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Cryptic Diversity in the Aphid-Parasitizing Wasp *Protaphidius nawaii* (Hymenoptera: Braconidae): Discovery of Two Attendant-Ant-Specific mtDNA Lineages

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The parasitoid wasp *Protaphidius nawaii* parasitizes the aphid *Stomaphis japonica*, which is obligatorily attended by several species of ants of genus *Lasius*. Subgenus *Lasius* or *Dendrolasius* ants use different defense strategies to protect the aphids that they attend (*Lasius*, shelter building; *Dendrolasius*, aggressive attack). We performed molecular phylogenetic analysis based on partial mitochondrial DNA sequences of *P. nawaii* and found that the parasitoid wasp consists of two highly differentiated genetic lineages. Although these two lineages distributed sympatrically, one tends to parasitize aphids attended by ants of subgenus *Lasius*, and the other parasitizes aphids attended by ants of subgenus *Dendrolasius*. The two lineages of *P. nawaii* appear to exhibit different oviposition behaviors adapted to the different aphid-protection strategies of the two ant subgenera.

Key words: ant–aphid mutualism, ant–parasitoid interaction, Aphidiinae, earthen shelters, host specificity, oviposition behavior, parasitism

INTRODUCTION

In the face of the global biodiversity crisis (Brooks et al., 2006), cataloging of the earth's species is among the highest priorities. Several studies have highlighted the existence and importance of cryptic biodiversity, which may represent a substantial proportion of global biodiversity. Owing to the increasing amount of studies incorporating DNA-based techniques, the number of reported cryptic species is increasing (Beheregaray and Caccione, 2007).

Host specificity is one factor that generates parasitoid diversity (Hardy and Otto, 2014). In general, to increase the success rate of parasitism, parasitoids tend to specialize to specific hosts (Cheng, 1986; Godfray, 1994; Shaw, 1994; Althoff, 2003). Usually, characteristics of the host itself determines the host specificity of parasitoids, but sometimes, instead of the host, the surrounding environment determines host specificity of parasitoids. In particular, the host preference or specificity of parasitoids on aphids is determined by the attending ants of the host aphids. Sadeghi-Namaghi and Amiri-Jami (2018) found that the parasitoid wasp *Lysiphlebus fabarum* successfully attacks the aphids *Acyrtosiphon gossypii* and *Brachycaudus cardui* attended by particular ant species. In another case, the parasitoid wasps *Paralipsis eikoeae* and *Aclitus sappaphis*, respec-

tively, attack the host aphid *Sappaphis piri* attended by the ants *Pheidole fervida* and *Lasius niger* (Takada and Shiga, 1974; Takada and Hashimoto, 1985; Akino and Yamaoka, 1998).

The genus *Protaphidius* (Braconidae: Aphidiinae) is a group of parasitoid wasps parasitizing aphid genus *Stomaphis*. To date, only three species have been described: *P. wissmannii* from Europe (Starý, 1959), *P. belokobylskijii* from Russia (Davidian, 2007), and *P. nawaii* from Japan (Takada, 1983). Genus *Protaphidius* is characterized by a long pseudo-ovipositor that enables these wasps to parasitize host aphids hidden in crevices in tree trunks (Starsky, 1976). Previous studies confirmed that the genus *Protaphidius* is closely related to the genus *Diaeretus* (Shi and Chen, 2005), but the phylogenetic relationship within *Protaphidius* is unclear.

