain n-alkanes and alkenes. Among these CHCs, dimethyl alkanes were the most important recognition cues for *P. megacephala*, suggesting their strong association with nestmate recognition. Thus, in the present study, we focused on dimethyl alkanes and found their intercorrelations with genetic factors and the bacterial endosymbionts.

In social insects, each colony has a unique CHC profile (MARTIN & al. 2008, OPPELT & al. 2008). Differences in these CHC profiles often correspond to genetic distances between ant lineages (DRESCHER & al. 2010, BLIGHT & al. 2012, TESEO & al. 2014). In *Pheidole megacephala*, we observed a significant association between genetic differences and distances in dimethyl-alkane profiles. Specifically, when the genetic distance exceeded 0.25 ( $F_{ST} > 0.25$ ), both dimethyl alkane distances and aggression levels increased significantly. This result suggests that the variations in dimethyl-alkane profiles and the corresponding aggression behaviors among workers from 14 colonies in Taichung have a genetic basis.

Our findings demonstrated that variations in dimethyl-alkane profiles among *Pheidole megacephala* colonies are attributed to not only genetic differences but also the bacterial endosymbionts of workers in these colonies. The CoIA revealed a correlation between the *Wolbachia* symbiont and the dimethyl-alkane profiles of *P. megacephala*. Specifically, colonies F3, F4, F6, U2, U3, U5, and U7, which were infected with *Wolbachia* (Fig. 7), exhibited a high abundance of peaks corresponding to long-chain dimethyl alkanes (peaks no. 37, 45, 53, 57, 63, and 64), ranging from

carbon numbers C33 to C39. Although few studies have examined the effects of *Wolbachia* on agonistic behavior in ants, some inferences can be drawn from previous research. For instance, the formation of a unicolonial population of Argentine ants *Linepithema humile* in new regions has been associated with the absence of *Wolbachia* infection (REUTER & al. 2005). By contrast, a laboratory study indicated that the gut microbiota in Argentine ants significantly changed after antibiotic treatment, leading to increased intraspecific aggression (LESTER & al. 2017).

The genetic background of the host appears to be closely linked to the bacterial endosymbionts in ants (XIAO & al. 2023). Similarly, our results revealed a significant correlation between the host's mitochondrial DNA and OTU Bray-Curtis distances. We also observed that colonies infected by *Wolbachia* generally had low FST values ( $\leq 0.25$ ). The *Wolbachia* bacteria could be inherited across generations through the infection of germ stem cells in the larvae and ovaries of female adults (MARTINS & al. 2021). In the study sites, colonies of *Pheidole megacephala* have unrestricted gene exchange across the urban and forest areas (PENG & al. 2023). Based on significant associations observed among CHC profiles, ant genetics, and host bacterial endosymbionts, we postulate that ant CHCs, shaped by heritable bacterial endosymbionts such as *Wolbachia*, contribute to colony-level differences in dimethyl-alkane profiles, leading to the observed agonistic interactions in *P. megacephala* across different habitats.

### Conclusions

This study demonstrated that despite a substantial reduction in genetic diversity in Taiwan due to a genetic bottleneck, no supercolony formation was observed in *Pheidole megacephala*. Workers exhibited aggression toward conspecifics from different colonies regardless of their habitat type. This finding indicates that coevolution between the bacterial endosymbionts and ant host may be a key factor driving the variation in chemical profiles and leading to agonistic interactions in *P. megacephala*. Our approach differs from previous works on agonistic interactions of ants by explicitly considering the mutual relationship between genetic and chemical cues and bacterial endosymbionts. Such relationships, however, may be multifaceted and different across ant species and ecosystems, and more complex interactions can be expected.

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### Declaration on use of generative artificial intelligence tools

The authors declare that they did not utilize generative artificial intelligence tools in any part of the composition of this manuscript.

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