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Phylogenetic congruence between *Mollitrichosiphum* (Aphididae: Greenideinae) and *Buchnera* indicates insect–bacteria parallel evolution

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Abstract. We wanted to test whether *Mollitrichosiphum*, an aphid genus with life cycles on subtropical woody host plants, and *Buchnera*, the primary endosymbiont of aphids, evolve in parallel. We used three aphid genes (mitochondrial *COI*, cytochrome oxidase subunit I and *Cytb*, cytochrome b; nuclear *EF1α*, translation elongation factor 1 alpha) and two *Buchnera* genes (*16S rDNA*; *gnd*, gluconate-6-phosphate dehydrogenase) to reconstruct phylogenies. The congruence between the phylogenetic trees of aphids and *Buchnera* was then measured. The results present phylogenetic evidence for the parallel evolution of *Mollitrichosiphum* and *Buchnera* at the intraspecific as well as the interspecific levels. Our results support the possibility of using endosymbiont genes to study host evolutionary history and biogeographical patterns. We also investigated the usability of the *Buchnera gnd* gene as a barcoding marker for aphid identification.

Introduction

Numerous insect species live in close association with obligate symbiotic bacteria, especially primary endosymbionts. They are metabolically complementary partners that provide each other with nutrition, which is thought to be essential to their survival (Baumann *et al*., 1997, 2005; Moran *et al*., 2008). Studies on the phylogenetic relationships of cospeciation parallelism interactions help us understand the evolutionary history of obligate symbiosis. Aphids are among the insects obligately associated with mutualistic intracellular symbionts (Baumann *et al*., 1997). In recent years, aphids and their primary endosymbiont, *Buchnera aphidicola,* have been widely investigated and serve as a paradigm for the other studies (Moran & Baumann, 1993; Moran *et al*., 1993; Baumann & Moran, 2000; Baumann *et al*., 1995, 1997; Moran & Telang, 1998).

Buchnera, which belongs to the γ-subdivision of the Proteobacteria, is generally believed to exist in all aphid species and located in specialized cells, bacteriocytes (Buchner, 1965). *Buchnera* provides its hosts with essential nutrients that are limiting factors for growth and reproduction (Buchner, 1965; Munson *et al*., 1991; Douglas & Prosser, 1992; Baumann *et al*., 1995, 1997; Nakabachi & Ishikawa, 1997; Shigenobu *et al*., 2000). This primary endosymbiont follows a vertical mode of transmission through infection of eggs or embryos (Harrison *et al*., 1989; Brough *et al*., 1990). Theoretically, this type of cytoplasmic inheritance does not permit horizontal transmission between endosymbionts and their hosts; thus, both organisms that are part of the symbiotic relationship should undergo strict parallel evolution and conform to phylogenetic congruence (Funk *et al*., 2000; Nikoh *et al*., 2010).

Some previous studies at the higher taxonomic levels supported the strict parallel evolution between aphids and *Buchnera* (Moran & Baumann, 1993). Other studies have not validated the parallel evolution (van Ham *et al*., 1997; Martinez-Torres *et al*., 2001; Pérez-Brocal *et al*., 2006). The most common horizontal transfer of symbionts can be easily detected between closely related host species (Chen *et al*., 1999; Wernegreen & Riley, 1999; Clark *et al*., 2000). At higher taxonomic levels, the horizontal transfer may be neglected

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in phylogenetic pattern analyses, which would result in an overestimation of evolutionary congruence. Thus, there are several complicating factors in the previously performed higher level analyses.

The most direct and informative evidence for the fine-scale fidelity of parallelism arises from phylogenetic investigations of closely related species and intraspecific lineages (Via, 1999; Clark *et al*., 2000; Funk *et al*., 2000; Hawthorne & Via, 2001; Wernegreen *et al*., 2001; Jousselin *et al.*, 2009; Peccoud *et al*., 2009a, b). Some work showed that ambiguities in phylogenetic reconstructions (e.g. different tree-building methods) may lead to slightly phylogenetic incongruence (Clark *et al*., 2000; Jousselin *et al.*, 2009). In addition, different *Buchnera* genes may also lead to some incongruence with the aphid phylogeny (Martinez-Torres *et al.*, 2001). Besides, there is a possibility that variations in the extent of fidelity of parallelism exist among aphid lineages with different life cycles. Of the aphid groups investigated in the previous studies, *Uroleucon* Mordvilko is monoecious and holocyclic on herbaceous plants (Moran *et al.*, 1999); *Brachycaudus* van der Goot is heteroecious or monoecious and holocyclic, host-alternating with members of Rosales (Amygdalaceae) as primary hosts, and various herbs mostly belonging to Compositae and Boraginaceae as secondary hosts (Blackman & Eastop, 2006); pea aphid (*Acyrthosiphon pisum* Harris) is a monoecious (nonhost-alternating) species that overwinters, usually in the egg stage, on various perennial herbaceous legumes (Lees, 1989). The most stringent test for a historical association by matching branching patterns from phylogenies between hosts and their endosymbionts is needed across more groups of aphids with different life cycles and geographical scales.

Mollitrichosiphum is an aphid genus of the tribe Greenideini (Hemiptera: Aphididae: Greenideinae) with 18 known species, and traditionally divided into two subgenera: *Mollitrichosiphum* and *Metatrichosiphon* (Remaudière & Remaudière, 1997). Most species are monocious and holocyclic on woody plants, such as Fagaceae, Betulaceae, Meliaceae, Proteaceae, Rosaceae, Myrtaceae, Sabiaceae, Elaeagnaceae and Simaroubaceae. They typically infest the new shoots and buds of their hosts, and heavily damage cash crops and ornamental plants (Zhang & Qiao, 2010). *Mollitrichosiphum* is mainly distributed in Southeast Asia. In China, species of the genus are mostly restricted to southern regions. The genus is a good model for testing the phylogenetic congruence between *Buchnera* and aphids with life cycles on subtropical woody host plants.