The aphid *Stomaphis japonica* (Aphididae: Lachninae) is attacked by the parasitoid wasp *Protaphidius nawaii* (Takada, 1983). *Stomaphis japonica* has an obligate mutualistic relationship with ants, and in Matsumoto (Nagano, Japan), where this study was conducted, it is attended mainly by ants of genus *Lasius*: *Lasius* (*Lasius*) *japonicus*, *Lasius* (*Dendrolasius*) *fuji*, and *Lasius* (*Dendrolasius*) *nipponensis* (Formicidae: Formicinae) (Yamamoto et al., 2015). Ants of the subgenera *Lasius* and *Dendrolasius* protect aphids in different ways. Those of subgenus *Lasius* build earthen shelters over both their trunk trails and the aphids they attend (Takada, 1983). These earthen shelters

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may protect the aphids from parasitoid wasps (Gibernau and Dejean, 2001). In contrast, ants of subgenus *Dendrolasius* rarely build earthen shelters, but they aggressively defend “bare” aphids. Because the subgenera *Lasius* and *Dendrolasius* employ different strategies to defend aphids, we hypothesized that *P. nawaii* may use different oviposition strategies to overcome the defense by *Lasius* or

Dendrolasius. Therefore, there may be cryptic diversity in this parasitoid wasp determined by the ant species attending the aphids that it parasitizes.

To explore cryptic attending-ant-specific diversity in the parasitoid wasp *P. nawaii*, we undertook an extensive sampling of the wasps, performed molecular phylogenetic analysis, and identified two distinct wasp lineages, each specific

Table 1. Details of the DNA sequenced samples of the aphid-parasitizing wasp *Protaphidius nawaii*. ‘Lineage’ refers to the mtDNA lineage in Fig. 2. All samples were collected in Matsumoto (Nagano, Japan) in 2016.

