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Fine-scale cospeciation between *Brachycaudus* and *Buchnera aphidicola*: bacterial genome helps define species and evolutionary relationships in aphids

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Aphids harbour an obligatory symbiont, *Buchnera aphidicola*, providing essential amino acids not supplied by their diet. These bacteria are transmitted vertically and phylogenic analyses suggest that they have 'cospeciated' with their hosts. We investigated this cospeciation phenomenon at a fine taxonomic level, within the aphid genus *Brachycaudus*. We used DNA-based methods of species delimitation in both organisms, to avoid biases in the definition of aphid and *Buchnera* species and to infer association patterns without the presumption of a specific interaction. Our results call into question certain 'taxonomic' species of *Brachycaudus* and suggest that *B. aphidicola* has diversified into independently evolving entities, each specific to a 'phylogenetic' *Brachycaudus* species. We also found that *Buchnera* and their hosts simultaneously diversified, in parallel. These results validate the use of *Buchnera* DNA data for inferring the evolutionary history of their host. The *Buchnera* genome evolves rapidly, making it the perfect tool for resolving ambiguities in aphid taxonomy. This study also highlights the usefulness of species delimitation methods in cospeciation studies involving species difficult to conceptualize—as is the case for bacteria—and in cases in which the taxonomy of the interacting organisms has not been determined independently and species definition depends on host association.

Keywords: aphids; endosymbiont; cospeciation; species delimitation; phylogeny

1. INTRODUCTION

Specialized interspecific interactions between two organisms maintained over evolutionary time periods may lead to simultaneous speciation of the lineages involved—a mode of diversification known as cospeciation (Hafner *et al.* 1994; Page 1994). This process may be frequent in host–symbiont interactions in which the symbiont is strictly vertically transmitted. In such cases, a speciation event in the host may result in the isolation and concomitant diversification of the symbiont. Cospeciation may be investigated by comparing the phylogenetic trees of each lineage. If cospeciation has occurred, the topologies of the phylogenetic trees should be congruent and the timing of speciation in both lineages, inferred from these trees, should be correlated (Page 1991). Such demonstrations of shared common history not only provide information about the evolution of the association and the biological processes governing it, but also validate the use of genetic information from symbionts to complement information from their hosts for the reconstruction of evolutionary history (Anderson *et al.* 2004; Nieberding *et al.* 2004; McCoy *et al.* 2005; Nieberding & Olivieri 2007).

Interspecific interactions potentially leading to cospeciation include associations between phytophagous insects and the bacterial symbionts living in their cells. The seminal paper by Buchner (1965) suggested that some of these bacteria provide essential nutrients to their hosts (Buchner 1965; Moran 2001; Moran *et al.* 2005*b*), which in turn supply a stable environment for their bacterial partners (Latorre *et al.* 2003). Buchner also suggested that such mutualistic associations were very common in phloem feeders, which require amino acid supplementation of their restricted sugar-based diet (Buchner 1965; Moran *et al.* 2005*b*). The interaction between aphids (Hemiptera: Aphididae) and a descendant of free-living γ-proteobacteria, *Buchnera aphidicola* (Moya *et al.* 2008), is the most widely studied example of such an interaction, probably due to the agronomic importance of aphids. Most of the 4400 species of aphids described to date (Remaudière & Remaudière 1997) harbour these symbiotic bacteria in specialized cells called bacteriocytes, located in the abdominal haemocoel of the aphid. *Buchnera* transmission seems to be exclusively vertical, with aphids obtaining the symbiont from their mother while still at the egg stage (Moran *et al.* 1995; Wilkinson *et al.* 2003). The relationship between *Buchnera* and their hosts is obligate, and *Buchnera* are therefore considered to be primary symbionts. Several physiological and genomic studies have investigated Buchner's thesis concerning the role of *B. aphidicola* in aphid nutrition. These studies have shown that *B. aphidicola* synthesizes certain vitamins, sterols and amino acids absent from the phloem sap consumed by its hosts (Douglas 1998; Shigenobu *et al.* 2000; Wilkinson *et al.* 2003; Moran *et al.* 2005*a*; Moran & Degnan 2006; Moya *et al.* 2008). Experimental studies of

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bacterium-free aphids have also shown that these bacteria are important for the growth and reproductive function of aphids (Baumann & Baumann 1994; Baumann 2005).

Phylogenetic reconstructions of aphid families have suggested that the association between aphids and *B. aphidicola* is approximately 150–200 million years old. *Buchnera* seems to have been acquired by aphids only once, and this symbiont seems to have cospeciated with its host ever since (Moran *et al.* 1995; Martinez-Torres *et al.* 2001). Only one study has investigated cospeciation at a fine taxonomic scale, within an aphid genus. Clark *et al.* (2000) reported significant cospeciation between the aphid genus *Uroleucon* Mordvilko 1914 and its primary symbiont. However, their study was limited to only 14 species of the 100 described *Uroleucon* species. It is widely accepted that the mode of transmission of *Buchnera* leads to significant cospeciation between these bacteria and their hosts, and cospeciation between vertically transmitted bacteriocyte-associated symbionts has been demonstrated in other systems (Bauman & Bauman 2005; Downie & Gullan 2005; Hosokawa *et al.* 2006; Gruwell *et al.* 2007). Nonetheless, we cannot entirely exclude the possibility of occasional horizontal transmission between closely related aphids on the same host plant. Such transmission might occur through hybridization or parasitoid stings (Clark *et al.* 2000). The loss of the primary symbiont is also possible (Perez-Brocal *et al.* 2006) and experimental studies have shown that secondary symbionts can compensate for the loss of *Buchnera* (Koga *et al.* 2003).

