

# Side-stepping secondary symbionts: widespread horizontal transfer across and beyond the Aphidoidea

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## Abstract

To elucidate the co-evolutionary relationships between phloem-feeding insects and their secondary, or facultative, bacterial symbionts, we explore the distributions of three such microbes — provisionally named the R-type (or PASS, or S-sym), T-type (or PABS), and U-type — across a number of aphid and psyllid hosts through the use of diagnostic molecular screening techniques and DNA sequencing. Although typically maternally transmitted, phylogenetic and pairwise divergence analyses reveal that these bacteria have been independently acquired by a variety of unrelated insect hosts, indicating that horizontal transfer has helped to shape their distributions. Based on the high genetic similarity between symbionts in different hosts, we argue that transfer events have occurred recently on an evolutionary timescale. In several instances, however, closely related symbionts associate with related hosts, suggesting that horizontal transfer between distant relatives may be rarer than transmission between close relatives. Our findings on the prevalence of these symbionts within many aphid taxa, along with published observations concerning their effects on host fitness, imply a significant role of facultative symbiosis in aphid ecology and evolution.

**Keywords:** aphid, PASS, psyllid, R-type, T-type, U-type

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## Introduction

Many insects and bacteria engage in intimate associations, or symbioses. Molecular analyses such as DNA sequencing and comparative sequence analyses, which have allowed for additional approaches such as *in situ* hybridization and diagnostic polymerase chain reaction (PCR), have played a key role in the identification of the various microbial participants. These techniques have also been used to describe a number of intriguing patterns regarding the diversity of bacterial symbionts within a given host clade, the distributions of closely related symbionts, the modes of transmission which shape these distributions, and the ages of associations between hosts and symbionts.

Aphids (Hemiptera: Aphidoidea) represent an ideal group of organisms for studies of symbiosis. Nearly all members of the Aphidoidea harbour obligate endosymbionts known as *Buchnera aphidicola* (Munson *et al.* 1991; but see Fukatsu *et al.* 1994) — vertically transmitted (i.e. from parent to offspring) bacteria housed within specialized

host cells known as bacteriocytes (or mycetocytes). *Buchnera aphidicola* supplement the phloem-sap diets of their aphid hosts through the synthesis of essential amino acids (Douglas & Prosser 1992; Baumann *et al.* 1995; Sandström & Moran 1999). The distributions of these symbionts can be explained by a pattern of strict vertical transmission (Munson *et al.* 1991; Rouhbachsh *et al.* 1996; Clark *et al.* 2000; Funk *et al.* 2000) since the time of a common ancestor, 84–164 million years ago (von Dohlen & Moran 2000).

In addition, many aphids harbour vertically transmitted bacteria that are not required for growth or reproduction. These are often referred to as secondary endosymbionts. Molecular and microscopy studies of several aphid species have revealed that these symbionts reside in a variety of locations inside their hosts, are sporadically distributed within and across host taxa, and comprise a smaller proportion of an embryo's symbiotic flora than does the primary symbiont, *B. aphidicola* (Buchner 1965; Unterman *et al.* 1989; Chen *et al.* 1996; Chen & Purcell 1997; Fukatsu *et al.* 2000; Fukatsu 2001; Fukatsu *et al.* 2001; Sandström *et al.* 2001). Secondary symbionts of aphids belong to a number of distinct lineages within the  $\gamma$ - and  $\alpha$ -Proteobacteria (Unterman *et al.* 1989; Chen *et al.* 1996; Chen & Purcell 1997;

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Fukatsu 2001; Sandström *et al.* 2001), and the Mollicutes (Fukatsu *et al.* 2001). Close relatives of aphid secondaries, with 16S rDNA sequence identities ranging from 98% to over 99%, have been found in distantly related arthropod species (Chen *et al.* 1996; Fukatsu *et al.* 2001; Sandström *et al.* 2001). Thus, for the few hosts that have been examined, the distributions of several secondary symbionts appear to be partly the result of horizontal transmission (i.e. movement between hosts other than that from parent to offspring) between host species. However, some evidence suggests that certain associations between aphids and secondary symbionts are specialized and have co-evolved (Fukatsu 2001).

To date, most DNA sequence-based studies of aphid secondary symbionts have focused on hosts within the tribe Macrosiphini. Moreover, although these studies provided some evidence for horizontal transfer (Chen & Purcell 1997; Darby *et al.* 2001; Sandström *et al.* 2001), few have employed statistical analyses to demonstrate this point, and little effort has been made to assess the extent to which horizontal transfer has shaped secondary symbiont distributions. Here we use diagnostic molecular screening techniques and DNA sequencing, along with pairwise divergence and phylogenetic analyses, to explore the distributions of three facultative  $\gamma$ -Proteobacterial secondary symbionts of aphids, known as the R-type (or S-sym, or Pea Aphid Secondary Symbiont – PASS), T-type (or Pea Aphid *Bemisia*-like Symbiont – PABS), and U-type (Chen & Purcell 1997; Unterman *et al.* 1989; Darby *et al.* 2001; Sandström *et al.* 2001), across a variety of aphid and psyllid (Hemiptera: Psylloidea) species. Specifically, we compare the phylogenies of hosts and symbionts to determine whether there is any evidence for co-speciation. Also, we compare 16S rDNA divergence values between primary and secondary symbionts residing within pairs of hosts sharing the same most recent common ancestor. Differences in these values are used to determine the likelihood that current secondary infections could possibly be the result of exclusive vertical transmission since the time of this common host ancestor. We also compare the rates of substitution in the 16S rDNA genes of R-, T- and U-type symbionts to those of free-living bacteria and *B. aphidicola*, and estimate the ages of symbiont common ancestors through the use of a molecular clock. Finally, we use data on symbiont prevalence within and across host taxa, along with observations of symbiont-associated fitness effects, to assess the potential importance of these microbes in aphid biology.

## Materials and methods

### Samples

Members of 75 species, spanning 15 subfamilies and tribes across the Aphidoidea (Remaudière & Remaudière 1997)

were collected from a variety of locations in the United States and Eurasia. In addition, members of four aphid species were obtained from laboratory stocks. A list of all aphid species involved in the survey, their taxonomic affiliations, and information regarding their collection is presented in Table 1.

Representatives of 27 psyllid species, from North and South America, along with Europe (see Thao *et al.* 2000b for collection information), were also surveyed as psyllids have been shown to harbor a number of secondary symbionts (Thao *et al.* 2000a), yet have not been screened for the R-, T-, or U-types. The samples included were *Acizzia uncatoides* (two samples), *Aphalaroida inermis*, *Bactericera cockerelli*, *Blastopsylla occidentalis*, *Boreioglycaspis melaleucae*, *Cacopsylla brunneipennis*, *Cacopsylla myrthi*, *Cacopsylla peregrina*, *Cacopsylla pyri*, *Calophya schini*, *Ctenarytaina eucalypti*, *Ctenarytaina longicauda*, *Ctenarytaina spatulata*, *Diaphorina citri*, *Glycaspis brimblecombei*, *Heteropsylla cubana*, *Heteropsylla texana*, *Neotriozella hirsuta*, *Psylla buxi*, *Trioza urticae*, *Pachyopsylla celtidismamma*, *Pachyopsylla venusta*, *Parasitopelma* sp., *Psylla* sp., *Russelliana intermedia*, *Spanioneura fonscolombii* and *Trioza eugeniae*.