According to previous studies (Moran *et al.*, 1995, Moran, 1996; Clark *et al.*, 1999; Jousselin *et al.*, 2009), *Buchnera* has faster evolutionary rates than its cospeciating aphid hosts. More rapidly evolving genes have been used to infer the relationships among groups with more recent shared ancestry (Pleijel *et al.*, 2008). Thus, if the parallel evolution hypothesis can be demonstrated among closely related taxa, it would be advantageous to use *Buchnera* markers to replace or supplement aphid markers for intrapopulation, intraspecific and interspecific phylogenetic analyses, as well as to assess genetic variation in species of aphids as shown in Jousselin *et al*. (2009), Lozier *et al*. (2009) and Peccoud *et al*. (2009b). In addition, mitochondrial *COI* (cytochrome coxidase subunit I) has recently been widely used as an aphid barcoding marker (Sabater-Munoz *et al.*, 2005; Valenzuela *et al*., 2007; Coeur d'acier *et al*., 2008; Foottit *et al*., 2009; Wang & Qiao, 2009; Wang *et al*., 2011). Although most aphid species can be successfully identified by *COI* barcode, previous studies have also indicated that it is problematic for the identification of recently diverged aphid species (Foottit *et al*., 2008; Lee *et al*., 2011). To address this problem, those *Buchnera* genes with faster evolutionary rates may be good barcode candidates for aphid identification.

In the present paper, to test whether *Mollitrichosiphum* and *Buchnera* have parallel evolution, we used three aphid genes (*COI*, *Cytb*, *EF1α*) and two *Buchnera* genes (*16S rDNA*, *gnd*) to reconstruct phylogenies, and then measured the congruence between the phylogenetic trees of aphids and *Buchnera* as well as their divergence times. We also investigated the usability of *Buchnera* genes as barcoding markers for aphid identification.

Materials and methods

Sampling and data collection

There are 11 known species of *Mollitrichosiphum* in China, and 7 of them were sampled in the present study. The 34 samples of these species are distributed from the Himalayas to southern China. For the aphids, we chose three genera as outgroups, specifically *Greenidea* Schouteden (Greenideini), *Cervaphis* van der Goot (Cervaphidini), and *Kurisakia* Takahashi (Thelaxinae). To test for parallel evolution using the *Buchnera* sequences, we chose the same aphid species as outgroups (see Table S1). All samples were preserved in 95 or 100% ethanol. All samples and voucher specimens were deposited in the National Zoological Museum of China, Institute of Zoology, Chinese Academy of Sciences, Beijing, China. Collection information, host plants and voucher numbers of the samples are shown in Table S1.

Total DNA from each aphid sample was extracted using the standard phenol-chloroform method (Sambrook *et al.*, 1989). Sequences of *Buchnera 16S rDNA* were amplified with sense primer 16sF (5′–AGAGTTTGATCATGGCTCAGATTG–3′) and anti-sense primer 16sR (5′–TACCTTGTTACGACTTCAC CCCAG–3′) (van Ham *et al.*, 1997). Sequences of *Buchnera gnd* (gluconate-6-phosphate dehydrogenase) fragments were amplified with the primers 5′-CGCGGATCCGGWCCWWSW ATWATGCCWGGWGG-3′ (BamHI) and 5′-CGCGGGCCCG TATGWGCWCCAAAATAATCWCKTTGWGCTTG-3′ (ApaI) (Clark *et al*., 1999). Mitochondrial *COI* and *Cytb* sequences of the aphid hosts were obtained in a previous study (Zhang *et al.*, 2011). In order to eliminate the maternally inherited influence of mitochondria and *Buchnera* genomes, we used a nuclear marker, *EF1α*. PCR amplification of *EF1α* used primers EF3 (5′-GAACGTGAACGTGGTATCAC-3′) and EF2

(5′- ATGTGAGCAGTGTGGCAATCCAA-3′) (Palumbi, 1996; von Dohlen *et al*., 2002).

PCR amplification was performed in a 30 μL reaction mixture containing 20 μL dd H_2O, 0.4 U Taq DNA polymerase, 3 μL 10× buffer, 2.4 μL dNTPs and 0.6 μL of both 10 μM forward and reverse primers (TransGen Biotech). The PCR conditions were as follows: 95°C for 5 min; 35 cycles consisting of denaturation at 95°C for 1 min, annealing temperatures (65°C for *16S rDNA*, 55°C for *gnd* and 51°C for *EF1α*) for 0.5 min and extension at 72°C for 2 min; and a final extension period at 72°C for 7 min.

PCR products were purified and ligated into the vector pMD19-T (TakaRa), and at least three clones from each individual were sequenced. Sequencing was performed on an ABI 3730 automated sequencer (ABI, USA). Both strands of the plasmids were sequenced using universal primers (M13+, M13−) with forward and reverse reads. The GenBank accession numbers for sequences are given in Table S1.

Alignment and phylogenetic analyses

Sequences were assembled using SeqManII module of Lasergene v5.0 (DNASTAR, Madison, WI, USA). Multiple alignments were made with MEGA v5.05 (Tamura *et al*., 2011) using default parameters and verified manually. Gaps were not required in alignments. The substitution saturation for gene segments was checked using DAMBE v5.2.31 (Xia & Xie, 2001), by calculating the transition and transversion versus divergence plot. Before combining gene fragments, the partition-homogeneity test in PAUP* v4.0b10 (Swofford, 2002) was used to estimate congruence between the datasets.

Aphid and bacterial datasets were analysed using maximum parsimony (MP), maximum likelihood (ML) and Bayesian methods. Each nucleotide was treated as an unordered character with four alternative states, and the gaps in all analyses and the fragments that were not amplified in combined datasets were all considered as missing data. The software jModelTest v0.1.1 (Posada, 2008) was applied to search for the best-fit model and parameters of DNA substitution for each gene and combined datasets. The most appropriate selections for the subsequent analysis were conducted through Bayesian Akaike information criterion (*AIC*).

