Co-obligate symbioses have repeatedly evolved across aphids, but partner identity and nutritional contributions vary across lineages

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ABSTRACT

Aphids are a large family of phloem-sap feeders. They typically rely on a single bacterial endosymbiont, *Buchnera aphidicola*, to supply them with essential nutrients lacking in their diet. This association with *Buchnera* was described in model aphid species from the Aphidinae subfamily and has been assumed to be representative of most aphids. However, in two lineages, *Buchnera* has lost some essential symbiotic functions and is now complemented by additional symbionts. Though these cases break our view of aphids harbouring a single obligate endosymbiont, we know little about the extent, nature, and evolution of these associations across aphid subfamilies. Here, using 16S rRNA amplicon sequencing on 223 aphid samples (147 species from 12 subfamilies) and metagenomics on 25 aphid species from nine subfamilies, we show that dual symbioses have evolved anew at least six times. We also show that these co-obligate symbionts have typically evolved from facultative symbiotic taxa. Genome-based metabolic inference confirms interdependencies between *Buchnera* and its partners for the production of essential nutrients but shows contributions vary across pairs of co-obligate associates. Lastly, fluorescent *in situ* hybridisation microscopy shows a common bacteriocyte localisation of newly acquired symbionts. Lastly, patterns of *Buchnera* genome evolution reveal that small genome reductions affecting a few key genes can be the onset of these dual systems, while large gene losses can occur without any co-obligate symbiont acquisition. Hence, the once thought exclusive *Buchnera*-aphid association appears fragile, with a few metabolic losses having recurrently promoted the establishment of a new co-obligate symbiotic partner.

Keywords: symbiont replacement, metabolic complementarity, aphid, nutritional symbiosis, co-obligate symbiont, *Buchnera*.

Introduction

Nutritional symbioses between animals and microorganisms are widespread in nature, and are particularly present in hemipteran insects with a nutrient-restricted diet. Aphids (Hemiptera: Apdididae) are a family of around 5,200 species organised into 23 extant subfamilies (Favret, 2022). Their diet consists entirely of plant phloem, which is rich in sugars, but poor in essential amino acids and B vitamins (Douglas, 2006; Sandstrom and Moran, 1999; Ziegler, 1975). In order to overcome this limitation, aphids are associated to an obligate vertically-transmitted endosymbiotic bacteria, *Buchnera aphidicola* that synthesises nutrients lacking in their diet, namely essential amino acids (EAAs) and B vitamins (Bermingham *et al.*, 2009; Blow *et al.*, 2020; Douglas, 1998; Hansen and Moran, 2011). The long-term association of *Buchnera* and aphids is evidenced by the high degree of *Buchnera* genome synteny and general congruence of aphid and symbiont phylogenies (Baumann *et al.*, 1995; Funk *et al.*, 2000; Jousselin *et al.*, 2009; Nováková *et al.*, 2013; van Ham *et al.*, 2003). As a result of this ancient association, *Buchnera* strains have evolved highly reduced, AT-rich, and gene-dense genomes (Chong and Moran, 2018). While genome erosion has affected many functional categories, extant *Buchnera* strains have markedly retained genes involved in the biosynthesis of essential amino acids and B vitamins. However intimate the aphid-*Buchnera* symbiosis is, long-lived associations can break down, leading to symbiont replacement or complementation.

While ancient symbiont replacement and complementation has been well documented in hemipteran taxa such as the Auchenorrhyncha (*e.g.* cicadas, treehoppers, leafhoppers, and planthoppers; Koga and Moran, 2014; Łukasik *et al.*, 2018; Matsuura *et al.*, 2018; Michalik *et al.*, 2021), Pseudococcinae (mealybugs; Husnik and McCutcheon, 2016; Szabó *et al.*, 2017), Adelgidae (adelgids; Dial *et al.*, 2022; Szabó *et al.*, 2022; Toenshoff *et al.*, 2012; Weglarz *et al.*, 2018), and Psylloidea (psyllids; Nakabachi *et al.*, 2013, 2020; Sloan and Moran, 2012), these associations have not been widely reported in aphids. The mutualism between aphids and *Buchnera* has generally been seen as stable and quite exclusive. However, most of our knowledge on this symbiosis comes from one aphid lineage: the Aphidinae subfamily. This subfamily encompasses about 3,150 species (around 60% of aphid diversity) including most aphid pests, and thus, has been the one that has been over-studied. Yet, microbial metagenomic data from aphids outside Aphidinae is revealing that the aphid/*Buchnera* relationship might not as stable as previously thought. In *Geopemphigus* and within Cerataphidini aphids, *Buchnera* has been replaced by *Bacteroidota* (Chong and Moran, 2018) and yeast-like symbionts (Fukatsu and Ishikawa, 1992; Fukatsu *et al.*, 1994), respectively. These new symbionts have taken over the nutrient provisioning role of the now defunct *Buchnera* (Chong and Moran, 2018; Vogel and Moran, 2013), allowing the aphids to continue thriving on their nutrient-deficient diet. In addition to symbiont replacement, symbiont complementation can arise if a co-existing microbe has the metabolic capacity to rescue or take over one or more of the roles of the pre-existing associate. Such co-obligate symbioses have indeed arose in at least two aphid groups: the Lachninae (Manzano-Marín *et al.*, 2017) and the *Periphyllus* genus (Chaitophorinae: Chaitophorini) (Monnin *et al.*, 2020; Renoz *et al.*, 2022a). These co-obligate symbionts now complement evolved auxotrophies of their corresponding *Buchnera* partners, most commonly those for biotin and riboflavin

and less often those for tryptophan and histidine.

In the remaining aphid subfamilies, there is no genomic evidence for the occurrence of multi-partner symbioses. However, a series of microscopic studies have revealed co-occurring bacteriocyte-associated bacteria outside of the Lachninae subfamily and *Periphyllus* genus which show co-obligate like characteristics. Early microscopic evidence showed co-existing bacteria in separate bacteriocytes to those of *Buchnera* in the aphids *Drepanosiphum* sp. (Drepanosiphinae), *Periphyllus testudinaceus* (Chaitophorinae: Chaitophorini), and *Panaphis juglandis* (Calaphidinae: Panaphidini) (Buchner, 1953). More recent work has also shown co-obligate like organisms in *Yamatocallis* spp. (Drepanosiphinae) (Fukatsu, 2001; Fukatsu and Ishikawa, 1993), *Sipha maydis* (Chaitophorinae: Siphini), *Anoecia corni* (Anoeciinae), and *Glyphina betulae* (Thelaxinae) (Kot, 2012; Michalik, 2010; Michalik *et al.*, 2014). In most above-mentioned cases, the co-obligate symbionts are not only inhabiting their own bacteriocytes, but also show a spherical cell shape, which is characteristic of many obligate symbionts of aphids and other insects with drastically reduced genomes (Dial *et al.*, 2022; Lamelas *et al.*, 2008, 2011b; Manzano-Marín *et al.*, 2016, 2017, 2020; Michalik *et al.*, 2021; Szabó *et al.*, 2022; Toenshoff *et al.*, 2012, 2014).

In this work we sought to explore the extent, nature, identity, and metabolic capabilities of nutritional endosymbiotic consortia across aphids. For this purpose, we assembled the most comprehensive and diverse set of aphid symbionts to date. This dataset included 25 newly sequenced symbiont genomes as well as 20 re-assembled and/or re-annotated previously sequenced ones. Through high-throughput 16S rRNA gene amplicon sequencing of 147 species of aphids, we were able to corroborate the fixed status of these symbionts in their respective hosts. We additionally explored whether new obligate symbionts necessarily emerge from frequent symbionts in the microbiota. In addition, through genome-based metabolic inference we explored the co-dependency of the co-existing symbionts and collaboration for the production of their hosts' essential nutrients. Through phylogenetic analyses we investigated the origin of co-obligate symbiont lineages. Lastly, using of Fluorescence *in situ* hybridisation (FISH), we corroborate the identity and bacteriocyte-specific localisation of distantly-related co-obligate symbionts in selected aphid species.

Materials and Methods

Aphid collection and sequencing

Twenty-five different species of aphids were sourced from the *CBGP - Continental Arthropod Collection* (Centre de Biologie pour la Gestion des Populations, 2018; supplementary table S1, Supplementary Material online), where the specimens were preserved in ethanol 70% at 6°C until extraction. In this collection, a specimen (with its unique voucher) corresponds to individuals from a single aphid colony. Bacteria-enriched DNA extractions were performed following Jousselin *et al.* (2016). When possible, 10-15 individuals were used for extraction.

For genomic sequencing, extracted DNA was used to prepare two custom paired-end libraries in Genoscope. Briefly, 5ng of genomic DNA were sonicated using the E220 Covaris instrument (Covaris, USA). Fragments were end-repaired, 3'-adenylated, and NEXTflex PCR free barcodes adapters (Bioo Scientific, USA) were added by using NEBNext Ultra II DNA library prep kit for Illumina (New England Biolabs, USA). Ligation products were purified by Ampure XP (Beckman Coulter, USA) and DNA fragments (>200bp) were PCR- amplified (2 PCR reactions, 12 cycles) using Illumina adapter-specific primers and NEBNext Ultra II Q5 Master Mix (NEB). After library profile analysis by Agilent 2100 Bioanalyser (Agilent Technologies, USA) and qPCR quantification using the KAPA Library Quantification Kit for Illumina Libraries (Kapa Biosystems, USA), the libraries were sequenced using 251 bp paired-end reads chemistry on a HiSeq2500 Illumina sequencer. In addition, Nanopore sequencing was done for the aphid species *Anoecia corni*. For long-read sequencing, the Rapid Low Input by PCR Sequencing Kit (SQK_RLI001) was used. The library was sequenced on an R9.4 flow cell. In addition to the newly produced sequencing reads, data for an additional 19 species was downloaded from the NCBI's Short Read Archive (supplementary table S2, Supplementary Material online).

For amplicon sequencing, we investigated symbiont diversity in 223 aphid samples, comprised of 147 species of 75 genera belonging to 12 subfamilies sourced from the aforementioned Aphididae collection. These species included those used for symbiont genome data as well as species closely related to them. DNA was extracted from single individuals and a 251bp portion of the V4 region of the 16S rRNA gene was chose for amplification following Mizrahi-Man *et al.* (2013). The 16S fragment was amplified using primers 16S-V4F (5'-GTGCCAGCMGCCGCGGTAA-3') and 16S-V4R (5'-GGACTACHVGGGTWTCTAAT-3') following the dual-index sequencing strategy developed by Kozich *et al.* (2013) and the protocol described in Jousselin *et al.* (2016). Each DNA extract was amplified twice along with negative controls (DNA extraction and PCR controls) using distinct 96-well microplates for PCR replicates. We obtained a total of 485 PCR products, which were pooled together and subjected to gel electrophoresis. The bands corresponding to the PCR products were excised from the gel, purified with a PCR clean-up and gel extraction kit (Macherev-Nagel) and quantified with the Kapa Library Quantification Kit (Kapa Biosystems). The DNA pool was then paired-end sequenced on an Illumina MiSeq flowcell with a 500-cycle Reagent Kit v2 (Illumina).

