Aphid Genotype Determines Intensity of Ant Attendance: Do Endosymbionts and Honeydew Composition Matter?

N. KATAYAMA,[1,2,3] T. TSUCHIDA,[4] M. K. HOJO,[5] AND T. OHGUSHI[1]

Ann. Entomol. Soc. Am. 106(6): 761–770 (2013); DOI: http://dx.doi.org/10.1603/AN13089

ABSTRACT Ant–aphid interactions are well-studied mutualisms, but surprisingly, the intraspecific variation in the degree of ant attendance of aphids has been less appreciated than interspecific variation. Honeydew composition is a key factor in determining aphids' traits in relation to the intensity of ant attendance, and composition may be altered by endosymbiotic bacteria. Here, we evaluate relationships among the intensity of ant attendance, honeydew composition, and aphids' endosymbionts. We found differences in the intensity of ant attendance and endosymbiont composition between clones of cowpea aphids (*Aphis craccivora* Koch). To investigate whether aphid genotype, endosymbionts, or both, influence honeydew composition, and the relative influence of these factors on the intensity of ant attendance, a secondary symbiont of the aphid, *Arsenophonus*, was removed from the clone exhibiting a lower degree of ant attendance. Then, the sugar and amino acid composition of honeydew were compared between different aphid genotypes and between same genotype with and without *Arsenophonus*. The experiments demonstrated that there was a difference in the sugar composition of honeydew between clones, but that *Arsenophonus* did not influence this difference. Furthermore, the intensity of ant attendance of the clone did not change after removing *Arsenophonus*. These results suggest that variation in the degree of ant attendance of this aphid is caused by aphid genotype, not by endosymbionts.

KEY WORDS *Aphis craccivora*, *Arsenophonus*, endosymbiont, honeydew, mutualism

Mutualism, an interaction between two or more species in which all participants receive a net benefit, is widespread in nature (Boucher et al. 1982, Herre et al. 1999). Because natural selection favors individuals that have maximum fitness, the species interactions can be variable in the presence of intraspecific variation in degree of association, and the mutualism may sometimes break down (Bronstein 1994, Sachs and Simms 2006). Hence, intraspecific variation in association with partners is critical to understanding the spatiotemporal patterns of mutualism.

Ant–aphid interactions are well-studied mutualistic associations in which ants exchange services (e.g., protection against natural enemies) for resources (i.e., aphid honeydew) (Way 1963). Aphids are small sap-feeding insects that are distributed worldwide, especially in temperate regions (Dixon 1998). Nymphs and adults feed on phloem sap, which is rich in sugar but is typically lacking in essential amino acids (Douglas 1998). To compensate for the nitrogen shortage, aphids ingest large amount of phloem sap, excrete surplus sugars as honeydew after absorbing nitrogen, and harbor endosymbiotic bacteria that synthesize essential amino acids (Dixon 1998, Douglas 1998). Ants tend aphids to collect their honeydew as food, and in return, provide aphids several services, such as promotion of reproductive performance (e.g., El-Ziady and Kennedy 1956, El-Ziady 1960, Buckley 1987), increased developmental rate (e.g., El-Ziady 1960) or colony growth (El-Ziady and Kennedy 1956, Buckley 1987), and reduction in the risk by predators, parasitoids, and parasitic fungi (Nixon 1951; Buckley 1987; Katayama and Suzuki 2002, 2003).

Intraspecific variation in the intensity of ant attendance is a key driver in the evolution and maintenance of the mutualism in aphids (Vantaux et al. 2012). Surprisingly, the intraspecific variation in ant attendance of aphids has been less appreciated than interspecific variation (but see Vantaux et al. 2011, 2012). The ecological importance of the ant–aphid mutualism is well accepted (Wimp and Whitham 2001, Ohgushi et al. 2007) because aphid-tending ants reduce the abundance, diversity, or both, of other herbivorous insects (Wimp and Whitham 2001, Suzuki et al. 2004, Ando and Ohgushi 2008). Therefore, the variation in ant attendance of aphids can greatly influence the community organization of insects.

[1] Center for Ecological Research, Kyoto University, 3-509 Hirano 2-chome, Otsu, Shiga 520-2113, Japan.
[2] Current address: Field Science Center for Northern Biosphere, Hokkaido University, Toikanbetsu 131, Horonobe-cho, Hokkaido 098-2943, Japan.
[3] Corresponding author, e-mail: noborukata1913@gmail.com.
[4] Frontier Research Core for the Life Sciences, University of Toyama, 3190 Gofuku, Toyama, Toyama 930-8555, Japan.
[5] Faculty of Agriculture, Department of Environmental Sciences and Technology, University of the Ryukyus, Senbaru 1, Nishihara, Okinawa 903-0213, Japan.

Honeydew composition is a key factor in determining aphids' traits in relation to the intensity of ant attendance. Sugar and amino acid composition of honeydew have been shown to vary greatly among aphid species, clones, or both (Hendrix et al. 1992; Völkl et al. 1999; Fischer et al. 2001; Woodring et al. 2007; Vantaux et al. 2011, 2012). Some, though not all, aphid clones can synthesize specific oligosaccharides from sugars in their diet (phloem sap), and excrete them in honeydew (Woodring et al. 2007). In particular, melezitose is often considered to be a key oligosaccharide affecting ants' attendance of aphids (Völkl et al. 1999, Fischer et al. 2001, but see Blüthgen and Fiedler 2004, Vantaux et al. 2011). Furthermore, amino acids in honeydew can influence ant attendance because most ants prefer mixed solutions containing sugar and amino acids to sugar alone (Lanza 1991, Wada et al. 2001, Blüthgen and Fiedler 2004, Hojo et al. 2008).

Honeydew composition may be altered by endosymbiotic bacteria. All aphids harbor a primary symbiont, *Buchnera. Buchnera* do not synthesize sugars, but synthesize essential amino acids from nonessential amino acids (mainly asparagine and glutamine) found in phloem sap, and provide them to their hosts (Buchner 1965, Sasaki and Ishikawa 1993, Douglas 1998). Therefore, aphids experimentally lacking *Buchnera* cannot use excess nonessential amino acids from phloem sap and instead excrete them in the honeydew (Sasaki et al. 1990), suggesting that amino acid concentration in honeydew can increase or decrease depending on *Buchnera* activity. In addition to *Buchnera*, many aphid clones harbor other facultative symbionts (also referred to as secondary symbionts or S-symbionts). The S-symbionts affect many ecological traits of the host aphid, for example, conferring heat tolerance, providing resistance against parasitoids and parasitic fungi, changing insect–host plant relationships, and modifying insect body color (Oliver et al. 2010, Tsuchida et al. 2010). Hence, it is possible that symbionts influence the honeydew composition of their host aphids, with the result that the intensity of ant attendance may be affected.

Both aphid genotype and endosymbionts may influence honeydew composition. However, little is known about which has a greater effect on the variation in ant attendance of aphid clones because the endosymbionts are vertically transmitted from mother to offspring. The aim of this study is to examine and compare the effects of genotype and endosymbionts on honeydew composition and degree of ant attendance.