Lineage	Voucher number	Attending ant species	Collection site	Latitude	Longitude	Altitude (m)	Collection date	GenBank accession number
A	w4	<i>Lasius (Lasius) japonicus</i>	Arigasaki	N36°14'44"	E137°57'35"	617	26. vi. 2016	LC507765
A	w19	<i>L. (L.) japonicus</i>	Hara	–	–	–	6. viii. 2016	LC507757
A	w21	<i>L. (L.) japonicus</i>	Hara	N36°16'15"	E137°59'11"	691	6. viii. 2016	LC507759
A	w22	<i>L. (L.) japonicus</i>	Hara	N36°16'15"	E137°59'11"	691	7. viii. 2016	LC507760
A	w24	<i>Lasius (Dendrolasius) fuji</i>	Souza	N36°15'00"	E137°59'35"	624	8. viii. 2016	LC507761
A	w25	<i>L. (L.) japonicus</i>	Hara	N36°16'15"	E137°59'11"	691	6. viii. 2016	LC507762
A	w29	<i>L. (L.) japonicus</i>	Hara	N36°16'14"	E137°59'07"	685	7. viii. 2016	LC507764
A	w41	<i>L. (D.) fuji</i>	Souza	N36°15'00"	E137°59'35"	624	19. viii. 2016	LC507766
A	w43	<i>L. (L.) japonicus</i>	Hara	N36°16'26"	E137°59'07"	698	24. viii. 2016	LC507768
A	w44	<i>L. (L.) japonicus</i>	Hara	N36°16'27"	E137°59'07"	709	24. viii. 2016	LC507769
A	w47	<i>L. (L.) japonicus</i>	Hara	N36°16'14"	E137°59'07"	685	28. viii. 2016	LC507770
A	w56	<i>L. (L.) japonicus</i>	Omura	N36°15'20"	E137°59'25"	648	10. ix. 2016	LC507775
A	w63	<i>L. (L.) japonicus</i>	Omura	N36°15'20"	E137°59'25"	648	13. ix. 2016	LC507778
A	w79	<i>L. (L.) japonicus</i>	Hara	N36°16'26"	E137°59'07"	698	6. x. 2016	LC507780
A	w80	<i>L. (L.) japonicus</i>	Hara	N36°16'26"	E137°59'07"	698	6. x. 2016	LC507782
A	w81	<i>L. (L.) japonicus</i>	Hara	N36°16'15"	E137°59'11"	691	6. x. 2016	LC507783
A	w82	<i>L. (L.) japonicus</i>	Hara	N36°16'15"	E137°59'11"	691	6. x. 2016	LC507784
A	w83	<i>L. (L.) japonicus</i>	Hara	N36°16'15"	E137°59'11"	691	6. x. 2016	LC507785
A	w85	<i>L. (L.) japonicus</i>	Hara	N36°16'15"	E137°59'11"	691	30. ix. 2016	LC507786
A	w86	<i>L. (L.) japonicus</i>	Hara	N36°16'14"	E137°59'07"	685	6. x. 2016	LC507787
A	w88	<i>L. (L.) japonicus</i>	Hara	N36°16'15"	E137°59'11"	691	6. x. 2016	LC507788
A	w89	<i>L. (L.) japonicus</i>	Omura	N36°15'20"	E137°59'25"	648	8. x. 2016	LC507789
A	w91	<i>L. (L.) japonicus</i>	Hara	N36°16'26"	E137°59'07"	698	9. x. 2016	LC507791
B	w5	<i>Camponotus obscuripes</i>	Asamaonsen	N36°15'35"	E137°59'29"	674	1. vii. 2016	LC507771
B	w6	<i>L. (D.) fuji</i>	Satoyamabe	N36°14'58"	E137°59'56"	662	1. vii. 2016	LC507777
B	w7	<i>L. (D.) fuji</i>	Souza	N36°15'00"	E137°59'35"	624	2. vii. 2016	LC507779
B	w8	<i>L. (D.) fuji</i>	Souza	N36°15'00"	E137°59'35"	624	2. vii. 2016	LC507781
B	w9	<i>L. (D.) fuji</i>	Souza	N36°15'00"	E137°59'35"	624	2. vii. 2016	LC507790
B	w10	<i>L. (D.) fuji</i>	Souza	N36°15'00"	E137°59'35"	624	6. vii. 2016	LC507751
B	w11	<i>L. (D.) fuji</i>	Souza	N36°15'00"	E137°59'35"	624	6. vii. 2016	LC507752
B	w12	<i>L. (D.) fuji</i>	Souza	N36°15'00"	E137°59'35"	624	10. vii. 2016	LC507753
B	w13	<i>L. (D.) fuji</i>	Souza	N36°15'00"	E137°59'35"	624	10. vii. 2016	LC507754
B	w14	<i>L. (D.) fuji</i>	Souza	N36°15'00"	E137°59'35"	624	10. vii. 2016	LC507755
B	w15	<i>L. (D.) fuji</i>	Souza	N36°15'00"	E137°59'35"	624	10. vii. 2016	LC507756
B	w20	<i>L. (D.) fuji</i>	Souza	N36°15'00"	E137°59'35"	624	5. viii. 2016	LC507758
B	w27	<i>L. (D.) fuji</i>	Souza	N36°15'00"	E137°59'35"	624	5. viii. 2016	LC507763
B	w42	<i>L. (L.) japonicus</i>	Hara	N36°16'15"	E137°59'11"	691	21. viii. 2016	LC507767
B	w52	<i>Lasius (Dendrolasius) nipponensis</i>	Omura	N36°15'20"	E137°59'25"	648	6. ix. 2016	LC507772
B	w54	<i>L. (D.) nipponensis</i>	Omura	N36°15'20"	E137°59'25"	648	7. ix. 2016	LC507773
B	w55	<i>L. (L.) japonicus</i>	Omura	N36°15'20"	E137°59'25"	648	10. ix. 2016	LC507774
B	w59	<i>L. (D.) nipponensis</i>	Omura	N36°15'20"	E137°59'25"	648	9. ix. 2016	LC507776
Outgroup		<i>Aphidius matricariae</i> (Aphidiinae; Braconidae)						EF077526
Outgroup		<i>Lysiphlebus orientalis</i> (Aphidiinae; Braconidae)						KC237748
Outgroup		<i>Orussus abietinus</i> (Orussidae)						EF032236

to the aphids attended by ants of subgenus *Lasius* and to aphids attended by ants of subgenus *Dendrolasius*.