16S amplicon sequencing and analysis

16S amplicon sequences were first filtered through Illumina's quality control procedure. We then used a pre-processing script from Sow *et al.* (2019) which uses FLASH v1.2.11 (Magoč and Salzberg, 2011) and CUTADAPT v1.9.1 (Martin, 2011) to merge paired sequences into contigs and trim primers, respectively. We then used the FROGS pipeline (Escudié *et al.*, 2018) to generate an abundance table of symbiont lineages across samples. Briefly, to generate the tables, we first filtered out sequences >261 and <241 bp. We then clustered variants with Swarm (Mahé *et al.*, 2014) using a maximum aggregation distance of 1. Lastly, we identified and removed chimeric variants with VSEARCH (Rognes *et al.*, 2016).

Taxonomic assignments of clusters was carried out using RDPtools v2.0.3 (https://github.com/rdpstaff-/RDPTools, last accessed July 18, 2022; Cole *et al.*, 2014) and BLASTn+ (Camacho *et al.*, 2009) against the Silva database release 138 (Quast *et al.*, 2013) as implemented in FROGS. Following taxonomic affiliation, we aggregated clusters when they shared the same taxonomy with at least 98% of identity (FROGS' affiliation_postprocess step). From the abundance table of clusters across samples, we transformed read numbers per aphid samples into percentages and sequences accounting for <0.5% of all the reads for a given sample were excluded using an R script following Jousselin *et al.* (2016). Clusters were kept only if present in both PCR replicates of the same sample. For final description of endosymbiont diversity we only kept the PCR replicate that yielded the highest number of reads. We refined ambiguous taxonomic assignations using BLASTn+ against 16S rRNA gene sequences extracted from whole endosymbiont genomes from this study and retrieved from GenBank. We also used the webtool leBIBI IV (https://umr5558-proka.univ-lyon1.fr/lebibi/lebibi.cgi, last accessed July 18, 2022) that provides automatic phylogenetic placement of bacterial 16S sequences (Flandrois *et al.*, 2015). The resulting relative abundance table (supplementary table S3, Supplementary Material online) was used to produced a heatmap in R of presence/absence of endosymbiotic taxa across aphid subfamilies.

Genome assembly

For all Illumina datasets, reads were right-tail clipped (using a minimum quality threshold of 20) using FASTX-Toolkit v0.0.14 (http://hannonlab.cshl.edu/fastx_toolkit/, last accessed July 18, 2022) and those shorter than 75 after were dropped. PRINSEQ v0.20.4 (Schmieder and Edwards, 2011) was used to remove reads containing undefined nucleotides as well as those left without a pair after the filtering and clipping process. Clean reads were assembled using SPAdes v3.11.1 (Bankevich *et al.*, 2012) with the --only-assembler option and k-mer sizes of 33, 55, 77, 99, and 127 (depending on the read length). Assembled contigs shorter than 200 bps were dropped. The surviving contigs were binned using results from a BLASTx (Altschul *et al.*, 1997) search (best hit per contig) against a database consisting of the proteomes of the Pea aphid and a selection of aphid's symbiotic and expected free-living bacteria (supplementary table S4, Supplementary Material online). When no genome was available for a certain lineage, closely related bacteria were used.

The binned contigs were manually screened using the BLASTx web server (*vs.* the nr database) to insure correct taxonomic assignment. The resulting contigs were then used as reference for read-mapping and individual genome re-assembly using SPAdes, as described above. Resulting contigs were checked for circularisation through assembly graphs. For those *Buchnera* strains that have evolved a low-complexity replication start, we closed the ends with "N" stretches after checking for the conserved proteins around the end and truncation of low-complexity sequencing artefacts. Details of any sequence modification post-assembly is captured in the GenBank-formatted annotation files.

Genome annotation

The resulting *Buchnera* genomes were annotated as follows. First, open reading frame (ORF) prediction was done using Prokka v1.14.6 (Seemann, 2014). This ORF prediction was followed by non-coding RNA prediction with infernal v1.1.2 (Nawrocki and Eddy, 2013) (against the Rfam v14.1 database; Kalvari *et al.*, 2021), tRNAscan-SE v2.0.9 (Chan *et al.*, 2021), and ARAGORN v1.2.36 (Laslett, 2004). This annotation was followed by careful manual curation of the genes on UGENE v37.1 (Okonechnikov *et al.*, 2012) through on-line BLASTx searches of the intergenic regions as well as through BLASTp and DELTA-BLAST (Boratyn *et al.*, 2012) searches of the predicted ORFs against NCBI's nr database. The resulting coding sequences (CDSs) were considered to be putatively functional if all essential domains for the function were found or if a literature search supported the truncated version of the protein as functional in a related organism, or if the the CDS displayed truncations but retained identifiable domains (details of the literature captured in the annotation file). Pseudogenes were further searched for based on synteny with closely related *Buchnera* strains on which previous manual curation was done. This prediction was performed based on nucleotide sequence using a combination of sequence alignment (with m-coffee; Wallace et al. 2006) and BLASTx searches against the NCBI's nr database. This last check allowed for the identification of pseudogenes missed by the previous searches. Once a *Buchnera* was manually curated, it was used as a Prokka reference protein set for the next *Buchnera* in order to maintain naming congruent across genomes. Following experimental evidence presented in Tamas *et al.* (2008), genes interrupted by a frameshift in a low complexity A- or T-homopolymeric region, were annotated as coding for a functional protein, with special attention to conservation of frameshifted region and amino acid sequence within closely related *Buchnera* strains.

For the genomes of co-obligate symbionts, draft Prokka annotations were performed and genes of interest to metabolic complementarity for nutrient provisioning underwent a manual curation as described above. For genome statistics, an estimated count for pseudogene features was done as follows. Contiguous proteins detected as truncated and with same assignment as well as proteins under 100 amino acids, were considered pseudogenes. tRNAs, ncRNAs, and rRNAs were predicted as described above.

Phylogenetics

In order to reconstruct a phylogenetic hypothesis for *Buchnera*, we used OrthoMCL v2.0.9 (Chen *et al.*, 2007; Li, 2003) to build clusters of orthologous proteins. To the newly acquired, reassembled, and re-annotated symbiont genomes; we added three already annotated ones from Lachninae (supplementary table S2, Supplementary Material online). These orthologous groups were then manually curated using the annotation to keep same genes together in clusters. *Escherichia coli* K-12 MG 1655 and a selection of *Pantoea* and *Eriwnia* strains were used as outgroups (supplementary table S5, Supplementary Material online). We then retrieved the single copy-core proteins of the selected genomes for phylogenetic reconstruction. We aligned the single-copy core protein sets, gene by gene, using MAFFT v7.453 (Katoh and Standley, 2013) (L-INS-i algorithm). Divergent and ambiguously aligned blocks were removed

using Gblocks v0.91b (Talavera and Castresana, 2007). The resulting alignments were concatenated for phylogenetic inference. Bayesian inference was performed in MrBayes v3.2.7a (Ronquist *et al.*, 2012) running two independent analyses with four chains each for up to 300,000 generations and checked for convergence with a burn-in of 25%. JModelTest v2.1.10 (Darriba *et al.*, 2012) was used to select the best model for phylogenetic reconstruction based on the Akaike's Information Criterion (cpREV+I+G4).

To analyse the phylogenetic relations of newly sequenced aphid co-obligate endosymbionts, we built two phylogenies. We first reconstructed a phylogeny for *Yersinia*- and *Serratia*-related endosymbionts. For this, we used the dataset built by (Rouïl *et al.*, 2020) and added data for *Serratia symbiotica* symbionts and the newly sequenced symbiotic strains. Given that many *Se. symbiotica* genomes lacked an intact *hrpA*, this gene was excluded from our dataset. Each gene set was individually aligned using MUSCLE v3.8.31 (Edgar, 2004). Then, we removed divergent and ambiguously aligned blocks with Gblocks. Bayesian inference was performed in MrBayes, using the GTR+I+G4 substitution model running two independent analyses with four chains each for 1,000,000 generations and checked for convergence. The second phylogeny was reconstructed for *Sodalis*-like endosymbionts. Given that many of these endosymbionts remain without a sequenced genome, we used 16S rRNA gene sequences following (Manzano-Marín *et al.*, 2017). We used SSU-ALIGN v0.1 (Nawrocki, 2009) to align the rRNA gene sequences. GBlocks v0.91b was then used to eliminate poorly aligned positions and divergent regions with the option '-b5=h' to allow half of the positions with a gap. MrBayes v3.2.7 was used for phylogenetic inference under the GTR+I+G4 model running two independent analyses with four chains each for up to 10,000,000 generations and checked for convergence, discarding the first 25% as burn-in. Visualization and tree-editing for all analyses was done in FigTree v1.4.1 (http://tree.bio.ed.ac.uk/software/figtree/, last accessed July 18, 2022).

Fluoresence *in situ* hybridisation microscopy

Live aphid specimens from *Sipha maydis* and *Anoecia corni* were fixed in modified Carnoy's fixative (6 chloroform: 3 absolute ethanol: 1 glacial acetic acid) and left overnight, following the protocol of (Koga *et al.*, 2009). Individuals were then dissected in absolute ethanol to extract embryos and transferred into a 6% solution of H_2O_2 diluted in absolute ethanol and were then left in this solution for two weeks (changing the solution every three days). Embryos were then washed twice with absolute ethanol. Hybridization was performed overnight at 28°C in standard hybridisation buffer (20 mM Tris-HCl [pH 8.0], 0.9M NaCl, 0.01% SDS, and 30% formamide) and then washed (20 mM Tris-HCl [pH 8.0], 5 mM EDTA, 0.1M NaCl, and 0.01% SDS) before slide preparation. Competitive probes were adapted for this specific symbionts from (Manzano-Marín *et al.*, 2017) (supplementary table S6, Supplementary Material online).

All files relating to orthologous protein grouping as well as phylogenetic reconstructions can be found at https://doi.org/10.5281/zenodo.6394198.