Materials and Methods

Materials. Cowpea aphid, *Aphis craccivora* Koch (Hemiptera: Aphididae), is a black small aphid (1 mm in body length), which feeds on several legume herbs, such as *Vicia angustifolia* L. and *Vicia faba* L. (Leguminosae). The aphid is tended by several ant species, including *Lasius japonicus* Santschi, *Pristomyrmex punctatus* (Mayr), and *Tetramorium tsushimae* Emery (Hymenoptera: Formicidae), all of which collect aphids' honeydew (Katayama and Suzuki 2002, 2003; Suzuki et al. 2004).

Ant Attendance in Natural Population. We conducted a field survey to examine the frequency of ant attendance of *A. craccivora* colonies at two sites: a common garden of the Center for Ecological Research (CER), Kyoto University (JAPAN: Shiga Prefecture: Otsu City, 35° 12′ N, 136° 08′ E), and the campus of Saga University (JAPAN: Saga Prefecture: Saga City, 33° 18′ N, 130° 01′ E), from early May to early June in 2006. Each site was ≈500- by 200-m area, and there were several ant and aphid colonies present in each. We observed 213 and 195 aphid colonies at Otsu and Saga, respectively, and checked whether colonies from the two sites were tended by ants.

Ant Attendance in Otsu and Saga Aphid Clones. In July 2004, a laboratory experiment was carried out to compare the intensity of ant attendance between the aphid clones collected in Otsu and Saga. One *A. craccivora* colony was collected from *V. angustifolia* growing at the common garden of CER (Otsu clone), and the other was collected from the campus of Saga University (Saga clone) in April of 2004. To make isofemale lines, we separately reared one clone from each aphid colony on seedlings of *V. faba* in a laboratory at 25°C under a photoperiod of 16:8 (L:D) h. *V. faba* used for this study was cultured in polyethylene pots (9 cm in diameter and 8 cm in depth) in an experimental chamber at 22–25°C under natural light conditions before aphid inoculation.

Two ant colonies of *L. japonicus* were collected from the common garden of CER (Otsu ant colony) and from the campus of Saga University (Saga ant colony) in May of 2004. Workers of Otsu and Saga colonies were transferred to 12 and 10 test tubes (1.2 cm in diameter and 18 cm in length), with 500 workers per tube, respectively. All replicates came from the same colonies at Otsu and Saga, respectively. The different numbers of ant colonies were created simply because different numbers of workers were collected. Wet cotton wool, ≈3 cm in depth, was placed at the bottom of the tubes to maintain the appropriate humidity. The tubes were covered with aluminum foil to create dark conditions, simulating natural ant nests. Tubes were connected to each other with a vinyl chloride tube (6 mm inner diameter and 10 cm in length) as an entrance. The transferred ants were provided a 10% sucrose solution from a test tube (1.2 cm in diameter and 12 cm in length) plugged with cotton wool. Before the experiment, ants were starved for 4 d to increase sensitivity to aphid honeydew.

Two seedlings of *V. faba* that were ≈20 cm in height with five leaf nodes were selected, and soil was removed from the roots. The seedlings were transplanted individually into plastic pots (10 cm in diameter and 4.5 cm in height) containing water. Each plastic pot was covered with a petri dish lid with a 15-mm hole in the center to allow the plant stem to penetrate. To exclude ant attraction to extrafloral nectaries (EFNs) of *V. fava*, EFNs were removed with scissors. Twenty individuals each from Otsu and Saga clones of aphids were placed on each seedling, re-

Table 1. Primers for detection of endosymbiotic bacteria used in this study

Target symbiont	Target gene	Primer name	Primer sequence (5′-3′)	Product size (kb)	References
Whole eubacterial symbionts	16S rRNA	16SA1	AGAGTTTGATCMTGGCTCAG	1.5	Tsuchida et al. 2006
		16SB1	TACGGYTACCTTGTTACGACTT		
Buchnera	16S rRNA	BuF700n	GAATTCTAGGTGTAGCGGTGA	0.66	Tsuchida et al. 2006
		BuR1363n	GCGATTCCGACTTCGTGGA		Tsuchida et al. 2006
Serratia	16S rRNA	16SA1	AGAGTTTGATCMTGGCTCAG	0.48	Tsuchida et al. 2006
		PASScmp	GCAATGTCTTATTAACACAT		Tsuchida et al. 2006
Hamiltonella	16S rRNA	PABSF	AGCACAGTTTACTGAGTTCA	0.2	Tsuchida et al. 2006
		16SB4	CTAGAGATCGTCGCCTAGGTA		Tsuchida et al. 2006
Regiella	16S rRNA	U99F	ATCGGGGAGTAGCTTGCTAC	0.2	Tsuchida et al. 2006
		16SB4	CTAGAGATCGTCGCCTAGGTA		Tsuchida et al. 2006
Rickettsia	16S rRNA	16SA1	AGAGTTTGATCMTGGCTCAG	0.2	Tsuchida et al. 2006
		Rick16SR	CATCCATCAGCGATAAATCTTTC		Tsuchida et al. 2006
Spiroplasma	16S rRNA	16SA1	AGAGTTTGATCMTGGCTCAG	0.51	Tsuchida et al. 2006
		TKSSspR	TAGCCGTGGCTTTCTGGTAA		Tsuchida et al. 2006
Arsenophonus	16S rRNA	16SA1	AGAGTTTGATCMTGGCTCAG	0.96	Tsuchida et al. 2002
		Ars16SR	TTAGCTCCGGAGGCCACAGT		Tsuchida et al. 2006

spectively. These potted seedlings were then placed in an experimental container (length: 19 cm, width: 25 cm, and height: 9 cm) covered with plaster 5 cm in depth (see Katayama and Suzuki 2010). The container was set at 25°C under light conditions. The inner side of the container wall was plastered with a talc powder to prevent ants from moving out. One ant-nest tube of *L. japonicus* was connected to the container to allow ants to forage on the plants freely. After 90 min, when ants acclimated to their surroundings, the number of ants tending aphids was counted 10 times every 10 min. We regarded ants tapping aphids by their antenna as tending ants. The average number of ants tending aphids throughout each 10-min survey was used for analysis. The experiment was replicated 12 and 10 times for Otsu and Saga ants (one replicate per ant tube), respectively.