To determine the phylogenetic proximity of related symbionts found within the same host species, the 16S rRNA genes of R-, T-, or U-type symbionts from several *Acyrtosiphon pisum* clones, maintained in the Moran laboratory at the University of Arizona, were sequenced directly from PCR products as described below. These aphids were not included in the symbiont survey, as it was known which symbionts they harboured prior to the start of the study. Their 16S rDNA sequences were deposited in GenBank under the following accession numbers: AY136138 (U-type of *A. pisum* clone 2a from New York), AY136139 (R-type of *A. pisum* clone 2BB from Wisconsin), AY136140 (R-type of *A. pisum* clone 9-2-1 from New York), AY136141 (T-type of *A. pisum* clone 8-2b from New York).

### DNA extractions

A modified protocol of Bender *et al.* (1983) was used to extract DNA from American and Israeli aphids. First, a single aphid was placed in a 1.5-mL tube, which was dipped into liquid nitrogen. The aphid was then crushed with a plastic pestle. Next, 100–200  $\mu$ L of 'lysis buffer' [0.1 M NaCl, 0.2 M sucrose, 0.1 M Tris-HCl (pH 9.1), 0.05 M ethylenediaminetetraacetic acid (EDTA), and 0.5% sodium dodecyl sulphate] was added to each tube, and the contents were further homogenized. Samples were incubated at 65 °C for 30 min, then 8 M potassium acetate was added to give a final concentration of 1 M. Samples were kept on ice for a minimum of 30 min, then centrifuged at 16,435 g for 15 min. The supernatant was decanted into a new 1.5-mL tube and an equal volume of 100% ethanol was added. After 5 min at room temperature,

**Table 1** Diagnostic molecular screening results reveal the distributions of aphid secondary symbionts

Species	Code	Taxonomy	Collection location*	R+	T+	U+	Accession number
<i>Tamalia coweni</i>	Tco	Tamaliinae	Arizona	-	-	-	
<i>Thelaxes suberi</i>	Tsu	Thelaxinae	Spain	-	-	-	
<i>Stegophylla</i> sp.	Ste	Phyllaphidinae	Arizona	-	-	-	
<i>Chaitophorus populeti</i>	Cpe	Chaitophorini	Spain	-	-	+	AY136167
<i>Chaitophorus populifolii</i>	Cpi	Chaitophorini	Arizona	A	-	-	
<i>Chaitophorus leucomelas</i>	Cle	Chaitophorini	Spain	-	-	-	
<i>Periphyllus bulgaricus</i>	Pbl	Chaitophorini	Spain	+	+	-	AY136157, AY136161
<i>Cinara (Cupressobium) cupressi</i>	Ccu	Cinarini	Spain	+	-	-	AY136144
<i>Cinara (Cinara) maritimae</i>	Cma	Cinarini	Spain	+	-	-	
<i>Cinara (Cupressobium) tujafilina</i>	Ctu	Cinarini	Spain	+	-	-	AY136146
<i>Essigella californica</i>	Eca	Cinarini	Arizona	A	A	A	
<i>Eulachnus pallidus</i>	Epa	Cinarini	Spain	-	-	-	AY136147
<i>Lachnus</i> sp.	Lac	Lachnini	Arizona	-	-	-	
<i>Pterochloroides persicae</i>	Ppe	Lachnini	Spain	+	-	-	AY136155
<i>Tuberolachnus salignus</i>	Tsa	Lachnini	Spain	-	-	-	
<i>Drepanosiphum oregonensis</i>	Dor	Drepanosiphini	Spain	-	-	A	
<i>Hamamelistes spinosus</i>	Hsp	Hormaphidini	Washington, D.C.	-	-	-	
<i>Hormaphis cornu</i>	Hco	Hormaphidini	Georgia	A	A	A	
<i>Acyrtosiphon lactucae</i>	Ala	Macrosiphini	Arizona	A	-	A	
<i>Acyrtosiphon pisum</i>	Api F	Macrosiphini	France*	-	-	-	
<i>Acyrtosiphon pisum</i>	Api E	Macrosiphini	England*	-	-	-	
<i>Brachycaudus cardui</i>	Bca	Macrosiphini	Spain	-	-	+	AY136143
<i>Brevicoryne brassicae</i>	Bbr	Macrosiphini	Arizona	-	-	-	
<i>Corylobium avellanae</i>	Cav	Macrosiphini	Spain	-	-	-	
<i>Diuraphis noxia</i>	Dno	Macrosiphini	Poland	-	-	-	
<i>Hyperomyzus lactucae</i>	Hla	Macrosiphini	Arizona	-	A	-	
<i>Macrosiphum euphorbiae</i> 1999	Meu 99	Macrosiphini	Arizona	A	A	+	
<i>Macrosiphum euphorbiae</i> 2001	Meu 01	Macrosiphini	Arizona	-	+	+	AY136148, AY136149
<i>Macrosiphum rosae</i>	Mro A	Macrosiphini	Arizona	-	A	+	AY136150
<i>Macrosiphum rosae</i>	Mro S	Macrosiphini	Spain	+	-	-	AY136152
<i>Megoura viciae</i>	Mvi	Macrosiphini	Spain	-	-	-	
<i>Myzus (Myzus) cerasi</i>	Mce	Macrosiphini	Spain	-	-	-	
<i>Macrosiphoniella helichrysi</i>	Mhe	Macrosiphini	Spain	+	-	-	AY136151
<i>Myzus (Nectarosiphon) persicae</i>	Mpe	Macrosiphini	Spain	-	-	-	
<i>Roepkea marchali</i>	Rma	Macrosiphini	Spain	-	-	-	
<i>Sitobion fragariae</i>	Sfr	Macrosiphini	Spain	-	-	-	
<i>Uroleucon atripes</i>	Uat	Macrosiphini	Arizona	-	+	A	
<i>Uroleucon nigrotuberculatum</i>	Uni	Macrosiphini	Wisconsin	-	+	-	AY136162
<i>Uroleucon pielouii</i>	Upi	Macrosiphini	Wisconsin	-	+	-	AY136163
<i>Uroleucon reynoldense</i>	Ure	Macrosiphini	Wisconsin	-	+	-	AY136164
<i>Uroleucon rudbeckiae</i>	Uru a	Macrosiphini	Arizona	-	A	+	AY136165
<i>Uroleucon rudbeckiae</i>	Uru b	Macrosiphini	Arizona	-	+	A	AY136166
<i>Uroleucon sonchi</i>	Uso	Macrosiphini	Spain	-	-	-	
<i>Wahlgreniella nervata</i>	Wne	Macrosiphini	Arizona	-	-	-	AY136168
<i>Aphis craccivora</i>	Acr S	Aphidini	Spain	+	-	-	AY136137
<i>Aphis craccivora</i>	Acr A	Aphidini	Arizona	A	+	A	AY136136
<i>Aphis fabae</i>	Afa	Aphidini	Spain	-	-	-	
<i>Aphis gossypii</i>	Ago A	Aphidini	Arizona	-	-	-	
<i>Aphis gossypii</i>	Ago S	Aphidini	Spain	-	-	-	
<i>Aphis nerii</i>	Ane S	Aphidini	Spain	-	-	-	
<i>Aphis nerii</i>	Ane A	Aphidini	Arizona	-	-	-	
<i>Aphis ruborum</i>	Aru	Aphidini	Spain	-	-	-	
<i>Aphis spiraeicola</i>	Asp	Aphidini	Spain	-	-	-	AY136142
<i>Hyalopterus pruni</i>	Hpr	Aphidini	Spain	-	-	-	
<i>Hysteroneura setariae</i>	Hse	Aphidini	Arizona	A	A	A	
<i>Rhopalosiphum padi</i>	Rpa	Aphidini	France*	-	-	-	
<i>Pemphigus betae</i>	Pbe 87	Pemphigini	Utah	+	-	-	