Results

Buchnera genome has repeatedly undergone further genome reduction

In order to reconstruct a comprehensive phylogeny of *Buchnera aphidicola* (hereafter *Buchnera*) to aid in our evolutionary interpretations, we assembled a genomic dataset of 48 strains housed by different aphid species (table 1). This dataset represents aphid symbionts from 13 different subfamilies, including the most speciose ones, and is, to our knowledge, the largest and most diverse dataset assembled for *Buchnera*.

Aphid taxonomy				*Buchnera*			
subfamily	tribe	species	source	strain	gnm	CDSs	GC
Eriosomatinae	Pemphigini	*Pemphigus immunis*	**NS**	3699	598	493	24.11
		Pemphigus populi	**NS**	3700	611	485	24.78
Aphidinae	Macrosiphini	*Cavariella theobaldi*	**NS**	2779	587	515	25.87
Aphidinae	Macrosiphini	*Muscaphis stroyani*	RAs	Mst	619	558	25.75
Aphidinae	Aphidini	*Hyalopterus amygdali*	**NS**	3475	632	583	25.04
		Rhopalosiphum padi	RAs	Rpa	644	592	25.23
		Protaphis terricola	**NS**	2569	607	564	22.71
		Aphis nasturtii	RAs	Ana	630	583	24.85
		Aphis nerii	RAs	Ane	631	586	24.16
	Macrosiphini	*Brevicoryne brassicae*	RAs	Bbr	643	590	25.03
		Hyadaphis tataricae	RAs	Hta	634	579	27.02
		Brachycaudus tragopogonis	**NS**	B_tra	643	591	25.65
		Artemisaphis artemisicola	RAs	Aar	633	576	24.54
		Hyperomyzus lactucae	RAs	Hla	644	590	26.06
		Sitobion avenae	RAs	Sav	636	572	25.96
		Acyrthosiphum lactucae	RAs	Ala	642	572	24.47
		Macrosiphum gaurae	RAs	Mga	644	581	25.86
Eriosomatinae	Fordini	*Baizongia pistaciae*	Ran	Bp	616	511	25.34
		Melaphis rhois	RAs	Mrh	624	533	25.62
Eriosomatinae	Eriosomatini	*Tetraneura ulmi*	**NS**	3693	531	465	22.72
		Eriosoma grossulariae	**NS**	3692	607	501	22.44
		Eriosoma lanigerum	**NS**	2421	627	502	22.46
Mindarinae	-	*Mindarus abietinus*	**NS**	3704	540	478	23.24
Neophyllaphidinae	-	*Neophyllaphis podocarpi*	**NS**	3733	559	512	22.33
Hormaphidinae	Hormaphidini	*Hormaphis cornu*	RAs	80	630	453	23.80
	Nipponaphidini	*Nipponaphis monzeni*	RAs	Nmo	588	450	22.34
Anoeciinae	-	*Anoecia corni*	**NS**	2928	551	437	23.13
		Anoecia oenotherae	RAs	Aoe	549	437	22.71
Thelaxinae	Thelaxini	*Thelaxes californica*	RAs	Tca	523	455	22.55
		Thelaxes suberi	**NS**	3717	530	472	23.33

Lachninae	Tuberolachnini	*Tuberolachnus salignus*	PSA	BTs	421	382	21.60
	Eulachnini	*Cinara splendens*	PSA	3004	445	378	23.62
		Cinara strobi	PSA	3249	440	376	23.90
Drepanosiphinae	-	*Drepanosiphum platanoidis*	**NS**	3702	449	378	18.37
Chaitophorinae	Siphini	*Chaetosiphella stipae*	**NS**	2659	462	402	21.63
		Sipha maydis	**NS**	3493	462	409	21.96
	Chaitophorini	*Periphyllus testudinaceus*	**NS**	2671	458	415	19.64
		Chaitophorus sp.	**NS**	3695	472	413	20.29
		Chaitophorus populicola	**NS**	3609	474	418	20.53
Calaphidinae	Calaphidini	*Symydobius americanus*	**NS**	3610	453	409	24.39
Phyllaphidinae	-	*Phyllaphis fagi*	**NS**	3703	431	369	22.73
		Stegophylla sp.	RAs	Ssp	413	349	22.99
Calaphidinae	Pterocallidini	*Pterocallis alni*	**NS**	3691	422	381	23.58
	Myzocallidini	*Myzocallis carpini*	**NS**	3696	442	394	23.83
	Panaphidini	*Panaphis juglandis*	**NS**	2786	434	389	23.76
	Therioaphidini	*Therioaphis trifolii*	RAs	Tma	420	390	20.20
		Sarucallis kahawaluokalani	RAs	Ska	429	389	24.80
		Takecallis arundicolens	**NS**	3697	435	399	24.53

Table 1. Characteristics of analysed *Buchnera* genomes. * Taxonomic status uncertain. NS="Newly sequenced", RAs="Re-assembled", RAn="Re-annotated", PSA="Previously sequenced and annotated". In bold, newly sequenced strains are highlighted. chr="chromosomal size" (rounded in Mbp), CDSs="Coding sequences", GC="G+C content" (rounded to two significant digits). Taxa have been organised to match the order of the displayed *Buchnera* phylogeny in figure 1.

After thorough manual curation of the *Buchnera* genomes, we extracted the single-copy core proteins and used them to reconstruct a concatenated protein phylogeny for *Buchnera* (figure 1). As previous works (Chen *et al.*, 2017; Li *et al.*, 2014; Nováková *et al.*, 2013; Ortiz-Rivas and Martínez-Torres, 2010), we found support for the non-monophyly of the Eriosomatinae subfamily. Also, the symbionts of both sequenced Phyllaphidinae species were recovered nested within those of Calaphidinae, which corroborates previous works recovering members of this subfamily as closely related to species or tribes of Calaphidinae (Nováková *et al.*, 2013; von Dohlen and Moran, 2000).

The phylogenetic tree, when combined with the genomic characteristics of Table 1, shows that the genome of *Buchnera* has undergone multiple events of genome reduction after diversification. The largest *Buchnera* genomes (>= 580 kbp) are retained in strains from Pemphigini, Aphidinae, Eriosomatini, Fordini, and Hormaphidinae. On the other side, small genomes of under 580 kbp have evolved once within the Eriosomatini (in *Tetraneura ulmi*), and three times in the branches leading to the Mindarinae + Neophyllaphidinae, Anoeciinae + Thelaxinae, and Lachninae + Drepanosiphinae + Chaitophorinae + Calaphidinae. After manual curation of the orthologous protein clusters of *Buchnera*, we found that the last common ancestor (LCA) of this endosymbiont coded for at least 653 proteins. Similarly to genome

reduction events in *Buchnera*, marked CDS losses (>=40) have independently occurred repeatedly in many aphid lineages. These genomic reductions are almost always accompanied by a drastic loss of protein-coding genes (CDSs). One marked exception are both *Buchnera* strains from Hormaphidinae, which hold rather large genomes (630 and 580 kbp) with a strikingly low number of CDSs (453 and 450). Surprisingly, both of these genomes keep a small number of pseudogenes (10 and 1), revealing large portions of their genomes are "deserted" (28.9% and 25.3%). The LCA of both *Buchnera* from Hormaphidinae is predicted to have had at least 472 CDs and a large genome, revealing small CDS losses leading to each of the Hormaphidini and Nipponaphidini branches.

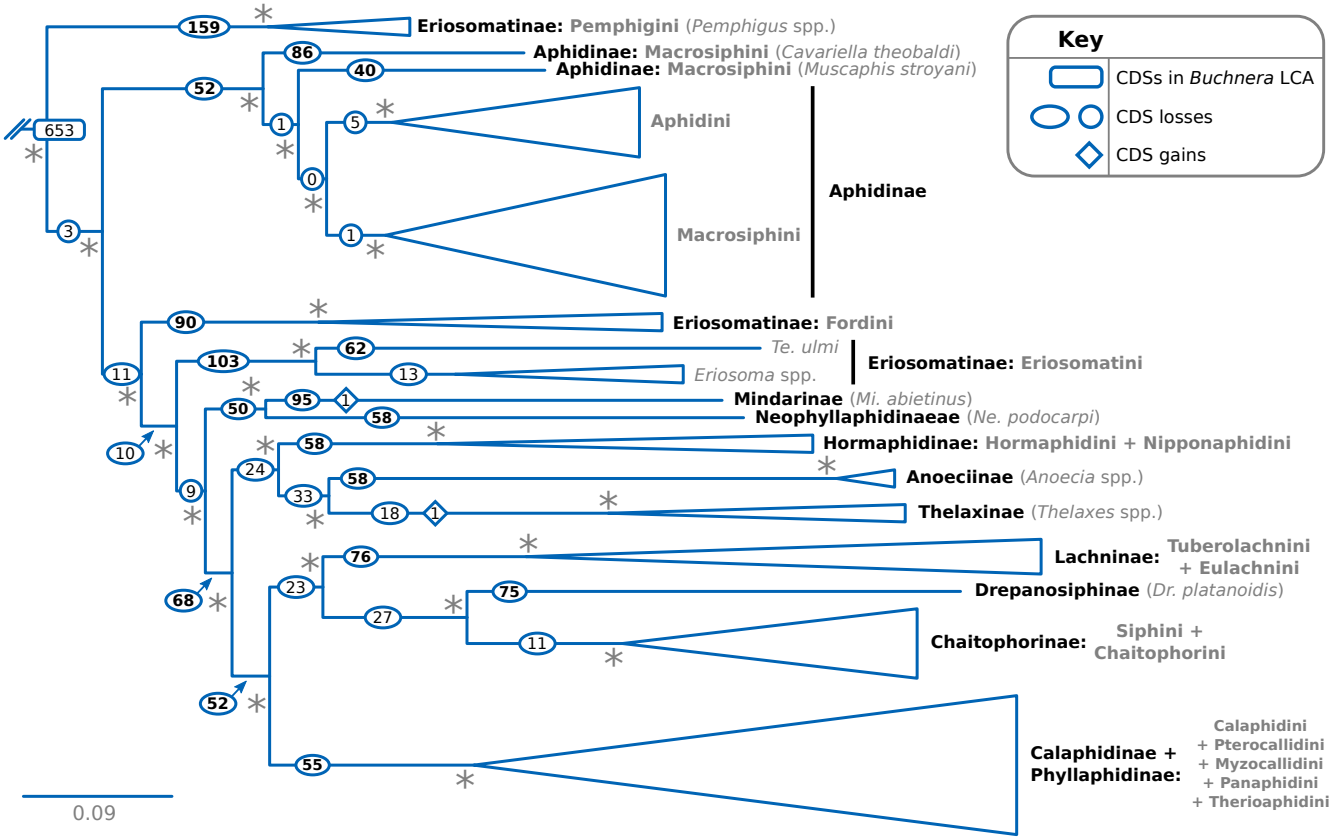

Figure 1. Phylogenetic relationships of *Buchnera*. Truncated Bayesian phylogenetic tree based on concatenated single-copy core proteins of selected *Buchnera*. Branches were collapsed on the basis of their aphid hosts' subfamily classification. At the right of leaves and clades, names of aphid subfamilies ans tribes are shown in the format "**family:** tribe" in black and grey, respectively. When a subfamily is only represented by the *Buchnera* strain of one aphid species or genus, it is indicated in between parentheses.Given that *fliM+fliN* and *fliO+fliP* often occurred as fused genes, they were each counted as one protein cluster instead of two. An asterisk at nodes stands for a posterior probability of 1. Full phylogenetic tree can be found at (supplementary figure S1, Supplementary Material online)