MATERIALS AND METHODS

Sample collection

Parasitoid wasps were sampled at seven sites in Matsumoto (Nagano, Japan) (Fig. 1). We collected all parasitized *Stomaphis japonica* mummies that we found on the trunks of 21 sawtooth oak trees (*Quercus acutissima*) from 5 June to 9 October 2016 (Table 1). All of the aphid colonies from which we collected parasitized mummies were attended by ants. The collected mummies were kept in the laboratory until the adult parasitoid wasps emerged. Forty-one wasps eventually emerged and were preserved in 70% ethanol. Three legs were cut from each wasp individual and preserved in 99.5% ethanol for molecular analysis. Attending ants from each colony were also collected and preserved in 99.5% ethanol for later identification by DNA barcoding. Voucher specimens, except for w85, w86, w88, w89, and w91, will be deposited as Hajimu Takada's Collection at the Hokkaido University Museum in Sapporo (Hokkaido, Japan).

DNA extraction, PCR amplification, and sequencing

We used a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) to extract DNA from the legs of the wasps and from the entire bodies of the ants, following the manufacturer's protocols. Then we amplified the mitochondrial COI gene by polymerase chain reaction (PCR) using Takara Ex Taq polymerase (Takara Bio, Shiga, Japan) and the primers Ron (5'-GGATCACCTGATATAGCATTCCC-3') and Nancy (5'-CCTGGTAAAATTTAAAATATAAACTTC-3') for the parasitoid wasps, and *Lasius*-L (5'-TAYCCGCCATTAGCTTCAAA-3') and *Lasius*-R (5'-TGAAATTAAGGATCCAATWGA-3') for the ants

(Maruyama et al., 2008). PCR was carried out for 30 cycles of 98°C for 10s, 50°C for 30s, and 72°C for 60s. Both strands of the amplified DNA were sequenced with a BigDye Terminator v1.1 Cycle Sequencing Kit (ABI, Weiterstadt, Germany) on an ABI 3130 Genetic Analyzer.

Phylogenetic analysis

The mitochondrial COI sequences were edited and aligned with SeqScape v. 2.6 software (ABI, Weiterstadt, Germany). In the phylogenetic analysis of the wasps, we used the following sequences as outgroups (*Aphidius matricariae* EF077526, *Lysiphlebus orientalis* KC237748, *Orussus abietinus* EF032236). We constructed a maximum likelihood phylogenetic tree based on the Tamura 3-parameter model with MEGA7 software (Kumar et al., 2016), and determined the lineage of the wasps from each colony. The robustness of the tree was assessed by nonparametric bootstrapping with 500 replicates. The genetic distance (Kimura two-parameter (K2P) model) between the lineages was obtained by using MEGA7. The GenBank accession numbers of the COI gene sequences are listed in Table 1. To determine the species of the ants, we conducted a BLAST search on the NCBI website (<http://www.ncbi.nlm.nih.gov>) for highly similar sequences to the ant DNA sequences.

RESULTS

Phylogenetic analysis

The partial COI gene of *Protaphidius nawaii* consisted of 422 nucleotide sites, of which 80 were parsimony informative. The topology of the maximum likelihood phylogenetic tree revealed two well-resolved lineages, designated A and B, within *P. nawaii* (Fig. 2). The genetic distance between the two lineages was 0.133–0.136 in K2P model. Both of the two lineages were collected within a limited area (Fig. 1), and even at the same sampling site (but from different tree) of 100 m between 614 m and 709 m. Both lineages distributed in the same altitudinal range (Table 1).

