Zoological Journal of the Linnean Society, 2018, **182**, 604–613. With 3 figures.

Historical and cospeciating associations between Cerataphidini aphids (Hemiptera: Aphididae: Hormaphidinae) and their primary endosymbiont *Buchnera aphidicola*

TING-TING XU[1,2†], JING CHEN[1†], LI-YUN JIANG[1] and GE-XIA QIAO[1*]

[1]*Key Laboratory of Zoological Systematics and Evolution, Institute of Zoology, Chinese Academy of Sciences, No. 1 Beichen West Road, Chaoyang District, Beijing 100101, P.R. China*
[2]*College of Life Sciences, University of Chinese Academy of Sciences, Shijingshan District, Beijing 100049, P.R. China*

Received 13 October 2016; revised 5 April 2017; accepted for publication 26 June 2017

Almost all viviparous aphid species harbour *Buchnera aphidicola* as their primary endosymbiont. Some species of the tribe Cerataphidini, however, possess yeast-like symbionts instead. Based on the multiple aphid and *Buchnera* genes, we construct the phylogenies of Cerataphidini aphid species and their corresponding *Buchnera*, respectively. Phylogeny building and ancestral state reconstructions suggest that in the evolutionary course of Cerataphidini, *Buchnera* has been lost several times and replaced by a eukaryotic symbiont. The *Buchnera* phylogeny generally mirrors that of aphid hosts, and strong aphid–*Buchnera* cospeciation signals are detected by statistical tests. This study therefore presents a good example of parallel evolution between aphids and *Buchnera* at the level of an entire aphid tribe, and the results suggest that the historical changes in symbiotic associations appear to have no effect on the pattern of aphid–*Buchnera* codiversification.

ADDITIONAL KEYWORDS: character reconstruction – parallel evolution – phylogenetic relationship – statistical testing.

INTRODUCTION

Endosymbiosis is found in many insect groups, and more than 10% of insect species exhibit an obligate mutualistic association with intracellular bacteria (Douglas, 1989; Moran & Telang, 1998; Moran, McCutcheon & Nakabachi, 2008). These intimate symbiotic associations are ideal systems in which to study the patterns of bacterial evolution, the origin of organelles and host–parasite coevolution. Many studies have addressed the evolutionary relationships between different insect groups and their endosymbionts (Lo *et al.*, 2003; Degnan *et al.*, 2004; Allen *et al.*, 2007; Gruwell, Morse & Normark, 2007; Toju *et al.*, 2013). Aphid–*Buchnera* symbiosis represents a classic and well-known example of obligate mutualism, and it has been widely investigated (Baumann *et al.*, 1995; Baumann, Moran & Baumann, 1997; review in Liu, Huang & Qiao, 2013).

Buchnera aphidicola (Gammaproteobacteria: Enterobacteriaceae) is the primary endosymbiont of almost all aphid species, and it inhabits specialized aphid cells called bacteriocytes (Buchner, 1965; Unterman, Baumann & Mclean, 1989; Munson, Baumann & Kinsey, 1991). *Buchnera* supplies aphids with nutrients (e.g. amino acids and vitamins) that are important to their growth and development but lacking in phloem sap (Douglas & Prosser, 1992; Douglas, 1998; Nakabachi & Ishikawa, 1999; Shigenobu *et al.*, 2000; Moran & Degnan, 2006; Wilson *et al.*, 2010). *Buchnera* is maternally transmitted between aphid generations and cannot live independently without its aphid host (Buchner, 1965; Baumann *et al.*, 1995).

Theoretically, symbionts with such a mode of strict vertical transmission should show a pattern of

*Corresponding author. E-mail: qiaogx@ioz.ac.cn
†These authors contributed equally to this work.

diversification that is parallel to their hosts and consequently leads to phylogenetic concordance between these two partners. Munson *et al.* (1991) reconstructed the phylogeny of *Buchnera* of 11 aphid species from four Aphididae subfamilies using 16S rRNA sequences, resulting in congruent phylogenetic relationships with the morphology-based tree of corresponding aphid hosts (Heie, 1987). Higher-level phylogenetic correspondence of aphids and *Buchnera* was then reconfirmed by Moran *et al.* (1993) and Baumann, Moran & Baumann (1997), and the establishment of this symbiotic association was estimated to date back to 160–280 Mya followed by long-term cospeciation of aphids and *Buchnera* (Moran *et al.*, 1993). Martinez-Torres *et al.* (2001) tested their phylogenetic congruence based on more gene sequences and broader taxonomic sampling. Some disagreements between the *Buchnera* phylogeny and the classic taxonomy and molecular phylogeny of aphids were revealed, which suggested that the aphid phylogeny should be revised. At finer phylogenetic scales, codiversification of *Buchnera* and their aphid hosts has been documented within certain aphid groups [e.g. *Uroleucon ambrosiae* (Thomas), *Brachycaudus* van der Goot, *Mollitrichosiphum* Suenaga, *Uroleucon* Mordvilko, *Pemphigus* Hartig and allied genera] (Clark *et al.*, 2000; Funk *et al.*, 2000; Jousselin, Desdevises & Coeur d'acier, 2009; Liu *et al.*, 2013, 2014). Some studies also used molecular data derived from *Buchnera* to investigate the phylogeny and phylogeographic history of aphids (Peccoud *et al.*, 2009; Jousselin, Genson & Coeur d'acier, 2010; Nováková *et al.*, 2013).

Previous studies concerning aphid–*Buchnera* relationships have mainly been focussed on aphid lineages that were in stable symbiotic associations with this obligate mutualist. However, *Buchnera* has been reported to be absent in some species of Cerataphidini (Fukatsu & Ishikawa, 1992; Fukatsu *et al.*, 1994). The aphid tribe Cerataphidini (Aphididae: Hormaphidinae) is heteroecious, seasonally obligately alternating between primary host plants, *Styrax* (Styracaceae), on which morphologically diverse galls are formed, and secondary host plants such as Compositae, Gramineae, Loranthaceae, Palmaceae and Zingiberaceae (Chen & Qiao, 2009; Aoki & Kurosu, 2010; Chen, Jiang & Qiao, 2014). Cerataphidini aphid species produce specialized sterile soldiers (Aoki, Yamane & Kiuchi, 1977; Aoki & Miyazaki, 1978; Stern & Foster, 1996) and are mainly distributed in eastern and south-eastern Asia (Ghosh, 1985, 1988). Within Cerataphidini, most species harbour *Buchnera* as their primary endosymbiont. However, some cerataphidine aphids have been reported to possess a different kind of symbiont (Fukatsu & Ishikawa, 1992; Fukatsu *et al.*, 1994). Fukatsu *et al.* (1994) surveyed the symbiotic systems of 39 Cerataphidini species using a histochemical method. *Buchnera* was found to be absent in 12 species, including *Cerataphis* Lichtenstein (excl. *Cerataphis bambusifoliae* Takahashi), *Glyphinaphis* van der Goot and *Tuberaphis* Takahashi, which harboured eukaryotic yeast-like symbionts (YLSs) instead.