The phylogenies of both groups must be reconstructed reliably to determine the extent of shared evolutionary history between the two organisms, and this requires accurate species delineation. However, species delineation is ambiguous in several groups of aphids, with species not always corresponding to phylogenetically independent lineages (Coeur d'acier *et al.* 2007). It is difficult to determine whether the ambiguities are due to taxonomic uncertainties and/or a lack of resolution of DNA markers. Species definition is even more problematic in bacteria as assessments of gene flow are not relevant in these organisms (Cohan 2002). In bacteria, species are defined on the basis of phenotypic and genotypic clusters of strains and/or arbitrary levels of genetic differentiation (Lerat *et al.* 2003; Achtman & Wagner 2008). Previous studies addressing the question of cospeciation between aphids and their obligatory bacteria delimited strains of *B. aphidicola* as a function of host association (Moran *et al.* 1995; Clark *et al.* 2000; Martinez-Torres *et al.* 2001), comparing phylogenies of aphid specimens with phylogenies of associated *Buchnera* strains. In these studies, each aphid species and, therefore, each strain of *Buchnera* was represented by a single specimen. This approach implies total confidence in the validity of aphid species definition and *Buchnera* host specificity. The use of species delimitation methods independently in aphids and their obligatory symbionts therefore seems to be an essential step for inferring patterns of association between aphid species and *Buchnera* strains and for the rigorous assessment of cospeciation between the two organisms.

This study builds on previous studies of the genus *Brachycaudus* Van der Goot 1913 (Coeur d'acier *et al.* 2008), and aims to reconstruct the phylogeny of *Buchnera* associated with *Brachycaudus* species, as a means of investigating cospeciation in this system. The genus *Brachycaudus* contains 50 valid species (Remaudière & Remaudière 1997; Andreev 2004). Some aphid species alternate between *Prunus* (Rosaceae) host species and secondary hosts, mostly from the Caryophyllaceae, Polygonaceae, Scrophulariaceae and Compositae. Other species are monoecious, living either on Rosales or on herbaceous plants of families acting as the secondary hosts of heteroecious species (Blackman & Eastop 2006). Several closely related *Brachycaudus* species share a host plant species (*Prunus* sp.) for part of their life cycle and have overlapping geographical distributions, maximizing the chances of horizontal transfer of *Buchnera* if this is possible. In a previous study (Coeur d'acier *et al.* 2008), we questioned the validity of some species of *Brachycaudus*. We therefore used sequence information to establish species boundaries for the aphids in this study. Furthermore, for inferences relating to the patterns of *Buchnera–Brachycaudus* associations, we used the same method to assess 'strain' delimitation in *B. aphidicola*.

We addressed two key questions: (i) does each aphid species have a specific bacterial strain and (ii) is there any evidence for fine-scale cospeciation between aphids and their obligatory symbionts? In addition to demonstrating host specificity and cospeciation at a fine taxonomic level, positive responses to both these questions would validate the use of the *Buchnera* genome as a source of information for resolving evolutionary relationships in aphids.

2. MATERIAL AND METHODS

(a) *Sampling and DNA amplification*

We sampled 56 specimens of *Brachycaudus*, representing 27 species (table S1 in the electronic supplementary material). For oligophagous and polyphagous species, we sampled aphid specimens associated with different host plant species. We also tried to take into account geographical variation in bacterial strains by sampling over a large geographical area.

Three DNA markers were used to reconstruct the phylogenetic relationships of *B. aphidicola* associated with *Brachycaudus* species. We used the tryptophan biosynthetase (*TrpB*) gene used in previous phylogenetic studies of *Buchnera* (Clark *et al.* 2000). Using the genome sequences of *B. aphidicola* available from GenBank, we defined degenerate primers binding to the flanking regions of this gene and directly amplified *TrpB* from total DNA extracted from aphids. We also targeted two neutrally evolving regions, the intergenic region between the *hupA* (DNA-binding protein hup-alpha) and *rpoc* (DNA-directed RNA polymerase beta subunit) genes (Gomez-Valero *et al.* 2007) and the intergenic region between the *ssb* (single-strand binding protein) and *dnaB* (replicative DNA helicase) genes, using primers defined by J. Carletto (2006, personal communication; primer sequences provided in table S2 of the electronic supplementary material).

The DNA extractions and aphid specimens used for *B. aphidicola* DNA amplification were those described by Coeur d'acier *et al.* (2008). PCR conditions are given in the electronic supplementary material.

(b) *Phylogenetic reconstructions*

The phylogeny of *Brachycaudus* was based directly from the results obtained by Coeur d'acier *et al.* (2008); we used a maximum-likelihood (ML) analysis of the combined DNA dataset (CytB, COI and ITS2; figure 1).

We used several tree-building methods to reconstruct the phylogeny of *Buchnera*: ML; maximum parsimony (MP); and Bayesian inference (BI). Details of the analyses are given in the electronic supplementary material 1.

(c) *Species delimitation*

For *Brachycaudus* species, we first followed taxonomic divisions and randomly picked one specimen per described species to represent each *Brachycaudus* species and derived a species tree from the ML *Brachycaudus* specimen tree by pruning branches in TreeEdit (http://evolve.zoo.ox.ac.uk; Rambaut & Charleston 2001). We then applied DNA-based species delimitation methods. For the *Buchnera* species tree, as no prior information concerning strain definition was applicable, we used only DNA-based species delimitation methods.

Many methods for the use of DNA data to delimit species have recently been proposed (Wiens & Penkrot 2002; Sites & Marshall 2003; Monaghan *et al.* 2005; Pons *et al.* 2006; Vogler & Monaghan 2007). We first applied a simple clustering method (following Dettman *et al.* 2003; Le Gac *et al.* 2007; Marussich & Machado 2007). We only considered as 'phylogenetic species' monophyletic groups of specimens: (i) present in all single-locus phylogenetic reconstructions, and (ii) for which clustering was strongly supported by all phylogenetic reconstructions (ML and MP bootstrap support > 70, posterior probabilities (pp) > 90). The clades identified had to be well differentiated from each other, to prevent minor tip clades from being recognized as distinct species. For *Brachycaudus*, we applied a threshold of at least two per cent difference in the COI sequence between individual specimens of a given clade and those of the nearest branch. For *Buchnera,* we applied a threshold of 4 per cent difference in the *TrpB* sequence between individual specimens of a given clade and those of the nearest branch (a 2% difference in the COI sequence is the smallest difference generally found between unambiguously identified aphid species, whereas a 4% difference in the *TrpB* sequence is the smallest difference generally found between *Buchnera* strains from unambiguously identified aphid species). Reciprocally, we arbitrarily set a maximum divergence between specimens included in the same phylogenetic species, to prevent the lumping together of too many specimens within the same species (less than 1% difference in the COI data between a given specimen of a species in the *Brachycaudus* dataset, less than 1% in the *TrpB* data between a given specimen of a species for the *Buchnera* dataset). We then randomly selected one specimen from each phylogenetic species to represent that species in cophylogenetic analyses. The ML trees used to establish the topologies were simply derived by pruning specimens from the global ML trees with TreeEdit.