Species specificity of the parasitoid wasps

Twenty-one of the 23 specimens of lineage A, and two of the 18 specimens of lineage B emerged from aphids attended by *Lasius* (*Lasius*) *japonicus* ants, which build earthen shelters over the aphids they attend; two specimens of lineage A and 12 specimens of lineage B emerged from aphids attended by *L. (Dendrolasius)* *fuji*; and three specimens of lineage B emerged from aphids attended by *L. (D.) nipponensis*. *L. (D.) fuji* and *L. (D.) nipponensis* do not build earthen shelters over the aphids. In addition, one specimen of lineage B emerged from aphids attended by *Camponotus obscuripes*, which also does not build earthen shelters over the aphids (Table 1, Fig. 2). The *P. nawaii* wasps of lineage A were significantly more likely to emerge from aphids attended by *Lasius* (*Lasius*), and those

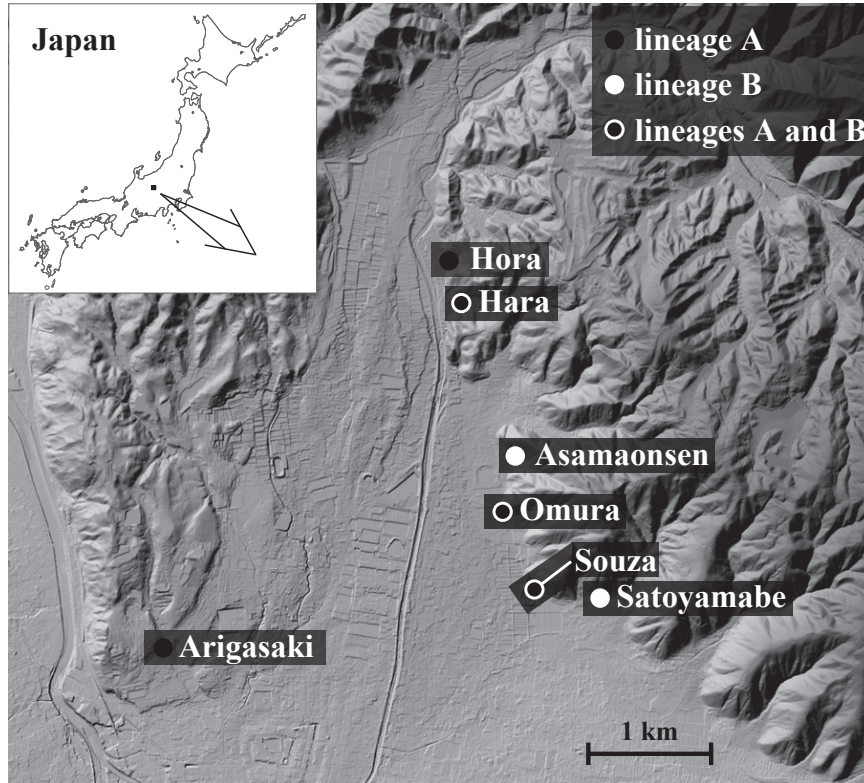


Fig. 1. The seven sampling sites of this study (see Table 1 for details). Black and white circles indicate the sites where parasitoid *Protaphidius nawaii* wasps were collected (black: lineage A; white: lineage B). White circles (inside black) indicate the sites where both lineages A and B were collected. For lineages A and B, see Fig. 2.

of lineage B were significantly more likely to emerge from aphids attended by *Lasius* (*Dendrolasius*) or *C. obscuripes* ($P < 0.05$; Fisher's exact test).

