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# **Supplemental information**

**Complex host/symbiont integration** 

of a multi-partner symbiotic system

## in the eusocial aphid Ceratovacuna japonica

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# Figure S1. Diagnostic PCR detection of *Buchnera*, *Arsenophonus*, and *Hamiltonella* of *Ce. japonica*, related to Figure 1.

(A) Symbiont detection in *Ce. japonica* populations. Each sample ID number corresponds to that of Figure 1B–D.
(B) Symbiont detection in *Ce. japonica* individuals. Samples of s1–3, t1–3, n1–3, and m1–3 were derived from Sakata-koen (populations #2–4 in Figure 1), Tsushima (#24–26), Norikura (#14), and Matsudaira (#18–20), respectively.

B, A, H, M, p.c., and n.c. means *Buchnera*, *Arsenophonus*, *Hamiltonella*, DNA size marker, positive control, and negative control, respectively. Each bacterial symbiont was detected using specific primers targeting a single-copy gene, *dnaK*. PCR product sizes of *dnaK* of *Buchnera*, *Arsenophonus*, and *Hamiltonella* are 186, 192, and 117 bp, respectively. DNA size markers shows 3,000, 1,500, 1,000, 900, 800, 700, 600, 500, 400, 300, 200, and 100 bp, from top to bottom.





		(191	0)
	····· Riesia pthiripubis (Pthirus pubis)	1	0.53
	98 98 98 98 98 98 98 98 98 98 98 98 98 9		0.58
	100 Riesia pediculicola (Pediculus humanus humanus BR-HL)		0.57
	Aschnera chinzeii (Nycteribia allotopa)		0.76
	LutaAschnera chinzeii (Basilia nattereri)		
	Aschnera chinzeii (Penicillidia jenynsii)	Group A	
	Arsenophonus sp. (Trichobius parasiticus)	-	
	Arsenophonus sp. ( <i>Trichobius caecus</i> )		
	70 Arsenophonus sp. (Trichobius longipes)		
	87 Arsenophonus sp. (Ceratovacuna japonica)		0.85
	Arsenophonus lipoptenae (Lipoptena cervi)		0.84
	Arsenophonus sp. (Platypleura kaempferi)	1	
	Arsenophonus sp. (Aleurodicus dispersus)	1	0.66
	—······arsenophonus nasoniae (Nasonia vitripennis)		4.99
	Arsenophonus sp. (Stomaphis fagi)		
	Arsenophonus sp. (Stomaphis takahashii)		
	Arsenophonus sp. (Macrosteles laevis)		
	Arsenophonus triatominarum (Triatoma infestans)		3.86
	Arsenophonus sp. (Bemisia tabaci)	Group B	1.86
	Arsenophonus sp. (Tetraleurodes mori)		
	Arsenophonus sp. (Trialeurodes vaporariorum)		
	······		
	······Arsenophonus sp. (Entylia carinata)		
	Arsenophonus sp. (Aphis craccivora)		2.42
	Arsenophonus sp. (Nilaparvata lugens)	I	2.95
	Proteus mirabilis		
	— Escherichia coli		
	0.03 Psocodea Diptera Hymenoptera Hemiptera		
в	Arsenophonus	Genome	size (Mb)
		0	



#### Figure S2. Phylogenetic analysis of Arsenophonus, related to Figure 2.

(A) Maximum likelihood (ML) tree of *Arsenophonus* based on the length of 1,451 bp of 16S rRNA sequences. *Escherichia coli* and *Proteus mirabilis* were used as outgroups.

(B) ML tree of Arsenophonus based on concatenated 204 single-copy orthologous protein sequences composed of

54,777 amino acid residues with recoding by the Dayhoff6 matrix. E. coli and P. mirabilis were used as outgroups.

Target symbionts in this study are highlighted in red on each tree. Bootstrap values no less than 70% are indicated on each node. Scale bars represent 0.03 and 0.1 substitutions per site.