Most notably, we found one gene unique to each of the *Buchnera* from Mindarinae and Thelaxinae, hinting at gene acquisition in these lineages. The first gene is Tn3-family DNA-resolvase/invertase in the pYqhA plasmid of *Mi. abietinus*. This gene is similar to *Escherichia coli*'s serine recombinase PinE (prom prophage origin), which catalyzes the inversion of an 1800-bp DNA fragment (the P region), which can exist in either orientation (Plasterk and van de Putte, 1985).The second gene is a predicted

amidinotransferase (FN0238 type), with best hits against Bacteroidota, present in a *Thelaxes*-specific plasmid coding for this and a *repA1* protein, similar to that present in pLeu and pYqhA plasmids (van Ham *et al.*, 1997).

Secondary symbionts complement metabolic deficiencies in six aphid lineages

Most known co-obligate symbiotic lineages have facultative counterparts (Manzano-Marín *et al.*, 2018, 2020; Meseguer *et al.*, 2017)), and thus, knowing the common secondary symbiotic microbiota from a large diverse aphid pool can provide clues into the available potential source of co-obligate symbionts. Our 16S rRNA amplicon survey of 223 aphid samples yielded ∼5.4 million sequencing reads with an average of ∼11,300 reads per aphid sample kept. Those reads were distributed into 287 clusters, with most of these assigned to *Buchnera aphidicola* and the nine known facultative or co-obligate symbionts of aphids (figure 2 and supplementary figure S2, Supplementary Material online). In addition, some clusters were designated as *Acetobacteraceae*- and *Gillamella*-related bacteria, known as gut-associated symbionts of honeybees, fruitflies, and ants (Brown and Wernegreen, 2019; Pais *et al.*, 2018; Smith and Newton, 2020). The remaining reads were assigned to ubiquitous bacteria that could be of an environmental source and not representative of the aphid microbiota. In fact, these bacterial taxa were not always found across PCR replicates of the same sample and were sometimes found in the controls.

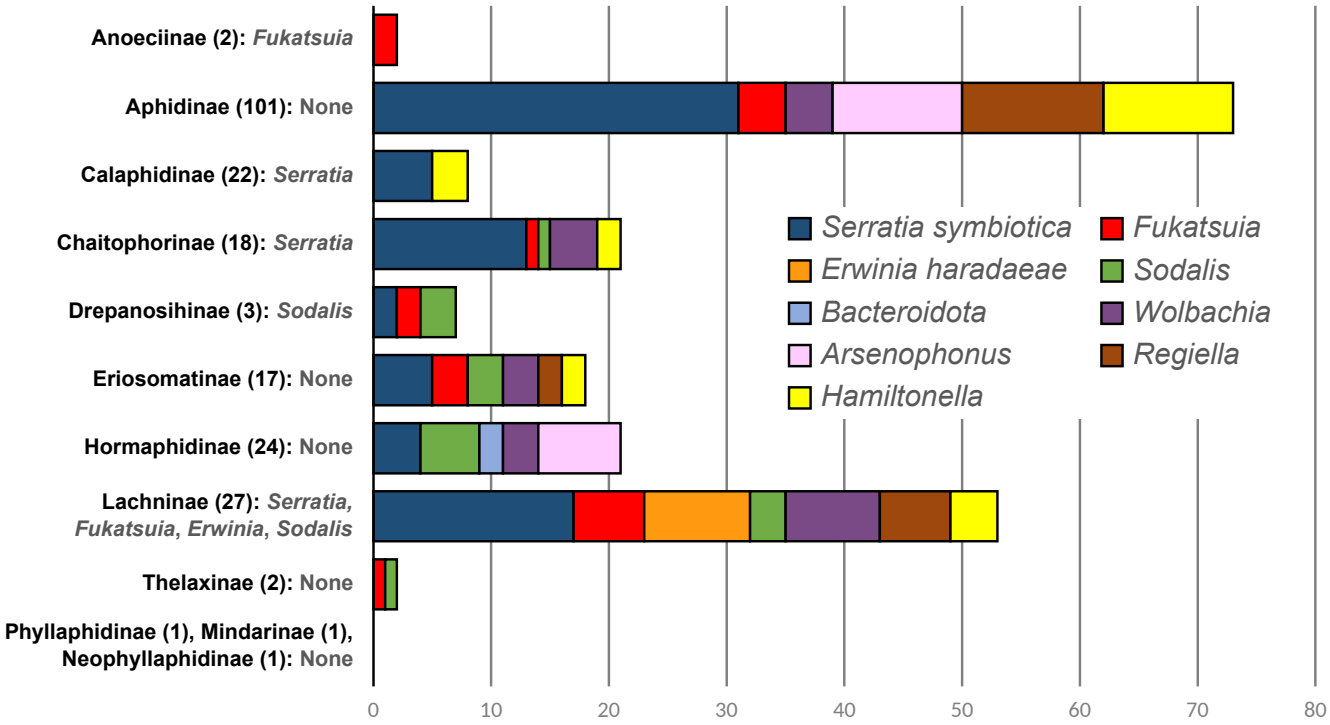

Figure 2. Secondary symbiont diversity across aphid subfamilies. Stacked barplot displaying absolute count of symbiont taxa found across samples of specific subfamilies. To the left of the bars, the subfamily name is indicated followed by the number of samples included in this survey in parentheses. Family names and counts are followed by the putative and known co-obligate symbiont taxa as inferred from the present study or previous genomic analysis studies.

This 16S amplicon survey is in agreement with previous analyses of genomic data: when a co-obligate association has been inferred in an aphid species by genome-based metabolic inference, the symbiont taxon was found in all samples of that species and often in closely related ones. For example, we confirmed that *Erwinia* is fixed within a specific clade of *Cinara* (Lachninae: Eulachnini; Manzano-Marín *et al.*, 2020) as well as *Se. symbiotica* within *Periphyllus* spp. (Chaitophorinae: Chaitophorini; Monnin *et al.*, 2020) and various Lachninae species (Lamelas *et al.*, 2011b; Manzano-Marín and Latorre, 2014; Manzano-Marín *et al.*, 2016, 2017, 2018; Meseguer *et al.*, 2017). From the bacterial taxa, *Se. symbiotica* was the most common secondary endosymbiont detected in our sampling, being present in 77 out of 223 samples. In addition to *Se. symbiotica*; *Hamiltonella*, *Wolbachia*, *Fukatsuia*, and *Sodalis*-like bacteria were found in 23, 22, 18, and 15 samples, respectively.

Our analysis also corroborated the absence of *Buchnera* in the one sample of *Cerataphis brasiliensis* (Hormaphidinae: Cerataphidini), a species in which the ancient obligate symbiont has been replaced by a so-called ”Yeast-like symbiont” (Vogel and Moran, 2013). Our data also suggests an absence of fixed secondary symbionts in all three tribes of Eriosomatinae. Although a *Bacteroidota* symbiont has been found as replacing *Buchnera* in a member of the Fordini (*Geopemphigus* spp.; Chong and Moran, 2018), we did not recovered a secondary bacterial 16S rRNA sequence belonging to this bacterial taxon in the selected Fordini. In fact, a *Bacteroidota* 16S rRNA sequences were only found in two samples of Hormaphidinae aphids in our survey (*Astegopteryx bambusae* ACOE3753 and *Reticulaphis mirabilis* ACOE3769). Similarly to Eriosomatinae; Hormaphidinae, Thelaxinae (*Thelaxes suberi*), Mindarinae (*Mindarus abietinus*), and Neophyllaphidinae (*Neophyllaphis podocarpi*) samples did not appear to be hosting a fixed secondary symbiont. However, the latter three were only represented by one or two samples, and thus, results should be taken with caution. While Aphidinae hosted many secondary symbionts, none appeared fixed at least at the tribe or even at genus level. Remarkably, we found *Se. symbiotica*, *Fukatsuia*, and *Sodalis*-like bacteria fixed in three groups of aphids, Siphini, Anoeciinae, and Drepanosiphinae (respectively), hinting at the co-obligate status of these bacterial taxa in their host species.

Given the well-established role of *Buchnera* as an essential amino acid- and vitamin B-provider, we analysed the genes coding for enzymes involved in the biosynthesis of these nutrients across *Buchnera* (figure 3 and supplementary figure S3, Supplementary Material online). We found that *Buchnera* strains belonging to six aphid lineages have evolved genomes potentially unable to meet the nutritional requirements of their host. These six lineages include the previously reported Lachninae and *Periphyllus* spp. (Chaitophorinae: Chaitophorini) aphids, as well as the newly identified *Anoecia* spp. (Anoeciinae), *Dr. platanoidis* (Drepanosiphinae), *Ch. stipae* and *Si. maydis* (Chaitophorinae: Siphini), and *Pa. juglandis* (Calaphidinae: Panaphidini). In all of the afore-mentioned aphid lineages, we were able to recover a symbiont genome belonging to a taxon identified as fixed by our 16S gene amplicon survey in the corresponding aphid taxa: *Se. symbiotica* in *Periphyllus* spp., Siphini, and *Pa. juglandis*; *Fukatsuia* in *Anoecia* spp., and *Sodalis*-like in *Dr. platanoidis*. Genomes of all symbionts showed clear evidence of genome reduction, with those of *Anoecia* spp., *Dr. platanoidis*, and *Pa. juglandis* showing the most reduced