Table 1 Continued

Species	Code	Taxonomy	Collection location*	R†	T†	U†	Accession number
<i>Pemphigus betae</i>	Pbe 89	Pemphigini	Utah	-	-	+	AY136154
<i>Pemphigus bursarius</i>	Pbr	Pemphigini	Spain	-	-	-	
<i>Prociphilus (Meliarhizophagus) fraxinifolii</i>	Pfr	Pemphigini	Arizona	-	-	-	
<i>Pemphigus noptonii</i>	Pno	Pemphigini	Ohio	-	-	-	
<i>Pemphigus populi</i>	Ppi	Pemphigini	Spain	-	-	-	AY136158
<i>Pemphigus populicaulis</i>	Ppc	Pemphigini	Arizona	A	A	A	
<i>Pemphigus populiramulorum</i>	Ppr	Pemphigini	Arizona	-	-	-	
<i>Pemphigus spyrothecae</i>	Psp	Pemphigini	Spain	-	+	-	AY136156
<i>Thecabius populimonilis</i>	Tpo	Pemphigini	Arizona	-	-	-	
<i>Eriosoma lanigerum</i>	Eli	Eriosomatini	Arizona	-	-	-	
<i>Eriosoma (Schizoneura) lanuginosum</i>	Elu	Eriosomatini	Spain	-	-	-	
<i>Eriosoma (Schizoneura) ulmi</i>	Eul	Eriosomatini	Spain	-	-	-	
<i>Tetraneura (Tetraneurella) akirine</i>	Tak	Eriosomatini	Spain	-	-	-	
<i>Tetraneura caerulescens</i>	Tca	Eriosomatini	Spain	-	-	-	
<i>Tetraneura ulmi</i>	Tul	Eriosomatini	Spain	-	-	-	AY136160
<i>Baizongia pistaciae</i>	Bpi	Fordini	Spain	-	-	-	
<i>Forda formicaria</i>	Ffo	Fordini	Spain	-	-	-	
<i>Geoica utricularia</i>	Gut	Fordini	Spain	-	-	-	
<i>Geopemphigus</i> sp.	Geo	Fordini	Texas	A	+	A	
<i>Melaphis rhois</i>	Mrh	Fordini	Arizona	A	A	A	
<i>Schlechtendalia chinensis</i>	Sch	Fordini	Japan	A	A	A	
<i>Smynthuroides betae</i>	Sbe	Fordini	Israel	+	N	N	AY136159
<i>Chromaphis juglandicola</i>	Cju	Myzocallidini	Spain	-	-	-	
<i>Melanocallis caryaefoliae</i>	Mca	Myzocallidini	Arizona*	A	A	A	
<i>Monellia caryella</i>	Mcl	Myzocallidini	Arizona*	A	A	A	
<i>Myzocallis</i> sp.	Myz	Myzocallidini	Arizona	-	-	-	AY136153
<i>Panaphis juglandis</i>	Pju	Myzocallidini	Spain	A	-	-	
<i>Therioaphis trifolii</i> f. <i>maculata</i>	Ttr	Myzocallidini	New York	-	-	-	
<i>Tinocallis</i> sp.	Tin	Myzocallidini	Spain	-	-	-	
<i>Pterocomma populeum</i>	Ppo	Pterocommatinae	Spain	-	-	-	

\*Samples derived from laboratory stocks.

†A = ambiguous results for this symbiont; N = sample was not screened for this symbiont.

samples were again centrifuged at 16,435 g for 15 min. The supernatant was discarded, and the pellet was washed in ice-cold 70% ethanol, then in ice-cold 100% ethanol. Samples were dried in a SpeedVac® and were then suspended in 'low-TE' (1 mM Tris-HCl and 0.1 mM EDTA).

For European aphids, DNA was extracted from single individuals as previously described (Latorre *et al.* 1986). Psyllid DNA samples were derived from multiple individuals (Thao *et al.* 2000b).

#### Diagnostic screening and DNA sequencing

To test for the presence of secondary symbionts, DNA samples were subjected to several diagnostic PCR reactions, each involving a diagnostic forward and 'universal' reverse primer, which amplify a fragment of the 16–23S rRNA operon. PCR reactions were carried out as previously

described (Moran *et al.* 1999, US and Israeli aphids; van Ham *et al.* 1997, European aphids), and all reactions included a 'negative' control (sterile water instead of DNA) so as to spot any possible contamination. A 'positive' control (DNA from an aphid known to harbour the relevant symbiont) was included to prevent false negatives. The diagnostic primers used here (Table 2) have been previously published or were newly designed based on an alignment of R-, T- and U-type 16S rDNA sequences with those of several closely related  $\gamma$ -Proteobacteria.

Primers spanning the intergenic spacer between the 16 and 23S rRNA genes generated PCR products that were of characteristic lengths for the R-, T- and U-type symbionts, which facilitated our assessment of symbiont presence/absence. Diagnostic PCR products (obtained from R250F, T99F, or U99F with 480R) from selected samples were subjected to restriction digests with the enzymes *Sst*I/*Sac*I, *Xba*I and *Cl*aI, to further confirm infection status (Sandström

Primer name*	Primer sequence (5' → 3')	Gene and position†	Uses‡
R1279F	CGAGAGCAAGCGGACCTCAC	16S; 1267–1286	DP
T1279F	CGAGGAAAGCGGAACCTCAG	16S; 1267–1286	DP
U1279F	CGAACGTAAGCGAACCTCAT	16S; 1267–1286	DP
R250F§	GGTAGGTGGGGTAACGGCTC	16S; 250–269	DP; AS; S
T99F¶	AGTGAGCGCAGTTTACTGAG	16S; 75–87	DP; AS; S
U99F§	ATCGGGGAGTAGCTTGCTAC	16S; 71–90	DP; AS; S
480R§	CACGGTACTGGTTCACCTATCGGTC	23S; 453–476	DP; AS
1502R	GTTACGACTTCACCCAG	16S; 1484–1502	S
10F§	AGTTTGATCATGGCTCAGATTG	16S; 10–31	S; AS
559F	CGTGCCAGCAGCCGGTAATAC	16S; 514–536	S

**Table 2** Primer sequences, their positions in 16S or 23S rRNA genes, and their uses in this study

\*Names of diagnostic primers begin with an 'R', 'T', or 'U', depending on the symbiont they were designed for.

†Positions of primers within the 16S and 23S rRNA genes are with respect to the sequences of *Escherichia coli* (GenBank accession numbers AE000460 and AE000453, respectively).

‡DP = diagnostic PCR; S = sequencing; AS = amplification for products to be sequenced.

§Previously described in Sandström *et al.* (2001).

¶Primer spans a six base pair insertion unique to the T-type.

*et al.* 2001). All PCR and restriction digest products were run on agarose gels, stained in ethidium bromide and visualized with UV light.

PCR products, spanning portions of the 16S and 23S rRNA genes, were obtained for samples that amplified in diagnostic screening reactions. These products were purified, and sequenced directly (Sandström *et al.* 2001, American aphids), or after cloning (Marchuk *et al.* 1992; Martinez-Torres *et al.* 2001, European aphids). All sequencing was performed at the University of Arizona GATC Sequencing Facility. Primers used in the sequencing reactions are described in Table 2.