Detection of Endosymbiotic Bacteria. To identify microbial endosymbionts of Otsu and Saga aphid clones, these clones were preserved in acetone for molecular analysis (Fukatsu 1999). Otsu and Saga clones were individually subjected to DNA extraction by conventional phenol extraction method. The purified DNA was dissolved in 200 μl of TE buffer (10 mM tris-HCl [pH 8.0] and 0.1 mM EDTA). Endosymbiotic bacteria of Otsu and Saga clones were examined with two independent methods: diagnostic polymerase chain reaction (PCR) detection and 16S ribosomal RNA (rRNA) gene clone library analysis as described in Fukatsu et al. (2001). Diagnostic PCR targeted seven bacteria (*Buchnera*, *Serratia*, *Regiella*, *Hamiltonella*, *Rickettsia*, *Spiroplasma*, and *Arsenophonus*), which were previously detected from various aphid species (Oliver et al. 2010), using specific primer sets (Table 1) on 16 individuals randomly chosen from Otsu and Saga clones. PCR reactions were conducted using Ampli*Taq* Gold DNA polymerase (Roche, Basel, Switzerland), and its supplemented buffer system under a temperature profile of 95°C for 10 min followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Total DNA preparations from aphids whose endosymbiotic bacteria had been identified definitely were used as control samples (Tsuchida et al. 2002). For the clone library method, 16S rRNA genes of whole eubacteria in the whole-insect DNA were amplified by PCR using universal primers 16SA1 in combination with 16SB1 (Table 1). The PCR products were cloned, and 24 clones for each of the aphid strains were subjected to restriction fragment length polymorphism (RFLP) genotyping and sequenced as previously described (Fukatsu and Nikoh 1998). The sequence similarity of detected symbionts was analyzed using BLAST (Altschul et al. 1990) on nucleotide sequences deposited in the DNA Data Bank of Japan (DDBJ), National Center for Biotechnology Information (NCBI), and GenBank databases. Candidate taxa for phylogenetic analysis were selected from a variety of bacterial 16S rRNA gene sequences including those known for bacteria isolated from insects and those showing high scores of similarity in BLAST search. Phylogenetic analysis of the S-symbionts was conducted using a maximum likelihood method with the program MEGA 5 (Tamura et al. 2011).

Antibiotic Treatment. To examine whether secondary endosymbionts influence honeydew composition and ant attendance, we experimentally eliminated secondary endosymbionts (i.e., *Arsenophonus*) from Saga clone in July 2005. As the Otsu clone did not have *Arsenophonus* (see Results), we used only the Saga clone for this experiment. Ten-day-old adults of Saga aphids were treated with ampicillin using a selective elimination technique (Koga et al. 2003). The dosage of ampicillin was 1 μg/mg aphid body weight. Five ampicillin-medicated aphids were reared individually on *V. faba* plants and allowed to produce nymphs for 24–48 h after treatment. The nymphs were designated as generation one (G1). Three newly emerged G1 adults from each ampicillin-medicated line were allowed to produce nymphs (G2), and then checked for *Buchnera* and *Arsenophonus* infection by using diagnostic PCR analysis. Of the 15 G1 lines examined, *Arsenophonus* was successfully eliminated from only one line. To confirm selective elimination of *Arsenophonus*, four insects from each generation were examined by diagnostic PCR over three successive generations. The line infected with *Buchnera* only was reared on *V. faba* as an *Arsenophonus*-removed strain ($Saga^{ars-}$), and used in subsequent experiments.

Honeydew Analysis. In September 2006, to examine honeydew components of Otsu, Saga, and Saga^{ars-} clones, individuals of each clone were reared at 20°C under a photoperiod of 16:8 (L:D) h in an environmental chamber. *V. faba* seedlings with two leaf nodes were cultivated in an outdoor experimental chamber at 20–25°C under natural light conditions. Leaves were clipped from the seedlings, and one leaf per plant was placed on a sheet of wet cotton wool (2 by 2 cm) in a petri dish. One adult of each clone was placed on separate leaves and reared in the environmental chamber. After 1 d, the mother and all newborn nymphs were removed, and the nymphs were kept until they reached the adult stage. Then, each aphid was placed on a new leaf disk (12 mm in diameter). Droplets of honeydew were collected using a 0.5-μl microcapillary tube under microscopy (Drummond Scientific Company, Broomall, PA) for a maximum of 1 h or until the tube was filled.

Honeydew samples (Otsu clone: $n = 8$, Saga clone: $n = 10$, and Saga^{ars-} clone: $n = 12$) were each dissolved in 15 μl of milli-Q-water containing 5 μg/μl xylose as an internal standard. In all, 10 and 5 μl volumes of the sample solution were used for sugar and amino acid analysis, respectively.

Sugars in honeydew were analyzed using high-pressure liquid chromatography (HPLC), using a Wakosil 5NH_2-MS packed column (4.6 by 150 mm; Wako Pure Chemical, Osaka, Japan) and an 80% acetonitrile mobile phase at room temperature. The flow rate was 1 ml/min. Peak sizes for the various sugars present in the honeydew samples were evaluated using a refractive index detector (RID; Shimadzu Corp., Kyoto, Japan). The sugar composition in each sample was determined using six sugar standards (xylose, fructose, glucose, sucrose, maltose, and melezitose) according to the internal standard method. For amino acid analysis, each sample was adjusted with 0.02 N HCl to a final volume of 100 μl, and analyzed using an automated amino acid analyzer L-8800 (Hitachi, Tokyo, Japan).

The differences in sugar and amino acid composition among clones were evaluated using analysis of similarity (ANOSIM) and expressed as two-dimensional scores obtained by nonmetric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarity index values. Similarity percentage analysis (SIMPER) was used to examine the relative contribution of each sugar to the dissimilarities of overall sugar composition among aphid clones.

Effects of *Arsenophonus* on Performance of Saga Clone. In September 2005, to examine whether *Arsenophonus* influences performance of aphids, we compared developmental rate and nymph production between Saga and Saga^{ars-} clones. *V. faba* seedlings with two leaf nodes were cultivated in an outdoor experimental chamber at 20–25°C under natural light conditions. Leaves were clipped from the seedlings, and one leaf of each was placed on the piece of wet cotton wool (2 by 2 cm) in a petri dish. One adult from the Saga or Saga^{ars-} clone was placed on the leaf and reared in an environmental chamber at 20°C under a photoperiod of 16:8 (L:D) h. After 1 d, the mother and all but one newborn nymphs were removed, and the remaining nymph was reared to adulthood in the chamber. Every 4 d, the leaf in the petri dish was replaced with a fresh one. The survival and nymph production of the aphid were checked daily. When the aphid produced nymphs, the newborn nymphs were removed after the number was counted. The aphid performance, including the developmental period and total nymph production, was measured. This experiment was replicated 18 times each for Saga and Saga^{ars-} clones.

Effects of *Arsenophonus* on Ant Attendance of Saga Clone. In October 2008, to examine the effect of *Arsenophonus* on ant attendance of Saga aphids, we carried out a laboratory experiment with *L. japonicus* ants, using a similar method as previously described. Two colonies of *L. japonicus* were collected from the common garden of CER on 1 October 2008 (ant colony A and B, hereafter). Workers of colonies A and B were transferred to 18 and 15 test tubes (prepared as above), respectively (1.2 cm in diameter and 18 cm in length), with 300 workers per tube.

Two seedlings of *V. faba* that were ≈10 cm in height with three leaf nodes were transplanted individually into plastic pots (10 cm in diameter and 4.5 cm in height) containing water. Ten individuals each from Saga and Saga^{ars-} colonies were placed on separate seedlings. All EFNs of *V. faba* were removed to exclude any effect of EFNs on ant behavior. These potted seedlings were then placed in the same experimental container as mentioned earlier, with one ant nest connected to the container. After 90 min, the number of ants tending aphids was counted 10 times every 10 min. The experiment was replicated 18 and 15 times for colony A and B, respectively.