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	Ceratovacuna japonica Lipoptena cervi Trichobius parasiticus	1 TI GAAGAGITI GAI CAI GOCI CAGATI GAACGCI GGCGGCA <mark>GGI</mark> CI AACACAI GCAAGI CGAGCGGCA [IGGAAAGAAGAGI] IAC. 1 TAATI GAAGAGITI GAI CAI GGCI CAGATI GAACGCI GGCGGCAAGCGI TACACACI IGCAAGGGGCAACGII TAAAACAGI GAACAGI 1	84 100 59
	Ceratovacuna japonica Lipoptena cervi Trichobius parasiticus	85 TITTTT GID GC GC GC GC GC GC GG GT GAGT A A <mark>n</mark> GT AT GG GG AT CIACCIAA A GG A GO GG GA DA ACTATT GG GA AC GAT A GCT A A TA CC GC AT A A 101 TA CITTTTTTTTT GA CG GC GA GC GG CG GA GG GG A GT A A TA CG GG GA TC I CC GC GG GG A GA GA GA ACTATT GG GA A 60 CTTTTT GT A GC GA CG GC A A C GG CTA GT A A TA CC GG GG AT CIACCIA TT GG GA AC GA TA CC CG CG A T	180 200 155
	Ceratovacuna japonica Lipoptena cervi Trichobius parasiticus	181 Ο ΓΓΑΑ GGA COAA GTA GGGGA CTTT	274 299 255
	Ceratovacuna japonica Lipoptena cervi Trichobius parasiticus	775 CAAC <mark>A</mark> ATCTCTAGCTGGTCTGAGAGGATGCTCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGATA <mark>T</mark> TGGACAAT 300 CAACCATCTCTAGCTGGTCTGAGAGGATCTCACCACCACTGGGACTGAGACTGGGACCAGACTCCTAGGGGAGGAGCAGTGGGGATATTGCACAAT 256 CAACCATCTTAGCTGGTCTGAGAGGACCATTGGCACTGGGACTGGAGACAGGCCCAGACTCCTAGGGGAGGAGGAGGAGTGGGGAATTGTTGGCACAT	374 399 355
	Ceratovacuna japonica Lipoptena cervi Trichobius parasiticus	375 GGCGAAAGCCTGAT GCAGCTAT C <mark>T</mark> CGCGTGTAT GAAGAAGGCCTTAGGGTTGTAAAGTACTTTCAGTTGTGAAGAAAT <mark>TTAA</mark> TTAATAATATAT 400 GGCGAAAGCCTGAT GCAGCTATCCCGCGTGTAT GAAGAAGGCCTT AGGTTGTAAAGTACTTTCAGTTGTAGAAAATATAATAT	474 499 455
	Ceratovacuna japonica Lipoptena cervi Trichobius parasiticus	475 <mark>A</mark> TT GACGTTA CCAACA AAAGAACACCGGCT AACTCCGTGC CAGCAGCCGCGGTAATACGGAGGGTGC <mark>G</mark> AGCGTTA <mark>A</mark> TCGCAATTACTGGGCGTAAAG KMM GCTTGACGTTA CCAACA GAAGAACCACCGGCT AACTCCGTGC CAGCAGCCGCGGTAATACGGAGGGGGAACGCTAGTCGAGTATTACTGGGCGTAAAG 695 DT GT GACGTTA ACACAAAGAAGACACGGCT AACTCCGTGC CAGCAGCCGCGGTAATACGGAGGGTGGAACGCTGGCATTACTGGGCTAAAG	574 599 555
	Ceratovacuna japonica Lipoptena cervi Trichobius parasiticus	575 GCACCAGGCGTTAATTAAGTTGGATGTGAAATCCCCCGGCCTTAACCTGGGAATGGCATTCAAAACTGCTTAGCTAGAGTTTTGAAGAGGGGGGGAGAA 600 GCACCAGGCGGTAATTAAGTTGGATGTGAAATCCCTGGCGCTGAACGTAGGATGCCATTCAAAACTGATTAGCTAGGGTGGAG 556 GCATTAGCGCGTTAATTAAGTTGGATGTGAAATCCCTAGGCTTAACTTAGCAATAGCATTCAAAACTGATTATCTGAGGGTTGGAGTGAGA	674 699 655
	Ceratovacuna japonica Lipoptena cervi Trichohius parasiticus	675 TI CCAT GT GT AGCGGT GAAA T GC GA GA <mark>T</mark> A T <mark>G</mark> T GC A GGAA T ACCAGT GGC GAA <mark>G</mark> GG GG CC CC T G GACAAA T A CT GA GGCT CAT GT GC GAAA AG CG T GG 700 TI CCAT GT GT AGCGGT GAAA T G GGT AGA GAT AT GC A GGAAT ACCAGT GG GG AA AG G GC CC CC T GT GACAGT CAT GT GC GAAA GG G GC 656 TI CCAT GT GT AG GGT GAAA T G GGT AGA GAT AT GC A T GGGT AA AG G G GAC CC CC T G GT GAAA GA T G GC GC CG AG	773 798 754