We also used the species delimitation approach described by Pons *et al.* (2006). This method statistically differentiates the shift in the branching patterns of a phylogenetic tree from interspecific long branches to intraspecific short polytomous branches using likelihood approaches, and identifies clusters of specimens corresponding to species. It first checks that the specimens do not belong to a single population following a coalescent process. It then selects an optimal threshold under this assumption, such that nodes before this threshold are considered as species diversification events, whereas branches beyond the threshold define clusters following a coalescent process. This method required the reconstruction of ultrametric trees for both *Brachycaudus* and *Buchnera*. Given the lack of a molecular clock (assessed for each gene using a likelihood ratio test (LRT)), we used the relaxed Bayesian method for multilocus data to obtain ultrametric trees (Thorne & Kishino 2002). We followed the step-by-step manual of Rutschmann (2005; see the electronic supplementary material for details). Some aphid fossils have been described (Heie 1967), but none of these fossils is recent enough for calibrating the *Brachycaudus* phylogeny. Evidently, no fossils exist for *Buchnera*. As our aim was simply to obtain ultrametric trees, we arbitrarily assigned prior ages of 1.0 (s.d. = 1) to both lineages (see Hughes *et al.* (2007) for a similar approach). We then tested the fit of a general mixed Yule coalescent (GMYC) model (assuming two branching patterns) against a null model (assuming a single branching process for the tree), using the R code provided by T. G. Barraclough. When the GMYC model was preferred over the null model, groups of sequences identified by the shift in branching pattern were considered to belong to different species. Species trees were derived from global phylogenies by choosing a single representative specimen for each putative species with TreeEdit.

(d) *Cospeciation tests*

We used the topology-based method known as reconciliation analysis (Page 1994) as implemented in TreeMap, the distance-based method ParaFit (Legendre *et al.* 2002) and LRT as suggested by Huelsenbeck & Bull (1996). Details of the analyses are given in electronic supplementary material 1.

(e) *Timing of speciation events*

The timing of speciation events can be compared by plotting coalescence time at cospeciating nodes from ultrametric trees (Page 1991). If *Buchnera* and aphids have cospeciated, the depth of cospeciation nodes in the two lineages should be correlated and the intercept of the regression line should not differ significantly from 0, indicating synchronous speciation in hosts and symbionts (Page 1996). The correlations between divergences occurring in the *Brachycaudus* and *Buchnera* lineages were calculated with TreeMap v. 1, using species phylogenies derived from ultrametric trees obtained with Multidivtime and the species delimitation method described by Pons *et al.* (2006). As branch lengths in ultrametric trees are not independent, we tested the significance of the correlations using the randomization test implemented in TreeMap (with 10 000 randomizations).

3. RESULTS

(a) Buchnera aphidicola *sequence analyses and phylogenetic reconstructions*

TrpB sequences were 547 bp long and were unambiguously aligned. The sequences were checked using Mega3 (Kumar *et al.* 2004) and no frameshift or nonsense codons were identified. *HupA–rpoc* sequence lengths ranged from 800 to 950 bp. Their alignment was problematic due to a highly divergent segment in the sequences. We excluded this zone from further analyses (250 bp). A 728 bp alignment of *ssb–dna* sequences was generated. Again, there was an ambiguously aligned segment of approximately 200 bp that we chose to exclude from the analysis. An alignment of 1808 bp of concatenated bacterial DNA fragments was generated, excluding ambiguously aligned parts. The sequences obtained have been deposited in the GenBank database under accession numbers EU998747–EU998911. Alignments are available in the electronic supplementary