Nucleotide Sequence Accession Numbers. The 16S rRNA gene sequence of the *Arsenophonus* symbiont from *A. craccivora* was deposited in the DDBJ, NCBI, and GenBank nucleotide sequence database under the number AB823665.

Results

Ant Attendance in Natural Population. The proportion of ant-tended aphid colonies largely differed between two sites (χ^2 test, $\chi^2 = 115.37$; $P < 0.001$). While 20.5% of Saga colonies ($n = 195$) were tended by ants, 73.6% of Otsu colonies ($n = 213$) were tended by ants. The aphid colony size did not differ between the two sites, nor between ant-tended and unattended colonies within each site (sites: $F = 0.01$, df = 1, 404, $P = 0.926$; ants: $F = 0.41$, df = 1, 404, $P = 0.524$; sites × ants: $F = 0.43$, df = 1, 404, $P = 0.511$), indicating that the difference in ant attendance between the two sites was not because of aphid colony size.

Ant Attendance in Otsu and Saga Clones. Average numbers of Otsu and Saga ants tending aphid clones were 1.76 ± 0.32 (mean ± SE; $n = 24$) and 0.51 ± 0.13 ($n = 20$), respectively. The average number of ants attending the Otsu clone was significantly higher than the Saga clone (Wilcoxon signed rank test; $z = -2.845$; $P = 0.004$; Fig. 1a). The same was true for Saga ants

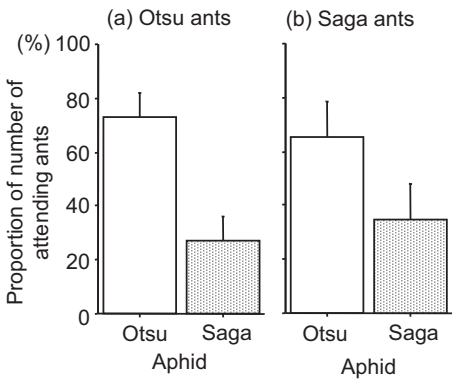

Fig. 1. Proportion of number of ants attending *A. craccivora.* Open and dotted columns indicate Otsu and Saga aphid clones, respectively. (a) *L. japonicus* ants collected at Otsu, and (b) *L. japonicus* ants collected at Saga. Error bars show SE.

(Wilcoxon signed rank test; $z = -2.030$; $P = 0.042$; Fig. 1b).

Detection of Endosymbiotic Bacteria. Diagnostic PCR analysis detected the essential endosymbiont *Buchnera* from all the samples in both Otsu and Saga strains as expected. Besides *Buchnera, Arsenophonus* was detected from only the Saga strain at 100% infection (16 out of 16 individuals). Other endosymbiotic bacteria (*Serratia, Regiella, Hamiltonella, Rickettsia,* and *Spiroplasma*) were not detected in either clone.

Endosymbionts in the both aphid strains were also analyzed by the clone library method. The RFLP analysis revealed the presence of two sequence types, the predominant type A and the relatively minor type B. The Otsu strain only possessed type A (24 out of 24 clones), while the Saga strain possessed both type A (15 out of 24 clones) and type B (9 out of 24 clones). Three clones of each type from each sample were sequenced, and type A and B sequences were found to be identical to each other. The type A sequence showed the high sequence similarity to 16S rRNA gene sequences of *Buchnera* from various aphids. However, the type B sequence exhibited high sequence similarity to 16S rRNA gene sequences of *Arsenophonus* from various insects. Molecular phylogenetic analysis confirmed that type B sequence from Saga strain belongs to the clade of *Arsenophonus* (Fig. 2). Thus, two independent methods yielded the same result: the Otsu clone possessed *Buchnera* only, while the Saga clone harbored *Arsenophonus* in addition to *Buchnera.*

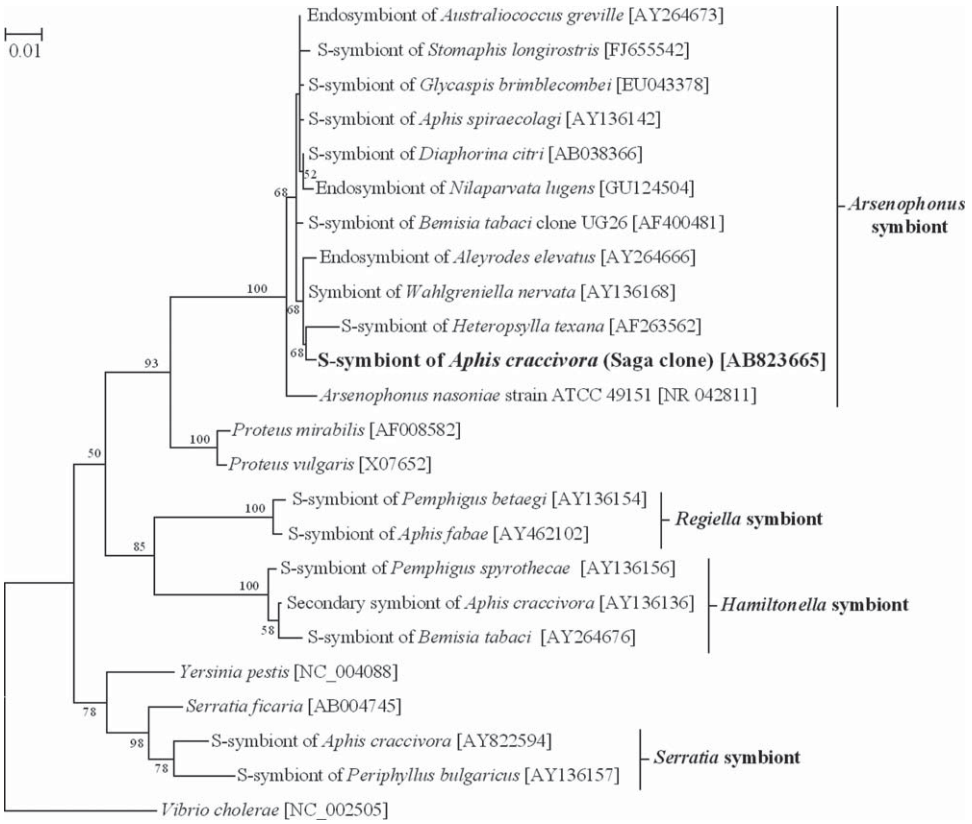

Fig. 2. Phylogenetic analysis of *Arsenophonus* symbiont from *A. craccivora* on the basis of 16S rRNA gene sequences. A maximum likelihood tree of 1,128 unambiguously aligned nucleotide sites is shown. The bootstrap values (in percentage) are shown at the nodes, although values <50% are not shown. Nucleotide sequence accession numbers are shown in brackets. S-symbiont clades of aphids are shown on the right.