Therefore, the aphid tribe Cerataphidini is an ideal model to understand the evolutionary dynamics of aphid–*Buchnera* symbiosis. In the present study, we aimed to trace the possible evolutionary scenarios for the association between cerataphidine aphids and *Buchnera* and to investigate the consequences of a partner changing in the obligate symbiosis for aphid–*Buchnera* codiversification.

We reconstructed detailed phylogenetic relationships of Cerataphidini aphid species and corresponding *Buchnera* based on largely expanded data sets, which contained six aphid and four *Buchnera* gene sequences of broadly sampled species from all known cerataphidine genera. We then traced the evolutionary history of the aphid–*Buchnera* association using ancestral character state reconstruction and tested the phylogenetic congruence between them. The results of this study will be helpful for understanding the patterns of aphid–*Buchnera* symbiosis and the evolution of interactions between aphids and their symbionts.

MATERIAL AND METHODS

Sampling and data collection

Twenty-six aphid species belonging to all ten known genera of Cerataphidini were sampled in this study. Five species of the other two tribes of Hormaphidinae (i.e. Hormaphidini and Nipponaphidini) and one species of Eriosomatinae, which is the traditional sister group of Hormaphidinae (Heie, 1967; Ghosh, 1985, 1988; Wojciechowski, 1992; Zhang *et al.*, 1999), were chosen as outgroups. Aphid samples were preserved in 95 or 100% ethanol for molecular experiments. Samples stored in 75% ethanol were used to make slide voucher specimens. All aphid voucher specimens and samples were deposited in the National Zoological Museum of China, Institute of Zoology, Chinese Academy of Sciences, Beijing, China. Detailed voucher information of all samples is provided in Supporting Information, Table S1.

Total DNA, including the *Buchnera* genome, was extracted from single aphid individuals using DNeasy Blood & Tissue Kit (Qiagen, Germantown, MD, USA). To reconstruct reliable phylogenetic relationships within Cerataphidini, six genes of aphids were amplified: four

mitochondrial genes (the standard *COI* barcode, *COI*/*COII*, *Cytb*, and 12S/16S rRNA) and two nuclear genes (*EF-1α* and *LWO*). Four gene fragments of *B. aphidicola* were amplified, including 16S rRNA, *dnaB*, *groEL* and *ilvD*. Some published gene sequences were taken from previous studies (see Supporting Information, Table S1). All primers used in this study are provided in Supporting Information, Table S2. PCR amplification was performed in a 25-μL reaction mixture containing 10× EasyTaq DNA Polymerase Buffer ($+Mg^{2+}$), 1.5 U EasyTaq DNA Polymerase, 2.5 mM each dNTP, 5 pmole each primer (TransGen Biotech, Beijing, China) and 1-μL DNA extract. The PCR conditions were as follows: 2- to 5-min initial denaturation at 94–95 °C followed by 35–40 cycles of denaturation at 92–95 °C for 20–90 s, annealing at 48–55 °C for 30–90 s, extension at 68–72 °C for 1–2 min, and a 7- to 10-min final extension at 72 °C. The annealing temperatures of each specific primer set were as follows: 48 °C for *Cytb* and *LWO*, 53 °C for *ilvD* and 50 °C for all the other genes. For the amplifications of *Cytb*, *LWO*, *EF-1α* and *ilvD* genes of some samples, a second nested PCR was necessary on a 1-μL aliquot from the first PCR. Conditions were identical except for the increase of the annealing temperature to 50 °C for *Cytb* and *LWO*, 52 °C for *EF-1α* and 55 °C for *ilvD*. PCR products of some fragments were cloned using pMD19-T Vector System (TaKaRa, Dalian, China) and Trans5α Chemically Competent Cell (TransGen Biotech) following the manufacturer's instructions. The PCR products and clones were sequenced on an ABI 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA).

Raw sequences were assembled by SeqMan II (DNAStar, Madison, WI, USA). Introns of *EF-1α* and *LWO* genes were removed by the GT-AG rule and aligning with the cDNA sequences from *Aleurodaphis blumeae* van der Goot (GenBank accession numbers JX489689 and JX489722) and only exons were used for further analyses. All sequences have been deposited in GenBank (Supporting Information, Table S1). Multiple alignments were performed with MEGA 6.0 (Tamura *et al.*, 2013) and MAFFT 7 (Katoh & Standley, 2013). Gblocks 0.91b (Castresana, 2000) was used to eliminate the poorly aligned positions of *COI*/*COII*, 12S/16S rRNA and 16S rRNA sequences.

Phylogenetic analysis

We constructed phylogenetic trees of aphids and corresponding *Buchnera* based on a combined aphid gene data set and a combined *Buchnera*-derived gene data set, respectively. Two data sets were produced for aphids: one contained all sampled aphid species and the other only included species harbouring *Buchnera*. For Bayesian analysis, the best model of nucleotide substitution for each gene was selected by jModelTest 2.1.10 (Guindon & Gascuel, 2003; Darriba *et al.*, 2012). The Bayesian information criterion (Schwarz, 1978) favoured TIM1 + I + G for *COI*, TIM2 + I + G for *COI*/*COII* and 12S/16S rRNA, GTR + I + G for *Cytb* and *dnaB*, TIM2 + G for *EF-1α*, TPM2uf + G for *LWO*, TVM + I + G for 16S rRNA, TIM3 + I + G for *groEL* and TPM3uf + I + G for *ilvD*. The Bayesian analysis was implemented in MrBayes 3.2.6 (Ronquist *et al.*, 2012) under default priors with each gene partition unlinked for parameter estimations. Four chains were run starting from a random tree with 1 million Markov chain Monte Carlo generations and sampled every 100 generations. The first 25% of trees were discarded as burn-in samples. The remaining trees were used to construct a 50% majority-rule consensus tree and to compute the posterior probabilities (PP). Maximum likelihood (ML) analysis was carried out using RAxML v8.2.8 (Stamatakis, 2014) with the GTRCAT model for each gene partition, and all parameters were estimated during the ML search. Bootstrap analysis was performed with 1000 replicates.