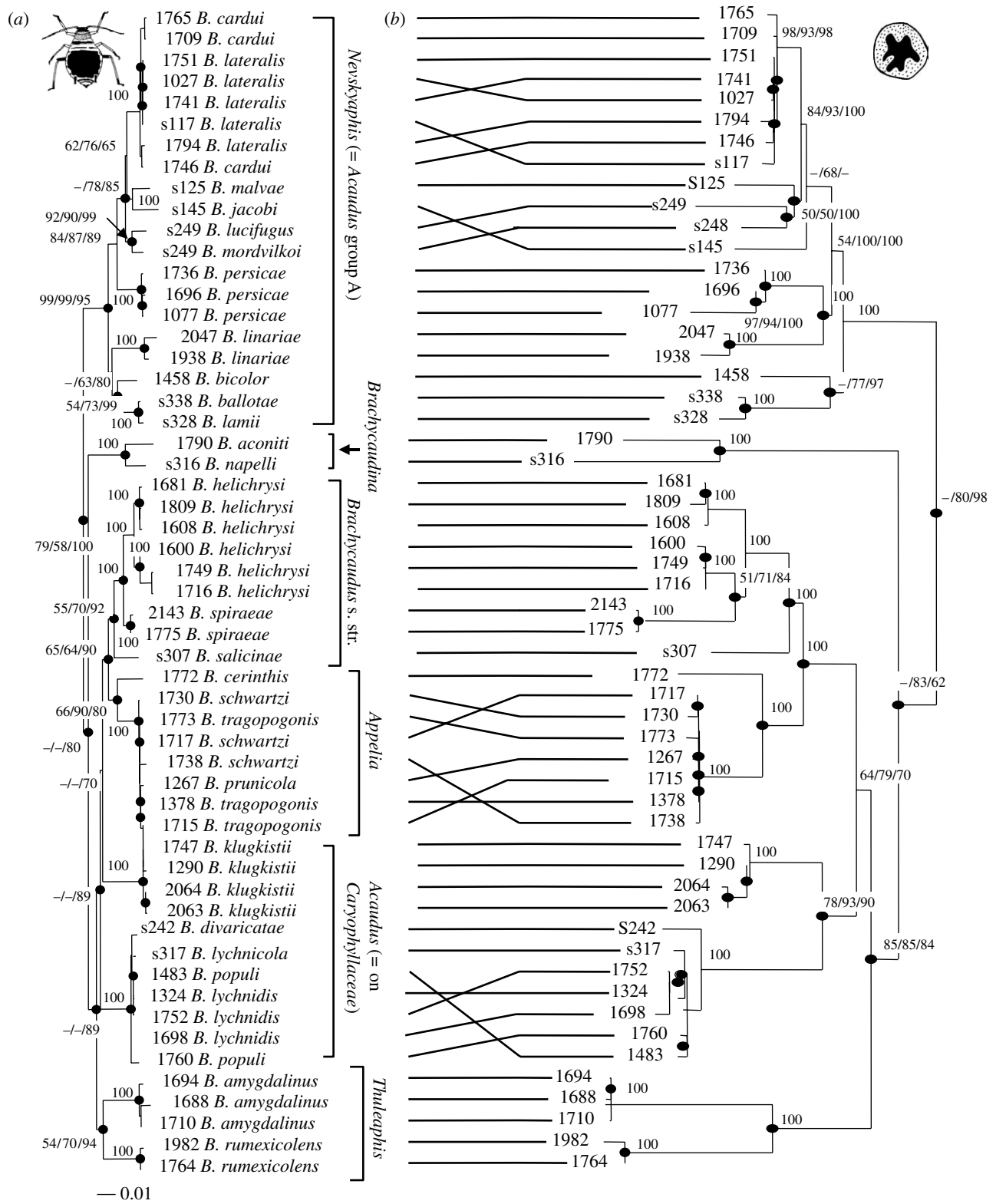

Figure 1. Comparison of *Brachycaudus* and *Buchnera* phylogenies. The phylogenetic reconstruction of (*a*) *Brachycaudus* is adapted from Coeur d'acier *et al.* (2008); it is based on the analysis of the combined dataset (CytB, COI and ITS2), and the ML topology and branch lengths are shown. The phylogenetic reconstruction of (*b*) *Buchnera* associated with *Brachycaudus* was obtained from the combined DNA dataset analysis, and the ML topology and branch lengths are shown. For both trees, values above branches are ML/MP bootstrap values for 50 per cent majority rule consensus/Bayesian pp, respectively. Bootstrap or pp values below 50 are not indicated. If only one value is given, then this value is the same for all measures of support. Aphid host species names are given for each specimen and aphids host subgenera (following Coeur d'acier *et al.* 2008) are indicated on the right-hand side of the aphids' phylogeny. Outgroups are not indicated.

material 2 and have been submitted to TreeBase. No significant differences in base composition were found between taxa for the three DNA fragments used (χ^2-test, $p=0.99$ for all fragments).

For *Buchnera*, the phylogenetic relationships inferred from the three genes were congruent, with the same clades strongly supported in each case (figures not shown). This confirmed the results of the incongruence