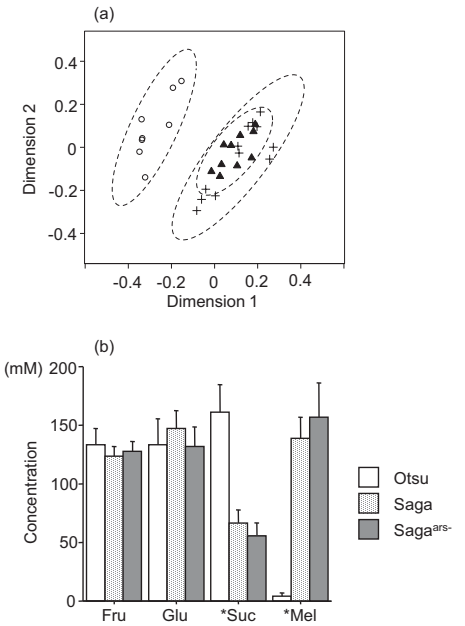

Fig. 3. Sugar composition in honeydew among clones. (a) NMDS ordination of the sugar composition in honeydew based on Bray–Curtis similarity index. Circles, triangles, and crosses indicate Otsu, Saga, and Saga^{ars-} clones, respectively. (b) Sugar concentrations of honeydew from Otsu (open columns), Saga (dotted columns), and Saga^{ars-} (solid columns) clones (Fru, fructose; Glu, glucose; Suc, sucrose; Mel, melezitose). Asterisks indicate significant difference among aphid clones (ANOVA; $P < 0.05$). Error bars show SE.

Sugar and Amino Acid Compositions in Honeydew. Four sugars (fructose, glucose, sucrose, and melezitose) were detected in honeydew from the two clones. The sugar composition is graphically portrayed by NMDS (stress value: 0.043; Fig. 3a). The sugar composition of honeydew excreted by Otsu aphids was significantly different from that excreted by Saga aphids (ANOSIM, global $R = 0.711$; $P = 0.001$) and Saga^{ars-} clones (global $R = 0.606$; $P = 0.001$). Melezitose and sucrose explained 40 and 30% of the overall dissimilarity in sugar composition, respectively. However, there was no difference in sugar composition of honeydew between Saga and Saga^{ars-} clones (ANOSIM, global $R = -0.060$; $P = 0.796$). Although no significant differences in concentrations of fructose and glucose among aphid clones were found (fructose: $F = 0.247$, df = 2, 27, $P = 0.783$; glucose: $F = 0.1996$, df = 2, 27, $P = 0.820$; Fig. 3b), concentrations of sucrose and melezitose significantly differed among aphid clones (analysis of variance [ANOVA]; sucrose: $F = 13.551$, df = 2, 27, $P < 0.001$; melezitose: $F = 11.5314$, df = 2, 27, $P < 0.001$; Fig. 3b). Sucrose in the Otsu clone honeydew was significantly greater than Saga and Saga^{ars-} clones (Tukey–Kramer test; $P < 0.05$). Melezitose in honeydew from the Otsu clone was significantly less than that of Saga and Saga^{ars-} clones (Tukey–Kramer test; $P < 0.05$). Sucrose and melezitose did not differ between Saga and Saga^{ars-} clones (Tukey–Kramer test; $P > 0.05$).

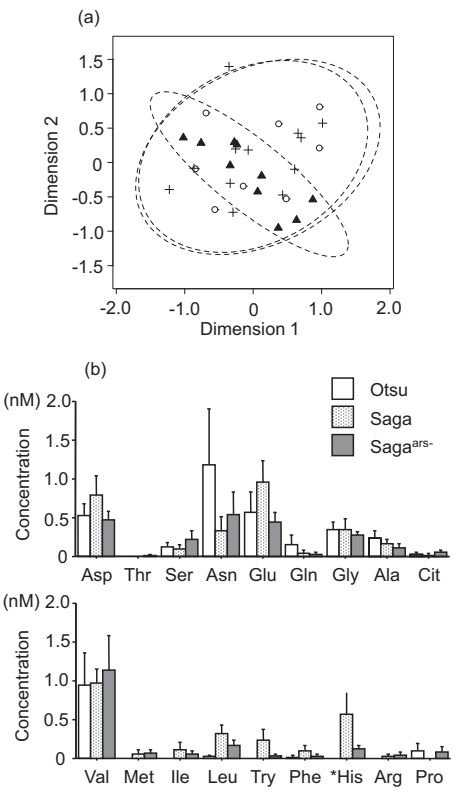

Fig. 4. Amino acid composition of honeydew among clones. (a) NMDS ordination of the amino acid composition in honeydew based on Bray–Curtis similarity index. Circles, triangles, and crosses indicate Otsu, Saga, and Saga^{ars-} clones, respectively. (b) Amino acid concentrations in honeydew of Otsu (open columns), Saga (dotted columns), and Saga^{ars-} (solid columns) aphid clones (Asp, aspartic acid; Thr, threonine; Ser, serine; Asn, asparagines; Glu, glutamic acid; Gln, glutamine; Gly, glycine; AL, alanine; Cit, citruline; Val, valine; Met, methionine; Ile, isoleucine; Leu, leucine; Try, tyrosine; Phe, phenylalanine; His, histidine; Arg, arginine; Pro, proline). Asterisks indicate significant differences among aphid clones (ANOVA; $P < 0.05$). Error bars show SE.

Eighteen amino acids (aspartic acid, threonine, serine, asparagine, glutamic acid, glutamine, glycine, alanine, citruline, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, arginine, and proline) were detected in *A. craccivora* honeydew. The amino acid composition is shown by NMDS analysis (stress value: 0.157; Fig. 4a), and did not differ among clones (ANOSIM, global $R = 0.014$; $P = 0.359$). The concentration of histidine significantly differed among clones ($F = 3.455$; df = 2, 27; $P = 0.0461$; Fig. 4b), but post hoc tests did not support significant differences (Tukey-Kramer test; $P > 0.05$). Concentrations of other amino acids did not differ among aphid clones ($P > 0.05$; Fig. 4b).

Effects of *Arsenophonus* on Performance and Ant Attendance of Saga Clone. The developmental period of Saga^{ars-} clone was slightly shorter than that of the Saga clone (Mann–Whitney U test, $z = -1.836$; $P =$

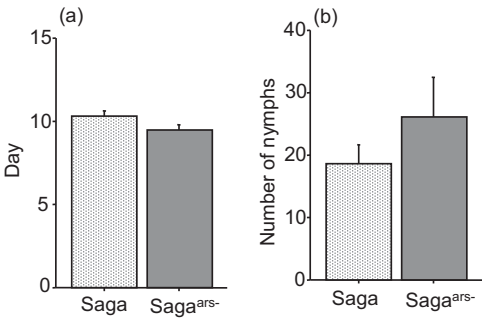

Fig. 5. Performance of aphids with and without *Arsenophonus*. (a) Developmental period, and (b) total nymph production by individual aphids. Dotted and solid columns indicate Saga and Saga^{ars} aphid clones, respectively. Error bars show SE.

0.066; Fig. 5a). The number of nymphs produced per aphid did not differ between Saga and Saga^{ars-} clones (Mann–Whitney *U* test, nymph production: $z = -0.4702$; $P = 0.638$; Fig. 5b).