Ancestral state reconstruction

To trace the historical changes of the aphid–*Buchnera* relationship within Cerataphidini, we performed ancestral state reconstruction using a ML approach. Whether *Buchnera* was present or absent was coded as 1 or 0, respectively. Reconstructions were performed in Mesquite 3.10 (Maddison & Maddison, 2016) under Mk1 and AsymmMk models using the Trace Character Over Trees option. All reconstructions were integrated over 1000 randomly selected trees from the post-burn-in aphid Bayesian trees, and the ancestral states were summarized on the Bayesian consensus tree.

Phylogenetic congruence testing

In this study, we applied three widely used statistical methods to test the phylogenetic concordance between aphids and *Buchnera* within Cerataphidini. The outgroup-pruned ML trees of *Buchnera*-harbouring aphid species and corresponding *Buchnera* were used.

TreeMap 1.0 (Page, 1994) is a topology-based program and reconciles the parasite and host trees by introducing four types of events: cospeciation (C), duplication (D), host switching (H) and sorting (S). We used both exact and heuristic searches to identify the optimal reconstructions that maximize the number of cospeciations and minimize the number of non-cospeciation events. Using the proportional-to-distinguishable option, 1000 random *Buchnera* trees were generated, and randomization tests were conducted to assess whether the observed numbers of cospeciation events were greater than expected by chance.

The distance-based program ParaFit (Legendre, Desdevises & Bazin, 2002) tests the significance of a global hypothesis of coevolution between parasites and their hosts as well as individual host–parasite association links. In the present study, the null hypothesis that the evolution of Cerataphidini aphid species and *Buchnera* has been independent (i.e. they are randomly associated) was tested through a permutation procedure. The patristic distances of *Buchnera* and aphids were calculated by Patristic (Fourment & Gibbs, 2006) from their outgroup-pruned ML trees and then were transformed to principal coordinates called matrix B and matrix C by DistPCoA (Legendre & Anderson, 1998). The aphid–*Buchnera* associations were described in matrix A. If the aphid–*Buchnera* link is observed in nature between the aphid species in the column and the *Buchnera* in the row, 1 was written. Otherwise, 0 was written. The test was performed for 999 permutations using ParaFit.

Jane 4.0 (Conow *et al.*, 2010) is an event cost-based program that uses a heuristic approach with a genetic algorithm to find optimal solutions. The event cost regime followed the default settings: cospeciation (0), duplication (1), host switching (2), loss (1) and failure to diverge (1). We performed analyses with 100 generations, a population size of 300 and a maximum of 99 999 stored solutions in each run. Statistical tests were then conducted to compare the resulting costs to the cost of original associations by randomizing the tip of trees and randomizing the parasite tree topology with a sample size of 500.

RESULTS

Phylogenetic relationships

For all sampled Cerataphidini aphid species, ML and Bayesian analyses yielded identical ingroup topology (Fig. 1). The monophyly of Cerataphidini was strongly supported (bootstrap = 99%, PP = 1.00). All genera represented by multiple species were recovered as monophyletic with high support values, except *Tuberaphis* and *Cerataphis*. *Ktenopteryx* + *Aleurodaphis* split off earliest from other taxa. *Tuberaphis* and *Cerataphis* formed a monophyletic clade as a whole, followed by *Glyphinaphis* and the remaining five genera. *Pseudoregma* and *Ceratovacuna* formed a sister group. The phylogenetic tree of cerataphidine species harbouring *Buchnera* (excl. *Glyphinaphis*, *Tuberaphis* and *Cerataphis brasiliensis*) (Fig. 2A) showed an ingroup topology that was essentially identical to Figure 1.

Buchnera is absent from several of the sampled aphid species of Cerataphidini, which resulted in negative

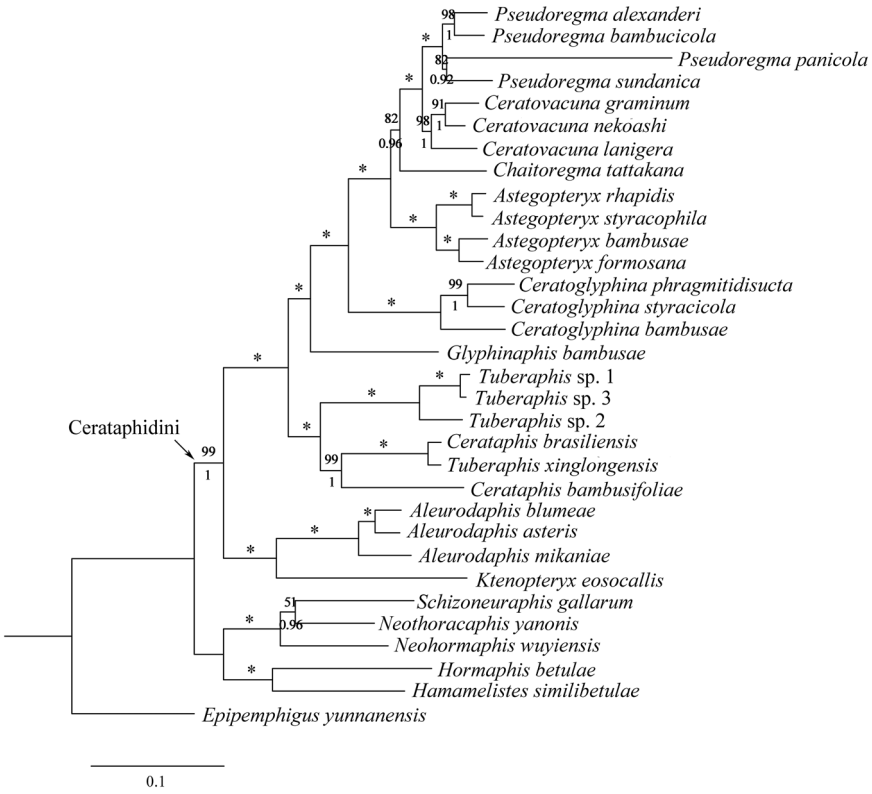

Figure 1. Cerataphidini phylogeny obtained from ML analysis. ML bootstrap values (> 50%) and Bayesian PP values (> 0.70) are shown above and below the branches, respectively. *Represents 100% ML bootstrap and 1.00 PP.