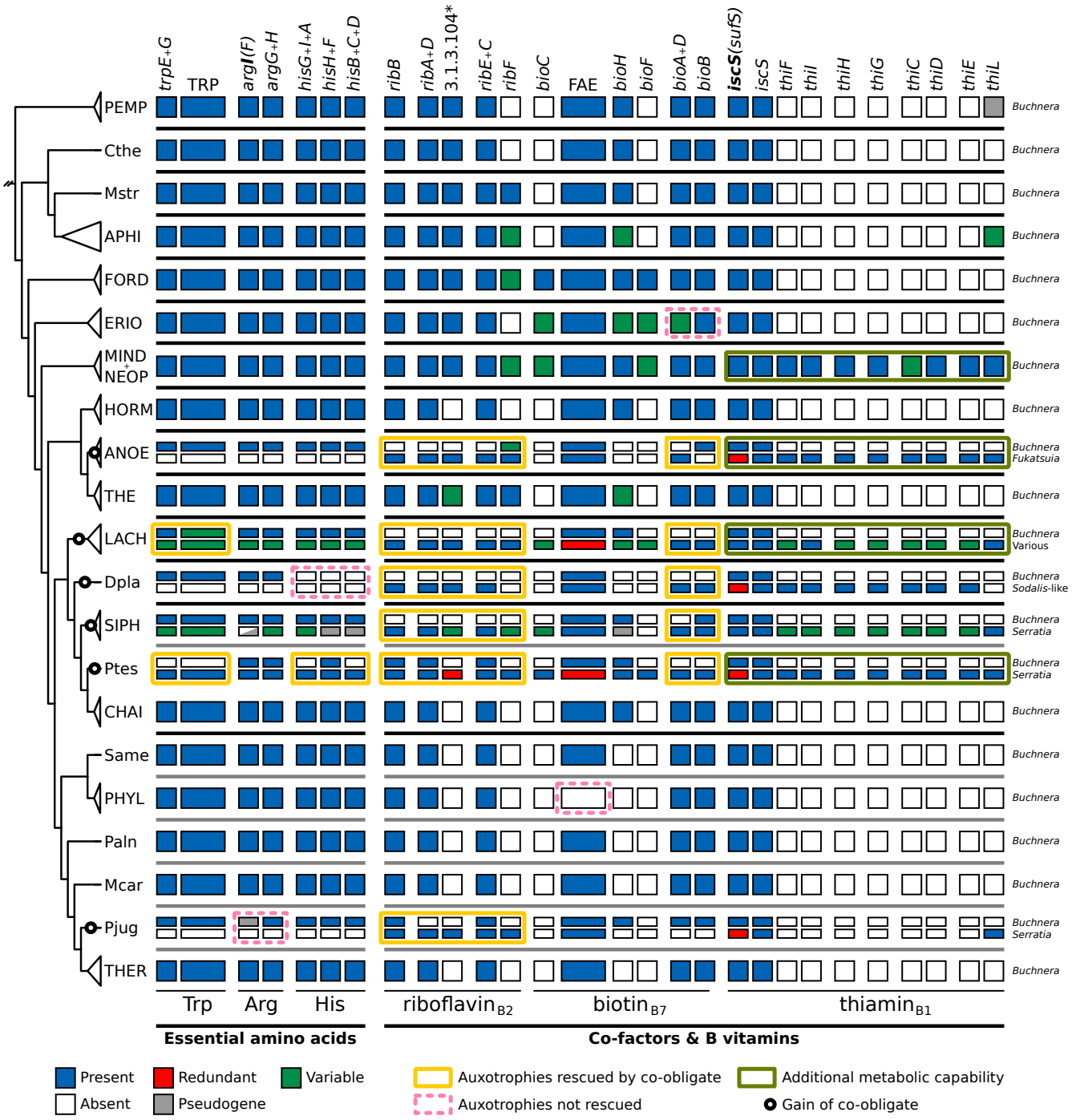

Figure 3. Metabolic complementarity of co-obligate symbiotic systems of aphids. Matrix summarising key metabolic capacities of obligate symbionts of different aphid species. On the left, dendrogram displaying phylogenetic relationships of *Buchnera* strains (see supplementary figure S1, Supplementary Material online). An empty circle at a node represent the acquisition of a co-obligate symbiont. At the leaves, abbreviation for aphid taxa. At the top of each box column, the name of the enzymes or pathway catalysing a reaction. At the bottom, the name of the compound synthesised by the enzymatic steps. At the right of each row of boxes, symbiont taxon name. Black and grey bars between boxes separate aphid subfamilies and tribes. TRP= anthranilate to tryptophan (*trpDCBA*), FAE= fatty acid elongation (*fab(B/F)GZ(I/V)*), PEMP= *Pemphigus*, Cthe= *Ca. theobaldi*, Mstr= *Mu. stroyani*, APHI= Aphidinae, FORD= Fordini, ERIO= Eriosomatini, MIND= Mindarinae, NEOP= Neophyllaphidinae, HORM= Hormaphidinae, ANOE= *Anoecia*, THE=Thelaxinae, LACH= Lachninae, Dpla= *Dr. platanoidis*, SIPH= Siphini, Ptes= *Pe. testudinaceus*, CHAI= *Chaitophorus*, Same= *Sy. americanus*, PHYL= Phyllaphidinae, Paln= *Pt. alni*, Mcar= *My. carpini*, Pjug= *Pa. juglandis*, THER= Therioaphidini. * EC number for reaction catalysed by promiscuous enzymes.

genomes (supplementary table S7, Supplementary Material online). The *Se. symbiotica* genomes of both Siphini and *Periphyllus* aphids were more similar in size to those of facultative and early co-obligate strains of Aphidinae and Lachninae aphids. Manual curation of the genes involved in essential amino acid and B vitamin biosynthesis revealed these co-obligate endosymbionts are in fact generally capable of rescuing auxotrophies evolved in their *Buchnera* partners.

In regards to B vitamins, riboflavin- and biotin-biosynthesis is often observed to be lost in *Buchnera* and being taken over/rescued by the new co-obligate symbiont. The two exceptions are in the biotin biosynthesis in *Anoecia* spp. and *Pa. juglandis*. In the former, enzymes from both symbionts are needed: *bioA*+*bioD* from the *Fukatsuia*-related symbiont and *bioB* from *Buchnera*. In the latter, the new *Se. symbiotica* symbiont has lost all three genes needed to produce this compound from KAPA, and thus *Buchnera* has retained this role. As reported for other *Periphyllus* spp. by Monnin *et al.* (2020), we corroborate the tryptophan- and histidine-biosynthetic role of the *Se. symbiotica* endosymbiont in the newly sequenced genome from *Pe. testudinaceus*. Nonetheless, we did not observed the phenylalanine biosynthetic role being lost in *Buchnera* from *Pe. testudinaceus*. Upon closer inspection of the *Buchnera* draft genomes and annotations of *Periphyllus* spp. analysed by Monnin *et al.* (2020), we found the presence of a conserved poly(A) region across *Buchnera* from *Periphyllus* spp. in the *pheA* gene. Due to the conserved nature of this region, we considered it as likely rescued by transcriptional frameshifting, as this rescue mechanism has been experimentally demonstrated in other *Buchnera* and *Blochmannia* strains (Tamas *et al.*, 2008). In addition, we found similar poly(A) regions disrupting the *pheA* gene to be present in all other sequenced *Buchnera* strains from Chaitophorinae. We found the thiamin-biosynthetic capacity (vitamin B_1) to be largely retained in the symbiotic consortia from Mindarinae, Neophyllaphidinae, Anoeciinae, Lachninae, Drepanosiphinae, Siphini, and *Pe. testudinaceus*. Most surprisingly, in the case of Mindarinae we found the retention of thiamin biosynthetic genes in newly sequenced *Buchnera* from Mindarinae (all) and Neophyllaphidinae (no *thiC*). The genes are present in four syntenic regions: between the *purH* and *rpoC* (*thiCEFSGH*), *dcd* and *metG* (*thiD*), *ribE* and *ribD* (*thiL*), and *dxs* and *yajR* (*thiI*). The location of the *thi* genes is syntenic with *Erwinia* spp., suggesting these genes were present in the LCA of *Buchnera* and have been repeatedly lost. Supporting this hypothesis, we observed the presence of a *thiL* gene/pseudogene in *Buchnera* from Pemphiginae and many Aphidinae, with top BLASTp hits *vs.* NCBI's nr to *Erwnia* and *Pantoea* bacteria, which are the closest free-living relatives of *Buchnera* (Husník *et al.*, 2011). Finally, *Buchnera* from Phyllaphidinae have lost the genes involved in fatty acid elongation, which would make *Buchnera* dependant on the host for the production of its own membrane. However, as is the case for other strains, it would be able to synthesise biotin from KAPA.

Regarding essential amino acids, we corroborated the collaboration of both symbiotic partners in the production of tryptophan in some Lachninae (Lamelas *et al.*, 2011b; Manzano-Marín *et al.*, 2016) as well as the takeover of this role and the histidine biosynthesis by the co-obligate *Se. symbiotica* in *Peryphillus* spp. (Monnin *et al.*, 2020). In addition, we uncovered the complete loss of histidine-biosynthetic genes in *Buchnera* from Drepanosiphinae and a loss of the *argI* gene, rendering *Buchnera* and potentially the aphid

host dependant on histidine and/or L-citruline for the production of these two essential amino acids.