Bayesian analyses (Larget & Simon, 1999) were conducted using MrBayes3.0 (Ronquist & Huelsenbeck, 2003), and the best models were selected from jModelTest for host and bacterial individual or combined datasets. For each dataset, chains were run starting from a random tree and followed by 1 million Markov chain Monte Carlo generations. The trees were sampled every 100 generations and the first 25% were discarded as burn-in sets when the chains reached the stable state (the average standard deviation of split frequencies ≤ 0.01). The posterior probabilities were obtained after assigning the consensus tree. The progress was run twice to test the reliability of the results.

Maximum likelihood phylogenetic analyses performed in PAUP were under the heuristic search strategy with ten random-addition sequences per replicate and TBR branch swapping. A bootstrap analysis was performed using the same model with Bayesian analyses and 100 pseudoreplicates for both host and bacterial individual or combined datasets. To obtain more precise and stable results, we also used RAxML (Stamatakis *et al*., 2005) and PHYML (Guindon & Gascuel, 2003) to analyse the host and bacterial datasets with the ML method. One thousand replicates for bootstrap analyses were run in RAxML and PHYML, and a 50% majority rule consensus was calculated to determine the support values for each node.

Equal weighting was used initially for all changes in the MP analyses (Mitchell *et al*., 1997). Parsimony analyses implemented a heuristic search, using tree-bisection and reconnection (TBR) branch swapping, with 1000 random-addition sequences for each taxa. The support for the resulting nodes was subsequently assessed using bootstrapping (Felsenstein, 1985) with 1000 pseudoreplicates under the TBR branch swapping, the heuristic search strategy and 100 random-addition sequences per replicate.

Test of the parallel evolution

In order to test whether *Mollitrichosiphum* and *Buchnera* have undergone parallel evolution, we chose the most commonly used software called TreeMap v1.0 (Page, 1994), and the more recent method developed by Conow *et al*. (2010) implemented in Jane v3.0. TreeMap is the standard program used to measure the degree of congruence between host and endosymbiont trees by estimating the number of codivergences, duplications, sorting events and host switching. Because the aphid and the *Buchnera* outgroups used were associates, TreeMap analyses included outgroups that were not pruned from both host and endosymbiont trees in the figures. An exact search or heuristic search was used to find the best reconstructions that attempt to maximize the number of cospeciations and minimize the number of noncospeciation events. One thousand random replicates were run on each reconstruction to test whether the observed two phylogenies contain more cospeciation events than the random expectation. Jane used a heuristic approach with genetic algorithm to exam the number of evolutionary events. The event costs, which were respected to Charleston's cost scheme, used here were 0 for cospeciation, 1 for duplication, 1 for host switching and 2 for loss. (Charleston, 1998). The analyses were performed with 500 generations and population size of 100. Both randomizing the tip of the trees and randomizing the parasite tree topology were run in a configuring statistical test using the sample size of 1000.

In order to provide further evidence of cospeciation, we also tested whether divergence times between host and endosymbiont were congruent. By plotting coalescence times (measured in units of genetic change) for codivergent nodes from ultrametric trees (obtained with gnd/16S and *COI*/*Cytb*/*EF1α* combined datasets from ML analyses), the temporal congruence between *Mollitrichosiphum* and *Buchnera* were calculated

with TreeMap. Furthermore, as branch lengths are not independent, we used a randomization test with 1000 replicates to assess for significant correlation of coalescence time in ultrametic trees.

Test of the usability of Buchnera gene as barcode marker for aphids

Interspecific and intraspecific pairwise distances of *16S rDNA*, *gnd* and *COI* were estimated with the Kimura-2-parameter (K2P) genetic model using the program MEGA v5.05. To check the barcoding gap, the frequency distribution of the distances was determined. Mean intra- and interspecific divergences were also calculated.

The success rates of PCR amplification and sequencing of the *16S rDNA*, *gnd* and *COI* barcodes were evaluated. A single band of PCR product on an electrophoresis gel was considered a successful amplification.

Results

Sequence analyses

We obtained *gnd* sequences for *Buchnera* from all 34 aphid samples; 817 bp of aligned sites were used for the analyses, which excluded primer and unambiguous sites. The *gnd* fragments were rich in variable (34.27%) and parsimony-informative (31.46%) sites. The nucleotide composition of the *gnd* sequences was on average 76.0% A + T, much higher than that of the free-living Enterobacteriaceae (Clark *et al*., 1999). *16S rDNA* fragments were successfully sequenced in 26 samples, whereas the rest failed to amplify due to nonspecific amplification. The number of aligned sites was 1354 bp, of which 1121 were conserved, 102 (7.53%) were variable and 69 (5.10%) were parsimony informative. Mean proportions of T: C: A: G were 22.3: 20.9: 28.7: 28.0. The *16S rDNA* sequences were highly conserved. The average G + C portion was 48.9%, nearly identical to the results in previous studies (Moran, 1996; von Dohlen, 1998; Martinez-Torres *et al.*, 2001). The aphid dataset included a 633-bp alignment of *COI*, 663 bp of *Cytb* and 785 bp of *EF1α*. Additional sequence-related information for the two *Buchnera* genes, two aphid host mitochondrial genes and one nuclear gene are listed in Table S2. All sequences have been deposited in the GenBank (accession numbers shown in Table S1).

The interspecific genetic divergences of *16S rDNA* for *Buchnera* from individual aphid species ranged from 0 to 5.8%. For the intraspecific divergences, it was 0–1.4% for *Mollitrichosiphum tenuicorpus* samples, but 0–0.4% for the other species. The interspecific genetic divergences of *gnd* ranged from 1.0 to 27.3%. The intraspecific divergences ranged from 1.3 to 9.4% for *M. tenuicorpus* samples, which was much higher than among samples of other species (0–0.4%). The interspecific sequence divergences of *COI* for aphids were 0.3–14.2%; the intraspecific divergences were 0–6.4% for *M. tenuicorpus* samples, but 0–0.5% for the six other species. According to Zhang *et al*. (2011), three cryptic species may be included in *M. tenuicorpus* samples. This may cause the higher operational threshold of intraspecific divergences. So we include these three cryptic species of *M. tenuicorpus* as three different species in the following interspecific and intraspecific genetic distance relative distribution analyses (see Fig. 1). *16S rDNA* was dropped from further barcode analysis because it had the lowest interspecific variation. Both *gnd* and *COI* could form species-specific clusters under different methods (MP, ML and Baysian). Success rates for both the PCR amplification and sequencing of *COI* and *gnd* fragments were up to a 100%. In addition, the sequences of *gnd* were easier to obtain than *COI* during the experiment, which meets the requirement for a DNA barcode.