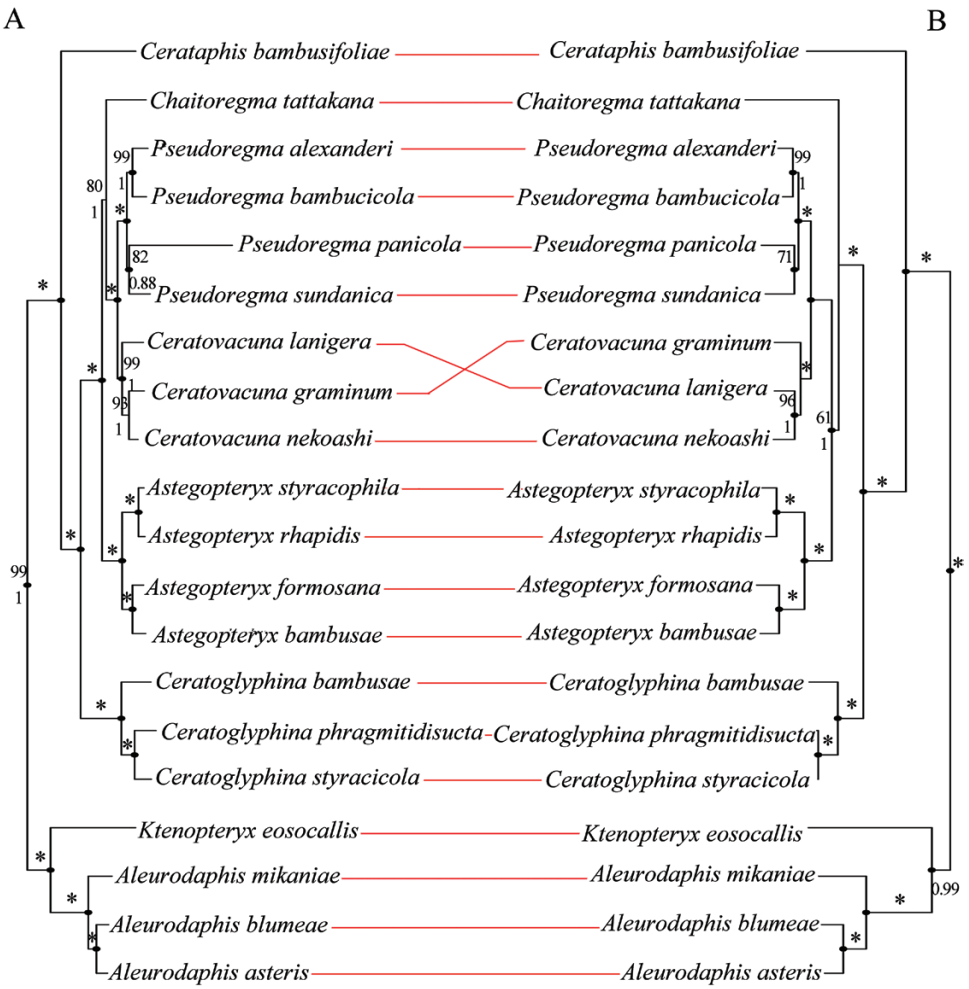

Figure 2. Tanglegram for Cerataphidini aphids and *Buchnera*. Each symbiont is connected to its host by a line. A, phylogeny of *Buchnera*-harbouring aphid species of Cerataphidini. B, *Buchnera* phylogeny (for *Buchnera*, the names of corresponding aphid host species are used). ML bootstrap values (> 50%) and Bayesian PP values (> 0.70) are shown above and below the branches, respectively. *Represents 100% ML bootstrap and 1.00 PP.

amplifications of *Buchnera* genes in this study. ML and Bayesian trees of *Buchnera* from the remaining cerataphidine representatives resulted in a congruent ingroup topology (Fig. 2B). The monophyly of *Buchnera* within Cerataphidini was well recovered (bootstrap = 100%, PP = 1.00), and the tree highly supported clustering of different *Buchnera* lineages into aphid genera. The phylogenetic relationships of *Buchnera* were generally congruent with the relationships of corresponding aphid hosts (Fig. 2A), with the exception of the positions of *Chaitoregma tattakana* and *Ceratovacuna lanigera*.

Character evolution

The results of ancestral state reconstruction for the aphid–*Buchnera* association within Cerataphidini are presented in Figure 3. *Buchnera* present was favoured for the ancestor of Cerataphidini under both Mk1 and AsymmMk models. In Mk1 reconstruction, *Buchnera* present was shown to be the ancestral state for most internal nodes, except for nodes 6–10, for which the states were clearly reconstructed as *Buchnera* absent. AsymmMk model analysis resulted in consistent reconstructions with the Mk1 model, except for nodes 5 and 11, whose ancestral states were *Buchnera* absent.

Ancestral state reconstructions implied that there had been several changes in *Buchnera* infection for the sampled cerataphidine representatives. Three alternative possible scenarios were indicated: (1) *Buchnera* had been lost at least three times independently, once, respectively, in node 8, node 9 and *Glyphinaphis* (reconstructions under both models); (2) two losses of *Buchnera* (one in node 6 and one in *Glyphinaphis*), followed by one reacquisition of *Buchnera* in *C. bambusifoliae* (Mk1 model, Fig. 3A); and (3) a single loss of *Buchnera* in node 5, followed by two reacquisitions