#### Assessment of diagnostic methods

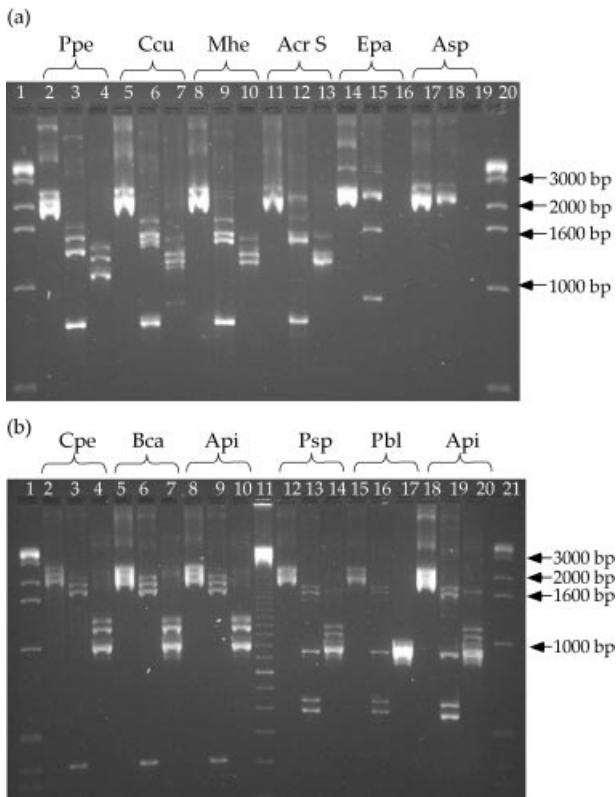
Diagnostic PCR reactions gave amplification in a number of aphid and psyllid species. However, not all amplified products were identical in length to those of positive R-, T- and U-type controls from *A. pisum* or to samples in which these bacteria were diagnosed through DNA sequencing (Fig. 1). In particular, the primer R250F exhibited several cases of nonspecific amplification, which resulted in the discovery of 16S rDNA sequences similar to those of several free-living bacteria (these were considered likely contaminants) along with symbionts of other insects. Sequencing and BLASTn searches revealed that the combination of diagnostic PCR and restriction digest reactions was sufficient to identify symbionts, as no samples tested positive with both of these techniques yet lacked a 16S rDNA sequence more than 95% similar to previously described R-, T-, or U-type symbionts (data not shown). Thus, samples were scored as positive (Table 1) only if they tested positive with both of these methods, or if associated

16S rDNA sequences were at least 95% similar to a previously described R-, T-, or U-type symbiont. Similarly, we will refer to all bacteria discovered here as R-, T-, or U-type symbionts only if they are at least 95% similar to all previously described members of these clades as revealed by BLASTn searches. We use this 95% cut-off criterion as all sequences that fell into a monophyletic group with the R-, T-, or U-types were less than 5% divergent from all other sequences within their respective clades.

In contrast, samples were scored as negative if they did not amplify, or yielded improperly sized products, in two relevant diagnostic PCR reactions (e.g. no amplification with R250F and 480R or with R1279F and 480R indicated a sample that was negative for the R-type). Alternatively, samples were declared negative if the partial 16S rDNA sequence of a diagnostic PCR product was less than 95% similar to previously described secondary symbionts. To avoid potential misdiagnosis, the few samples not meeting each of the criteria required to be declared negative or positive were considered ambiguous.

#### 16S rDNA phylogeny

Overlapping contigs from sequencing reactions were assembled using SEQMANII (DNASTAR 1997). Sequences were then compared to those in GenBank using BLASTn searches. Sequences at least 95% similar to all previously described R-, T-, or U-type 16S rDNA sequences were assigned to the relevant symbiont group. The results of BLASTn searches were also used to choose other symbiotic and free-living bacteria for inclusion in subsequent phylogenetic analyses. Completed sequences were submitted to GenBank and their accession numbers are presented in Table 1.



**Fig. 1** Results of diagnostic molecular surveys. Brackets encompass lanes corresponding to different aphids, and abbreviations above brackets are as listed in Table 1. (a) Gel picture of diagnostic R-type PCR and restriction digests. Lanes 1 and 20 contained a 1000-bp ladder (Gibco BRL) size standard. Products from R250F and 480R are found in lanes 2, 5, 8, 11, 14 and 17 (undigested), and lanes 3, 6, 9, 12, 15 and 18 (digested with *SacI/SstI*). Products from R1279F and 480R are found in lanes 4, 7, 10, 13, 16 and 19 (undigested). Note that Asp and Epa samples did not amplify with R1279F and 480R, nor were their R250F and 480R products cut with *SacI/SstI* to yield products of the expected sizes. This was consistent with our sequencing results, which indicated the absence of the R-type. (b) Gel of diagnostic T- and U-type PCR and restriction digests. Lanes 1 and 21 contained the 1000-bp size standard, while lane 11 contained a 100-bp ladder (Amersham BioSciences). Products obtained with U99F and 480R are found in lanes 2, 5 and 8 (undigested), and lanes 3, 6 and 9 (digested with *Clal*). Undigested products obtained with the primers U1279F and 480R are found in lanes 4, 7 and 10, while those obtained with the primers T1279F and 480R are found in lanes 14, 17 and 20. Products from reactions with T99F and 480R are found in lanes 12, 15 and 18 (undigested) and in lanes 13, 16 and 19 (digested with *XbaI*).

The 16S rDNA sequences obtained here, along with those of other insect symbionts (including all previously published aphid R-, T- and U-type sequences) and several free-living bacteria, were aligned in SEQUENCE NAVIGATOR 1.01 (Applied Biosystems 1994). Aligned sequences were imported into MACCLADE 4.03 (Maddison & Maddison 2000) where the alignment was adjusted manually. Published 16S rDNA sequences were included from the sym-

bionts *B. aphidicola* (primary symbiont of the aphid *Myzus persicae*, M63249), *Sodalis glossinidius* (secondary symbiont of tsetse flies, U64867), SOPE (the primary endosymbiont of the weevil *Sitophilus oryzae*, AF005235), and *Arsenophonus triatominarum* (a symbiont of the kissing bug *Triatoma infestans*, U91786). Also included were 16S rDNA sequences from secondary symbionts of the psyllids *G. brimblecombei* (AF263561), *H. texana* (AF263562), *B. cockerelli* (AF263557), and *B. occidentalis* (AF263558); the whiteflies (Hemiptera: Aleyrodoidea) *Aleurodicus dugesii* (AF286129) and *Bemisia tabaci* (Z11926); the aphids *Uroleucon ambrosiae* (AF293621, AF293622), *Uroleucon rudbeckiae* (AF293626), *A. pisum* (AF293617, AF293616, AF293618, AB033779, AB033778, AB033777, AJ297720, M27040), *Uroleucon helianthicola* (AF293625), *Uroleucon astronomus* (AF293623), *Macrosiphoniella ludoviciana* (AF293619), *Uroleucon solidaginis* (AF293627), *Uroleucon aenum* (AF293620), and *Uroleucon caligatum* (AF293624); along with a symbiont of the beetle *Chilomenes sexmaculatus* (AJ272038). 16S rDNA sequences from the following free-living bacteria were also included: *Pseudomonas aeruginosa* (AF094720), *Vibrio cholerae* (X74695), *Pantoea ananatis* (Z96081), *Escherichia coli* (AB045731), *Enterobacter cloacae* (Y17665), *Serratia entomophila* (AJ233427), *Serratia ficaria* (AJ233428), *Yersinia enterocolitica* (Z75316), *Klebsiella pneumoniae* (Y17657), and *Proteus vulgaris* (AJ233425).

Two analyses were performed in PAUP\* 4.0b8 (Swofford 2001) — an heuristic search using parsimony and a neighbour-joining search using distance (with the Kimura two-parameter model of nucleotide substitution). One thousand bootstrap replicates were performed for each search, to estimate the level of support for nodes. Trees were rooted using *P. aeruginosa* as the outgroup.