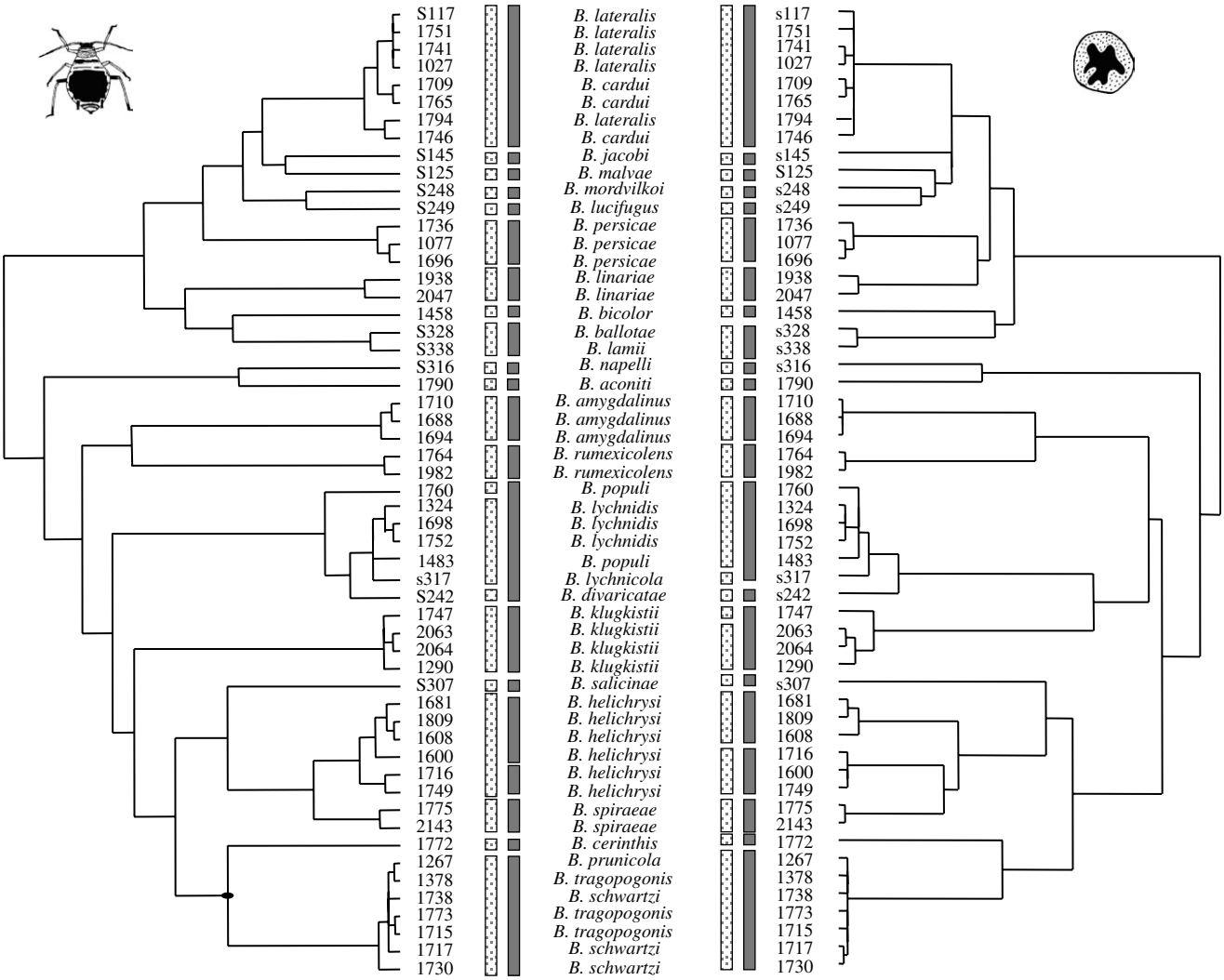

Figure 2. Aphids and *Buchnera* ultrametric trees obtained with MULTIDIVTIME and clusters of specimens recognized as different species by our phylogenetic method (grey bars) and the method of Pons *et al.* (2006) (white bars) in *Brachycaudus* and associated *Buchnera* specimens.

length difference test, which did not reject the null hypothesis of sequence data homogeneity ($p > 0.05$).

MP heuristic searches conducted on the combined dataset resulted in 960 MP trees of 2224 steps, based on 634 parsimony informative characters (CI = 0.651, RI = 0.822). We obtained one ML tree (Ln = −13224.113), which was globally congruent with the 50 majority rule MP trees. For BI, for each partitioning strategy, the independent runs converged on similar likelihood scores and reached stability at approximately $2–3 \times 10^4$ generations. The most complex strategy partitioning strategy was optimal (see table S3 in the electronic supplementary material).

We present the ML tree with pp and bootstrap supports for equivalent nodes in BI and MP trees. This bacterial phylogeny retrieved the major clusters found in the host *Brachycaudus* (Coeur d'acier *et al.* 2008; figure 1).

(b) *Species delimitation and* Buchnera–Brachycaudus *association patterns*

Our initial sample included 27 *Brachycaudus* species according to classical taxonomy. Our clustering method based on phylogenetic trees and genetic distances detected 21 species of *Brachycaudus* (figure 2). For the *Brachycaudus* ultrametric tree obtained with MULTIDIVTIME, the GMYC model was preferred over the null model of uniform branching patterns ($2\Delta L = 25.04$, χ^2-test, $p \ll 0.0001$) and the shift in the branching patterns retrieved 22 species of *Brachycaudus*. These groups of specimens matched closely with those identified with our simple clustering method (figure 2). We observed several mismatches between classical taxonomic species and these phylogenetic species. Several closely related *Brachycaudus* species were never assigned to distinct clusters. This was the case for three species in the subgenus *Appelia*—*Brachycaudus schwartzi* (Börner 1931), *Brachycaudus prunicola* (Kaltenbach 1843) and *Brachycaudus tragopogonis* (Kaltenbach 1843)—which were lumped together by both species delimitation methods. *Brachycaudus lateralis* (Walker 1848) and *Brachycaudus cardui* (Linnaeus 1758) were also consistently lumped together, as were *Brachycaudus ballotae* (Passerini 1860) and *Brachycaudus lamii* (Koch 1854). Four *Acaudus* species associated with Caryophyllaceae—*Brachycaudus lychnidis* (Linnaeus 1758), *Brachycaudus divaricatae* (Shaposhnikov 1956), *Brachycaudus populi* (del Guercio 1911) and *Brachycaudus lychnicola* (Hille Ris Lambers 1966)—were also identified as a single species with our clustering method, whereas the method of Pons *et al.* (2006) retrieved three species in this group—*B. divaricatae*, *B. lychnicola* and *B. lychnidis*—plus one of the *B. populi* specimens. *Brachycaudus helichrysi*

Table 1. Ln likelihoods of aphids and *Buchnera* trees with alternative datasets. (Significance levels were determined in the SH test. *p*-values indicate the probability that the score of the ML tree for a given dataset is significantly higher than that for the alternative topologies. $^{*}0.01<p<0.05$, $^{**}p<0.01$. Only the 46 specimens for which all DNA fragments for the host and bacterial symbionts were obtained were used in these tests.)

	tree		
dataset	*Buchnera*	*Brachycaudus*	*p*
Buchnera	−12670.03	−12855.05	0.0001^{**}
Brachycaudus	−7323.625	−7188.10	0.0001^{**}

(Kaltenbach 1843) specimens seemed to fall into two groups that clustered as a single species with the method of Pons *et al.* (2006), whereas our clustering method identified two sister species.

The clustering method based on phylogeny and genetic distances retrieved 21 species of *Buchnera* (figure 2). For the *Buchnera* ultrametric tree obtained with MULTIDIVTIME, the GMYC model was preferred over the null model of uniform branching patterns ($2\Delta L=28.14$, χ^2-test, $p\ll 0.0001$). The method of Pons *et al.* (2006) retrieved 24 species of *Buchnera* and these clusters matched closely with those identified with our simple clustering method. Furthermore, when we compared the clusters found in *Buchnera* and their hosts, we observed grouping of the same specimen/species together in all but a few cases (figure 2).

These results have implications for the pattern of association of aphids and *Buchnera*. The comparison of aphid taxonomy and sequence-based species delimitation in *Buchnera* suggests that the same strain of *Buchnera* is found in several closely related *Brachycaudus* species (figure 2). However, when a phylogenetic method of species delimitation is applied to both partners, an almost perfect one-to-one association pattern is observed (figure 2).