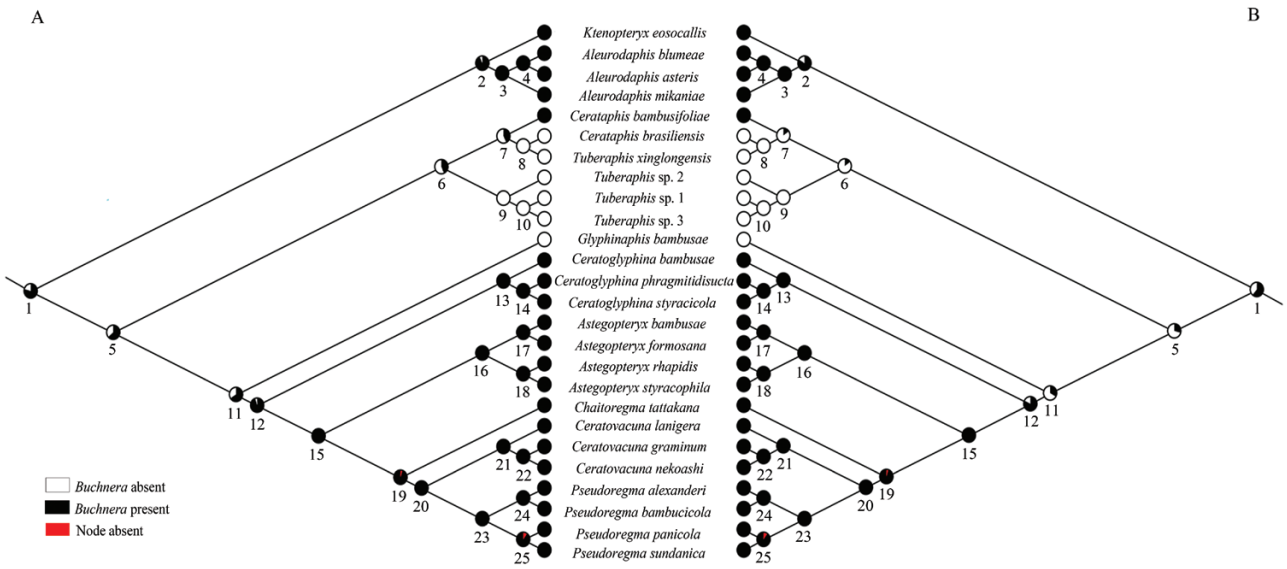

Figure 3. Ancestral state reconstructions for *Buchnera* infection in Cerataphidini under Mk1 model (A) and AsymmMk model (B). Pie charts at nodes indicate the percentages of trees for which a given state is reconstructed as the uniquely best state for that node.

of *Buchnera* (one in *C. bambusifoliae* and one in node 12) (AsymmMk model, Fig. 3B).

Phylogenetic congruence

A tanglegram for the association between Cerataphidini aphids and *Buchnera* generated by TreeMap is presented in Figure 2. The heuristic search introduced 17 cospeciations, two duplications and six sorting events. Eight best reconstructions were identified in the exact search with 17 cospeciations, zero or one duplication, one or two switches, and two or four sorting events. Randomization test results suggested that the observed number of cospeciation events was significantly greater than expected by chance ($P < 0.001$). The ParaFit analysis rejected the null hypothesis that the phylogenetic trees of aphids and *Buchnera* are randomly associated (ParaFitGlobal = 0.0851, $P = 0.001$). Twelve of 20 individual host–parasite association links contributed to the global trace statistic ($P < 0.05$). The result of Jane analysis also showed significant phylogenetic concordance between aphids and *Buchnera* within Cerataphidini ($P < 0.001$) with 17 cospeciations, two host switches and two losses.

DISCUSSION

Evolution of aphid–*Buchnera* symbiosis within Cerataphidini

Buchnera aphidicola is commonly found among almost all aphid species of Aphididae as their obligate primary endosymbiont (Buchner, 1965; Baumann, Moran & Baumann, 1997). It has been suggested that this endosymbiotic association had a single origin in the common ancestor of all modern viviparous aphid species, dating back to 160–280 Mya (Munson *et al.*, 1991; Moran *et al.*, 1993). Therefore, the common ancestor of Cerataphidini should have harboured *Buchnera*, especially considering the fact that most extant cerataphidine species are infected with *Buchnera*. In the present study, the character reconstructions using different models strongly suggested that the ancestor of Cerataphidini had established a symbiotic association with *Buchnera*, which provides clear evidence for the ancestrality of *Buchnera* infection within the Cerataphidini.

Using a histochemical method, Fukatsu *et al.* (1994) found that six *Tuberaphis* species, five *Cerataphis* species and *Glyphinaphis bambusae* did not harbour the typical intracellular symbiotic bacteria, *Buchnera*. In this study, we examined all sampled cerataphidine species using specific PCR amplification and discovered that *C. brasiliensis* and *G. bambusae*, which had been detected in Fukatsu *et al.* (1994), as well as four species of *Tuberaphis* newly sampled in our study were not infected with *Buchnera*. Ancestral state reconstructions suggested that several changes for the aphid–*Buchnera* association had occurred during the evolution of Cerataphidini. Alternative scenarios could be proposed based on the results of character reconstruction, which included multiple independent losses of *Buchnera* or losses followed by reacquisitions of *Buchnera*. However, considering the long history

of aphid–*Buchnera* symbiosis (Moran *et al.*, 1993) and the intimate nutritional interactions between these two partners within this obligate mutualism (Shigenobu *et al.*, 2000; Tamas *et al.*, 2002; Wilson *et al.*, 2010), there seems to be little possibility of regaining *Buchnera* after losing. Therefore, we infer that at least three *Buchnera* losses have taken place for the sampled cerataphidine representatives.

Previous studies discovered that the Cerataphidini aphid species without *Buchnera* harboured eukaryotic YLSs (Fukatsu & Ishikawa, 1992; Fukatsu *et al.*, 1994). PCR amplifications of 18S rRNA of YLS from the *Buchnera*-free aphid samples in our study confirmed this finding (data not shown). The genome sequencing of YLS from *C. brasiliensis* revealed that the YLS possesses genes for the full biosynthetic pathways of essential amino acids (Vogel & Moran, 2013). We consequently hypothesize that in *Glyphinaphis* and certain species of *Cerataphis* and *Tuberaphis*, of which *Buchnera* are lost, YLSs have replaced the nutritional functions of *Buchnera*.

Fukatsu *et al.* (1994) speculated that in the evolutionary course of Cerataphidini, the replacement of *Buchnera* by YLS occurred only once in the common ancestor of *Cerataphis*, *Glyphinaphis* and *Tuberaphis* because such replacement was extremely rare in aphids. However, neither our study nor previous phylogeny reconstructions of Cerataphidini (Stern, 1994, 1998; Huang *et al.*, 2012; Chen *et al.*, 2014) supported clustering of these three genera into a monophyletic clade. Additionally, it is worth noting that both our study and Fukatsu *et al.* (1994) discovered that *C. bambusifoliae* did harbour *Buchnera*. The high morphological similarity of *C. bambusifoliae* to other *Cerataphis* species (Takahashi, 1925), the formation of single-cavity galls (Kurosu *et al.*, 2008) and its phylogenetic position revealed by the present and previous studies (Stern, 1998; Chen *et al.*, 2014) confirm its affiliation to the genus *Cerataphis*. Therefore, even if the monophyly of *Cerataphis*, *Glyphinaphis* and *Tuberaphis* as a whole is supported and *Buchnera* has been lost and replaced by YLS in their common ancestor, one reacquisition of *Buchnera* should have occurred in *C. bambusifoliae*. The probability of that reacquisition happening, however, is very small, as we stated above.