	Ceratovacuna japonica Lipoptena cervi Trichobius parasiticus	774 GAGCAAACAGGATTAGATACCCTGGTAGTCCATGCTGTAAAC <mark>T</mark> ATGTCGATTTGGAGGTTGTGCTTGAATTG <mark>A</mark> GCCTCCCGGAGCTAACGCGTAAAC 799 GAGCAAACAGGATTAGATACCCTGGTAGTCCA <mark>C</mark> GCTGTAAACGATGTGGATTTGGAGGTTGT <mark>A</mark> ATGATGCGTCCGGAGCTAACGCGTTAAA 755 GAGCAAACAGGATTAGATACCCTGGTAGTCAJGCTGTAAACGATGTGGAGTTGGAGTTGGTCAAAACGATGGTGTAA	873 898 853
	Ceratovacuna japonica Lipoptena cervi Trichobius parasiticus	100 FICE ACCE <mark>I</mark> CT GEGEGA GTA ACGEC CEAA BETTA AAACT CAAA TEAA TIGAC GEGEGCC GCACA ACCEATEGEGA CEATEGETTA ATT CEATEGA ACCEAA 1890 ECACCECT GEGEGA GTA CEGEC GEAA BETA AAACT CAAA TEAA TIGAC GEGEGCCC SCACA ACCEGETGA GEATT GETTA ATT CEATEGA ACCEAA 1890 ECACCECT GEGEGA GTA CEGEC GEAR BETA AAACT CAAA TEAA TIGAC GEGEGCCC SCACA ACCEGETGA GEATA TIGETTA ATT CEATEGA ACCEAA	973 998 953
	Ceratovacuna japonica Lipoptena cervi	94 BAACCTIACCTACTCTIGACACTIGAAAGAATCTITIAGAAATAGAAGAGTCCTTTCGGGAACTITIGATACAGGTGCTGCATGGCTGCATGGCTGCAG 999 BAACCTIACCTACTACTACTGTGACAGGCAGGGAATCICIGAGAAATAGAGGAGTCCTTCGGGAACGGTGAGAAAGGTGGTGATGGCTGCAGGACGCTGGGAGGAAGGTGCGCTGCGCTGCGCAGGAAGGCTGCGCGCAGGACAGGTGCGCGCGC	1073
	Ceratovacuna japonica Lipoptena cervi	TA ATACHT AGTA GATAGAT GALQAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG	1172 1197
	Ceratovacuna japonica Lipoptena cervi	1017 LA SEA A COTA DOCATEGA COT COCATA CACE CONCELLA A COTA CONCELLA CALLON CONCELLA ALCANDO CONCELLA	1272
	Ceratovacuna japonica Lipoptena cervi Trichobius parasiticus	1223 NGC GAACT COGAACTAC GO GAACTAC GO GATT COACTO CAACTO CACTO CATO AACTO GAAT CACTACTAC CAATA CO CAATA COATA	1372 1397 1353
	Ceratovacuna japonica Lipoptena cervi Trichobius parasiticus	1973 NT ACCTI CCCGGGCCTT GTACACACCCCCCGT CACACCATGGACTGCTT GTAAACAACTACGTACGT TACCTTT 1978 NT ACCTI CCCGGGCCTT GTACACACCCCCCGT CACACCATGGACCTCCTT GTAAACAACTACGTACGT TACCTTT 1986 NT ACCTI CCCGGGCCTT GTACACACCCCCCGT CACACCATGGACTCCTT GCAAACAACTACGTACGT TACCTTTTTAAACAATTGGATCGCTT	1461 1487 1453
	Comta anuna innenian		1539
	Lipoptena cervi Trichobius parasiticus	1488 TA CCACT TTGT GATT CAT GA CT GGGGT GAAGT CG TAACAAGG TAACC GT AGG GAACCT GCGGT TGGAT CACCT CCT TA 1454 TA CCACT TTGT GGT T CTAAT CT GGGT GAAA T GGAAT CAA GG TAACC GA GG GGAACCT GCGGT TGGAT CACCT CCT TA	1566 1511
в	Lipoptena cervi Trichobius parasiticus	1488 FACCACITIĞIGALTOATGACIGGGÜIGAAGIGCIAAGAAGIAACOGAAGGCAACCIGGGGAACCIGGGGIGGAICACCICCITA 1484 FACCACITIĞIGĞITOTAATCIGGGTIGAAATGAAAGIAACGAAGGGCGAATT	1566 1511
в	Ceratovacuna japonica Lipoptena cervi Trichobius parasiticus Ceratovacuna japonica Acyrthosiphon pisum	1488 FACEACT TO TOATGACT GOOD GAACT CONTACCAAGETAACCOTAGECGAACCT GCGGTT GGAT CACCT CCTTA 1454 FACCACTTTCTGCTTCTACTTCTGCTTAATCT GOOT GAAATGAAACGTACGAAGGCGAATT 1500 TTGAAGAGTTTGATCATGGCTCAGATTGAACGCTGGTGGCAGGCCTAACACATGCAAGTCGAGCGGCATCGAGTGAGCGCAGTTACTGAGTTCATG 1 GAATTGAAGAGTTTGATCATGGCTCAGATTGAACGCTGGTGGCAGGCCTAACACATGCAAGTCGAGCGGCATCGAGTGAGCGCAGTTACTGAGTTCATG	1566 1511 97 100
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#### Figure S3. Alignments of 16S rRNA sequences of Arsenophonus and Hamiltonella, related to Figure 2.