The average number of ants in colony A and B were 8.32 ± 0.73 (mean $\pm$ SE; $n = 36$) and 2.43 ± 0.37 ($n = 30$), respectively. Attendance behavior of the two ant colonies used in this experiment was similar. In both ant colonies, the average number of ants tending the Saga clone did not differ from that tending the Saga^{ars-} clone (Wilcoxon signed rank test, $z = -0.570$; ant colony A: $P = 0.2868$; colony B: $P = 0.2012$; Fig. 6).

Discussion

This study demonstrated that the Otsu clone was more attended by both Otsu and Saga ants than the Saga clone, and that the sugar composition of honeydew also differed between Otsu and Saga clones. These results clearly show that there is intraspecific variation in aphids' honeydew composition and the intensity of ant attendance. We also detected a difference in endosymbiont composition between the two clones. Aphids from the Saga clone harbored *Buchnera* and *Arsenophonus*, but those from the Otsu clone harbored *Buchnera* only. However, the sugar and amino acid composition of honeydew did not differ between Saga and Saga^{ars-} clones, suggesting that *Arsenophonus* did not influence the synthesis of sugars and amino acids in the host aphid. In addition, *Arsenophonus* did not affect performance of the host aphid or the intensity of ant attendance in the laboratory.

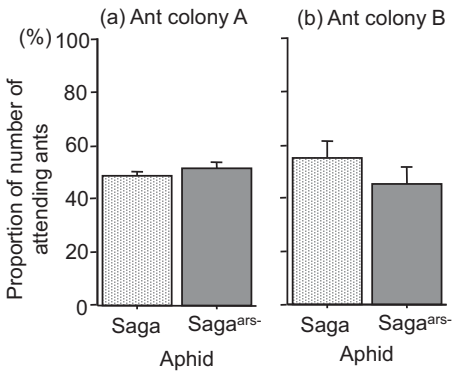

Fig. 6. Proportion of number of ants tending Saga and Saga^{ars} aphid clones. Dotted and solid columns indicate Saga and Saga^{ars} aphid clones, respectively. (a, b) We carried out this experiment with two colonies of *L. japonicus* ants (A and B) collected from Otsu. Error bars show SE.

Although we did not exchange *Buchnera* between clones, *Buchnera* is unlikely to influence the difference in the intensity of ant attendance observed between Otsu and Saga clones. It is well-known that *Buchnera* strongly promote their hosts' reproduction and may affect the amino acid composition of honeydew, but they do not synthesize sugars (Douglas 1998). If *Buchnera* activity differs between these clones, there would likely be a difference in the composition of amino acids, not that of sugars. We did not find a difference in amino acid composition of honeydew between these clones; although the concentration of histidine significantly differed among clones in initial tests, post hoc tests did not show significant differences. Hence, it is unlikely that *Buchnera* caused the observed difference in the intensity of ant attendance. Thus, we conclude that the observed variation in ant attendance is caused by aphid genotype, not by endosymbionts.

Secondary Symbiont: *Arsenophonus*. All *A. craccivora* clones harbor *Buchnera*, and some clones also have an endosymbiont of *Serratia* (Tsuchida et al. 2006). In addition to *Serratia*, we detected another endosymbiont, *Arsenophonus*, from the Saga clone. Our results demonstrated that *Arsenophonus* did not influence aphid performance, honeydew composition, or ant attendance. *Arsenophonus* is a secondary symbiont of a wide range of arthropods including aphids, psyllids, ticks, whiteflies, and parasitoid wasps (Gherna et al. 1991, Thao and Baumann 2004, Dale et al. 2006, Clay et al. 2008, Nováková et al. 2009). Duron et al. (2008) investigated 136 arthropod species, and reported that *Arsenophonus* infected six species (spider, cockroach, blowfly, louse fly, firebugs, and wasp). *Arsenophonus* is a male-killing symbiont in a parasitic wasp (*Nasonia virtipennis*) (Gherna et al. 1991), and reduced the growth rate of pea aphid *Acyrthosiphon pisum* (Harris) in laboratory experiments (Russell and Moran 2005). It is possible that *Arsenophonus* also affect their *A. craccivora* hosts, but at present their potential role is unknown. Additional field and laboratory studies will be necessary to examine the effects of *Arsenophonus* on *A. craccivora*.

Sugar Composition in Aphid Honeydew. Honeydew is one of the key factors maintaining ant–aphid mutualisms (Hölldobler and Wilson 1990, Völkl et al. 1999, Blüthgen and Fiedler 2004). Völkl et al. (1999) analyzed sugar composition of honeydew from four aphid species feeding on tansy *Tanacetum vulgare* (L.), and found that *Lasius niger* L. ants preferentially tended aphids that excreted melezitose-rich honeydew. They hypothesized that melezitose was a key

substance in increasing the intensity of ant attendance of aphids. Indeed, *L. niger* or *Myrmica rubra* (L.) prefer melezitose over monosaccharides such as glucose or fructose (Völkl et al. 1999, Fischer et al. 2001, Tinti and Nofre 2001, Woodring et al. 2007). However, there are several reports against this hypothesis as well (Blüthgen and Fiedler 2004; Vantaux et al. 2011, 2012). Many Australian ants prefer sucrose to melezitose (Blüthgen and Fiedler 2004), and the degree of ant attendance of black bean aphids *Aphis fabae* Scopoli is independent of melezitose concentration in honeydew (Vantaux et al. 2011, 2012). Our results also did not support their hypothesis, as the Otsu clone had a greater degree of ant attendance and excreted high levels of sucrose and low levels of melezitose. We conclude that melezitose may not be an essential element for attracting ants in Japanese aphids, and in contrast, that sucrose may be more important than melezitose for aphids to attract ants. In fact, ants in our field (Otsu City) are attracted to sucrose solution more than fructose, glucose, and melezitose solutions (N. K., unpublished data). Consequently, particular sugar components may not determine the general pattern of ant attendance, because different ant species have different preferences for sugars, amino acids, and their relative abundances in honeydew (Blüthgen and Fiedler 2004, Heil et al. 2005, Hojo et al. 2008).

Intraspecific Variation in Ant Attendance. The frequency of ant attendance of natural *A. craccivora* colonies differed between Otsu and Saga sites: Otsu colonies had a higher frequency of ant attendance than Saga colonies. The low frequency of ant attendance at the Saga site was not because of colony size or a lack of potential mutualistic ant species: there was no difference in aphid colony size between the two sites, and several ant species that typically tend aphids, such as *L. japonicus* and *P. punctatus*, are common at the Saga site (N. Katayama, personal observation). While the strength of ant–aphid mutualisms can be altered by biotic factors, such as host plant quality (Fischer et al. 2001, Stadler et al. 2002, Stadler and Dixon 2005), we experimentally demonstrated that the Otsu clone attracted more ants than the Saga clone using same host plant species in a laboratory. This result shows that there is hereditary variation in aphids' ability to attract ants within the aphid species, and suggests that the dominant aphid genotype may differ between sites.