Co-obligate symbionts have repeatedly evolved from facultative symbiont lineages

Phylogenetic placement of the newly sequenced *Serratia*-related co-obligate symbionts confirmed their taxonomic assignment as *Se. symbiotica* (figure 4A). The recovered phylogenetic tree suggests at least two independent origins of the co-obligate *Se. sybmiotica* endosymbionts in *Periphyllus* spp., with support for a single origin of this symbiont lineage in the LCA of *Pe. aceris* and *Pe. acericola*. Additionally, the resulting phylogeny does not support a common origin for the co-obligate symbionts of Siphini aphids, suggesting repeated replacements within this tribe. Regarding the co-obligate symbionts of *Anoecia* spp., they are recovered as a sister group to *Fu. symbiotica*. The 16S identity of the *Fukatsuia*-like co-obligate symbionts of Anoeciinae aphids falls below the recommended species threshold of 98.7% (95.79% and 96.04%; Chun *et al.*, 2018), but above the recommended minimum threshold for being classified as the same genus (Yarza *et al.*, 2014). Nonetheless, both *Anoecia* spp. symbionts show a 16S sequence identity

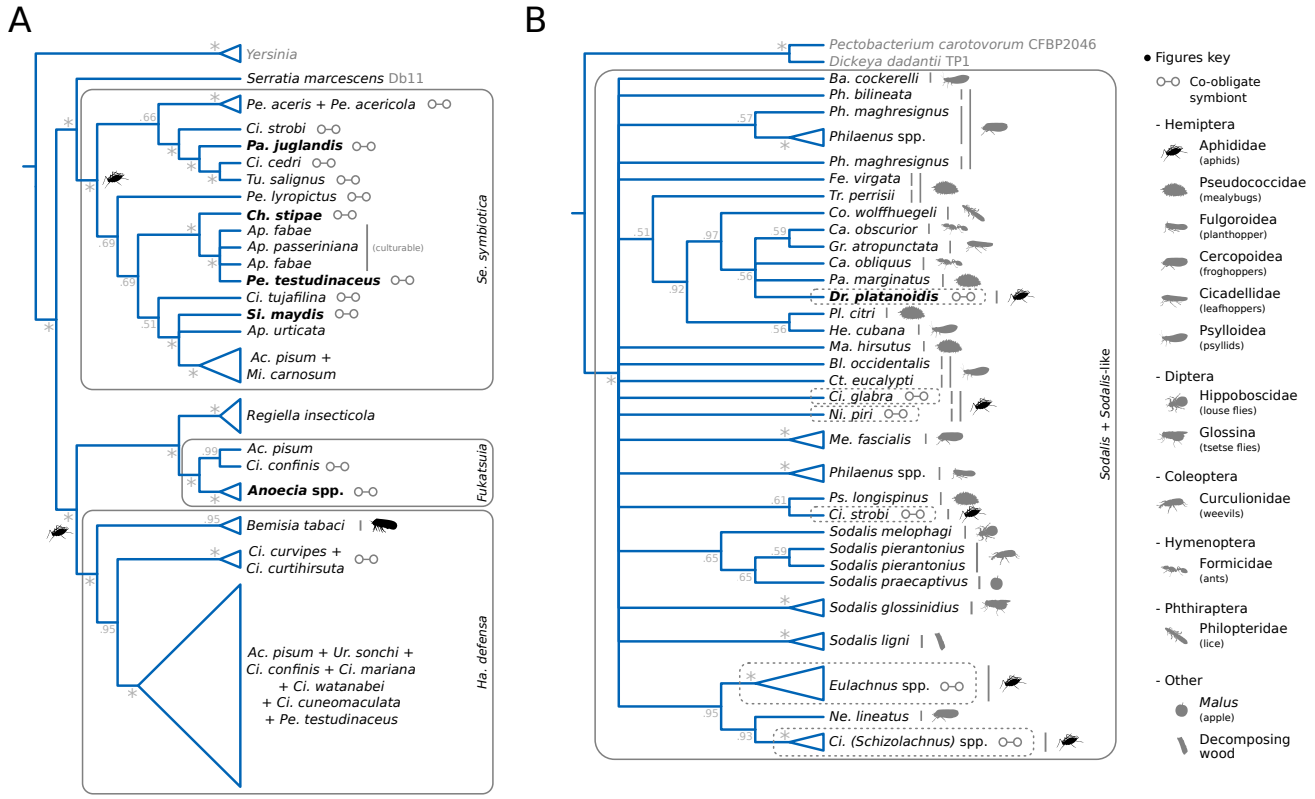

Figure 4. Phylogenetic relationships of aphid co-obligate endosymbionts. **(A)** Phylogenetic placement of *Yersinia*- and *Serratia*-related co-obligate endosymbionts. Silhouettes at nodes or at leaves indicate host relationship of clade. Grey boxes with binomial names on the side indicate aphid symbiotic taxa of interest. **(B)** Phylogenetic placement of *Sodalis*-related co-obligate endosymbionts. Grey box delimits the *Sodalis* + *Sodalis*-related clade. Silhouettes at branch ends indicate identity of host relationship of leaf/clade. Dotted boxes highlight *Sodalis*-like symbionts of aphids. For both A and B, abbreviated nomenclature is used when referring to the symbiont's host name. Names in bold, highlight newly sequenced endosymbiont lineages. Names in grey mark taxa used as outgroups.

of 98.77%, suggesting they belong to the same molecular species. Co-obligate *Sodalis*-like symbionts have been identified in several aphid species (Manzano-Marín *et al.*, 2017, 2018). The newly identified *Sodalis*-like co-obligate symbiont of *Dr. platanoidis* does not cluster with any of the previously identified aphid *Sodalis*-like symbionts. In fact, the tree suggests most co-obligate *Sodalis*-like endosymbionts from aphids have evolved independently, with those of *Eulachnus* and *Cinara (Schizolachnus)* spp. having likely evolved from closely related ancestor strains.

Co-obligate symbionts in *An. corni* and *Si. maydis* reside inside bacteriocyte cells

In order to investigate the tissue tropism of the newly identified co-obligate endosymbionts, we analysed available preserved specimens of *An. corni* and *Si. maydis* embryos through FISH miscroscopy using specific probes for each symbiont (figure 5). We corroborated and taxonomically identified the secondary symbionts previously observed by transmission electron microscopy by Michalik (2010); Michalik *et al.* (2014). The *Fukatsuia* symbiont of *An. corni* resides in separate bacteriocytes to those of *Buchnera* and shows a spherical pleomorphic shape. Similarly, we observed the *Se. symbiotica* symbiont of *Si. maydis* inhabiting different bacteriocytes to those of *Buchnera*. However, the co-obligate endosymbiont shows an elongated rod-shape, more typical of facultative and early co-obligate symbionts (Manzano-Marín and Latorre, 2014; Moran *et al.*, 2005).

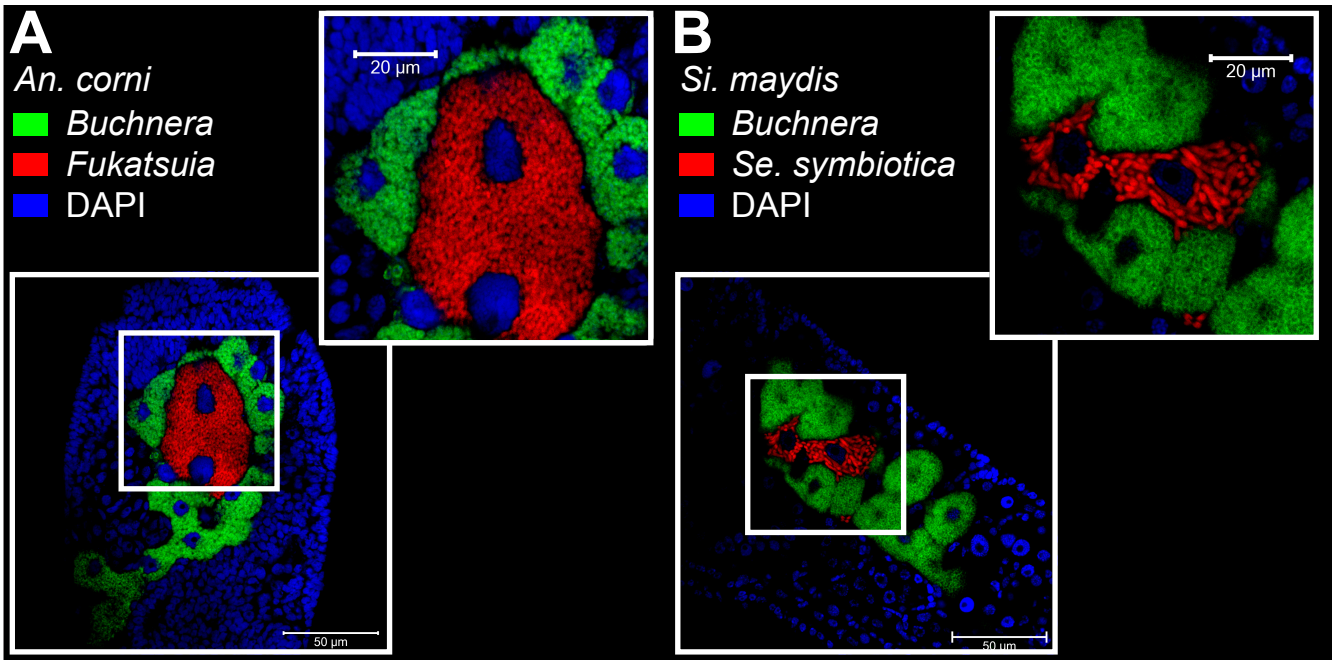

Figure 5. Location and morphology of co-obligate symbionts in selected aphid species. Merged FISH microscopic images of aphid embryos from **(A)** *An. corni* (lateral) **(B)** *Si. maydis* (tilted lateral). Co-obligate symbionts' signal is shown in red, *Buchnera*'s in green, and DAPI's (staining DNA, highlighting host nuclei) in blue. Thick white boxes indicate the magnified region depicted in the top-right of each panel. The scientific name for each species along with the false colour code for each fluorescent probe and its target taxon are shown at the top-left of each panel. Unmerged images can be found in https://doi.org/10.5281/zenodo.6394198.

'*Candidatus* Fukatsuia anoeciicola' sp. nov.

'*Candidatus* Fukatsuia anoeciicola' (an.oe'ci.i.co.la. N.L. fem. n. *Anoecia*, an aphid genus; L. masc./fem. n. suff. -cola, inhabitant, dweller; N.L. gen. n. *anoeciicola*, inhabiting *Anoecia* aphids).

We propose the specific name '*Candidatus* Fukatsuia anoeciicola' for the monophyletic lineage of enterobacterial endosymbionts from the '*Candidatus* Fukatsuia' genus hitherto exclusively found affiliated as co-obligate nutritional symbionts in *Anoecia* species (Hemiptera: Aphididae: Anoeciinae). In embryos of *Anoecia corni*, '*Candidatus* Fukatsuia anoeciicola' is found co-inhabiting the bacteriome intracellularly in different bacteriocytes to those of *Buchnera aphidicola* (figure 5; Michalik *et al.*, 2014). In oviparous *An. corni*, '*Candidatus* Fukatsuia anoeciicola' has a spherical shape (mean cell diameter of 2.08μm), it is located intracellularly in bacteriocytes surrounded by those of *Buchnera*, and can also be found in oocytes (Michalik *et al.*, 2014).

Discussion

Long-term associations with strict vertically-transmitted bacterial partners tends to have serious consequences for the genome of the symbiont: drastic genome reduction can eventually lead to impairment of symbiotic functions (*e.g.* host's nutrition) (McCutcheon *et al.*, 2019). Symbiont replacement is recognised as an important evolutionary force, since it has the potential of rapidly expanding the metabolic capacities of the symbiotic system (Sudakaran *et al.*, 2017). The nutritional association of *Buchnera aphidicola* with aphids has persisted for millions of years and has been seen, until very recently, as an exclusive co-evolutionary history. Throughout its history, *Buchnera*'s genome has remained extraordinary stable, generally keeping genes that are essential for their symbiotic function (Chong *et al.*, 2019). Here we add to the recent evidence that the aphid-*Buchnera* evolutionary history is marked by repeated losses of essential metabolic capacities and complementation by a new partner. We discovered seven new aphid species from four subfamilies in which *Buchnera* has lost one or several essential symbiotic functions. In all cases, *Buchnera*'s auxotrophies are rescued by a new bacterial partner. Symbiont identity and fixation within species was further confirmed by 16S rRNA gene amplicon sequencing and in two cases by FISH microscopy.