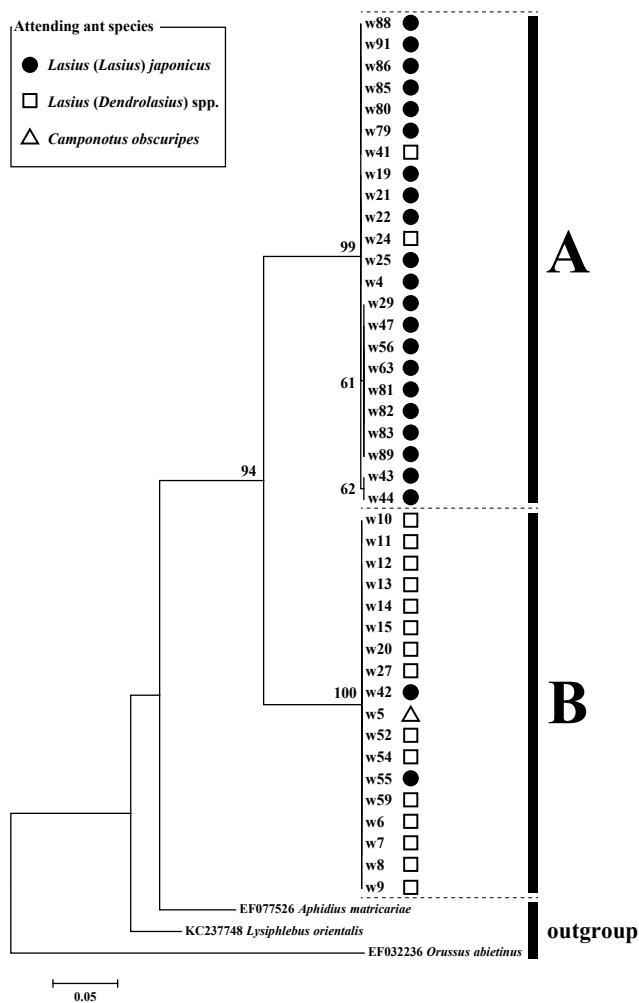


Fig. 2. Maximum-likelihood phylogenetic tree of *Protaphidius nawaiii* based on a partial mitochondrial DNA sequence of the COI gene. Numbers beside the branches represent maximum-likelihood bootstrap values (as a percentage; only those >50% are shown). See Table 1 for specimen codes. The attending ant species are indicated by graphical symbols. The scale shows the nucleotide substitution rate of 0.05.

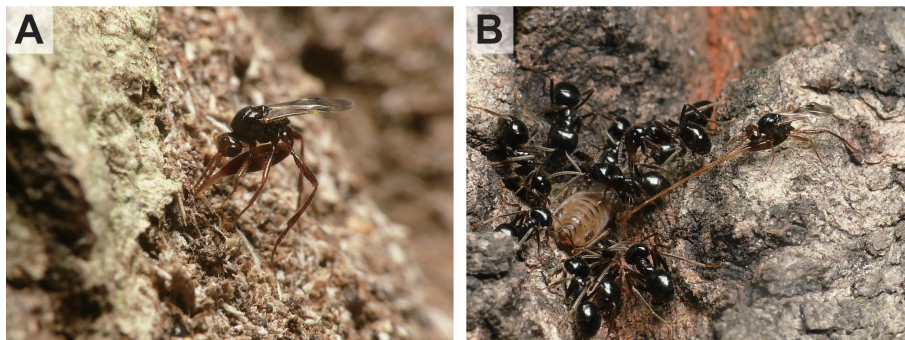


Fig. 3. Oviposition behavior of *Protaphidius nawaiii*: (A) *P. nawaiii* ovipositing in a *Lasius*-defended (shelter-covered) aphid (*Stomaphis japonica*); (B) *P. nawaiii* ovipositing in a *Dendrolasius*-defended aphid (*S. japonica*).

DISCUSSION

The results presented here show that the specificity of the parasitoid wasps studied is determined by the ant species attending the host aphids, rather than by the host itself. The results of the molecular analysis showed that two lineages of *Protaphidius nawaiii* parasitoid wasps parasitized *Stomaphis japonica*, and each lineage had tendency to parasitize aphids attended by different ant subgenus (*Lasius* or *Dendrolasius*). The genetic distance between the two lineages was 0.133–0.136 (COI), which is sufficiently large compared with 0.03 (the standard interspecific COI genetic distances in insects; Hebert et al., 2003), or with 0.04 (COI genetic distance between sister species in *Lysiphlebus*, a genus of Aphidiinae; Petrovč et al., 2015). Thus, the two lineages of *Protaphidius* appear to be well supported monophyletic groups presumably equivalent to different species. The horizontal and elevational distribution of the two lineages largely overlapped (Fig. 1), indicating that the two lineages do not distribute allopatrically, but cohabit and partition a resource (aphids).