To date, little is known about the detailed process and the mechanism of *Buchnera* loss and symbiont replacement in Cerataphidini. Assuming there is a higher transmission efficiency of YLS, Fukatsu *et al.* (1994) proposed that the replacement might have been completed within just a small number of aphid generations. However, we cannot rule out the possibility that *Buchnera* and YLS may have experienced a period of coexistence similar to *Serratia symbiotica* and *Buchnera* in *Cinara cedri* Mimeur (Lachninae), in which *Buchnera* is undergoing a process of genome degradation and functional complement by the coresident *S. symbiotica* (Pérez-Brocal *et al.*, 2006; Lamelas *et al.*, 2008, 2011). In certain lineages of Cerataphidini, *Buchnera* may completely lose its symbiotic capacity after YLS has taken over all its functions and eventually become lost from its aphid host.

Parallel evolution of *Buchnera* and Cerataphidini aphids

In the present study, we constructed the phylogenies of *Buchnera*-harbouring aphid species and corresponding *Buchnera* using six aphid genes and four *Buchnera* genes from broad taxonomic samples covering all known genera of the tribe Cerataphidini. Phylogenetic matching between aphids and *Buchnera* was well presented in Cerataphidini (Fig. 2). Aphid genera were defined with strong support in the phylogenetic tree of *Buchnera*. The bacterial phylogeny almost fully reflected the generic and interspecific relationships among aphid species, except for the positions of *C. tattakana* and *C. lanigera*. Several phylogenetic hypotheses revealed in the present and previous studies using aphid genes were confirmed by the genes derived from *Buchnera*, such as the basal position of *Ktenopteryx* + *Aleurodaphis* and the sister group relationship between *Ceratovacuna* and *Pseudoregma* (Stern, 1994, 1995, 1998; Huang *et al.*, 2012; Chen *et al.*, 2014). Moreover, statistical testing also revealed significant phylogenetic congruence between *Buchnera* and aphids and suggested that they were cospeciating. A total of 17 cospeciation events were detected by the analyses of TreeMap and Jane. The results of ParaFit also indicated a significant correlation between the phylogenies of aphids and *Buchnera*.

The pattern of codiversification between aphids and their primary endosymbiont *Buchnera* has been demonstrated in several aphid groups covering different taxonomic levels from the whole group of viviparous aphids (the Aphididae) to populations within a specific aphid species (Munson *et al.*, 1991; Moran *et al.*, 1993; Baumann, Moran & Baumann, 1997; Clark *et al.*, 2000; Funk *et al.*, 2000; Jousselin *et al.*, 2009; Liu *et al.*, 2013, 2014). The present study confirmed significant concordance of aphid and *Buchnera* phylogenies at the level of an entire aphid tribe, which would enrich the instances of aphid–*Buchnera* parallel evolution. Furthermore, our study showed that *Buchnera* had been lost and replaced by YLS within certain lineages of Cerataphidini. The significant cophylogenetic pattern between aphids and corresponding *Buchnera* corroborated their strict cospeciating relationship,

which was not affected by the historical changes of symbiotic associations between *Buchnera* and Cerataphidini aphids.

CONCLUDING REMARKS

In conclusion, our results indicated that during the evolutionary history of Cerataphidini, the obligate endosymbiont *Buchnera* had been lost several times and replaced by the eukaryotic YLS with similar nutritional functions. Both phylogeny comparison and results of statistical testing suggested there was significant concordance between the aphid and bacterial phylogenies, which confirms that *Buchnera* and their aphid hosts have diversified in parallel within the Cerataphidini, and their cospeciation pattern has not been affected by the evolutionary lability of this obligate symbiosis. To explore the origin of the aphid–YLS association, a comprehensive survey of the symbiont systems of all known species from *Cerataphis* and *Tuberaphis* is necessary. Further phylogenetic studies with a broad sampling of YLS-harbouring species and more information about YLSs (e.g. transmission mode, genome) are greatly needed to elucidate the mechanism underlying symbiont replacement and the evolution of symbiotic interactions between aphids and YLSs.

ACKNOWLEDGEMENTS

We thank Fen-Di Yang for making aphid slides and all collectors for collecting samples. The work was supported by the National Natural Sciences Foundation of China (nos 31430078, 31402000, 31561163002) and a grant from the Ministry of Science and Technology of the People's Republic of China (nos 2013FY111200, 2014FY110100).

REFERENCES

Allen JM, Reed DL, Perotti MA, Braig HR. 2007. Evolutionary relationships of "*Candidatus* Riesia spp.," endosymbiotic Enterobacteriaceae living within hematophagous primate lice. *Applied and Environmental Microbiology* **73:** 1659–1664.

Aoki S, Kurosu U. 2010. A review of the biology of Cerataphidini (Hemiptera, Aphididae, Hormaphidinae), focusing mainly on their life cycles, gall formation, and soldiers. *Psyche* **2010:** 380351.

Aoki S, Miyazaki M. 1978. Notes on the pseudoscorpion-like larvae of *Pseudoregma alexanderi* (Homoptera, Aphidoidea). *Japanese Journal of Entomology* **46:** 433–438.

Aoki S, Yamane S, Kiuchi M. 1977. On the biters of *Astegopteryx styraciola* (Homoptera, Aphidoidea). *Kontyû* **45:** 563–570.

Baumann P, Baumann L, Lai CY, Rouhbakhsh D, Moran NA, Clark MA. 1995. Genetics, physiology, and evolutionary relationships of the genus *Buchnera*: intracellular symbionts of aphids. *Annual Review of Microbiology* **49:** 55–94.

Baumann P, Moran NA, Baumann L. 1997. The evolution and genetics of aphid endosymbionts. *Bioscience* **47:** 12–20.

Buchner P. 1965. *Endosymbiosis of animals with plant microorganisms*. New York: Interscience Publishers, 297–332.

Castresana J. 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution* **17:** 540–552.

Chen J, Jiang LY, Qiao GX. 2014. A total-evidence phylogenetic analysis of Hormaphidinae (Hemiptera: Aphididae), with comments on the evolution of galls. *Cladistics* **30:** 26–66.