#### Phylogenetic comparisons

Phylogenies of several aphids in the tribe Macrosiphini and their symbionts were compared using the REL (Resampling Estimate of Log-Likelihood) method (Kishino & Hasegawa 1989), to determine the possibility of symbiont persistence through strict co-speciation. This test gives a bootstrap probability for candidate trees without performing maximum likelihood estimation for each resampled data set, and can be used to compare the likelihood of alternative tree topologies given the nucleotide sequence data. We specifically considered the likelihood that the phylogeny of several U-type symbionts is identical, or congruent, to that of their hosts. Included in these analyses were the hosts *A. pisum*, *M. ludoviciana*, *U. helianthicola*, *U. solidaginis*, *U. aenum*, *U. rudbeckiae* and *U. astronomus*, as well as their U-type associates. Parsimony searches were used to suggest a likely U-type symbiont tree topology for this subset of taxa —  $\{[(U. solidaginis, U. aenum) (U. rudbeckiae, A. pisum)] (U. astronomus, U. helianthicola),$

*M. ludoviciana*). All nodes in this tree were supported by bootstrap values of 80 or higher. We then compared the approximate bootstrap probability of this topology to those of three possible host trees based on mitochondrial and nuclear gene sequences (from Figs 1c in Moran *et al.* 1999) — {(*U. solidaginis*, *U. aenum* (*U. astronomus*, *U. rudbeckiae*)), *U. helianthicola*], *M. ludoviciana*, *A. pisum*). All REL analyses were executed in MOLPHY 2.3 (Adachi & Hasegawa 1996).

#### Relative rates and pairwise divergences

To determine the rates at which symbiont 16S rRNA genes are evolving, relative rates tests were performed. The 16S rDNA sequences included all exceeded 1300 nucleotides in length and were derived from the R-type symbionts of *A. pisum* (six individuals) and *U. caligatum*; the T-type symbionts of *Uroleucon nigrotuberculatum*, *U. ambrosiae* (two individuals), *U. rudbeckiae* (two individuals), *Uroleucon pieloui*, *Uroleucon reynoldense*, *Aphis craccivora*, *A. pisum* (two individuals), *Periphyllus bulgaricus*, *C. sexmaculatus* and *B. tabaci*; the U-type symbionts of *U. solidaginis*, *Brachycaudus cardui*, *Macrosiphum euphorbiae* (two individuals), *Macrosiphum rosae*, *M. ludoviciana*, *U. aenum*, *Pemphigus betae*, *A. pisum*, *U. astronomus* and *Chaitophorus populeti*; *B. aphidicola*; and the free-living bacteria *E. coli*, *S. entomophila*, *S. ficaria* and *V. cholerae*. This trimmed-down alignment file (otherwise identical to that used for the 16S rDNA phylogeny) was opened in PAUP\*<sup>b8</sup>, where missing and ambiguous sites were removed. *Vibrio cholerae* was defined as the outgroup, and a guide tree was constructed (using parsimony and 100 bootstrap replicates) for weighting in relative rates tests (Robinson *et al.* 1998). The bootstrap 60% majority-rule consensus tree used in relative rates tests had the following topology: (*V. cholerae*, (((*A. pisum* California R, *A. pisum* Japan R, *A. pisum* Japan R, *A. pisum* Japan R, *A. pisum* Wisconsin R, *A. pisum* Arizona R), *U. caligatum* R) (*S. ficaria*, *S. entomophila*)), *E. coli* ((((*U. solidaginis* U (*B. cardui* U, *C. populeti* U), *U. aenum* U, *P. betae* U) (*M. euphorbiae* U, *M. euphorbiae* U), *M. rosae* U, *A. pisum* U, *U. astronomus* U), *M. ludoviciana* U) (((*U. nigrotuberculatum* T, *U. ambrosiae* Arizona T, *U. reynoldense* T, *U. pieloui* T, *U. ambrosiae* Minnesota T, *C. pyri* T) (*U. rudbeckiae* T, *U. rudbeckiae* T, *A. pisum* New York T, *A. pisum* Wisconsin T), *P. bulgaricus* T, *C. sexmaculatus* T), *A. craccivora* T, *B. tabaci* T)), *Buchnera*)). The ungapped and unambiguous 1301 base pair alignment was opened in RRTREE (Robinson-Rechavi & Huchon 2000; <http://pbil.univ-lyon1.fr/software/rrtree.html>). 16S rRNA rates of substitution were compared between R-type, T-type, U-type, *B. aphidicola*, *E. coli* and *Serratia* species with *V. cholerae* defined as the outgroup. Divergences were computed using the Kimura two-parameter model of nucleotide substitution, and relative rates tests were

performed as described by Robinson *et al.* (1998). The alignment file created for relative rates tests was also used to compute pairwise divergences between the 16S rDNA sequences of free-living and symbiotic bacteria. Both the absolute number of differences and Kimura two-parameter corrected distances were computed using PAUP\* 4.0b8.

#### Primary and secondary symbiont pairwise divergence comparisons

Finally, pairwise divergence comparison analyses were performed in an attempt to help determine whether R-, T-, or U-type symbionts could have persisted within host lineages through strict vertical transmission since the time of a common host ancestor (i.e. whether they have undergone strict co-speciation). If such a scenario has occurred, genetic divergences between secondaries infecting a pair of host species from two distinct taxa should resemble those between *B. aphidicola* from the same taxa. Specifically, because *B. aphidicola* undergoes strict vertical transmission (Munson *et al.* 1991; Rouhbachsh *et al.* 1996; Clark *et al.* 2000; Funk *et al.* 2000), and because bacterial 16S rRNA substitution rates range from 1 to 4% per hundred million years (Moran *et al.* 1993; Ochman & Wilson 1987; Ochman *et al.* 1999), differences in divergences (between *B. aphidicola* and secondaries in equi-distant hosts) should not exceed four-fold under strict vertical transmission of secondary symbionts. Violations of these conditions indicate horizontal transfer; however, cases in which conditions are not violated do not imply co-speciation, as similar divergences could simply be chance. This approach does not require knowledge of phylogenetic relatedness and, thus, provides an alternative approach to examining the possibility of co-speciation.

We constructed an alignment including several R-, T- and U-type 16S rDNA sequences from symbionts associated with hosts from the tribes Chaitophorini, Macrosiphini, Pemphigini and Aphidini, and from the genera *Acyrtosiphon* and *Uroleucon*. Also included were 16S rDNA sequences of *B. aphidicola* symbionts from species in the same tribes and genera: *A. pisum* (M27039), *Diuraphis noxia* (M63251), *M. persicae* (M63249), and *Uroleucon sonchi* (M63250) of the Macrosiphini; *Chaitophorus viminalis* (M63252) of the Chaitophorini; *P. betae* (M63254) and *Pemphigus populi* (AJ296750) of the Pemphigini; and *Rhopalosiphum maidis* (M63247), *Rhopalosiphum padi* (M63248), and *Schizaphis graminum* (M63246) of the Aphidini. The alignment was performed as described above. Pairwise distances were then calculated over 1296 nucleotides (no missing or ambiguous sites) using the Kimura two-parameter model of nucleotide substitution, and compared between *B. aphidicola* and secondaries infecting one member, each, of the same two host taxa. For example, the 16S rDNA divergence

between *B. aphidicola* of *P. populi* (Pemphigini) and *M. persicae* (Macrosiphini) was compared to that between the U-type symbionts of *P. betae* (Pemphigini) and *M. ludoviciana* (Macrosiphini). When sequences were available for symbionts of more than one host species within a given taxon the smallest *B. aphidicola* divergence value was compared to the largest secondary symbiont divergence value. This minimized the differences in 16S rDNA divergences, making any conclusions of horizontal transfer quite conservative.