We tested substitution saturation of *gnd, COI, Cytb* and *EF1α* (introns removed) sequences via DAMBE (Xia & Xie, 2001) by estimating TN93 (Tamura & Nei, 1993) genetic distance. The obtained plot of the numbers of transitions and transversions showed linearity and suggested that no saturation patterns existed at any position, thus allowing for the determination of all nucleotide positions applied in the phylogenetic analysis. The result of the partition homogeneity test showed that the two datasets from the *gnd* and *16S rDNA* gene, *COI*, *Cytb* and *EF1α* sequences were not significantly incongruent and could therefore be combined.

Phylogenetic analyses

Maximum parsimony analysis of the *gnd/16S rDNA* combined dataset yielded four most-parsimonious trees, with tree length of 1171 (CI = 0.701, RI = 0.881). Similar procedures for the *gnd* gene yielded 12 MP trees (TL = 845, CI = 0.696, RI = 0.896), for *EF1α* 2 MP trees (TL = 318, CI = 0.692, RI = 0.818), for the *COI/Cytb* combined dataset 4 trees (TL = 666, CI = 0.658, RI = 0.877) and for the *COI/Cytb/EF1α* combined dataset 4 trees (TL = 915, CI = 0.677, RI = 0.871). For ML and Bayesian analyses, following the results of the jModelTest analysis, the TrN + I + G, GTR + I + G, GTR + G, TIM2 + I + G and GTR + I + G model was selected as the most suitable for the *gnd*, *EF1α*, *gnd/16S rDNA, COI/Cytb* and *COI/Cytb/EF1α* combined datasets, respectively.

For *Buchnera*, the phylogenetic relationships inferred from the *gnd* gene and *gnd/16S rDNA* combined dataset were almost identical, and the topology of each based on MP, ML and Bayesian analyses of the *gnd* gene and *gnd/16S rDNA* combined data were also highly congruent (Figs 2, 3). The only topological difference tended to occur at the nodes for sequences from *Buchnera-Mollitrichosiphum nigrofasciatum*, some of which had relatively low support in these analyses. The combined dataset yielded higher support topology due to more informative sites. However, for *16S rDNA*, a striking lack of phylogenetic structure at taxonomic levels higher than species was observed (comb topology); therefore, we kept this

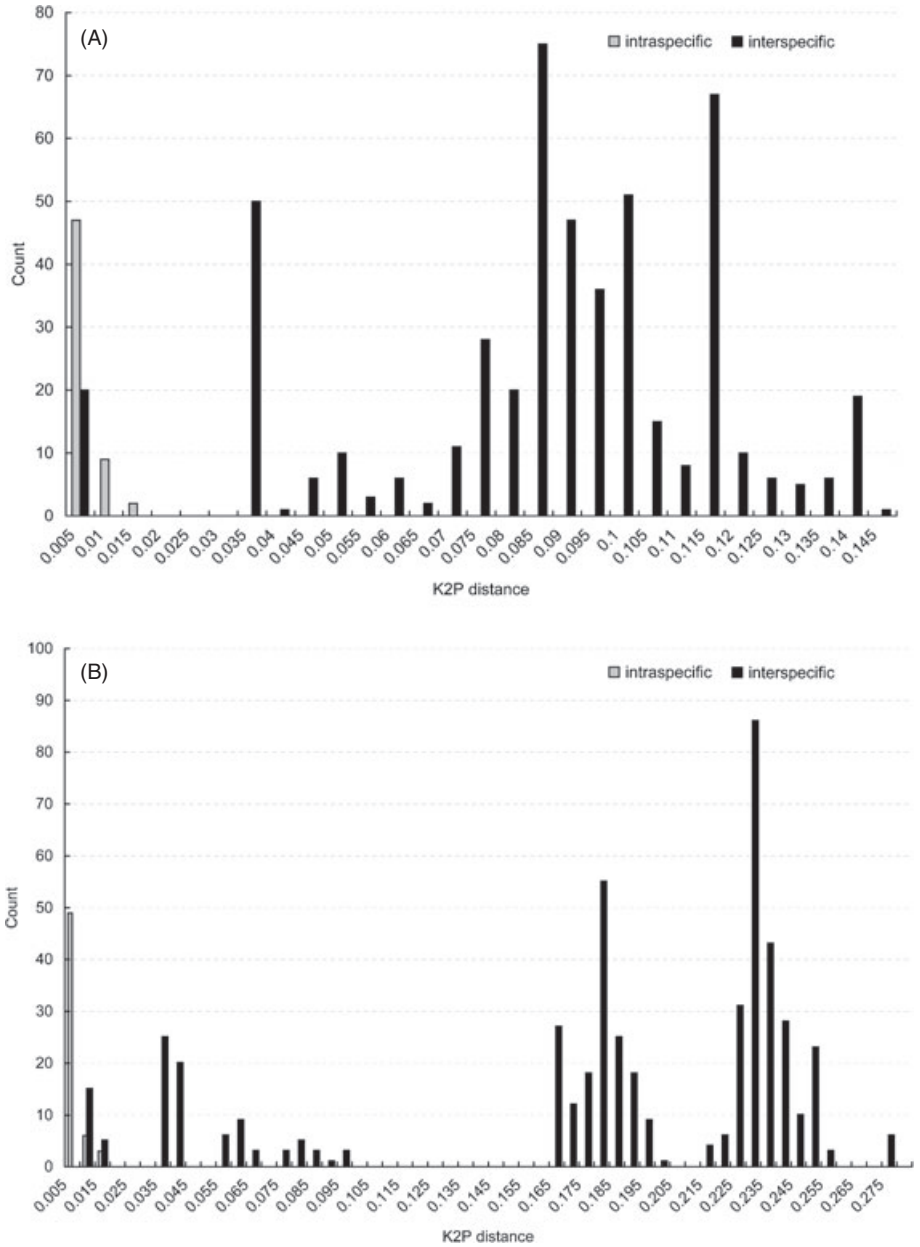

Fig. 1. Count-distribution of intra- and interspecific pairwise distances generated with MEGA and Excel. (A) For *gnd* analyses. (B) For *COI* analyses.

gene in analyses to increase phylogenetic information but did not separately discuss its phylogenetic inferences.