Chen J, Qiao GX. 2009. A study on diversity of aphid's galls of Hormaphidinae. *Acta Zootaxonomica Sinica* **34:** 269–276.

Clark MA, Moran NA, Baumann P, Wernegreen JJ. 2000. Cospeciation between bacterial endosymbionts (*Buchnera*) and a recent radiation of aphids (*Uroleucon*) and pitfalls of testing for phylogenetic congruence. *Evolution* **54:** 517–525.

Conow C, Fielder D, Ovadia Y, Libeskind-Hadas R. 2010. Jane: a new tool for the cophylogeny reconstruction problem. *Algorithms for Molecular Biology* **5:** 16.

Darriba D, Taboada GL, Doallo R, Posada D. 2012. jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods* **9:** 772.

Degnan PH, Lazarus AB, Brock CD, Wernegreen JJ. 2004. Host-symbiont stability and fast evolutionary rates in an ant-bacterium association: cospeciation of *Camponotus* species and their endosymbionts, *Candidatus* Blochmannia. *Systematic Biology* **53:** 95–110.

Douglas AE. 1989. Mycetocyte symbiosis in insects. *Biological Reviews* **64:** 409–434.

Douglas AE. 1998. Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. *Annual Review of Entomology* **43:** 17–37.

Douglas AE, Prosser WA. 1992. Synthesis of the essential amino acid tryptophan in the pea aphid (*Acyrthosiphon pisum*) symbiosis. *Journal of Insect Physiology* **38:** 565–568.

Fourment M, Gibbs MJ. 2006. PATRISTIC: a program for calculating patristic distances and graphically comparing the components of genetic change. *BMC Evolutionary Biology* **6:** 1.

Fukatsu T, Aoki S, Kurosu U, Ishikawa H. 1994. Phylogeny of Cerataphidini aphids revealed by their symbiotic microorganisms and basic structure of their galls: implications for host-symbiont coevolution and evolution of sterile soldier castes. *Zoological Science* **11:** 613–623.

Fukatsu T, Ishikawa H. 1992. A novel eukaryotic extracellular symbiont in an aphid, *Astegopteryx styraci* (Homoptera, Aphididae, Hormaphidinae). *Journal of Insect Physiology* **38:** 765–773.

Funk DJ, Helbling L, Wernegreen JJ, Moran NA. 2000. Intraspecific phylogenetic congruence among multiple symbiont genomes. *Proceedings of the Royal Society of London B: Biological Sciences* **267:** 2517–2521.

Ghosh AK. 1985. Hormaphidinae: distribution, phylogeny and systematics. In: Szelegiewicz H, ed. *Evolution and biosystematics of aphids. Proceedings of the International*

Aphidological Symposium at Jablonna. Warsaw: Polska Academia Nauk. 303–336.

Ghosh AK. 1988. *The fauna of India and the adjacent countries. Homoptera: Aphidoidea. Part 4. Subfamilies: Phloeomyzinae, Anoeciinae and Hormaphidinae*. Calcutta: Zoological Survey of India.

Gruwell ME, Morse GE, Normark BB. 2007. Phylogenetic congruence of armored scale insects (Hemiptera: Diaspididae) and their primary endosymbionts from the phylum Bacteroidetes. *Molecular Phylogenetics and Evolution* **44:** 267–280.

Guindon S, Gascuel O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **52:** 696–704.

Heie OE. 1967. Studies on fossil aphids (Homoptera: Aphidoidea) especially in the Copenhagen collection of fossils in Baltic amber. *Spolia Zoologica Musei Hauniensis* **26:** 1–274.

Heie OE. 1987. Paleontology and phylogeny. In: Minks AK, Harrewijn P, eds. *Aphids: their biology, natural enemies, and control, Vol. 2A*. Amsterdam: Elsevier Biomedical Press, 367–391.

Huang XL, Xiang-Yu JG, Ren SS, Zhang RL, Zhang YP, Qiao GX. 2012. Molecular phylogeny and divergence times of Hormaphidinae (Hemiptera: Aphididae) indicate Late Cretaceous tribal diversification. *Zoological Journal of the Linnean Society* **165:** 73–87.

Jousselin E, Desdevises Y, Coeur d'acier A. 2009. Fine-scale cospeciation between *Brachycaudus* and *Buchnera aphidicola*: bacterial genome helps define species and evolutionary relationships in aphids. *Proceedings of the Royal Society of London B: Biological Sciences* **276:** 187–196.

Jousselin E, Genson G, Coeur d'acier A. 2010. Evolutionary lability of a complex life cycle in the aphid genus *Brachycaudus*. *BMC Evolutionary Biology* **10:** 295.

Katoh K, Standley DM. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution* **30:** 772–780.

Kurosu U, Kutsukake M, Aoki S, Wang CC, Lee HJ, Fukatsu T. 2008. Galls of *Cerataphis bambusifoliae* (Hemiptera, Aphididae) found on *Styrax suberifolius* in Taiwan. *Zoological Studies* **47:** 191–199.

Lamelas A, Gosalbes MJ, Manzano-Marín A, Peretó J, Moya A, Latorre A. 2011. *Serratia symbiotica* from the aphid *Cinara cedri*: a missing link from facultative to obligate insect endosymbiont. *PLoS Genetics* **7:** e1002357.

Lamelas A, Pérez-Brocal V, Gómez-Valero L, Gosalbes MJ, Moya A, Latorre A. 2008. Evolution of the secondary symbiont "*Candidatus* Serratia symbiotica" in aphid species of the subfamily Lachninae. *Applied and Environmental Microbiology* **74:** 4236–4240.

Legendre P, Anderson MJ. 1998. *Program DistPCoA*. Montréal: Département de Sciences Biologiques, Université de Montréal.

Legendre P, Desdevises Y, Bazin E. 2002. A statistical test for host-parasite coevolution. *Systematic Biology* **51:** 217–234.

Liu L, Huang XL, Qiao GX. 2013. Trends in research on the primary endosymbiont of aphidis, *Buchnera aphidicola*. *Chinese Journal of Applied Entomology* **50:** 1419–1427.

Liu L, Huang XL, Zhang RL, Jiang LY, Qiao GX. 2013. Phylogenetic congruence between *Mollitrichosiphum* (Aphididae: Greenideinae) and *Buchnera* indicates insect-bacteria parallel evolution. *Systematic Entomology* **38:** 81–92.