We found *Se. symbiotica* associated as the new co-obligate partner of *Buchnera* in now three subfamilies with at least four independent acquisitions (figure 6). Our phylogenetic data on Siphini and previous analyses of *Se. symbiotica* associated with Lachninae suggests than within these groups, several independent acquisitions/replacements have occurred (Manzano-Marín *et al.*, 2016, 2017; Meseguer *et al.*, 2017). *Se. symbiotica* was the most common secondary symbiont detected in our 16s rRNA survey, confirming its predominance as a natural facultative symbiont of aphids (Pons *et al.*, 2022; Zytynska and Weisser, 2016). In fact, *Se. symbiotica* has not only been recorded in aphids, but has also been identified as a possible facultative endosymbiont in Japanese populations of the hemlock woolly adelgid *Adelges tsugae* (von Dohlen *et al.*, 2013). Strains of this symbiont have been recorded as protecting against

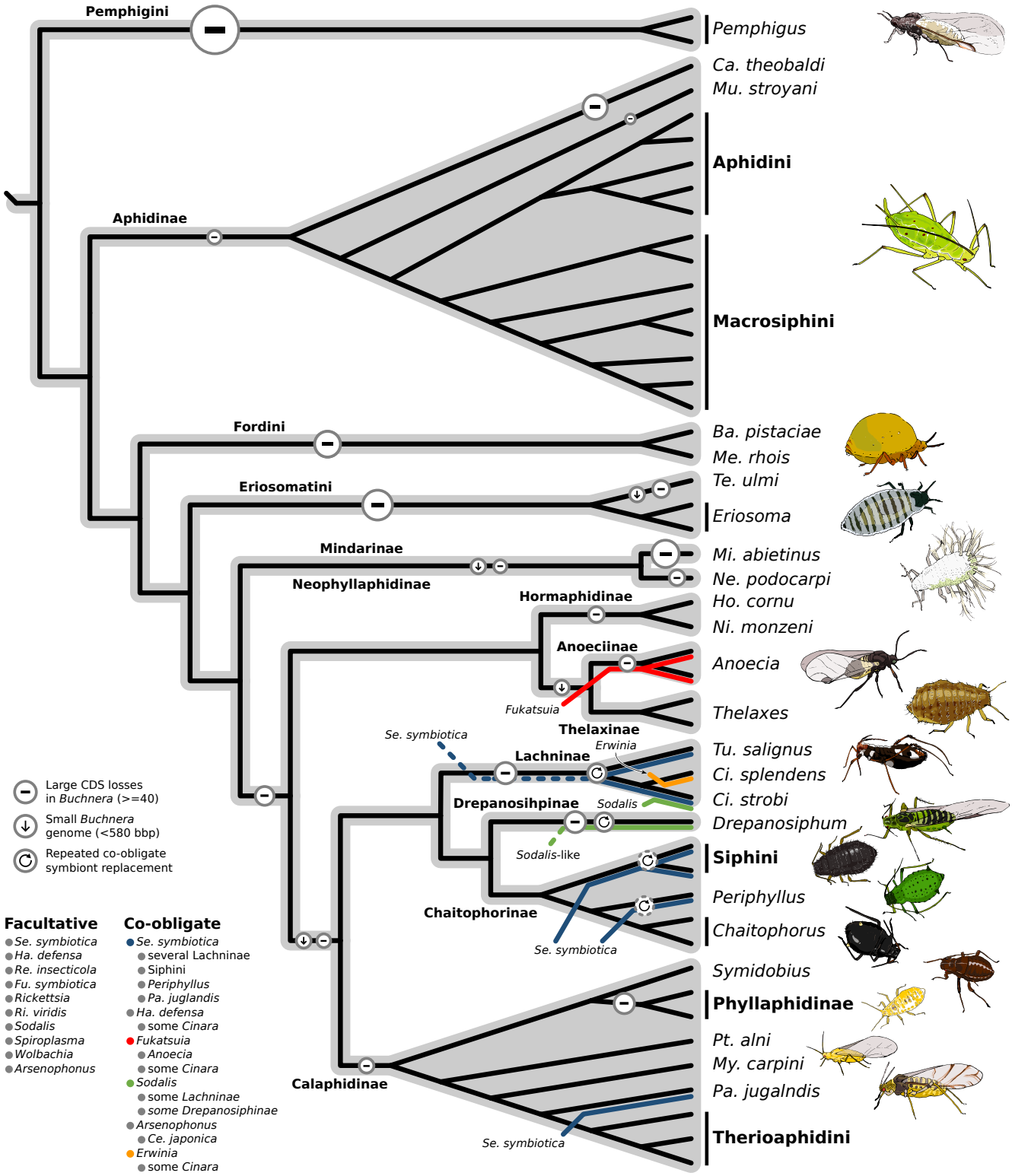

Figure 6. Evolutionary history of co-obligate symbioses in Aphididae. Diagram summarising results obtained from the genomic analysis presented in this work. Cladogram was made using the reconstructed phylogeny for *Buchnera* symbionts. Black lines in the cladogram represent *Buchnera*. Coloured lines are used to represent the acquisition of co-obligate symbionts. Dotted lines represent uncertainty in the acquisition of a specific taxa. Dotted circles represent uncertainty in symbiont replacement dynamics within the aphid lineage. On the right, aphid cartoons depicting variety of species across the Aphididae.

heat-stress (Chen *et al.*, 2000; Montllor *et al.*, 2002; Russell and Moran, 2006), providing resistance against parasitoid wasps (Oliver *et al.*, 2003), and even compensating for the elimination of the primary obligate endosymbiont *Buchnera* in the pea aphid *Acyrthosiphon pisum* (Koga *et al.*, 2003). Given the multiple benefits conferred by this endosymbiont, it is therefore not unexpected to find it widely present across aphids taxa and populations, where it might easily expected to become more common when the right environmental and biological conditions arise. The pervasiveness of *Se. symbiotica* strains in aphid populations has probably facilitated their adaptation as new co-obligate partners upon *Buchnera*'s gene losses. However, *Se. symbiotica* is not the only symbiont that has become essential to its aphid hosts. A *Fukatsuia*-related symbiont (hereafter *Fukatsuia anoeciicola*) was revealed as a co-obligate partner of Anoeciine aphids. Another species of this symbiotic lineage, *Fukatsuia symbiotica*, has already been reported as a co-obligate endosymbiont of Lachninae (Manzano-Marín *et al.*, 2017; Meseguer *et al.*, 2017). Our study revealed that *Fukatsuia*-related symbionts are found in aphid species from different subfamilies, but much less frequently than *Se. symbiotica*. *Fukatsuia* symbionts have been associated with several benefits to the pea aphid, however only when co-infecting with other symbiont species (Donald *et al.*, 2016; Doremus and Oliver, 2017; Heyworth and Ferrari, 2015). Additionally a *Sodalis*-like symbiont was identified as a co-obligate partner of Drepanosiphinae. Despite *Sodalis*-related strains being widely found across arthropods, they have been rarely reported in aphids. Some of the few strains found in aphids have been described as being in an obligate association with some Lachninae, mainly *Cinara* spp. (Manzano-Marín *et al.*, 2017, 2018; Meseguer *et al.*, 2017). We have nonetheless spotted this symbiont occasionally in four subfamilies. With limited knowledge on *Sodalis* and *Fukatsuia* potential benefits/costs as facultative symbionts, it is hard to speculate on the environmental conditions favouring their expansion in populations, and thus, its fixation as a co-obligate partner. In any case, our data shows that even relatively rare symbionts can eventually become essential. Among the speciose Aphidinae subfamily, which shelters a large diversity on facultative symbionts (figure 2), we found no evidence of putative co-obligate endosymbionts, including in the banana aphid *Pentalonia nigronervosa*, corroborating previous genomic analyses (Manzano-Marín, 2020).

A common pattern in the dual nutritional symbioses revealed here is the riboflavin biosynthetic-role takeover by the new co-obligate symbiont. The biosynthetic genes of this pathway have been found to be particularly upregulated in aphid embryos (Bermingham *et al.*, 2009), which suggests the importance of this nutrient in the early life stages of the aphid but not in later ones. This could relax selection for the retention of these genes in *Buchnera* populations at later life stages of aphids, and thus open a window for facilitating the takeover of the nutritional role by a co-existing symbiont. Biotin was the second most common nutrient predicted to be supplied by the secondary co-obligate endosymbionts: it was found in all cases except in the co-obligate association observed in *Pa. juglandis*. A similar biotin-biosynthetic role is found in the *Sodalis*-like co-obligate endosymbionts of the psyllids *Ct. eucalypti* and *He. cubana* (Sloan and Moran, 2012). To our knowledge, no information is available on the specific life stage on which the aphid might depend on this nutrient; which precludes elaborating scenarios on how this pathway can be lost in *Buchnera*. In addition, thiamin was found to be retained in different lineages of

co-obligate symbionts with a reduced genome, while lost in others as well as in all *Buchnera* genomes but the ones associated with Mindarinae and Neophyllaphidinae (figure 3). Consequently, complete and almost-complete pathways (lacking the thiamine-monophosphate kinase gene *thiL*) are found in obligate symbiotic systems of five subfamilies. The retention of this pathway in highly reduced genomes of endosymbionts suggest an important role for this nutritional role. This has been previously proposed in the endosymbiotic systems of a monophyletic group of *Cinara* spp., where the thiamin-biosynthetic genes have been serially horizontally transferred from a *Sodalis*-like bacteria to the now co-obligate *Er. haradaeae*, and further to a tertiary co-obligate *Ha. defensa* (Manzano-Marín *et al.*, 2020). Interestingly, the *Buchnera* strains that retain this pathway are associated exclusively with aphids feeding on conifers, as *Cinara* spp. Unfortunately, and to our knowledge, no comparative phloem sap analysis of conifers *vs.* angiosperms is available to infer whether this symbiotic function can be related to host plant association. Further, other di-symbiotic systems maintaining thiamin genes are generally associated with more diverse botanical families. On the other hand, the presence of this pathway in the *Se. symbiotica* of *Si. maydis* (Siphini) can be related to the recent acquisition of this co-obligate strain: its genome size is similar to that of facultative strains. Therefore, the retention of this pathway might not necessarily be related to any essential nutrient supplementation role. More studies elucidating the importance of thiamin during aphid development and its abundance in the phloem-sap of diverse host plant families are necessary to fully understand the evolutionary processes leading to its losses and gains throughout aphids diversification.