In the tree obtained using the *gnd* and *gnd*/*16S rDNA* combined dataset, all *Buchnera* ingroups constituted a monophylum. Relationships among the bacterial sequences reflected those inferred for their hosts (Hardy *et al.*, 2008; Gruwell *et al.*, 2010). We use the designation '*Buchnera*–aphid species name' for the *Buchnera* of corresponding aphid species in the following text, for example *Buchnera–M. tenuicorpus*.

In the *gnd* and combined dataset analyses, the obtained topologies clearly separated into two major clades: subgenera *Metatrichosiphon* and *Mollitrichosiphum*. *Buchnera* sequences from the aphid subgenus *Metatrichosiphon* were recovered as a clade including six aphid species. *Buchnera–M. nigrum* was always clustered with *Buchnera–M. luchuanum* and *Buchnera–M. rhuase*, supported by high K2P-values. A sister relationship between *Buchnera–M. nandii* and *Buchnera–M. montanum* was also strongly supported. The position of *Buchnera–M. nigrofasciatum* was not stable, which was first separated from the other representative five aphid species in the *gnd* analyses and clustered with *Buchnera–M. nandii* and *Buchnera–M. montanum* in the combined dataset analyses.

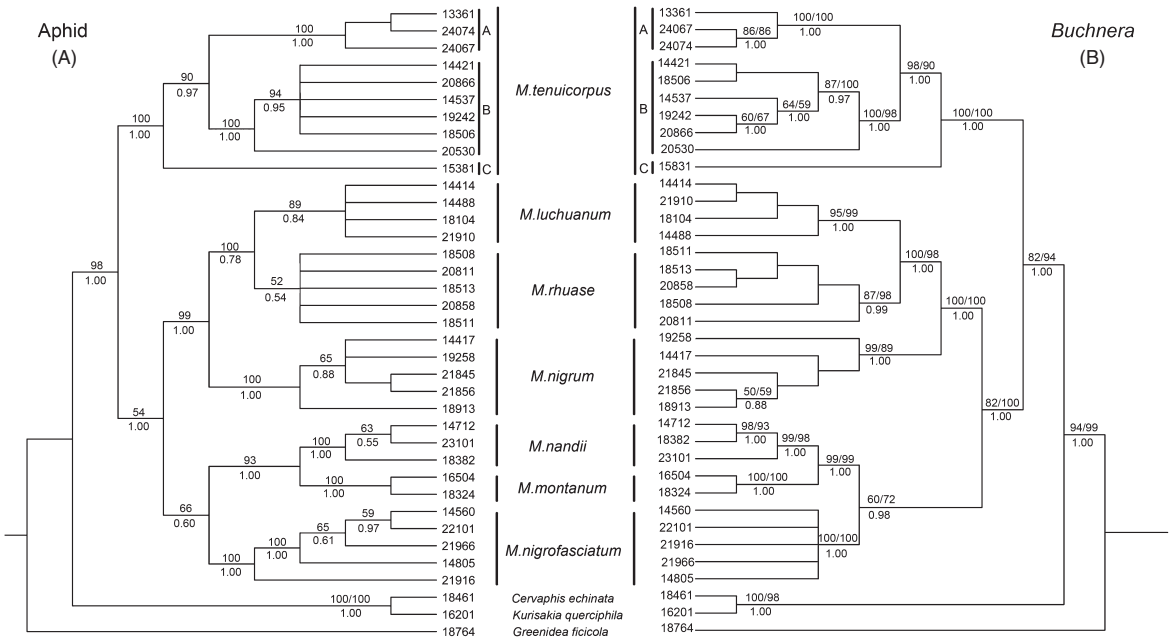

Fig. 2. Comparison of *Mollitrichosiphum* and *Buchnera* phylogenies. (A) Phylogenetic relationships between *Mollitrichosiphum* based on MP analysis of *COI* and *Cytb* datasets. Values above branches are MP bootstrap values, values below branches indicate posterior probablities of Bayesian inference. (B) MP phylogenetic trees inferred from the *Buchnera gnd/16S rDNA* combined dataset from the aphid species indicated. Values above branches are MP/ML bootstrap values; values below branches indicate posterior probablities of Bayesian inference. Bootstrap or bootstrap value below 50 is not indicated.

Buchnera sequences from the subgenus *Mollitrichosiphum* assembled as a sister branch to the representative subgenus *Metatrichosiphon* lineage. According to the geographical distributions of the aphid host, the clade of *Buchnera–M. tenuicorpus* was divided into three minor branches. The basal branch had one sample from Tibet, and the second branch contained three samples from Yunnan. A previous study indicated that *M. tenuicorpus* may consist of at least three cryptic species based on the phylogenetic patterns and genetic distances (Zhang *et al.*, 2011).

Significant congruence was observed among the aphid phylogenies derived from *EF1α*, *COI/Cytb* and *COI/Cytb/EF1α* combined datasets analyses using MP, ML and Bayesian methods. The aphid phylogenies also showed highly consistency with the tree created using the endosymbiont combined dataset. Our results were almost identical to those of Zhang *et al.* (2011), except for the position of *Buchnera–M. nigrofasciatum* (see Figs 2, 3).