## Results

### Symbiont distributions

The results of our diagnostic molecular screening survey are summarized in Table 1. Each of the R-, T- and U-type symbionts was found in one or more previously unscreened members of the Macrosiphini. The R-type was found in members of six new aphid tribes – the Aphidini, Chaitophorini, Cinarini, Fordini, Lachnini and Pemphigini. The T-type was found in one psyllid, *Cacopsylla pyri* (GenBank accession no. AY136145), and in four new aphid tribes – the Aphidini, Chaitophorini, Fordini and Pemphigini. Finally, the U-type was found in two new aphid tribes – the Chaitophorini and Pemphigini. In total, within the Aphidoidea the R-type was found in 10/79 surveyed species, the T-type in 11/78, and the U-type in 6/76. Within the Psylloidea, 0/27 surveyed species were found to associate with the R- or U-types, while only 1/27 psyllid species was positive for the T-type. Though more aphid and psyllid hosts tested negative than positive for each symbiont, it should be noted that negative results here do not imply that the symbionts of interest are absent from a species, as only one to a few individuals were surveyed.

These results indicate that R-, T- and U-type symbionts are certainly not confined to members of the Macrosiphini, or even to the superfamily Aphidoidea in the case of the T-type. Indeed, BLASTn searches using T-type 16S rDNA sequences revealed that such molecules share a high degree of similarity (over 98%) with that of a putative male-killing bacterium described from the ladybird beetle, *Chilomenes sexmaculatus* (MEN Majerus personal communication; GenBank accession no. AJ272038). Also, T-type 16S rDNA sequences have previously been shown to share over 98% identity with that from a secondary symbiont of the whitefly, *Bemisia tabaci* (Sandström *et al.* 2001).

Diagnostic screening revealed two cases of potentially stable associations between a symbiont type and a host clade. First, all three surveyed members of the genus *Cinara* were found to harbour the R-type (Table 1). Also, three previously unscreened Nearctic members of the subgenus *Uroleucon* – *U. pieloui*, *U. reynoldense* and *U. nigrotuberculatum* – were found to associate with the T-type

(Table 1), adding to previous findings of the T-type in the related North American species *Uroleucon ambrosiae*, *Uroleucon astronomus* and *Uroleucon rudbeckiae* (Sandström *et al.* 2001).

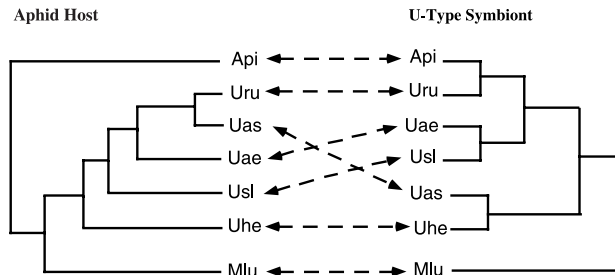
### Phylogenetic analysis

The 16S rDNA phylogeny of aphid- and psyllid-associated bacteria described here, plus their symbiotic and free-living relatives, is presented in Fig. 2. Several bacterial sequences obtained from aphids (and one from a psyllid) fell into one of three strongly supported clades termed R [parsimony (BS) = 72], T (parsimony BS = 100), and U (parsimony BS = 100). Within these clades, relationships were often unresolved because of high genetic similarity. As previously demonstrated (Sandström *et al.* 2001), the T- and U-types were sister clades (parsimony BS = 92), and members of the R-clade clustered with species of the genus *Serratia* (parsimony BS = 94).

Additionally, 16S rDNA sequences of aphid-associated bacteria obtained here fell into three other clades containing previously described insect endosymbionts (Fig. 2). First, bacteria from the distantly related aphids *Aphis spiraeicola*, a *Myzocallis* sp., and *Wahlgreniella nervata*, formed a strongly supported clade (parsimony BS = 100) which included *Arsenophonus triatominarum* (Hypsa & Dale 1997), a symbiont of the kissing bug *Triatoma infestans* (Hemiptera: Reduviidae), along with secondary symbionts of several psyllids (Thao *et al.* 2000a) and a whitefly (Spaulding & von Dohlen 2001). In our analysis, this clade was the sister taxon to the free-living bacterium *Proteus vulgaris*. We will refer to this symbiotic group as Ars – short for *Arsenophonus*, the genus name of the *T. infestans* symbiont and of the male-killing symbiont of *Nasonia vitripennis* (Gherna *et al.* 1991), to which our bacteria were also related (data not shown). Next, a bacterium associated with *Eulachnus pallidus* fell within a clade which included symbionts of tsetse flies, weevils and some psyllids (parsimony BS = 77). We will refer to this clade as So-So, short for *Sitophilus oryzae* Primary Endosymbiont (Heddi *et al.* 1999) and *Sodalis glossinidius* (Dale & Maudlin 1999) – names for two symbionts within this group. Finally, bacteria from *Pemphigus populi* and *Tetraneura ulmi* formed a monophyletic clade (parsimony BS = 100) nested within the clade including the T- and U-types (parsimony BS = 92). We will refer to these bacteria as the V-type. While we use the term symbiont when referring to newly described aphid associates of the Ars, So-So and V clades, we point out that further research on their transmission and localization is required for definitive proof and elucidation of their symbiotic lifestyles.

Symbiont 16S rDNA phylogenies conflicted with phylogenies of their hosts. For example, the T-type symbionts of several members of the genus *Uroleucon* were more closely related to a T-type symbiont from the psyllid *C. pyri*





**Fig. 3** Incongruency between host and symbiont trees reveals horizontal transfer of U-type symbionts of the Macrosiphini. The host phylogeny (left) is redrawn from Fig. 1(c) of Moran *et al.* 1999. The unrooted U-type symbiont phylogeny (right) was obtained through parsimony searches including only the U-type 16S rDNA sequences of the taxa shown here. Dashed arrows connect hosts to their respective symbionts (named after hosts), and suggest several inconsistencies. This host topology was one of just three possible alternatives because of a trichotomy involving (Uae) (Usl), and (Uru, Uas); the other topologies were also incongruent with that of the symbiont tree (not shown). Name abbreviations not presented in Table 1 are as follows: Mlu = *Macrosiphoniella ludoviciana*, Uhe = *Uroleucon helianthicola*, Usl = *Uroleucon solidaginis*, Uae = *Uroleucon aenum*, Uas = *Uroleucon astronomus*.

Finally, U-type 16S rDNA sequences evolved more quickly than did those of free-living bacteria, yet more slowly than *B. aphidicola* 16S rDNA.

Pairwise divergence analyses of symbiont and free-living bacteria revealed that secondary symbionts, of a given type, show high levels of sequence similarity (table available from corresponding author). Within the R-, T- and U-clades, all compared sequences were less than 0.5, 1.6 and 2.2% divergent, respectively. Strikingly, T-type symbionts in the psyllid *C. pyri* and several Nearctic aphids of the subgenus *Uroleucon* were identical over 1301 compared nucleotides. The U-clade was the most genetically diverse, with an average of 1.2% 16S rDNA divergence between symbionts in different host species compared to 0.4% for the T-type.