In this context, Vantaux et al. (2011) provided the first evidence that there is great intraspecific variation in sugar composition of honeydew of the black bean aphid *A. fabae*, but the strength of ant attendance was not affected by the amount of sugar components in honeydew (Vantaux et al. 2011). We checked honeydew components of 21 *A. craccivora* clones and found that the clones were divided into two types, that is, a "sucrose-type" (like the Otsu clone) and a "melezitose-type" (like the Saga clone), in terms of honeydew composition (N. K., unpublished data). These honeydew types were not consistent with secondary symbiont composition in the aphids. An additional experiment using six clones demonstrated that the "sucrose-type" clone was more attractive to ants than the "melezitose-type" clone (N. K., unpublished data). These findings suggest that there is intraspecific variation in honeydew composition and ant attendance of aphids, and this variation is correlated with sugar composition.

The ant–hemipteran mutualism is ecologically important because hemipteran-tending ants can have a prominent role in organizing the local community structure of insects (Wimp and Whitham 2001, Suzuki et al. 2004, Ando and Ohgushi 2008). There is some evidence that plant quality can affect ant attendance of hemipteran insects (Breton and Addicott 1992, Stadler et al. 2002, but see Morales and Beal 2006). On high-quality plants, the presence of hemipteran insects enhances ant attendance (Stadler et al. 2002), probably because of increases in the quality, quantity, or both, of rewards they provide. Mooney and Agrawal (2008) highlighted the importance of plant genotype in influencing ant–aphid interactions. Our findings provide further evidence for this: aphid genotype also affects honeydew quality and ant attendance. Plant–aphid genotypic interactions may be a strong driving force in shaping spatiotemporal patterns of ant–aphid associations. However, we know little about these interactions. Future work evaluating this issue will provide critical insight into how ant–aphid mutualisms organize community structure in the context of eco-evolutionary feedbacks.

Acknowledgments

We are deeply grateful to Ryohei Yamaoka for permission to use equipment at Kyoto Institute of Technology and for helpful advice. We are also grateful to Nobuhiko Suzuki, Toru Ide, Hideki Kagata, Yoshino Ando, Shunsuke Utsumi, and Takefumi Nakazawa for their helpful advice and support during this study. We also thank Melissa Whitaker for correcting the English of the text. The current study was supported by a JSPS Research Fellowship for Young Scientists to N. Katayama, a Ministry of Education, Culture, Sports, Science, and Technology (Japan) Grant-in-Aid for Scientific Research (A-15207003 and B-25291102) to T. Ohgushi, and the Grant for Biodiversity Research of the 21st Century COE Program (A14) and the Global COE Program (A06).

References Cited

Altschul, S. F., G. Warren, W. Miller, E. W. Myers, and D. J. Lipman 1990. Basic local alignment search tool. J. Mol. Biol. 215: 403–410.

Ando, Y., and T. Ohgushi 2008. Ant and plant-mediated indirect effects induced by aphid colonization on herbivorous insects on tall goldenrod. Popul. Ecol. 50: 181–189.

Blüthgen, N., and K. Fiedler 2004. Preference for sugars and amino acids and their conditionality in a diverse nectar-feeding ant community. J. Anim. Ecol. 73: 155–166.

Boucher, D. H., S. James, and K. H. Keeler 1982. The ecology of mutualism. Annu. Rev. Ecol. Syst. 13: 315–347.

Breton, L. M., and J. F. Addicott 1992. Does host-plant quality mediate aphid–ant mutualism? Oikos 63: 253–259.

Bronstein, J. L. 1994. Conditional outcomes in mutualistic interactions. Trends Ecol. Evol 9: 214–217.

Buchner, P. 1965. Endosymbiosis of animals with plant microorganisms. Wiley, New York, NY.

Buckley, R. C. 1987. Interactions involving plants, Homoptera, and ants. Annu. Rev. Ecol. Syst. 18: 111–135.

Clay, K., O. Klyachko, N. Grindle, D. Civitello, D. Oleske, and C. Fuqua 2008. Microbial community ecology of ticks: prokaryotic diversity, distribution and interactions in the lone star tick, *Amblyomma americanum.* Mol. Ecol. 17: 4371–4381.

Dale, C., M. Beeton, C. Harbison, T. Jones, and M. Pontes 2006. Isolation, pure culture, and characterization of "*Candidatus* Arsenophonus arthropodicus," an intracellular secondary endosymbiont from the hippoboscid louse fly *Pseudolynchia canariensis.* Appl. Environ. Microb. 72: 2997–3004.

Dixon, A.F.G. 1998. Aphid ecology. Chapman & Hall, London, United Kingdom.

Douglas, A. E. 1998. Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera.* Annu. Rev. Entomol. 43: 17–37.

Duron, O., D. Bouchon, S. Boutin, L. Bellamy, L. Zhou, J. Engelstädter, and G. D. Hurst 2008. The diversity of reproductive parasites among arthropods: *Wolbachia* do not walk alone. BMC Biol. 6: 27.

El-Ziady, S. 1960. Further effects of *Lasius niger* L. on *Aphis fabae* Scopoli. Proc. R. Entomol. Soc. Lond. A 35: 30–38.

El-Ziady, S., and J. S. Kennedy 1956. Beneficial effects of the common garden ant, *Lasius niger* L., on the black bean aphid, *Aphis fabae* Scopoli. R. Entomol. Soc. Lond. A 31: 61–65.

Fischer, M. K., K. H. Hoffmann, and W. Völkl 2001. Competition for mutualists in an ant-homopteran interaction mediated by hierarchies of ant attendance. Oikos 92: 531–541.

Fukatsu, T. 1999. Acetone preservation: a practical technique for molecular analysis. Mol. Ecol. 8: 1935–1945.

Fukatsu, T., and N. Nikoh 1998. Two intracellular symbiotic bacteria from the mulberry psyllid *Anomoneura mori* (Insecta, Homoptera). Appl. Environ. Microbiol. 64: 3599–3606.

Fukatsu, T., T. Tsuchida, N. Nikoh, and R. Koga 2001. *Spiroplasma symbiont* of the pea aphid, *Acyrthosiphon pisum* (Insecta: Homoptera). Appl. Environ. Microbiol. 67: 1284–1291.

Gherna, R. L., J. H. Werren, W. Weisburg, R. Cote, C. R. Woese, L. Mandelco, and D. J. Brenner 1991. *Arsenophonus nasoniae* gen. nov., sp. nov. the causative agent of the son-killer trait in the parasitic Wasp *Nasonia vitripennis.* Int. J. Syst. Bacteriol. 41: 563–565.

Heil, M., J. Rattke, and W. Boland 2005. Postsecretory hydrolysis of nectar sucrose and specialization in ant/plant mutualism. Science 308: 560–563.

Hendrix, D. L., Y. Wei, and J. E. Leggett 1992. Homopteran honeydew sugar composition is determined by both the insect and plant species. Comp. Biochem. Physiol. 101: 23–27.

Herre, E. A., N. Knowlton, U. G. Mueller, and S. A. Rehner 1999. The evolution of mutualisms: exploring the paths between conflict and cooperation. Trends Ecol. Evol. 14: 49–53.