Parallel evolution test

A tanglegram was built in TreeMap to reconcile the MP tree of the *Buchnera gnd/16S rDNA* combined dataset with the MP tree of the *COI/Cytb* combined host dataset (Fig. 4A, B). With the exact search, to minimize host-switching events, TreeMap introduced 20 cospeciation events, 12 duplications and 30 sorting events. With heuristic search, 21 cospeciation events, 10 duplications, 23 sorting events and 1 host switch were detected. The number of observed coevolution events was significantly greater than could be expected by chance ($P < 0.001$) when using a randomization test with 1000 randomly generated bacterial trees. The only host shift occurred from *Buchnera–M. nigrum* 21856 to *Buchnera–M. nigrum* 18913, which are the same aphid species at different collection location (*M. nigrum* 21856 collected from Guangdong; *M. nigrum* 18913 collected from Guangxi). Results obtained with Jane also show significant levels of cospeciation between *Mollitrichosiphum* and *Buchnera* ($P < 0.001$): 22 cospeciations, 10 duplications, 3 host switches and 32 losses were inferred.

When comparing the two ML ultrametric trees obtained from COI/Cytb/EF1α combined dataset and *Buchnera* and gene respectively, the linear relationship between the coalescence times for aphid and *Buchnera*, estimated from the maximum likelihood distance, showed strict cospeciation of the two lineages (Fig. 5). The branch length randomization test corroborated a strong correlation of aphid and *Buchnera* coalescence time ($r = 0.8992$), and thus we could not reject the hypothesis that strict cospeciation has occurred ($P = 0.001$).

Discussion

Phylogenetic congruence between Mollitrichosiphum and Buchnera

Our results based on different tests confirm the strict congruence between aphid and endosymbiont phylogenies. Genus

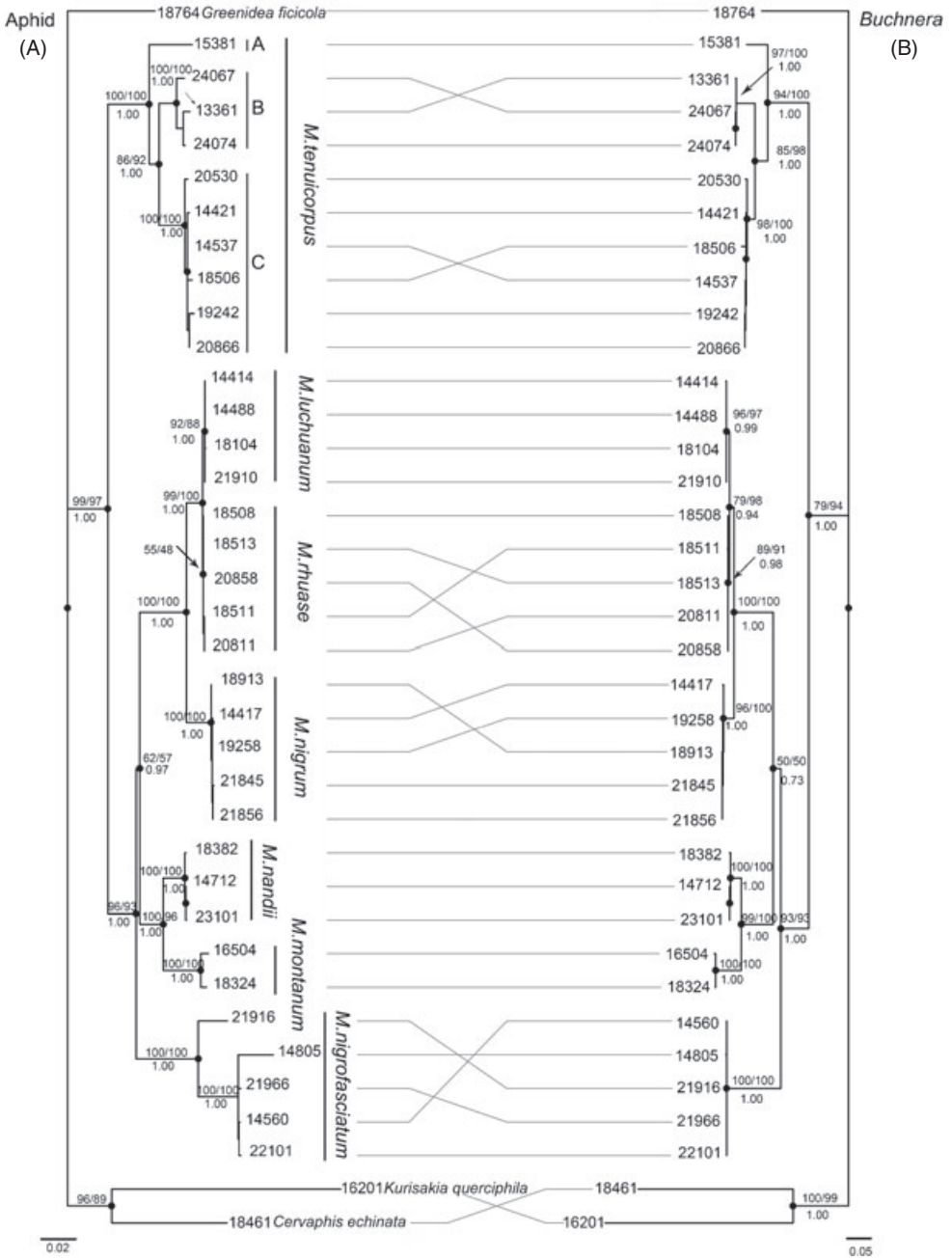

Fig. 3. Comparison of *Mollitrichosiphum* and *Buchnera* phylogenies. (A) Phylogenetic relationships between *Mollitrichosiphum* based on ML analysis of *COI, Cytb* and *EF1α* combined datasets. Values above branches are ML bootstrap. (B) ML phylogenetic trees inferred from *Buchnera gnd* sequences from the aphid species indicated. Values above branches are MP/ML bootstrap values; values below branches indicate posterior probablities of Bayesian inference. Bootstrap or bootstrap value below 50 is not indicated.

and species groups defined for the aphids are reflected in the bacterial phylogeny. The data based on the endosymbiont phylogeny implied the following results, which are consistent with previous studies: monophyly of genus *Mollitichosiphum*; subdivision of genus *Mollitrichosiphum* (two subgenera, *Mollitrichosiphum* and *Metatrichosiphon*); and three subclades of the clade *Mollitrichosiphum tenuicorpus* (Zhang *et al.*, 2011).