(c) *Cospeciation tests*

(i) Brachycaudus *specimens versus associated* Buchnera

Reconciliation analyses suggested almost perfect 'cospeciation' between aphid specimens and their associated *B. aphidicola* strains (figure 1). TREEMAP v. 1 suggested that 34 cospeciation events had occurred—a number significantly greater than would be expected by chance ($p<0.001$). We could not use TREEMAP v. 2.02b for this analysis due to computational complexity. The distance-based method, PARAFIT, also suggested significant cospeciation ($p<0.001$). The only association suggestive of a host shift was that of *Buchnera* with *Brachycaudus spiraeae* (Börner 1932) and *B. helichrysi* (figure 1).

The Shimodeira-Hasegawa (SH) tests indicated significant disagreement between the topology obtained with the combined analyses of *Buchnera* genes and the topology obtained with ML analyses of the *Brachycaudus* dataset (table 1).

The SH tests suggested significant disagreement between pairs of *Buchnera* loci. The aphid combined DNA dataset generally showed less disagreement within each individual *Buchnera* loci than between *Buchnera* loci (table 2). Accordingly, the *Brachycaudus* combined dataset topology had the highest summed likelihood across datasets (table 2). The LRT suggested that there was significant conflict between *Buchnera* loci and the combined *Brachycaudus* dataset. The summed likelihoods under the hypothesis that each *Buchnera* locus and the combined aphid dataset had a different topology (L_1) were significantly higher than the summed likelihoods under the hypothesis that all datasets have the same topology (i.e. *Brachycaudus* ML tree; table 2), as shown by the δ value higher (235.97) than any of the simulated values (δ values in the null distribution ranged from 16.05 to 203.40). In addition, the exclusion of individual loci from the calculation of δ showed that the *Brachycaudus* combined dataset contributed less to phylogenetic conflict than *Buchnera* genes. Thus, *Buchnera* loci are not more consistent with each other than with *Brachycaudus* combined data.

(ii) Brachycaudus *species trees versus* Buchnera *species trees*

Cospeciation was significant for the different pairs of species trees with all methods ($p<0.001$; see table S4 in the electronic supplementary material). However, a comparison of the *Brachycaudus* 'taxonomic species' tree with the *Buchnera* phylogenetic species tree suggested that either bacterial strains often fail to speciate with their host or that host shifts between closely related aphid species and lineage extinction occur regularly (figure 3*a*). These mismatches are probably due to the erroneous delimitation of some aphid species. When species were delimited by sequence-based methods, an almost perfect pattern of cospeciation was observed (figure 3*b*). The only strongly supported exception was the association of *B. helichrysi* and *B. spiraeae* with their *Buchnera* strains.

(d) *Timing of speciation events*

Comparisons of the ultrametric species trees obtained with the method developed by Pons *et al.* (2006) suggested that there was a strong correlation between the divergences observed in the aphid and *Buchnera* lineages, as indicated by the *R* value (figure 4) obtained in the branch length randomization test, which was significant. For both least-squares linear regressions, the *y*-intercept was not significantly different from 0 ($p<0.01$), consistent with synchronous speciation in the two clades.

4. DISCUSSION

(a) *Fine-scale cospeciation*

Using phylogenetic species delimitation methods independently in the aphid genus *Brachycaudus* and its obligatory symbiont *B. aphidicola*, we showed that: (i) *Buchnera aphidicola* has diversified into independently evolving and distinct lineages that could arguably be considered equivalent to species, (ii) several *Brachycaudus* species do not appear to be valid according to these methods, and (iii) each phylogenetic species of aphids identified by these methods hosts a distinct bacterial strain and, reciprocally, each strain of bacteria appears to be specific to a species of *Brachycaudus*. We also demonstrated significant cospeciation between these aphid species and their bacteria. These findings were confirmed by topology and distance-based cospeciation methods and tests for the temporal congruence of speciation events in the two lineages. The only method that rejected cospeciation was the LRT conducted on specimen-based phylogenies. This method is known to be highly liberal (whether used for testing for cospeciation or combining datasets in phylogenetic reconstructions; Clark *et al.* 2000). However, it clearly indicated that the genes of

Table 2. Ln likelihoods of *Brachycaudus* and *Buchnera* trees with alternative datasets. (Significance levels for pairwise comparisons were determined in the SH test. *p*-values indicate the probability that the score of the ML tree for a given dataset is significantly higher than that for alternative topologies. * $0.01<p<0.05$, ** $p<0.01$. $\delta=2((\ln L_1)-\ln(L_0))$ measures the difference in the likelihood between datasets being allowed to have different topologies ($\ln L_1=-19746.77$) and the highest likelihood obtained when all datasets are constrained to have the same topology ($(L_{0(Brachycaudus\ \mathrm{Ml\ topology})})=-19864.76$).)

dataset\tree	*Buchnera TrpB*	*Buchnera hupA–rpoc*	*Buchnera ssb–dna*	*Brachycaudus* combined	(L_1)	δ (excluding individual loci)
Buchnera TrpB	−3095.27	−3138.52*	−3186.3**	−3148.77*		128.9752
Buchnera hupA–rpoc	−5644.76**	−5581.0324	−5657.19**	−5613.37		171.3
Buchnera ssb–dna	−3754.79**	−3755.62**	−3696.64	−3728.79		171.6752
Brachycaudus combined	−7637.91**	−7514.317**	−7565.227**	−7373.83		235.9752
L sum	−20132.73	−19989.4894	−20105.357	−19864.76	−19746.77	

the endosymbionts were no more congruent with each other than with the *Brachycaudus* DNA dataset. Like Clark *et al.* (2000), we suggest that the lack of detection of cospeciation with this test does not reflect the biological reality. It is more likely to be due to the inadequacy of the models of evolution used and a high probability of conflict when numerous taxa are included in the analyses and when the tree topologies tested are strongly defined (Clark *et al.* 2000). Thus, overall, our results rule out the regular occurrence of host shifts throughout the evolution of *B. aphidicola*, even between closely related aphid species using the same host plants. *Buchnera aphidicola* is therefore similar in many ways to a maternally transmitted genetic element of the aphid, such as mitochondria. This interpretation is consistent with studies showing horizontal transfer of these bacteria (Wilkinson *et al.* 2003). Genomic studies, which demonstrate strong reduction of the *Buchnera* genome, also suggest that symbiont lateral transfer is highly unlikely except perhaps between closely related aphid species (Moran & Degnan 2006; Gomez-Valero *et al.* 2007). The only strongly supported exception to the one-to-one cospeciation scenario proposed is the association between *B. helichrysi*, *B. spiraeae* and their *Buchnera* strains. This result requires further confirmation, with denser sampling in this group of species.