The defense strategies used by the attending *Lasius* and *Dendrolasius* ants against oviposition by the parasitoid *P. nawaiii* wasp are both powerful, but different. *Lasius* (*Lasius*) ants defend the aphids by building shelters over them, whereas *Lasius* (*Dendrolasius*) ants defend the aphids not by building shelters but by aggressively attacking the parasitizing wasp. Note that *L. (Dendrolasius) nipponensis* sometimes builds rough shelters that cover only part of the aphid colony.

The correspondence of the two parasitoid wasp lineages to different attending ant species that use different defense strategies to protect aphids suggests that the two parasitoid wasp lineages may have adapted their oviposition behavior to circumvent these different defenses. Takada (1983) reported that *P. nawaiii* parasitizes aphids protected by shelters by first inserting its antennae into the shelter to make a hole; then it inserts its pseudo-ovipositor through the hole to oviposit in a host aphid body (Fig. 3A). In contrast, when *P. nawaiii* parasitizes bare aphids surrounded by numerous aggressive *Dendrolasius* ants, it stays at a distance from the aphids and uses its long pseudo-ovipositor to oviposit in a host aphid body (Maruyama et al., 2013) (Fig. 3B). These different oviposition behaviors so far reported may correspond to the two parasitoid wasp lineages revealed

in this study. In the future, the morphological characteristics of the two wasp lineages should be examined along with their comparative oviposition success in aphids defended by *Lasius* (*Lasius*) or *Lasius* (*Dendrolasius*) ants.

As noted above, the different ways in which ants defend aphids may have led to the specificity of the two parasitoid lineages. However, the aphid defenses by each ant subgenus vary from situation to situation. Shelters constructed by ant subgenus *Lasius* collapse after rain and aphids are temporarily

exposed. Additionally, the ant subgenus *Dendrolasius* sometimes covers some aphid individuals (but rarely covers all individuals). Such an occasional change in the way the ants defend aphids may have caused a partial discrepancy of the specificity of the two lineages of *P. nawaii* to the attending ant subgenera (w41 and w24 of lineage A parasitizing the *Dendrolasius*-attended aphids, and w42 and w55 of lineage B parasitizing the *Lasius*-attended aphids). In addition, *P. nawaii* of lineage B parasitized aphids attended by *Camponotus obscuripes*. This indicates that the lineage B of *P. nawaii*, which attacks aphids attended by the ant subgenus *Dendrolasius*, can also parasitize aphids attended by *C. obscuripes*, which does not build shelters.

Previous studies showed that the *S. japonica*-*P. nawaii* host-parasitoid system involves additional ectoparasitic hyperparasitoid wasp species *Euneura stomaphidis* and *Dendrocerus stomaphis* (Kamijo and Takada, 1983; Takada, 1983, 2009). Sanders and Van Veen (2010) showed that the relationship between aphids and their parasitoid and hyperparasitoid wasp communities varies with the presence of attending ants. As such, changes in the aphid parasitoid species (lineages) due to attending ant difference revealed in this study may affect the communities of the hyperparasitoids involved. Further investigation of this aphid–parasitoid wasp communities will shed light on the importance of ant association in the maintenance of host–parasitoid species-specific system.

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COMPETING INTERESTS

The authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

TY and TI designed this study. HH and TY performed field study and molecular phylogenetic analysis. TK observed oviposition behavior of parasitizing wasps and took pictures. TY, YN and TI wrote the article and all authors contributed the final manuscript.

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