The only unstable lineage was *Mollitrichosiphum nigrofasciatum* (or *Buchnera–M. nigrofasciatum*). As shown here, *M. nigrofasciatum* (or *Buchnera–M. nigrofasciatum*) was placed as a sister group to *Mollitrichosiphum montanum* (or *Buchnera–M. montanum*) + *Mollitrichosiphum nandii* (or *Buchnera–M. nandii*) in both the host *COI/Cytb* and *COI/Cytb/EF1α* MP and Bayesian combined dataset analyses

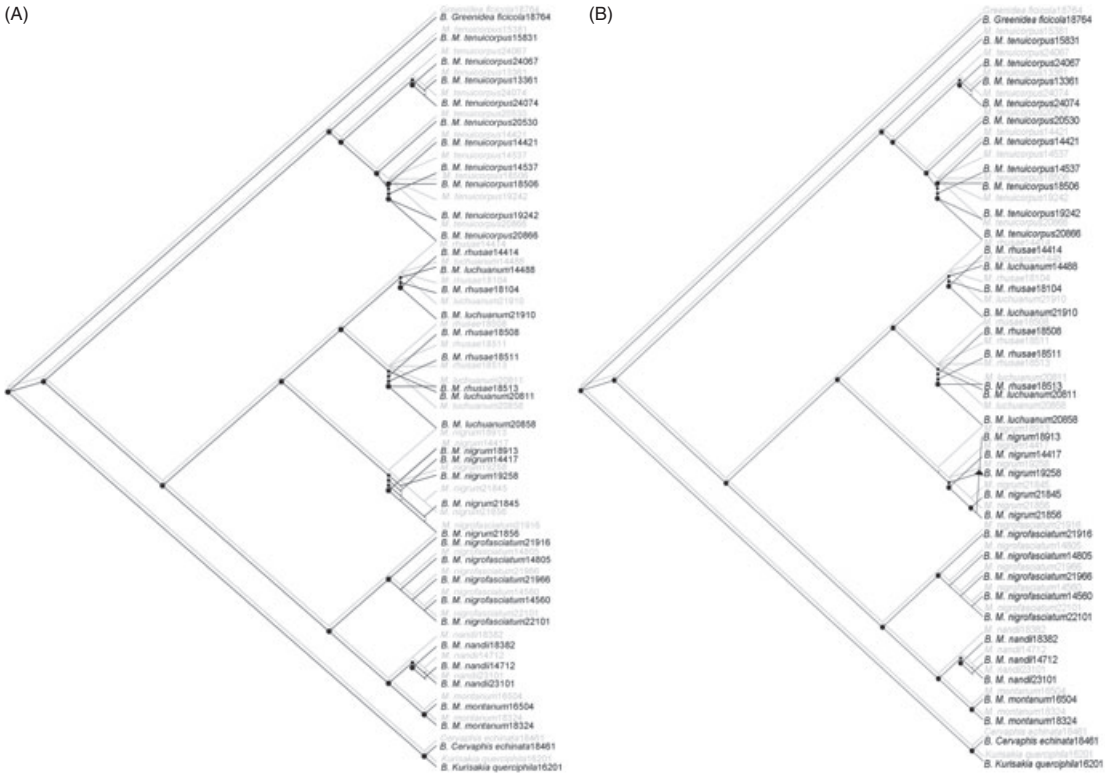

Fig. 4. Co-phylogenetic relationship between *Mollitrichosiphum* and *Buchnera* estimated using TreeMap. The reconciled trees were from MP aphid tree and the MP *Buchnera* tree. (A) The reconstruction using the exact search has the minimum number of inferred host switch. (B) The reconstruction using the heuristic search. Black and grey lines indicate the phylogenies of the *Buchnera* and aphid host, respectively. Circles represent co-divergence, square duplication, arrow switching and short truncated lines sorting events. Aphid names in grey and *Buchnera* names in black are followed by their respective voucher numbers.

and *Buchnera* MP, ML and Bayesian combined dataset analyses. However, *M. nigrofasciatum* (or *Buchnera–M. nigrofasciatum*) was the sister group to the *Metatrichosiphon* (or *Buchnera–Metatrichosiphon*) in the host *EF1α* ML, MP and Bayesian analyses, *COI/Cytb* and *COI/Cytb/EF1α* ML combined dataset analyses and the *Buchnera gnd* ML, MP and Bayesian analyses. Interestingly, the differences appeared in both the aphid and endosymbiont topologies and resulted in corresponding consistent topologies between the host and endosymbiont. This suggests that the data from *Buchnera* can produce similar results to the data from their aphid hosts. Although different methods of tree estimation (MP, ML and Bayesian) or different DNA markers containing diverse phylogenetic information may obscure the relationships (Clark *et al.*, 2000), the aphid dataset and *Buchnera* dataset in this study yielded topologically and temporally congruent phylogenies. This strongly indicates the parallel evolution between *Mollitrichosiphum* and *Buchnera*.

The possibility of using endosymbiont genes to study host evolutionary history and phylogeographical pattern

The association between aphids and *Buchnera*, which may have been involved in host adaptability, were one of the few potential examples of intimate metabolic symbiotic mutualism. This make aphid–*Buchnera* a potential example of a system that diversified by parallel evolution. The congruence in the phylogenetic trees of the endosymbionts and their hosts supports cospeciation of aphids and their primary endosymbionts following a single infection in the common ancestor of modern aphids due to stable vertical transmission of *Buchnera* from mothers to daughters (Houk & Griffiths, 1980; Moran & Baumann, 1993; Baumann *et al.*, 1997; Martinez-Torres *et al.*, 2001).