#### Pairwise divergence comparisons

Comparisons between the 16S rDNA distances of *B. aphidicola* and secondary symbionts infecting pairs of hosts sharing the same most recent common ancestor are presented in Table 4. In all but one case, the divergences between *B. aphidicola* sequences were at least twice as large as those between secondary symbionts of equidistant hosts. Only 4/12 comparisons were consistent with the possibility of strict secondary symbiont co-speciation, under the assumption that 16S rDNA substitution rates should not differ by more than four-fold: the U-type infections in *Pemphigus betae* and *Macrosiphoniella ludoviciana* (*Pemphigini* vs. *Macrosiphini*), *Chaitophorus populeti* and *M. ludoviciana* (*Chaitophorini* vs. *Macrosiphini*), and *A. pisum* and *U. astronomus* (*Acyrtosiphon* vs. *Uroleucon*); and the R-type

**Table 3** Relative rates tests on the 16S rRNA genes of secondary symbionts

Taxon 1	Taxon 2	$K_{13}^*$	$K_{23}^*$	$K_{13}-K_{23} \pm SD$
R-type	<i>E. coli</i>	0.0963	0.0985	-0.0023 $\pm$ 0.0075
R-type	U-type	0.0963	0.1233	-0.0271 $\pm$ 0.0087†
R-type	<i>Serratia</i>	0.0963	0.1001	-0.0039 $\pm$ 0.0048
R-type	T-type	0.0963	0.1265	-0.0302 $\pm$ 0.0084†
R-type	<i>B. aphidicola</i>	0.0963	0.1487	-0.0524 $\pm$ 0.0117†
<i>E. coli</i>	U-type	0.0985	0.1233	-0.0248 $\pm$ 0.0099†
<i>E. coli</i>	<i>Serratia</i>	0.0985	0.1001	-0.0016 $\pm$ 0.0070
<i>E. coli</i>	T-type	0.0985	0.1265	-0.0280 $\pm$ 0.0098†
<i>E. coli</i>	<i>B. aphidicola</i>	0.0985	0.1487	-0.0502 $\pm$ 0.0108†
<i>Serratia</i>	U-type	0.1001	0.1233	-0.0232 $\pm$ 0.0086†
U-type	T-type	0.1233	0.1265	-0.0031 $\pm$ 0.0083
U-type	<i>B. aphidicola</i>	0.1233	0.1487	-0.0254 $\pm$ 0.0115†
<i>Serratia</i>	T-type	0.1001	0.1265	-0.0263 $\pm$ 0.0086†
<i>Serratia</i>	<i>B. aphidicola</i>	0.1001	0.1487	-0.0486 $\pm$ 0.0112†
T-type	<i>B. aphidicola</i>	0.1265	0.1487	-0.0222 $\pm$ 0.0116

*B. aphidicola*, *Buchnera aphidicola*.

\* $K_{x3}$  = 16S rDNA distance between taxon *x* and the outgroup, taxon 3 (i.e. *Vibrio cholerae*).

†Difference is significant at  $\alpha = 0.05$ .

infection in *A. pisum* and *U. caligatum* (*Acyrtosiphon* vs. *Uroleucon*). Preliminary analyses suggested that many R-type 16S rDNA sequence divergences were inconsistent with co-speciation. For example, divergences between symbionts infecting hosts of different tribes were as low as 0.44% when compared over approximately 1000 base pairs. However, because these sequences were incomplete they were excluded from formal divergence analyses.

## Discussion

### Symbiont diversity

Previous studies that did not include DNA sequence information suggested that a number of aphid species harbour vertically transmitted facultative bacterial associates that reside in various host tissues (e.g. Buchner 1965; Fukatsu & Ishikawa 1998). Observations on their distributions revealed that they had been independently acquired by, and possibly lost from, aphid lineages. However, because the bacteria were not identified, no conclusions could be made regarding symbiont diversity, the phylogenetic breadth of the hosts infected by related microbes, or the likelihood of occasional co-speciation. Including the six groups of bacteria described here, aphids have been reported to associate with secondary symbionts spanning 10 distinct bacterial lineages (Unterman *et al.* 1989; Chen *et al.* 1996; Jeyaprakash & Hoy 2000; Darby *et al.* 2001; Fukatsu 2001; Fukatsu *et al.* 2001; Sandström *et al.* 2001). Given our serendipitous discovery of three

Host taxa	<i>B. aphidicola</i> 16S divergence*	Secondary 16S divergence†	Fold Difference
Pemphigini–Macrosiphini	0.0587 (Ppi-Mpe)‡	0.0188 (Pbe <sup>U</sup> -Mlu <sup>U</sup> )§	3.1
		0.0039 (Psp <sup>T</sup> -Api <sup>T</sup> )§	15.1
Pemphigini–Aphidini	0.0520 (Pbe-Sgr)‡	0.0031 (Psp <sup>T</sup> -Acr <sup>T</sup> )§	16.8
Chaitophorini–Macrosiphini	0.0572 (Cvi-Mpe)‡	0.0188 (Cpe <sup>U</sup> -Mlu <sup>U</sup> )	3.0
		0.0039 (Pbl <sup>T</sup> -Api <sup>T</sup> )§	14.7
Chaitophorini–Pemphigini	0.0692 (Cvi-Ppi)‡	0.0093 (Cpe <sup>U</sup> -Pbe <sup>U</sup> )	7.4
		0.0031 (Pbl <sup>T</sup> -Psp <sup>T</sup> )	22.3
Chaitophorini–Aphidini	0.0555 (Cvi-Rma)‡	0.0031 (Pbl <sup>T</sup> -Acr <sup>T</sup> )§	17.9
Aphidini–Macrosiphini	0.0180 (Rma-Mpe)‡	0.0039 (Acr <sup>T</sup> -Api <sup>T</sup> )§	4.6
<i>Acyrtosiphon–Uroleucon</i>	0.0200 (Api-Uso)	0.0062 (Api <sup>R</sup> -Uca <sup>R</sup> )	3.2
		0.0031 (Api <sup>T</sup> -Uam <sup>T</sup> )§	6.5
		0.0101 (Api <sup>U</sup> -Uas <sup>U</sup> )§	2.0

**Table 4** 16S rDNA divergences of *Buchnera aphidicola* vs. those of secondary symbionts for pairs of aphid hosts sharing same most recent common ancestor (see text for details)

\*Host species abbreviations not described in Table 1 or Fig. 3 are as follows: Sgr = *Schizaphis graminum*, Cvi = *Chaitophorus viminalis*, Rma = *Rhopalosiphum maidis*, Uso = *Uroleucon sonchi*, Uam = *Uroleucon ambrosiae* from Arizona, Uca = *Uroleucon caligatum*.

†The secondary symbiont types (i.e. R, T, or U) for which divergence values are presented are listed next to their host abbreviation codes.

‡The smallest distance between *B. aphidicola* infecting members of these taxa is presented here.

§The largest distance between secondary symbionts infecting members of these taxa is presented here.

new aphid associates, it is likely that a substantial number of the microbial guests residing in aphids have yet to be described.

Research on other members of the Sternorrhyncha has revealed similar patterns of symbiont diversity. For example, the psyllid *Diaphorina citri*, was found to associate with five distinct bacteria, which were occasionally all found to reside within the same host individuals (Subandiyah *et al.* 2000). In addition, at least four distinct bacterial symbionts have been found in whiteflies (Clark *et al.* 1992; Costa *et al.* 1995; Jeyaprakash & Hoy 2000; Spaulding & von Dohlen 2001), and at least five infect scale insects (Fukatsu & Nikoh 2000; Thao *et al.* 2002). Thus, these related plant sap feeders are common habitats for a number of independently derived symbionts.

#### Symbiont transmission

Within laboratory colonies, aphid secondary symbionts undergo highly efficient vertical transmission with no observed instances of horizontal transfer (Chen & Purcell 1997; Sandström *et al.* 2001). These observations suggest that vertical transfer plays a large role in the persistence of symbionts within populations. Despite this importance, our examination of a wide range of host species provides no definitive evidence for co-speciation, indicating that horizontal transfer is, in large part, responsible for R-, T- and U-type distributions across species. Given our observations of low 16S rDNA divergences between symbionts in different hosts, it appears that a number of horizontal transfer events occurred recently, with T-type

symbionts having undergone an especially recent radiation onto their hosts. Thus, interspecies movement is probably an ongoing process for these bacteria.