(A) A multiple alignment of Arsenophonus symbionts of Ce. japonica, L. cervi, and T. parasiticus is shown. Sequence identities of Arsenophonus symbionts between Ce. japonica and L. cervi, and those between Ce. japonica and T. parasiticus are 93.0% and 89.4%, respectively.

(B) A pair-wise alignment of Hamiltonella symbionts of Ce. japonica and A. pisum. The sequence identity between these two sequences is 99.8%.

See Table S3 for the accession number of 16S rRNA sequences used for these alignments.



### Figure S4. Structure of bacteriome, related to Figure 3.

Confocal microscopy images of bacteriomes are shown.

- (A) Hoechst 33342 signals.
- (B) FM 4-64FX signals.
- (C) A merged image of (A) and (B).
- (D) DAPI signals.
- (E) Phalloidin signals.

Scale bars show 50  $\mu m$  in (A–E).



Figure S5. Taxon-annotated GC-coverage plot for metagenomic assembly, related to Figure 4.

Each blob indicates the contigs in the assembly. The blob colors are based on the best match to the taxonomically annotated RefSeq protein database using DIAMOND. Annotated contigs as "Arthropoda" were shown as the label "Host" and the label "Others" means contigs do not hit *Buchnera*, *Arsenophonus*, *Hamiltonella*, or Arthropoda. The size of the blobs indicates the length of the contigs. *Buchnera* pLeu plasmid was not found in the initial assembly used in this analysis.



#### Figure S6. Synteny plots of chromosomes of Buchnera, Arsenophonus, and Hamiltonella, related to Figure 4.

Genomic synteny between symbionts is visualized.

(A) *Buchnera* of *Ce. japonica* and *A. pisum* (BA000003.2). The synteny is well conserved among the two *Buchnera* genomes, whereas several gaps are discernible.

(B) *Buchnera* of *Ce. japonica* and *Ci. cedri* (CP000263.1). The synteny is well conserved, whereas several gaps are discernible.

(C) Arsenophonus of Ce. japonica and N. vitripennis (CP038613.1). Little syntenic blocks are recognizable.

(D) Arsenophonus of Ce. japonica and L. cervi (CP013920.1). Little syntenic blocks are recognizable.

(E) *Hamiltonella* of *Ce. japonica* and *A. pisum* (CP001277.1). Syntenic blocks are observed with a number of inversions and rearrangements.

(F) *Hamiltonella* of *Ce. japonica* and *B. tabaci* (CP016303.1). The synteny looks well conserved with some deletions and inversions.



#### Figure S7. The infection process of *Hamiltonella* during embryogenesis, related to Figure 5.

(A) The developmental stage of an S-shape embryo. *Hamiltonella* is also infected into the embryo from the posterior part with *Arsenophonus*.

(B) The developmental stage of early germ band retraction after katatrepsis. Masses of *Hamiltonella* are observed around the bacteriome.

(A and B) Blue (DAPI), green (Cy5), and red (Cy3) indicate nuclei, *Hamiltonella*, and *Arsenophonus*, respectively. Scale bars show 50 µm in (A and B). hd, head.