Hojo, M. K., A. Wada–Katsumata, M. Ozaki, S. Yamaguchi, and R. Yamaoka 2008. Gustatory synergism in ants mediates a species-specific symbiosis with lycaenid butterflies. J. Comp. Physiol. 194: 1043–1052.

Hölldobler, B., and E. O. Wilson 1990. The ants. The Belknap Press of Harvard University, Cambridge, MA.

Katayama, N., and N. Suzuki 2002. Cost and benefit of ant attendance for *Aphis craccivora* (Hemiptera: Aphididae) with reference to aphid colony size. Can. Entomol. 134: 241–249.

Katayama, N., and N. Suzuki 2003. Bodyguard effects for aphids of *Aphis craccivora* Koch (Homoptera: Aphididae) as related to the activity of two ant species, *Tetramorium caespitum* Linnaeus (Hymenoptera: Formicidae) and *Lasius niger* L. (Hymenoptera: Formicidae). Appl. Entomol. Zool. 38: 427–433.

Katayama, N., and N. Suzuki 2010. Extrafloral nectaries indirectly protect small aphid colonies via ant-mediated interactions. Appl. Entomol. Zool. 45: 505–511.

Koga, R., T. Tsuchida, and T. Fukatsu 2003. Changing partners in an obligate symbiosis: a facultative endosymbiont can compensate for loss of the essential endosymbiont *Buchnera* in an aphid. Proc. R. Soc. Lond. B 270: 2543–2550.

Lanza, J. 1991. Response of fire ants (Formicidae: *Solenopsis invicta* and *S.gerninata*) to artificial nectars with amino acids. Ecol. Entomol. 16: 203–210.

Mooney, K. A., and A. A. Agrawal 2008. Plant genotype shapes ant-aphid interactions: implications for community structure and indirect plant defense. Am. Nat. 171: 195–205.

Morales, M. A., and A.L.H. Beal 2006. Effects of host plant quality and ant tending for treehopper *Publilia concave.* Ann. Entomol. Soc. Am. 99: 545–552.

Nixon, G.E.J. 1951. The association of ants with aphids and coccids. Commonwealth Institute of Entomology, London, United Kingdom.

Nováková, E., V. Hypša, and N. A. Moran 2009. Arsenophonus, an emerging clade of intracellular symbionts with a broad host distribution. BMC Microbiol. 9: 143.

Ohgushi, T., T. P. Craig, and P. W. Price 2007. Ecological communities: plant mediation in indirect interaction webs. Cambridge University Press, Cambridge, MA.

Oliver, K. M., P. H. Degnan, G. R. Burke, and N. A. Moran 2010. Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. Annu. Rev. Entomol. 55: 247–266.

Russell, J. A., and N. A. Moran 2005. Horizontal transfer of bacterial symbionts: heritability and fitness effects in a novel aphid host. Appl. Environ. Microbiol. 71: 7987–7994.

Sachs, J. L., and E. L. Simms 2006. Pathways to mutualism breakdown. Trends Ecol. Evol. 21: 585–592.

Sasaki, T., T. Aoki, H. Hayashi, and H. Ishikawa 1990. Amino acid composition of the honeydew of symbiotic and aposymbiotic pea aphids *Acyrthosiphon pisum.* J. Insect Physiol. 36: 35–40.

Sasaki, T., and H. Ishikawa 1993. Nitrogen recycling in the endosymbiotic system of the pea aphid, *Acyrthosiphon pisum.* Zool. Sci. 10: 779–785.

Stadler, B., and A.F.G. Dixon 2005. Ecology and evolution of aphid-ant interactions. Annu. Rev. Ecol. Syst. 36: 345–372.

Stadler, B., A.F.G. Dixon, and P. Kindlmann 2002. Relative fitness of aphids: effects of plant quality and ants. Ecol. Lett. 5: 216–222.

Suzuki, N., K. Ogura, and N. Katayama 2004. Efficiency of herbivore exclusion by ants attracted to aphids on the vetch *Vicia angustifolia* L. (Leguminosae). Ecol. Res. 19: 275–282.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28: 2731–2739.

Thao, M. L., and P. Baumann 2004. Evidence for multiple acquisition of *Arsenophonus* by whitefly species (Sternorrhyncha: Aleyrodidae). Curr. Microbiol. 48: 140–144.

Tinti, J., and C. Nofre 2001. Responses of the ant *Lasius niger* to various compounds perceived as sweet in humans: a structure-activity relationship study. Chem. Senses 26: 231–237.

Tsuchida, T., R. Koga, M. Horikawa, T. Tsunoda, T. Maoka, S. Matsumoto, J. Simon, and T. Fukatsu 2010. Symbiotic bacterium modifies aphid body color. Science 330: 1102–1104.

Tsuchida, T., R. Koga, M. Sakurai, and T. Fukatsu 2006. Facultative bacterial endosymbionts of three aphid species, *Aphis craccivora, Megoura crassicauda* and *Acyrthosiphon pisum*, sympatrically found on the same host plants. Appl. Entomol. Zool. 41: 129–137.

Tsuchida, T., R. Koga, H. Shibao, T. Matsumoto, and T. Fukatsu 2002. Diversity and geographic distribution of secondary endosymbiotic bacteria in natural populations of the pea aphid, *Acyrthosiphon pisum.* Mol. Ecol. 11: 2123–2135.

Vantaux, A., T. Parmentier, J. Billen, and T. Wenseleers 2012. Do *Lasius niger* ants punish low-quality black bean aphid mutualists? Anim. Behav 83: 257–262.

Vantaux, A., W. Van den Ende, J. Billen, and T. Wenseleers 2011. Large interclone differences in melezitose secretion in the facultatively ant-tended black bean aphid *Aphis fabae.* J. Insect Physiol. 57: 1614–1621.

Völkl, W., J. Woodring, M. Fischer, M. W. Lorenz, and K. H. Hoffmann 1999. Ant-aphid mutualisms: the impact of honeydew production and honeydew sugar composition on ant preferences. Oecologia 118: 483–491.

Wada, A., Y. Isobe, S. Yamaguchi, R. Yamaoka, and M. Ozaki 2001. Taste-enhancing effects of glycine on the sweetness of glucose: a gustatory aspect of symbiosis between the ant, *Camponotus japonicus,* and the larvae of the lycaenid butterfly, *Niphanda fusca.* Chem. Senses 26: 983–992.

Way, M. J. 1963. Mutualism between ants and honeydew-producing Homoptera. Annu. Rev. Entomol. 8: 307–344.

Wimp, G. M., and T. G. Whitham 2001. Biodiversity consequences of predation and host plant hybridization on an aphid-ant mutualism. Ecology 82: 440–452.

Woodring, J., R. Wiedemann, W. Völkl, and K. H. Hoffmann 2007. Oligosaccharide synthesis regulates gut osmolality in the ant-attended aphid *Metopeurum fuscoviride* but not in the unattended aphid *Macrosiphoniella tanacetaria.* J. Appl. Entomol. 131: 1–7.

Received 3 June 2013; accepted 23 August 2013.