(b) *Can the* Buchnera *genome help to refine aphid species delimitation and to elucidate the evolutionary history of these species?*

Each phylogenetic cluster found within *Brachycaudus* corresponds to a phylogenetic cluster in *Buchnera*. The few exceptions to this rule are not strongly supported and phylogenetic uncertainty can probably account for these mismatches. Furthermore, the shift in the branching pattern between intra- and interspecific divergence is more marked in the *Buchnera* phylogenetic tree than in the aphid tree. This similarity in the phylogenetic clusters between aphids and their symbionts and the higher interspecies divergence in *Buchnera* suggests that *Buchnera* sequences could be useful tools for determining whether conflicts between aphid species taxonomical division and phylogenetic clusters are due to low levels of sequence evolution or simply inaccurate species definition. This method can be directly applied to the *Brachycaudus* genus, in which several disagreements between taxonomy and phylogenetic reconstructions were found. For instance, *B. cardui* and *B. lateralis* consistently appear as a single cluster in all phylogenetic analyses (Coeur d'acier *et al.* 2008). Their species or subspecies status has been debated by taxonomists (Shaposhnikov 1964; Burger 1975; Heie 1992; Andreev 2004). Both species live on plants of the Compositae and their main distinguishing character is conspicuous: the length of femoral hair (Heie 1992). We show that the lack of DNA divergence between aphid specimens identified *a priori* as belonging to these two species is retrieved in the DNA of their symbiont. There are therefore now strong indications that these two morphospecies may actually correspond to a single biological species. Similarly, the species status of three *B.* (*Appelia*) species showing no significant divergence in aphid DNA data has been ambiguous (Heie 1992; Andreev 2004; Cocuzza *et al.* 2007): the identification of these species is based principally on biological characters, such as host–plant association and hybridization success. The lack of divergence in their *Buchnera* DNA might settle taxonomic discussions and suggests that these species could be grouped together. The group of species living for all or part of their life cycle on plants of the Caryophyllaceae has generated less taxonomic discussion. However, aphid and *Buchnera* genetic data again agree with morphological and biological data, indicating that these species form a homogeneous group (Burger 1975; Coeur d'acier *et al.* 2008). In summary, *Buchnera* DNA data confirm some of the taxonomic clusters suggested by phylogenetic analyses of *Brachycaudus*. Furthermore, these genetic clusters provide support for a 'lumper' approach to *Brachycaudus* taxonomy. They suggest that, in *Brachycaudus*, the use of the ecological species concept may on occasions have led taxonomists to describe a new species when a new host–plant association was found. When subtle morphological characters can be used to distinguish between these species, doubt may be cast on the plasticity of these characters, which may depend on the host–plant species on which the aphids have fed (Wool & Hales 1997; Ruiz-Montoya *et al.* 2005).

The significance of the cospeciation tests between *Brachycaudus* species and their *Buchnera* also confirmed that *B. aphidicola* DNA data can be used to infer the evolutionary history of the hosts (Coeur d'acier *et al.* 2008). The topologies of the trees are the same: *Buchnera* associated with *Appelia*, *Thuleaphis* and *Brachycaudus* subgenera all appear as monophyletic clades and *Buchnera* strains associated with the subgenus *Acaudus* (*sensu* Remaudière & Remaudière 1997) are divided into two differentiated paraphyletic clades, and these nodes are strongly supported (figure 1). Some polytomies were resolved with the *Buchnera* dataset: the *Buchnera* phylogeny clustered all specimens of the subgenus *Acaudus* associated with Caryophyllaceae together, whereas the phylogenetic reconstruction for *Brachycaudus* failed to resolve the phylogenetic position of *B. klugkisti* and its relationship to other *Brachycaudus* species living on Caryophyllaceae. A combined analysis of *Buchnera* and the *Brachycaudus* DNA dataset should therefore provide a robust *Brachycaudus* phylogeny.