Liu L, Li XY, Huang XL, Qiao GX. 2014. Evolutionary relationships of *Pemphigus* and allied genera (Hemiptera: Aphididae: Eriosomatinae) and their primary endosymbiont, *Buchnera aphidicola*. *Insect Science* **21:** 301–312.

Lo N, Bandi C, Watanabe H, Nalepa C, Beninati T. 2003. Evidence for cocladogenesis between diverse dictyopteran lineages and their intracellular endosymbionts. *Molecular Biology and Evolution* **20:** 907–913.

Maddison WP, Maddison DR. 2016. Mesquite: a modular system for evolutionary analysis. Version 3.10. Available at: http://mesquiteproject.org

Martinez-Torres D, Buades C, Latorre A, Moya A. 2001. Molecular systematics of aphids and their primary endosymbionts. *Molecular Phylogenetics and Evolution* **20:** 437–449.

Moran NA, Degnan PH. 2006. Functional genomics of *Buchnera* and the ecology of aphid hosts. *Molecular Ecology* **15:** 1251–1261.

Moran NA, McCutcheon JP, Nakabachi A. 2008. Genomics and evolution of heritable bacterial symbionts. *Annual Review of Genetics* **42:** 165–190.

Moran NA, Munson MA, Baumann P, Ishikawa H. 1993. A molecular clock in endosymbiotic bacteria is calibrated using the insect hosts. *Proceedings of the Royal Society of London B: Biological Sciences* **253:** 167–171.

Moran NA, Telang A. 1998. Bacteriocyte-associated symbionts of insects. *Bioscience* **48:** 295–304.

Munson MA, Baumann P, Clark MA, Baumann L, Moran NA, Voegtlin DJ, Campbell BC. 1991. Evidence for the establishment of aphid-eubacterium endosymbiosis in an ancestor of four aphid families. *Journal of Bacteriology* **173:** 6321–6324.

Munson MA, Baumann P, Kinsey MG. 1991. *Buchnera* gen. nov. and *Buchnera aphidicola* sp. nov., a taxon consisting of the mycetocyte-associated, primary endosymbionts of aphids. *International Journal of Systematic and Evolutionary Microbiology* **41:** 566–568.

Nakabachi A, Ishikawa H. 1999. Provision of riboflavin to the host aphid, *Acyrthosiphon pisum*, by endosymbiotic bacteria, *Buchnera*. *Journal of Insect Physiology* **45:** 1–6.

Nováková E, Hypša V, Klein J, Foottit RG, von Dohlen CD, Moran NA. 2013. Reconstructing the phylogeny of aphids (Hemiptera: Aphididae) using DNA of the obligate symbiont *Buchnera aphidicola*. *Molecular Phylogenetics and Evolution* **68:** 42–54.

Page RDM. 1994. Parallel phylogenies: reconstructing the history of host-parasite assemblages. *Cladistics* **10:** 155–173.

Peccoud J, Simon JC, McLaughlin HJ, Moran NA. 2009. Post-Pleistocene radiation of the pea aphid complex revealed by rapidly evolving endosymbionts. *Proceedings of the National Academy of Sciences of the United States of America* **106:** 16315–16320.

Pérez-Brocal V, Gil R, Ramos S, Lamelas A, Postigo M, Michelena JM, Silva FJ, Moya A, Latorre A. 2006. A small microbial genome: the end of a long symbiotic relationship? *Science* **314:** 312–313.

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* **61:** 539–542.

Schwarz G. 1978. Estimating the dimension of a model. *The Annals of Statistics* **6:** 461–464.

Shigenobu S, Watanabe H, Hattori M, Sakaki Y, Ishikawa H. 2000. Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. APS. *Nature* **407:** 81–86.

Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **30:** 1312–1313.

Stern DL. 1994. A phylogenetic analysis of soldier evolution in the aphid family Hormaphididae. *Proceedings of the Royal Society of London B: Biological Sciences* **256:** 203–209.

Stern DL. 1995. Phylogenetic evidence that aphids, rather than plants, determine gall morphology. *Proceedings of the Royal Society of London B: Biological Sciences* **260:** 85–89.

Stern DL. 1998. Phylogeny of the tribe Cerataphidini (Homoptera) and the evolution of the horned soldier aphids. *Evolution* **52:** 155–165.

Stern DL, Foster WA. 1996. The evolution of soldiers in aphids. *Biological Reviews* **71:** 27–79.

Takahashi R. 1925. *Aphididae of Formosa. part 4*. Report of the Department of Agriculture Government Research Institute Formosa **16:** 1–65.

Tamas I, Klasson L, Canbäck B, Näslund AK, Eriksson AS, Wernegreen JJ, Sandström JP, Moran NA, Andersson SG. 2002. 50 million years of genomic stasis in endosymbiotic bacteria. *Science* **296:** 2376–2379.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* **30:** 2725–2729.

Toju H, Tanabe AS, Notsu Y, Sota T, Fukatsu T. 2013. Diversification of endosymbiosis: replacements, co-speciation and promiscuity of bacteriocyte symbionts in weevils. *The ISME Journal* **7:** 1378–1390.

Unterman BM, Baumann P, McLean DL. 1989. Pea aphid symbiont relationships established by analysis of 16S rRNAs. *Journal of Bacteriology* **171:** 2970–2974.

Vogel KJ, Moran NA. 2013. Functional and evolutionary analysis of the genome of an obligate fungal symbiont. *Genome Biology and Evolution* **5:** 891–904.

Wilson ACC, Ashton PD, Calevro F, Charles H, Colella S, Febvay G, Jander G, Kushlan PF, Macdonald SJ, Schwartz JF, Thomas GH, Douglas AE. 2010. Genomic insight into the amino acid relations of the pea aphid, *Acyrthosiphon pisum*, with its symbiotic bacterium *Buchnera aphidicola*. *Insect Molecular Biology* **19** (Suppl 2): 249–258.

Wojciechowski W. 1992. *Studies on the systematic system of aphids (Homoptera, Aphidinea)*. Katowice: Uniwersytet Slaski.

Zhang GX, Chen XL, Qiao GX, Zhong TS, Li JH. 1999. *Fauna of agricultural and forestry aphids of northwest China (Insecta: Homoptera: Aphidinea)*. Beijing: China Environmental Science Press.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Voucher information and GenBank accession numbers for all aphid species used in this study.
Table S2. Primers used in this study.