The amino acid-biosynthetic genes are almost always retained exclusively in *Buchnera* (figure 3), with marked exceptions of the split pathway between the co-obligate symbionts of the Lachninae aphids *Cinara cedri* and *Tu. salignus* (Lamelas *et al.*, 2008; Manzano-Marín *et al.*, 2016) as well as the takeover of the biosynthesis of this compound plus histidine in *Periphyllus* spp. (Monnin *et al.*, 2020; Renoz *et al.*, 2022b). Most surprising was the identification of evolved auxotrophies in *Buchnera* that were not rescued by any co-existing symbionts. This was the case for arginine in *Pa. juglandis*, histidine in *Dr. platanoidis*, fatty acid elongation in Phyllaphidinae, and biotin in *Er. grossulariae*. This leads us to hypothesise that either the aphid is able to supplement precursors through horizontally transferred genes or the specific diet of the aphid is able to rescue these auxotrophies. In fact, horizontally transferred genes from diverse bacteria have been predicted and/or shown to support nutrient biosynthesis in Pseudococcinae (Husnik and McCutcheon, 2016; Husnik *et al.*, 2013; Szabó *et al.*, 2017), psyllids (Sloan *et al.*, 2014), and the whitefly *Bemisia tabaci* (Bao *et al.*, 2021; Ren *et al.*, 2020; Xie *et al.*, 2018). Another scenario would be the presence of an additional symbiont not detected by our molecular methods. However, in the case of *Dr. platanoidis* and *Pa. juglandis*, microscopic studies have revealed the presence of only two different bacteria (Buchner, 1953).

Our study also revealed patterns of *Buchnera* degradation: this primary symbiont has undergone multiple events of CDS losses, which accordingly is most commonly accompanied by a reduction in genome size. While the acquisition of co-obligate endosymbionts is often associated to a drastic gene loss (figure 6), there are also some exceptions to this pattern. In Calaphidinae and Phyllaphidinae, *Buchnera* are

generally below 450 Mb (as small as in Lachninae) but this reduction was not concomitant to dependency on a new partner, as gene losses did not affect symbiotic functions. On the other hand, very few genes are actually lost in the branch leading to *Pa. juglandis* (18, out of which 8 are related to essential amino acid- and B vitamin-biosynthesis). This shows that it is the inactivation of very few functional genes that actually triggers dependency on an additional symbiont and not necessarily large-scale genome decay. We also observed large genomes with a low coding density in Hormaphidinae aphids. Such large amounts of ”deserted” DNA are more commonly observed in some transitional symbiont genomes (Manzano-Marín and Latorre, 2016; Manzano-Marín *et al.*, 2018). To our knowledge, such a low coding density has only been reported for the primary obligate endosymbiont of mealybugs, *Tremblaya princeps* (McCutcheon and von Dohlen, 2011). This suggests that, although rarely observed, large gene losses can precede genome reduction, even in a small compact symbiont genome. More surprisingly, we found the recent acquisition of genes to *Buchnera* plasmids in *Mi. abietinus* and *Thelaxes* spp. The role these two gained genes might play are quite different. The DNA-resolvase/invertase found in the pYqhA plasmid of the *Buchnera* from *Mi. abietinus* might play a role in promoting inversions within this plasmid. On the other hand, the predicted amidinotransferase carried by *Buchnera* from *Thelaxes* spp. might play a yet unknown metabolic role. Regarding the DNA-resolvase/invertase, it keeps similarity to the PinE protein of *E. coli* which is responsible for a naturally occurring inversion of the so-called P-region (Plasterk and van de Putte, 1985). In fact, historical inversions are observable among pLeu plasmids of *Buchnera* from different aphid subfamilies (Gil *et al.*, 2006; Van Ham *et al.*, 2000). In addition, similar proteins have been found in the pBioThi plasmids of the co-obligate symbiont *Er. haradaeae*, which show a duplicated inverted region causing a duplication of biotin- and thiamin-biosynthetic genes. Therefore, these DNA-resolvase/invertases could play a role in rearrangement of plasmidic genes in different endosymbiotic lineages. Both of these novel genes were likely acquired through horizontal gene transfer (HGT) events, from a hitherto unknown origin. HGT events have been observed in the aphid endosymbiont *Er. haradae*, where biotin- and thiamin-biosynthetic genes have been likely horizontally transferred from a once co-existing symbiont related to *Sodalis* bacteria (Manzano-Marín *et al.*, 2020). Similar cases of HGT have been observed in other small vertically transmitted symbiont genomes. These include the aforementioned example of *Er. haradaeae*, the transfer of an antifungal lagriamide biosynthetic gene cluster in the *Burkholderia* symbiont of the beetle *Lagria villosa* (Waterworth *et al.*, 2020), and the acquisition of a biotin-biosynthetic gene cluster in *Wolbachia* (Driscoll *et al.*, 2020; Gerth and Bleidorn, 2017; Nikoh *et al.*, 2014), *Legionella polyplacis*, (Říhová *et al.*, 2017), and the *Midichloria* symbiont of the tick *Hyalomma marginatum* (Buysse *et al.*, 2021), among others. HGT events in *Buchnera* and other endosymbionts evidence that, despite a degraded machinery needed for homologous recombination and their highly compact and degraded genomes, these symbionts are still able to horizontally acquire genes from unrelated bacteria.

These new endosymbionts have almost always evolved from facultative endosymbiotic taxa, suggesting a common facultative-to-obligate route for most of these secondary co-obligate endosymbionts. As is the case for Lachninae aphids (Manzano-Marín *et al.*, 2017; Meseguer *et al.*, 2017), repeated replacement of co-obligate endosymbionts has likely occurred in *Periphuyllus* and Siphini aphids: the two *Se. symbiotica* from

these aphids do not form well-supported monophyletic clusters (figure 4). In addition, repeated replacement has also occurred within Drepanosiphinae: while we found *Drepanosiphum* and *Drepanosiphoniella* hosted *Sodalis*-like symbionts, *Yamatocallis* spp. harbour unrelated gammaproteobacterial obligate symbionts (Fukatsu, 2001).

In conclusion, we found that co-obligate symbiotic systems are more widespread in aphids than previously thought, potentially existing in at least 11.4% of aphid species, given known species diversity in aphid lineages that are now inferred as hosting a dual nutritional symbiosis. During the drafting of this manuscript, two works have been published: one corroborating our finding of a co-obligate *Se. symbiotica* endosymbiont in *Si. maydis* (Renoz *et al.*, 2022b) and another one showing the evolution of a co-obligate symbiotic system in *Ceratovacuna japonica* (Hormaphidinae: Cerataphidini; Yorimoto *et al.*, 2022). A few major aphid lineages are still missing from this reconstruction, most markedly members of two well-diversified subfamily Greenideinae and Hormaphidinae where more dual symbiotic systems might be revealed. Co-obligate endosymbionts most commonly evolve from facultative endosymbiotic taxa, but not necessarily the most common ones, as exemplified by the recruitment of *Er. haradaeae* in *Cinara* and *Sodalis*- and *Fukatsuia*-related symbionts in now three subfamilies. Our analyses also show that a few key gene losses, rather than large-scale ones, are at the onset of co-obligate symbioses and that genome reduction can be decoupled from additional symbiont acquisitions. It has sometimes been speculated that it is the pervasiveness of facultative symbionts that can relax selective pressure on *Buchnera* and make it plunge further down the rabbit hole of endosymbiosis leading to co-dependency on a third partner (Lamelas *et al.*, 2011a). Our data on *Buchnera* genome degradation, co-obligate symbiont acquisition, and symbiont prevalence do not seem to draft such an evolutionary scenario. It is likely that the inherent decay of *Buchnera*, when it affects essential functions, opens a niche for the establishment of a new obligate symbiont: likely any symbiont that is potentially capable of filling that niche.

Finally, the role of additional or lost metabolic capabilities in aphid symbiotic systems remains an open question. However, we expect that investigation into ingested phloem by specific aphid species on their host plants as well as the specific dependence on nutrients by aphids at different life stages will shed light on these gain/losses of metabolic potential. To better understand co-obligate symbiont replacement, the role of additional or lost metabolic capabilities in aphid symbiotic systems in relation to the nutritional needs of the aphids to be further explored. We expect that investigation into the ingested phloem by specific aphid species as well as the dependence on specific nutrients by aphids at different life stages will shed light on gain/losses of metabolic potential and the establishment of new obligate symbionts.

Supplementary Material & Data Availability

Supplementary figures S1-S3 and tables S1-7 and have been included in this submission. All auxiliary files for phylogenetics and other analyses, unmerged FISH microscopy images, as well as the genomes of *Buchnera* and co-obligate endosymbionts are available online at https://doi.org/10.5281/zenodo.6394198.

Newly sequenced and annotated genomes are in the process of being submitted for accessioning to the European Nucleotide Archive (ENA).

Acknowledgements

We would like to acknowledge the talented artist/scientist Jorge Mariano Collantes Alegre for the aphid cartoons in figure 6. This work was supported by the Marie-Curie AgreenSkills + fellowship programme co-funded by the EU's Seventh Framework Programme (FP7-609398) to A.M.M. The Marie-Skłodowska-Curie H2020 Programme (H2020-MSCA-IF-2016) to E.J., the Agropolis foundation/Labex Agro ("Cinara's microbiome") to E.J, the France Génomique National Infrastructure, funded as part of the *Investissement d'Avenir* program managed by the *Agence Nationale pour la Recherche* (ANR-10-INBS-0009) to E.J., C.C., and V.B. This publication has been written with the support of the AgreenSkills + fellowship programme which has received funding from the EU's Seventh Framework Programme under grant agreement No. FP7-609398 (AgreenSkills + contract) and the Horizon 2020 Programme under grant agreement 746189 (H2020-MSCA-IF-2016- MicroPhan). We are grateful to the genotoul bioinformatics platform Toulouse Midi-Pyrenees (Bioinfo Genotoul) for providing help and/or computing and/or storage resources. The authors are grateful to the CBGP-HPC computational platform and the staff maintaining the *Life Science Compute Cluster* (LiSC; http://cube.univie.ac.at/lisc) at the University of Vienna which were used for computational analysis. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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