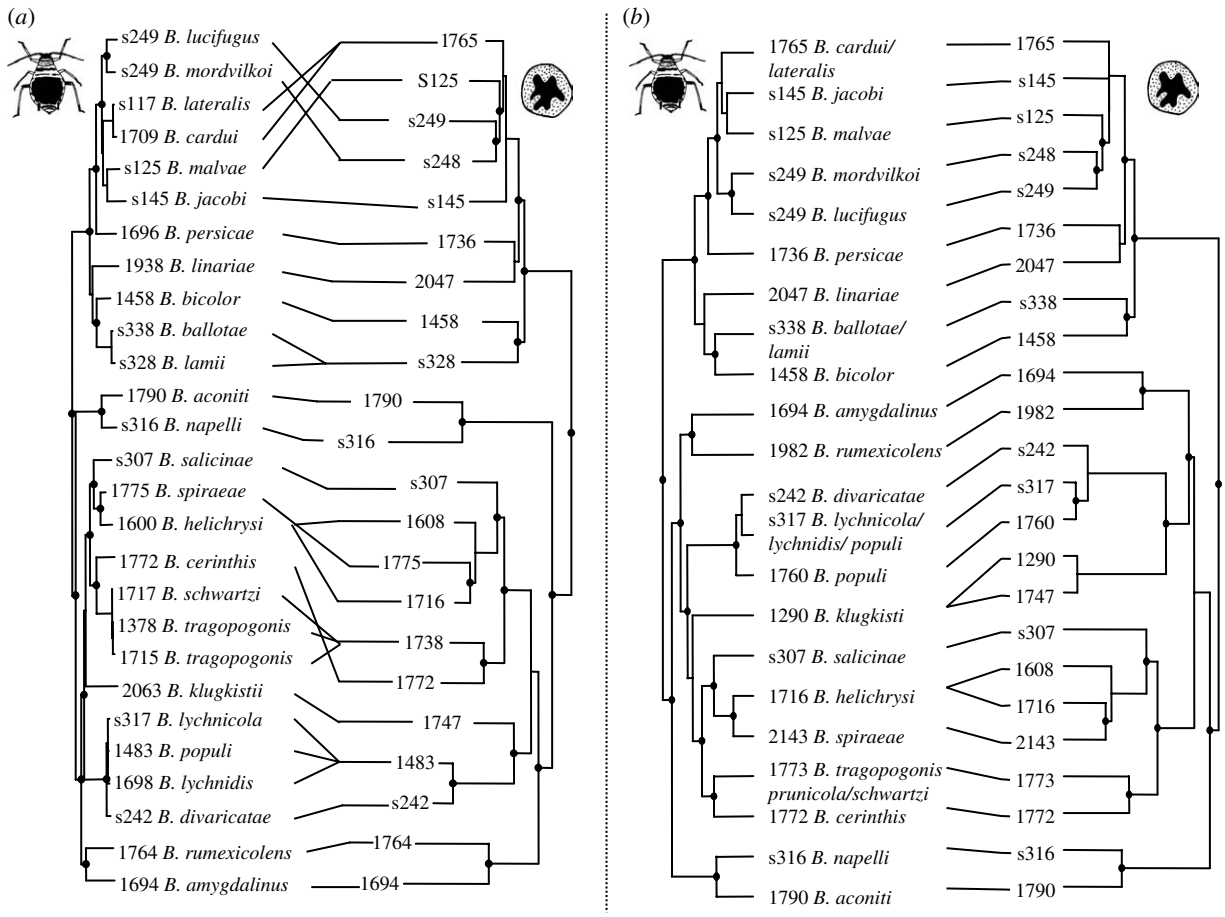

Figure 3. Pairwise comparisons of *Brachycaudus* and *Buchnera* species phylogenies. (*a*) *Brachycaudus* 'taxonomic' species tree versus *Buchnera* phylogenetic species tree (species delimitation based on the method of Pons *et al.* (2006)). (*b*) *Brachycaudus* 'phylogenetic' species tree versus *Buchnera* phylogenetic species tree (species delimitation based on the method of Pons *et al.* (2006) for both organisms).

(c) *Conclusion*

The idea that symbionts, whether parasites or mutualists, can be used as an additional source of data to infer the evolutionary history of their hosts is not new (Fahrenholz 1913) and has recently been applied to various systems (Nieberding *et al.* 2004; Criscione & Blouin 2007; Nieberding & Olivieri 2007). This approach has been applied to aphids before, but not explicitly, and with a conserved gene, the 16SrDNA (Lozier *et al.* 2007). The use of rapidly evolving *Buchnera* DNA fragments will be particularly useful in phylogenetic studies of aphids, a group that has probably evolved through rapid radiations (Von Dohlen & Moran 2000). The sequencing of the *B. aphidicola* genome (Moran & Degnan 2006) identified several genes and intergenic regions as potentially useful targets for phylogenetic inference. The difficulty, however, will lie in defining universal sets of primers for intergenic regions, particularly when bacterial genes have not been conserved throughout evolution (Gil *et al.* 2006).

The species delimitation analysis applied here has already been successfully used to define coherent species in asexual lineages (Fontaneto *et al.* 2007, 2008). We highlight here the advantages of this method for identifying bacterial species and suggest that the idea of *B. aphidicola* as a single traditional species should be reconsidered in the light of sequence-based species definitions. The small number of bacterial species described is not unique to *Buchnera* and, with the use of DNA methods, estimates of bacterial diversity are likely to increase (Cohan 2002; Achtman & Wagner 2008). Finally, such methods also make it possible to define host and symbiont species independently. This

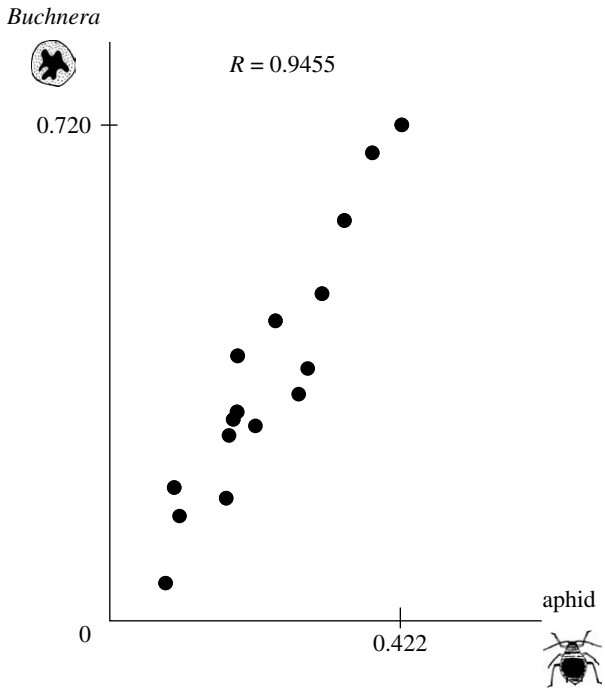

Figure 4. Plots of sequence divergence for cospeciating nodes based on the depth of the nodes of the ultrametric ML phylogenetic trees from figure 3*b*.

study demonstrates the usefulness of species delimitation for cophylogenetic studies, for interactions involving organisms in which species are difficult to conceptualize (bacteria, but also fungi and viruses), and also in interspecific interactions in which species may be defined arbitrarily on the basis of their host association.

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