Evidence similar to that presented here has been used to argue that a number of facultative symbionts, including *Wolbachia pipientis*, *Sodalis glossinidius*, and several psyllid associates, have been independently acquired by different host lineages (O'Neill *et al.* 1992; Werren *et al.* 1995; Aksoy *et al.* 1997; Thao *et al.* 2000a). Despite this evidence, the means by which facultative symbionts move between arthropod species remain obscure. Some researchers have proposed a role of transmission through host plants (Darby *et al.* 2001). Given the similarity of symbionts in aphids and ladybird beetles (Werren *et al.* 1994; Chen *et al.* 1996; Majerus *et al.* 1999; Fukatsu *et al.* 2001) it is tempting to propose the involvement of predator–prey interactions. Several investigations have found similar *W. pipientis* in hosts and their parasites, suggesting possible interspecific transmission through host–parasite interactions (Vavre *et al.* 1999; Cordaux *et al.* 2001; Noda *et al.* 2001). Accordingly, experimental studies have documented the transfer of *W. pipientis* between a host and its parasitoid and between conspecific parasitoids infecting the same host (Heath *et al.* 1999; Huigens *et al.* 2000). However, there is still no evidence of a means by which nonparasitoids can acquire symbionts. We note that even rare transfer events may be sufficient to explain the observed patterns.

In contrast to patterns suggesting interspecific horizontal transfer of secondary symbionts, several of our observations are suggestive of symbionts persisting within a single host species. These cases do not necessarily imply

strict vertical transmission; they could, rather, reflect horizontal transfer between only conspecifics. Overall, the phylogenetic and genetic similarities of symbionts in a single species would not be expected if rampant interspecific horizontal transfer was common. We therefore take these observations to indicate the existence of impediments to such lateral movement. Such barriers could include limited opportunities for interspecific transfer, limited host ranges influenced by survival in, and transmission to offspring, a new host, or frequent extinction of newly infected lineages as a result of maladaptive phenotypic effects caused by symbionts. Thus, despite the apparent recency of some horizontal transfer events apparently being recent, the persistence of symbionts within host species suggests that intraspecific transfer is more common than interspecific movement, enhancing opportunities for one-to-one coevolution between aphids and secondary symbionts.

### Symbiont distributions

Based on our observations of R-, T-, and U-type symbiont distributions across multiple aphid tribes and beyond, we can rule out the possibility that these clades are specialized on hosts within the tribe Macrosiphini, or that T-type symbionts are confined to the Sternorrhyncha. However, the rarity of these bacteria within the Psylloidea (relative to the Aphidoidea) suggests that the R-, T- and U-type symbionts may be a more significant component of aphid biology.

Several other facultative symbionts of arthropods have been found to infect even more phylogenetically diverse ranges of host species. For example, closely related *W. pipientis* symbionts have been found in species spanning the Arthropoda (Jeyaprakash & Hoy 2000; Werren & Windsor 2000) and in nematodes (Sironi *et al.* 1995). Closely related symbionts (over 98% 16S rDNA identity) of the genus *Spiroplasma* have been found in hosts of the insect orders Coleoptera (Majerus *et al.* 1999), Lepidoptera (Jiggins *et al.* 2000), Hemiptera (Fukatsu *et al.* 2001), as well as in ticks (Weisburg *et al.* 1989). Also, *Rickettsia* with over 99% 16S rDNA similarity infect beetles (Werren *et al.* 1994), aphids (Chen *et al.* 1996), and ticks (Philip *et al.* 1983). These observations indicate that transfer among distant host taxa is a common feature of facultative symbioses between bacteria and arthropods and suggest that some symbiont lineages retain a generalized ability to live in distant host species. Alternatively, gene acquisition by symbionts may permit the colonization of novel hosts; such acquisition could be mediated by bacteriophage that are known to associate with secondary symbionts of aphids (van der Wilk *et al.* 1999; Sandström *et al.* 2001). In contrast to these observations, high genetic similarity of symbionts within *Acyrtosiphon pisum* and within *Macrosiphum euphorbiae* suggests that some symbionts are adapted to a lifestyle

within their current hosts, lowering their potential for interspecific horizontal transfer. To address questions regarding the potential host ranges of secondary symbionts we propose an experimental approach involving microinjection of symbionts into novel host species.

### History of associations

The overall lack of phylogenetic congruency and the disparity in the divergences of secondary symbionts and obligate symbionts, as shown here, suggest that associations between specific hosts and their current secondary symbionts are quite young. While high genetic similarity between R-type symbionts and free-living *Serratia* (i.e. 2.3% divergence between the *Aphis craccivora* R-type and *Serratia entomophila* over 1135 nucleotides), suggests a recent origin of symbiosis, the story for the T- and U-types is more complicated. Given that T-type 16S rRNA substitution rates are similar to those of *Buchnera aphidicola* (Table 3), applying the rate calibrated for *B. aphidicola* (i.e. 2–4% substitution per hundred million years, Moran *et al.* 1993) suggests that those described in this study shared a common ancestor no more than 20–40 million years ago (based on the maximum divergence, of 1.6%, between the T-types of *Bemisia tabaci* and *Chilomenes sexmaculatus*). However, the monophyly of the T-, U- and V-types (Fig. 2) suggests that the age of symbiosis in this clade of microbes is ancient – average sequence divergence between T- and U-types was around 8%, revealing a minimum age of 100 million years. Also, the elevated substitution rates of T- and U-type bacteria, vs. those of free-living bacteria, are consistent with a long history of symbiosis. To determine more conclusively whether the ancestor of these bacteria was a symbiont, extensive screening of nonsymbiotic environments for bacteria in this clade would be necessary. It would also be useful to determine whether the similar lifestyles of T-, U- and, possibly, V-type symbionts rely on homologous pathways and whether the genes for these pathways were present in their common ancestor.

### Importance of facultative symbiosis

Only a few attempts have been made to estimate phenotypic effects associated with infection by secondary symbionts, and each of these has focused on symbionts of the pea aphid, *A. pisum*. Two bacteria, the R-type and symbionts of the genus *Spiroplasma*, have been shown to depress host fitness slightly under certain environmental conditions (Chen *et al.* 2000; Fukatsu *et al.* 2001). Yet, the R-type symbiont confers benefits at high temperatures (Chen *et al.* 2000; Montllor *et al.* 2002), possibly expanding the range of niches available to infected *A. pisum*. Furthermore, a recent study suggests that single isolates of

R- and T-type symbionts confer resistance to parasitism by the hymenopteran parasitoid, *Aphidius ervi* (Oliver *et al.* in press).

Several studies have revealed a high frequency of secondary symbionts within aphid populations. For example, the T-type was found in 40/40 *Uroleucon ambrosiae* collected from various regions throughout the USA (Sandström *et al.* 2001), while another  $\gamma$ -Proteobacterial symbiont, known as YSMS, was found to infect 100% of surveyed individuals belonging to two species within the aphid genus *Yamatocallis* (Fukatsu 2001). In addition, a survey of California *A. pisum* populations described the R-type in 50/57 individuals (Chen & Purcell 1997), while the U-type symbiont was found in 219/858 individuals surveyed across Japan (Tsuchida *et al.* 2002). Given the observations on their prevalence within, and incidence across, species along with their significant effects on host fitness in a variety of ecological and environmental contexts, we conclude that facultative symbionts have probably played a significant role in the ecology and evolution of aphids.

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