In previous studies, Funk *et al*. (2000) explored similar questions with *Uroleucon ambrosiae*. Using 20 multilocus haplotypes collected across U.S.A., they concluded there was evidence for strictly vertical transmission and cospeciation of symbiotic organisms at the intraspecific level. Similar results have also recently been found in European pea aphid (*Acyrthosiphon pisum*) complex and their primary symbiont *Buchnera*. Analyses of a *Buchnera* pseudogene for dating the radiation of the pea aphid complex were consistent with the previous results from the data of aphid nuclear genes (Peccoud *et al.*, 2009a, b). The topology and temporal congruence obtained from these studies may reflect the obligate nature of this intimate mutualism (Peccoud *et al.*, 2009a, b). In total, these studies have implied that there is parallel cladogenesis

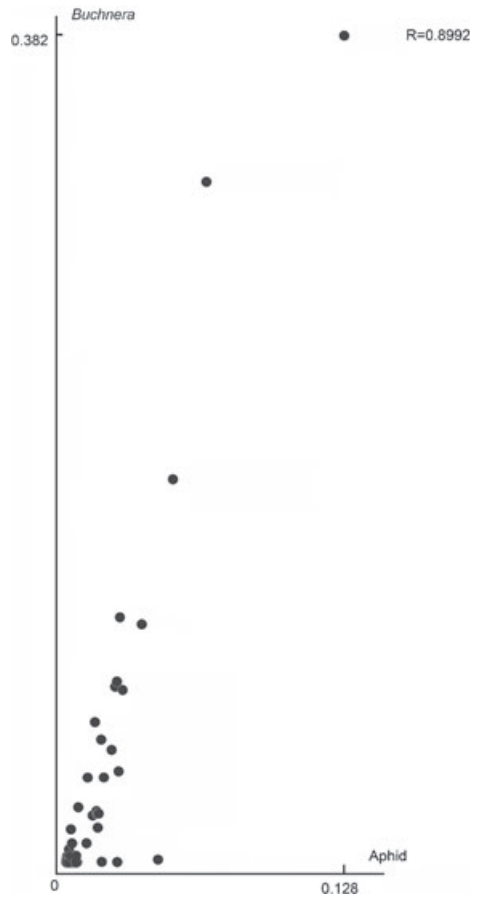

Fig. 5. Plots of coalescence times for the cospeciation events based on the depth of the nodes of ML phylogenetic trees.

between the hosts and the endosymbionts at lower taxonomic levels. In other words, horizontal transfer of endosymbionts has not been detected between the hosts. This indicates that it is possible to reconstruct the evolutionary history of the hosts based on the endosymbiont genes (Martinez-Torres *et al*., 2001; Maekawa *et al*., 2005; Jousselin *et al*., 2009).

Significant congruence was shown between the topology of the endosymbionts and their aphid host in the TreeMap and Jane analyses. The topologies of the trees are almost the same: seven *Buchnera* representative aphid species all appear as monophyletic clades and the branch of *Buchnera* associated with *M. tenuicorpus* are also divided into three differentiated subclades. This is also in agreement with aphid morphological characters, such as the antennal setae on the flagellum of the subgenus *Mollitrichosiphum*, which are similar lengths in all directions and have a nearly straight radial sector; in the subgenus *Metatrichosiphon* the setae are different in length and the radial sector is curved (Zhang & Qiao, 2010).

Dated about 100–250 Ma, *Buchnera* and the common ancestor of aphids began cospeciation; this means that they have radiated synchronously (Moran *et al*., 1993; Martinez-Torres *et al*., 2001). During the long-term mutualistic pattern, aphids and symbiotic bacteria have experienced a common evolutionary history. In addition, due to the consistent topology, the geographical distribution patterns of aphid hosts are also reflected in the phylogenetic trees of their endosymbionts. For example, *Buchnera–M. tenuicorpus* can be divided into three subclades: clade C, the first to split off, is in Tibet; clade A comprises a sample collected in Yunnan; and clade B is widely distributed in southern China (Hainan, Guangdong, Fujian, Yunnan). Phylogenetic patterns and genetic distance of the *gnd* gene indicate that the *Buchnera* representative host *M. tenuicorpus* may include three cryptic species. The uplift of the Tibetan Plateau, reorganization of major river catchments and the isolation of Hainan Island were most likely important factors contributing to the diversification of species in this genus according to a previous study (Zhang *et al*., 2011).

Evidence for the usability of Buchnera gnd gene for aphid barcoding

Because the phylogenetic relationships of aphid hosts are reflected in the *Buchnera* molecular data, it is possible to identify host species by using bacterial genes. Although based on a relatively small sequence set, our study suggests the potential usability of the *Buchnera gnd* gene as a marker candidate for aphid barcoding based on several assessable criteria. First, *gnd* sequences are easy to amplify and sequence with one pair of universal primers. The intracellular bacterium *Buchnera* is widespread in almost all aphids groups and exhibits a mutualistic association with its aphid host, allowing ready collection of *Buchnera* genes from aphids. Second, the genetic divergence estimated by the K2P-distance model showed that the *gnd* region possesses high interspecific divergence and is able to distinguish the selected aphid species. Finally, although the sample is not big enough, all of the seven species are accurately identified by the *gnd* gene. In addition, according to previous studies, synonymous sites in *Buchnera* genes evolve about twice as rapidly as those in the mitochondrial genes of their aphid host, which means that *Buchnera* genes are potentially informative in phylogenetic analyses (Clark *et al.*, 1999, 2000; Funk *et al.*, 2000).

Conclusion

The data presented in this study reveal significant phylogenetic congruence between *Buchnera* and their aphid hosts, which serves as strong evidence for parallel evolution and cospeciation. Our results provide strong additional evidence for the consistent vertical transmission of *Buchnera* through aphid maternal lineages and the absence of horizontal transfer at intraspecific and interspecific levels. The parallel phylogenetic relationships of *Mollitrichosiphum* and *Buchnera* suggest that *Buchnera* genes have the potential to be used to investigate aphid evolutionary history and biogeographical patterns. Although our sampling was limited, our study also indicates that the *Buchnera gnd* gene is just as good as *COI* as a barcoding marker for aphids.

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/j.1365-3113.2012.00647.x

Table S1. Information about the specimens used in this study.

Table S2. Summary of data on characteristics of the four genes analysed in this study.

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